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Rôle de l'endothélium dans les dommages radio-induits aux tissus sains

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Articles et Communications

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Liste des abréviations

3D-CRT :	3D-Conformal Radiotherapy
α-sma :	Alpha smooth muscle actin
Ang II :	Angiotensine II
ApoE :	Apolipoprotein E
bFGF :	basic Fibroblast Growth Factor
BMP :	Bone Morphogenic Proteins
CML :	Cellules Musculaires Lisses
Col I :	Collagen type I
Col III :	Collagen type III
CTGF:	Connective Tissue Growth Factor
EGF:	Epidermal Growth Factor
eNOS :	endothelial Nitric Oxide Synthase
ET :	endothéline
Gy :	Gray
HGF :	Hepatocyte Growth Factor
HSP27 :	Heat Shock Protein 27
HMVEC :	Human Microvascular Endothelial Cells
HUVEC :	Human Umbilical Vein Endothelial Cells
ICAM-1 :	Intercellular Adhesion Molecule 1
IGF-1 :	Insulin-like Growth Factor 1
IL- :	interleukine
IM-RT :	Intensity Modulated Radiotherapy
KGF :	Keratinocyte Growth Factor
L-NAME :	N w-nitro L-arginine methyl ester
LAP :	Latency associated peptide
LDLr :	Low Density Lipoprotein receptor
LTBP :	Latent TGF-β binding protein
MMP :	Matrix Metalloproteinase
NFkB :	Nuclear Factor kappa B
NO :	Nitric Oxide
PAI-1 :	Plasminogen Activator Inhibitor type 1
PAR :	Proteolytically Activated Thrombin Receptor
PDGF :	Platelet-Derived Growth Factor
PECAM :	Platelet/Endothelial Cell Adhesion Molecule
ROS :	Reactive Oxygen Species
SMAD :	mothers against decapentaplegic homolog
SOD :	Superoxide Dismutase
TGF-β :	Transforming Growth Factor beta
TIMP :	Tissue inhibitor of Metalloproteinase
TM :	Thrombomoduline
TNFα :	Tumor Necrosis Factor alpha
tPA :	tissue-type Plasminogen Activator
uPA :	urokinase-type Plasminogen Activator
VCAM :	Vascular Adhesion Molecule
VEGF :	Vascular endothelial Growth Factor

I Introduction

A Dommages radio-induits aux tissus sains.

1. Les rayonnements ionisants.

Les rayonnements ionisants sont exploités pour de nombreuses applications dans le domaine industriel, énergétique, militaire ou encore médical. Dans ce dernier, les examens diagnostiques comme la radiographie et la scanographie mais également les traitements curatifs, palliatifs ou symptomatiques des tumeurs cancéreuses par radiothérapie exploitent les propriétés des rayonnements ionisants. On distingue deux types de rayonnements ionisants : - les rayonnements électriquement chargés, directement ionisants (rayonnements α et β), émettant leur énergie sous forme de particules chargées, des noyaux d'hélium pour le rayonnement α et des électrons pour le rayonnement β. – les rayonnements non chargés, indirectement ionisants, dont les neutrons et les rayonnements électromagnétiques (rayonnements γ et X). Exploités dans le domaine médical et particulièrement la radiothérapie, les rayonnements électromagnétiques γ et X se caractérisent par l'émission de photons de faible énergie. Ces deux rayonnements ont comme caractéristique d'avoir une grande capacité de pénétration dans la matière. Les rayonnements X sont générés par la projection d'électrons sur une plaque métallique (tungstène, or) et les rayonnements γ sont émis à partir d'une source radioactive (ex : Cobalt ^{60}).

La dose reçue par la matière vivante se mesure en Gray (Gy), c'est-à-dire un transfert d'énergie de 1 joule à un kilogramme de matière. Très schématiquement, les effets cellulaires et moléculaires des particules ionisantes sont initiés par la formation d'espèces radicalaires et la création de lésions sur différentes molécules cellulaires. Ainsi, l'irradiation des tissus biologiques a pour conséquence la formation d'espèces radicalaires hautement réactives produites principalement par la radiolyse de l'eau. D'autre part, les particules ionisantes sont capables de créer directement des lésions aux lipides, glucides, protéines et surtout aux acides nucléiques. C'est principalement pour ces propriétés que les rayonnements ionisants sont utilisés dans les stratégies curatives des tumeurs par radiothérapie. Par des effets directs ou indirects, les rayonnements ionisants peuvent induire principalement différentes altérations de l'ADN (modifications de base, cassures simple-brin ou double brin). Si elles sont importantes et/ou si les mécanismes de réparation de ces lésions sont dépassés ou altérés, alors ces dommages à l'ADN sont létaux et provoquent la mort cellulaire.

Cependant, en fonction de la dose, de la radiosensibilité de chaque type cellulaire ou d'autres facteurs, les effets des rayonnements ionisants n'engendrent pas forcément des dommages irréversibles à l'ADN et dans ce cas une activation, une modification fonctionnelle et/ou phénotypique des cellules irradiées sont observées.

2. La Radiothérapie.

La radiothérapie est une méthode de traitement utilisant les rayonnements ionisants dont l'objectif est de délivrer à la tumeur une dose maximale tout en préservant l'intégrité des tissus sains adjacents. Plus de la moitié des patients atteints de cancer sont traités par radiothérapie seule ou en combinaison avec d'autres traitements comme la chirurgie et /ou la chimiothérapie. Ainsi, la radiothérapie est utilisée pour traiter différents types de cancers comme les leucémies, la maladie de Hodgkin, les carcinomes épidermoïdes, les adénocarcinomes, les carcinomes urothéliaux, les gliomes et les mélanomes. On distingue principalement trois techniques de radiothérapie. La plus utilisée est la radiothérapie externe ou transcutanée qui est une technique utilisant une source de rayonnement située en dehors de l'organisme. Très utilisée pour traiter les cancers gynécologiques, la curiethérapie est une technique utilisant des sources radioactives (iridium ^{192}, césium ^{137}) placées dans l'organisme. Enfin, la radiothérapie métabolique consiste à injecter des radioéléments qui vont se fixer sur les cellules tumorales (Iode ^{131} pour le traitement de certains cancers de la thyroïde).

Les protocoles de radiothérapie sont définis principalement en fonction du type de la tumeur, de sa localisation, de sa taille, de son extension et de son stade. Il est établi depuis très longtemps une relation de proportionnalité entre la radiosensibilité et la vitesse de prolifération cellulaire. Les cellules tumorales ont une capacité de prolifération plus importante que les cellules saines et c'est principalement sur cette différence que sont basés les protocoles de radiothérapie. Ainsi, la dose totale ne suffit pas pour définir un traitement par irradiation, mais il faut également prendre en compte la dose par fraction, le nombre de fractions et le nombre de fractions par jour ou par semaine. Une radiothérapie classique délivre la dose totale par fractions de 2 Gy, une fraction par jour, 5 jours par semaine et ce fractionnement de dose permet d'obtenir un meilleur ratio efficacité anti-tumorale /tolérance des tissus sains.

Les stratégies thérapeutiques par radiothérapie développées jusqu'à aujourd'hui sont associées à l'apparition d'effets secondaires précoces et/ou tardifs sur les tissus sains. L'incidence et la sévérité des effets secondaires observés dépendent de l'organe irradié et de la dose totale reçue mais également de nombreux autres facteurs comme le type de fractionnement, le volume irradié ou encore la radiosensibilité individuelle du patient. En clinique, la notion d'équilibre bénéfice/risque

repose sur le compromis entre l'efficacité maximale du contrôle tumoral et la minimisation des dommages aux tissus sains adjacents ***(Figure 1)***.

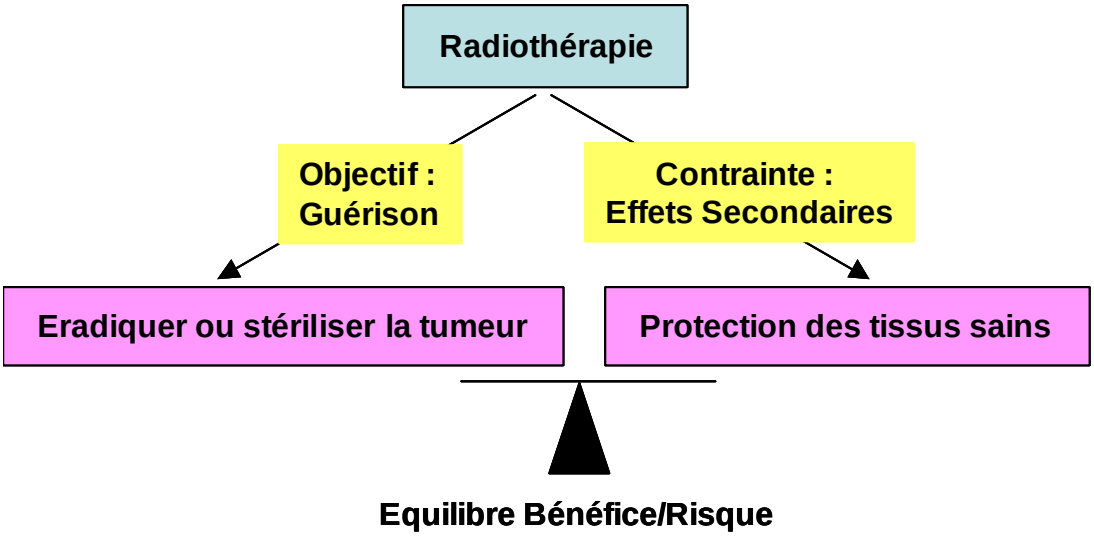

Figure 1 : Notion de balance Bénéfice /Risque en radiothérapie

La toxicité radio-induite aux tissus sains est non seulement un facteur limitant dans l'escalade de dose pouvant être délivrée à la tumeur mais sa sévérité peut affecter la qualité de vie des survivants du cancer qui sont de plus en plus nombreux. La réduction du volume de tissus normaux présents dans le champ d'irradiation par de nouvelles techniques de radiothérapie (3D-CRT, radiothérapie conformationnelle, IMRT : Radiothérapie à intensité modulée) est une stratégie visant à limiter les effets secondaires. Cependant la toxicité aux tissus sains reste, aujourd'hui, un problème incontournable lié à ce type de traitement (Stone et al., 2003). Ainsi, environ 1.5 million de personnes sont traitées par radiothérapie chaque année en Europe et on estime que la plupart des patients développent des effets aigus et que 5 à 10 % developpent des complications tardives.

Dans ce contexte, la compréhension des mécanismes moléculaires et cellulaires associés aux dommages radio-induits est indispensable dans l'objectif de mettre en place des stratégies thérapeutiques visant à protéger les tissus sains sans compromettre, et même améliorer, le contrôle tumoral (Bentzen, 2006).

3. Principes généraux des dommages radio-induits aux tissus sains.

Alors que les effets secondaires des chimiothérapies sont systémiques, ceux de la radiothérapie sont plutôt locaux ou loco-régionaux. Historiquement, les lésions radio-induites aux tissus sains ont été classées en dommages aigus, sub-aigus et tardifs selon la cinétique d'apparition des symptômes cliniques. Le processus pathologique des dommages radio-induits commence

pendant le protocole d'irradiation mais les lésions morphologiques et les symptômes cliniques surviennent des jours à des mois, voire des années, après l'irradiation.

Les concepts décrits dans la littérature concernant l'initiation, le maintien et la progression vers la fibrose des tissus après irradiation ont évolué depuis quelques années (Bentzen, 2006; Denham and Hauer-Jensen, 2002). L'hypothèse de la cellule cible suggère que la sévérité des effets tissulaires est principalement due à la déplétion cellulaire d'un compartiment cible par mort radio-induite ayant pour conséquence une déficience fonctionnelle de l'organe. Bien que cette hypothèse reste envisageable pour les effets précoces, elle semble difficile à appliquer aux effets tardifs. D'autre part, le concept d'effet conséquentiel qui se définit par un lien de causalité entre la sévérité de l'atteinte aiguë et le développement des séquelles tardives n'a pour l'instant toujours pas été démontré formellement (Dorr and Hendry, 2001). Ainsi, il est maintenant admis que la réponse tissulaire aux rayonnements est la résultante de l'activation et de l'implication intégrée de l'ensemble des compartiments qui composent le tissu (Bentzen, 2006). Bien entendu, la part de la mort cellulaire précoce et la sévérité des lésions aiguës sur un compartiment cible peut affecter la réponse globale progressive aux rayonnements mais la notion d'un continuum d'effets orchestré par l'ensemble des compartiments et des cascades chroniques de cytokines permet d'ouvrir les champs d'investigation sur diverses approches thérapeutiques. Ainsi, le processus cicatriciel radio-induit est un continuum d'effets où les séquences d'évènements comme la mort cellulaire précoce, l'activation du système de coagulation, la réponse inflammatoire, l'activation des compartiments vasculaires et mésenchymateux et les processus de remodelage matriciel sont autant de phénomènes imbriqués les uns aux autres et participant à l'initiation, la progression et la chronicité des lésions radio-induites ***(Figure 2)***.

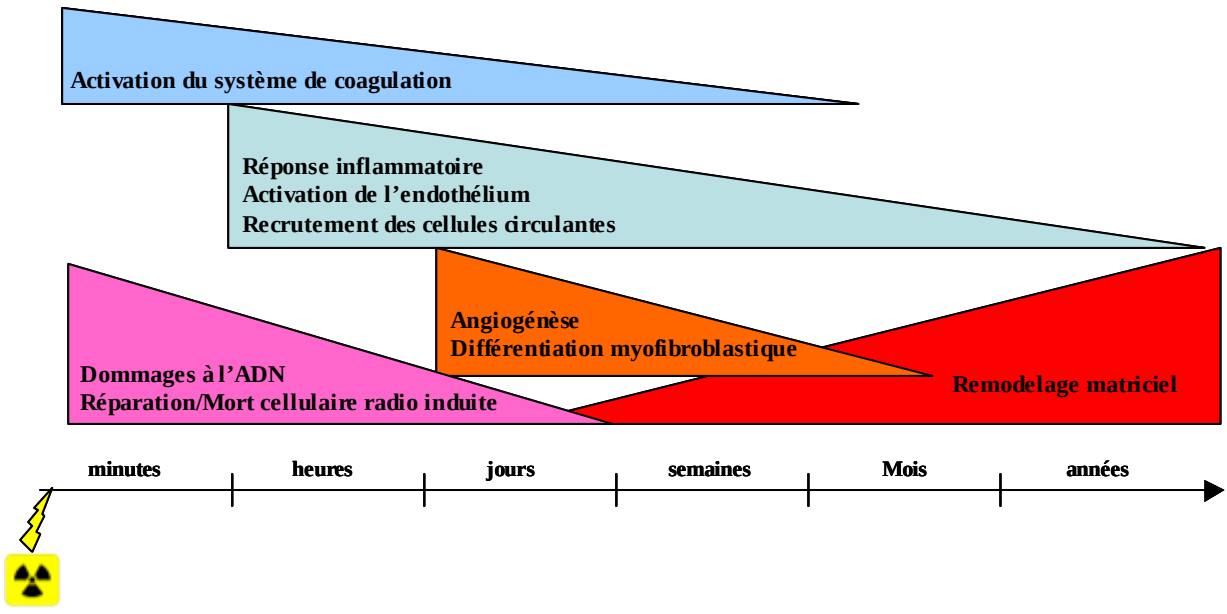

Figure 2 : Représentation de la cinétique des évènements impliqués dans la réaction tissulaire suite à un stress par rayonnements ionisants

Ce processus cicatriciel complexe fait intervenir de nombreuses cytokines, chémokines et facteurs de croissance conduisant finalement à des lésions chroniques comme les fibroses radiques. L'avancée des connaissances sur les mécanismes moléculaires impliqués dans les lésions radio-induites doit permettre de décrypter au mieux ce continuum d'effets et d'aborder de manière non restrictive le rôle de chaque compartiment dans ce processus.

D'autre part, l'obligation légale récente en France de déclarer tout incident ou accident de radiothérapie et en particulier les surdosages montre que de tels accidents, bien que rares, sont possibles. Par exemple, l'accident récent de l'Hôpital d'Epinal où au moins 23 patients traités par radiothérapie pour un cancer de la prostate ont subi un surdosage et ont présenté des dommages sévères au niveau de la portion de rectum présente dans le champ d'irradiation. A ce jour, il n'existe pas de traitement ciblé consensuel permettant de traiter de telles lésions sévères et une des missions de l'Institut de Radioprotection et de Sûreté Nucléaire (IRSN) est de développer des recherches afin de proposer des stratégies thérapeutiques visant à traiter les dommages radio-induits aux tissus sains après radiothérapie et en cas d'accidents d'irradiation, qu'ils soient de radiothérapie ou autre.

Les travaux du laboratoire de radiopathologie tentent d'aborder les différentes hypothèses et les rôles respectifs des compartiments muqueux, mésenchymateux ou encore de la composante inflammatoire (Bourgier et al., 2005; Francois et al., 2003; Linard et al., 2004). Les travaux présentés dans ce travail de thèse abordent le rôle du compartiment vasculaire et plus précisément l'influence des cellules endothéliales dans l'initiation des lésions vasculaires et tissulaires après irradiation.

4. La fibrose radio-induite.

4.1 Généralités.

Les fibroses radio-induites sont des complications des irradiations accidentelles ou thérapeutiques qui se caractérisent par une accumulation de tissu conjonctif à l'origine de dysfonctionnements de l'organe ou du tissu lésé. Les fibroses cutanées, pulmonaires, cardiaques, rénales, vasculaires ou intestinales résultant du traitement par radiothérapie constituent un problème clinique important car elles peuvent modifier, en fonction de leur sévérité et de l'organe touché, l'évolution clinique du patient avec au mieux une altération de sa qualité de vie et, au pire, l'engagement du pronostic vital. Par exemple, les fibroses intestinales radio-induites ou entérites radiques sont des complications tardives de la radiothérapie des cancers de la sphère abdomino-pelvienne. Elles peuvent entraîner la perte de fonctionnalité de la muqueuse intestinale à l'origine d'un syndrome de malabsorption et se caractérisent par une fibrose responsable d'une sténose

intestinale pouvant conduire à un syndrome d'occlusion et nécessiter une résection chirurgicale (Andreyev et al., 2005; Andreyev, 2005; Francois et al., 2005). De la même manière les pneumonies et les fibroses pulmonaires radio-induites sont fréquemment observées suite aux traitements par radiothérapie des cancers de la sphère thoracique (cancers du sein, du poumon et maladie de Hodgkin) (Tsoutsou and Koukourakis, 2006). Ainsi, on estime que 50 à 90 % des patients ayant subi une irradiation au niveau des poumons présentent des anomalies des tests fonctionnels pulmonaires (McDonald et al., 1995). Les symptômes cliniques sont plus ou moins sévères comme des dyspnées modérées ou sévères, des orthopnées, des cyanoses et des insuffisances respiratoires. Dans certains cas de fibroses sévères, ces affections de l'appareil respiratoire ou de la circulation pulmonaire peuvent conduire à des pathologies comme des hypertrophies-dilatations du ventricule droit ou « cœurs pulmonaires chroniques » avec, ou sans, insuffisance cardiaque (Seppenwoolde et al., 2004).

Les mécanismes moléculaires et cellulaires de la fibrose radio-induite sont comparables à un processus cicatriciel pathologique. Ils sont initiés dans les secondes qui suivent l'irradiation par la production d'espèces réactives de l'oxygène. Les espèces radicalaires générées vont alors déclencher de nombreux processus à l'origine des effets précoces radio-induits. Les phases précoces de la fibrogénèse radio-induite se caractérisent par une production rapide de cytokines pro-inflammatoires et de facteurs de croissance, une activation de l'endothélium vasculaire et un recrutement de cellules inflammatoires sur le site irradié. Ce processus de cicatrisation est régulé par une balance complexe entre les protéines pro et anti-fibrosantes ainsi que les cytokines pro et anti-inflammatoires. D'autre part, l'activation des cellules du mésenchyme comme les fibroblastes ou les cellules musculaires lisses en myofibroblastes de fibrose prend une part déterminante dans l'accumulation excessive de matrice extracellulaire (Delanian et al., 1998; Vozenin-Brotons et al., 2003). Les signaux moléculaires de réparation suite à un stress par rayonnements ionisants sont émis de manière chronique et déséquilibrée favorisant la progression de la fibrose. Ainsi, au contraire d'une cicatrisation normale, les mécanismes associés au processus de réparation tissulaire radio-induite se perpétuent des mois à des années après l'irradiation conduisant aux fibroses radiques.

4.2 La fibrose radio-induite est assimilable à une réparation tissulaire pathologique.

Mécanismes généraux de la cicatrisation.

Les phases précoces ou d'initiation des lésions radio-induites aux tissus sains sont comparables à un processus cicatriciel. C'est pourquoi une description succincte des mécanismes de

réparation tissulaire permettra de mieux comprendre ce concept d'assimilation des lésions radio-induites à une cicatrisation pathologique.

Une lésion tissulaire induit un processus de cicatrisation qui suit une cinétique d'évènements moléculaires et cellulaires bien caractérisés permettant la régénération tissulaire de manière plus ou moins parfaite. Ainsi, on considère une régénération tissulaire parfaite lors d'une restitution parfaitement normale de l'architecture et de la fonction des tissus et une régénération tissulaire imparfaite lorsque la lésion initiale est remplacée par une cicatrice fibreuse. On distingue plusieurs étapes dans le processus de réparation tissulaire. Tout d'abord, une phase de réaction inflammatoire stricte qui se caractérise par une congestion vasculaire, un œdème et la migration des leucocytes au site lésé. Ceci va permettre de constituer un granulome inflammatoire suivi d'une phase de détersion principalement assurée par l'action des macrophages par phagocytose qui vise à éliminer les tissus nécrotiques, les germes et l'exsudat inflammatoire. Enfin, cette phase de détersion est suivie par la phase de cicatrisation proprement dite caractérisée par la formation d'un tissu de granulation.

La formation du tissu de granulation.

La formation d'un tissu de granulation permet de former un tissu conjonctif transitoire visant à combler la perte de substance et qui se caractérise par une angiogénèse active et la différenciation myofibroblastique. Les mécanismes moléculaires impliqués sont complexes et font intervenir à la fois l'activation des cellules endothéliales, la production de cytokines pro-inflammatoires et des facteurs de croissance stimulant la différenciation cellulaire comme le Platelet-Derived Growth Factor (PDGF), le Fibroblast Growth Factor (FGF) et le Transforming Growth Factor-β1 (TGF-β1).

La formation d'un réseau de néo-vaisseaux est cruciale dans la constitution du tissu de granulation. Les néo-vaisseaux sont formés à partir de bourgeons endothéliaux situés en bordure du granulome inflammatoire en zone non lésée. Ce réseau vasculaire organisé progressivement permet l'approvisionnement du tissu de granulation en nutriments, hormones et gaz respiratoires. Ce réseau de capillaires est très dense dans les phases précoces et diminue progressivement pour se stabiliser en fonction de la maturation du tissu de granulation. Parallèlement au processus angiogénique, la formation du tissu de granulation conduisant à la cicatrisation nécessite une différenciation des fibrocytes/fibroblastes en myofibroblastes ou différenciation myofibroblastique (Tomasek et al., 2002). Ces cellules acquièrent un phénotype contractile (expression de α smooth muscle actine), proliférant et sécrétoire visant à compenser la perte de substance. Le TGF-β1 joue un rôle fondamental dans la différenciation myofibroblatique car il régule au niveau transcriptionnel

l'expression de l'α–sm actine (Desmouliere et al., 1993). Par exemple, l'injection sous cutanée de TGF-β1 induit la formation d'un tissu de granulation riche en myofibroblastes chez la souris (Roberts et al., 1986). D'autre part, comme nous le verrons plus tard, le TGF-β1 est capable de stimuler la synthèse de matrice extracellulaire et notamment celle de collagènes fibrillaires et d'influencer de nombreux acteurs moléculaires impliqués dans le remodelage matriciel (Verrecchia et al., 2001). L'évolution de la composition matricielle est déterminante également dans le processus de maturation du tissu de granulation. Très schématiquement, lors de la phase de constitution du tissu de granulation la matrice extracellulaire est riche en fibronectine, glycosaminoglycanes et collagène de type III. La composition matricielle évolue progressivement lors de la cicatrisation avec une diminution de la production de fibronectine et une dégradation progressive du collagène de type III au profit du collagène de type I. Les modifications qualitatives et quantitatives de la matrice extracellulaire sont principalement dues aux propriétés sécrétoires des myofibroblastes mais la propriété contractile de ces cellules est également déterminante. En effet, le phénotype contractile de ces cellules leur confère une forte capacité de migration et permet également au tissu néoformé d'acquérir des propriétés de contraction participant activement au processus de cicatrisation. L'étape ultime du processus cicatriciel est enclenchée lorsque la perte de substance est totalement comblée et se caractérise par une stabilisation du réseau vasculaire, du remodelage matriciel ainsi que l'inhibition de la prolifération et/ou la mort par apoptose des myofibroblastes.

Des dysfonctionnements des mécanismes de réparation tissulaire peuvent aboutir à des processus cicatriciels pathologiques notamment à des fibroses qui, en fonction de l'étendue, de la sévérité et de l'organe touché, peuvent être la cause d'importantes atteintes morphologiques et fonctionnelles. Ces lésions cicatricielles fibrotiques se caractérisent par une boucle d'activation chronique conduisant à l'accumulation de matrice extracellulaire. Cette production excessive de tissu conjonctif est due principalement à la persistance de signaux moléculaires impliqués dans la production de matrice extracellulaire et des dysfonctionnements des mécanismes d'inhibition de la différenciation, de la prolifération et de la mort des myofibroblastes.

4.3 Histo-pathologie des lésions radio-induites.

Les fibroses radiques sont observées dans tous les organes irradiés comme la peau (Delanian et al., 2003), les poumons (Tsoutsou and Koukourakis, 2006), le cœur (Adams et al., 2003), les reins (Cohen and Robbins, 2003), le rectum (Babb, 1996) ou encore l'intestin (Hauer-Jensen, 1990; Hauer-Jensen et al., 2003). Les phases précoces des lésions radio-induites se caractérisent par une

réaction inflammatoire importante, des atteintes de l'endothélium vasculaire et des épithéliums. Ainsi, la radio-sensibilité importante de l'épithélium lui confère une forte réponse suite au stress ionisant. Par exemple les lésions précoces intestinales sont caractérisées par des dommages muqueux extrêmement sévères avec parfois des ulcères très profonds (Bentzen et al., 2003; Francois et al., 2005; Hauer-Jensen et al., 2003) entraînant une atteinte fonctionnelle sévère (Francois et al., 2003) . Les phases aiguës ou sub-aiguës de fibrose active se caractérisent par une accumulation matricielle principalement produite par des fibroblastes différenciés en myofibroblastes sous l'effet de facteurs de croissance pro-fibrosants comme le TGF-β1. Durant les phases précoces et très actives, le tissu irradié est riche en cellules inflammatoires avec la présence d'œdème dû à l'extravasation des protéines sériques provenant de la perte de perméabilité vasculaire. Au niveau histologique, les fibroses radio-induites tardives se caractérisent par une accumulation excessive de matrice extracellulaire et particulièrement de collagènes fibrillaires. Cette accumulation pathologique de matrice extracellulaire provient des dérégulations des processus de synthèse et de dégradation matricielle (Strup-Perrot et al., 2004) où la différenciation myofibroblastique jouent un rôle important (Vozenin-Brotons et al., 2003). Les fibroses établies et très tardives sont relativement pauci-cellulaires, peu vascularisées et très riches en matrice extracellulaire.

5. TGF-β- Médiateur moléculaire clef de la fibrose.

Facteur pro-fibrosant le plus étudié, le TGF-β est un peptide de 25 kDa appartenant à la super famille TGF-β incluant les activines et les Bone Morphogenic Proteins (BMP). Il est exprimé par la plupart des types cellulaires et intervient dans la régulation de multiples fonctions cellulaires comme l'apoptose et la prolifération, la différenciation ou encore la production de matrice extracellulaire. Le TGF-β existe sous trois isoformes (TGF-β 1, 2,3) mais le TGF-β1 est considéré comme l'isoforme clef impliquée dans le processus de fibrogénèse. Ce processus qui s'initie immédiatement après le stress initial conduit à la fois à une sur-expression des constituants matriciels mais également à des modifications de la nature de ces constituants. Cette matrice extracellulaire est principalement produite par les cellules du mésenchyme comme les fibroblastes, les myofibroblastes et cellules musculaires lisses. Bien entendu de multiples cytokines sont capables d'influencer la production matricielle mais les effets sur la matrice extracellulaire sont principalement dus à l'action du TGF-β1. Comme nous venons de le voir le TGF-β1 est capable d'induire la différenciation myofibroblastique mais également de stimuler l'accumulation de matrice extracellulaire à la fois par une induction des collagènes au niveau transcriptionnel

(Verrecchia and Mauviel, 2002) et par une inhibition des mécanismes de dégradation matricielle (Eickelberg et al., 1999). Ainsi la synthèse des collagènes fibrillaires, des protéoglycanes, de la thrombospondine ou encore de la fibronectine est stimulée par le TGF-β1. D'autre part le TGF-β1 est capable d'inhiber l'activation ou la synthèse des protéases qui dégradent la matrice comme les MMPs ou de stimuler la synthèse des inhibiteurs de ces protéases comme les TIMPs ou PAI-1. L'ensemble des actions du TGF-β vis à vis des acteurs moléculaires impliqués dans le processus du remodelage matriciel fait pencher l'équilibre synthèse /dégradation vers une accumulation de matrice extracellulaire ***(Figure 3)***.

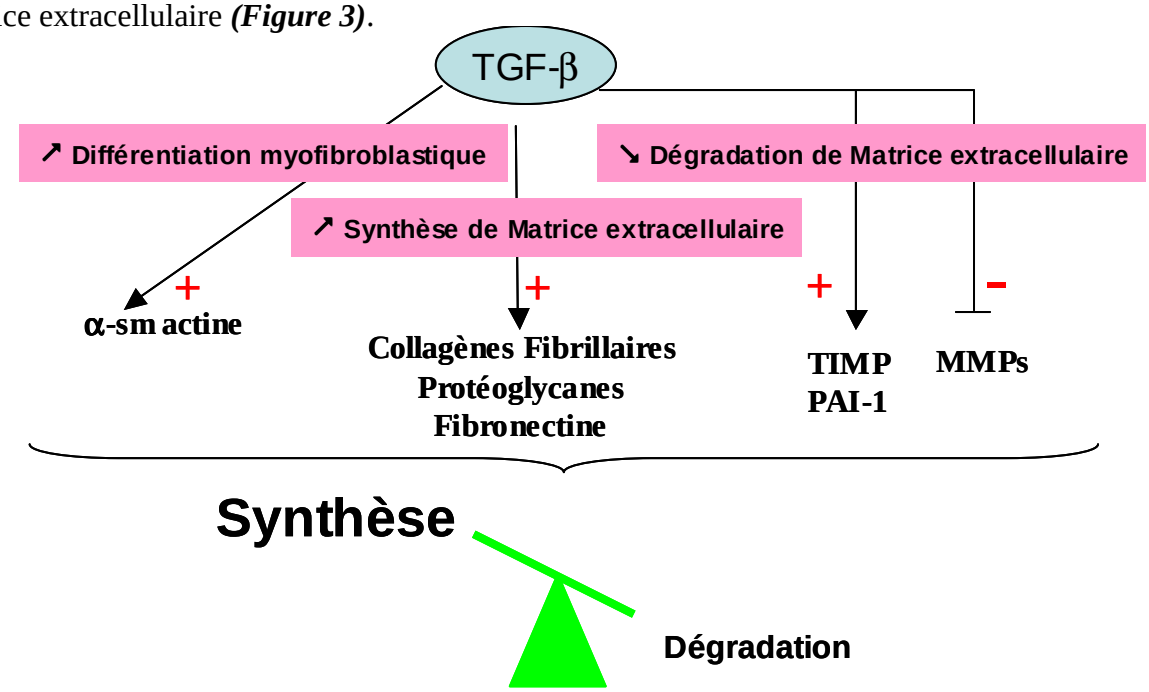

Figure 3 : Rôles du TGF-β dans le remodelage matriciel impliqué dans le processus de fibrogénèse

5.1 La voie de Signalisation TGF-β/SMAD.

Bien que le TGF-β1 ait été démontré comme étant capable de moduler plusieurs voies de signalisation comme les voies JUNK, PI3K/AKT, JAK/STAT, Rho/ROCK ou ERK/p38MAPK (Derynck and Zhang, 2003; Moustakas and Heldin, 2005), la voie de transduction par les protéines SMADs est la plus caractérisée (Massague, 2000). C'est principalement la voie TGF-β/SMAD que nous allons développer car elle est la principale impliquée dans les phénomènes de fibrogénèse.

Le TGF-β1 est sécrété sous forme inactive, latente, par de nombreux types cellulaires. Le TGF-β1 inactif est formé de deux peptides de 12,5 kDa liés par des ponts disulfure et peut former deux complexes latents : un complexe où le TGF-β1 est associé à une protéine nommée LAP (Latency Assiociated Peptide), un complexe où la forme TGF-β/LAP est elle-même associée à une autre protéine appartenant à la famille des LTBP (Latent TGF-β Binding Protein) (Fortunel et al.,

2000). Plusieurs processus sont capables d'activer le TGF-β après dissociation du LAP et/ou du LTBP comme l'acidification, les espèces réactives de l'oxygène, la plasmine ou encore la thrombospondine. Après activation de sa forme latente, le TGF-β1 se lie à son récepteur transmembranaire de type II (TGFβ–RII) possédant une activité kinase. La phosphorylation de TGFβ-RII par la liaison de son ligand va provoquer son hétérodimérisation avec le récepteur de type I (TGFβ-RI ou ALK5) et entraîner la phosphorylation de ce dernier. Ceci initie une cascade de signalisation impliquant des facteurs de transcription de la famille SMAD (Massague et al., 2005). Cette voie de signalisation intracellulaire est initiée par l'activation de R-SMADs (receptor-regulated Smads ; SMAD1, 2, 3, 5 et 8) qui sont directement phosphorylées par le recepteur au TGF-β de type I activé. Une co-SMAD (SMAD4) va alors former des complexes avec les R-SMADs phosphorylées et ces complexes hétérodimériques vont migrer dans le noyau cellulaire et se fixer à des séquences (CAGAC) présentes dans les promoteurs de gènes cibles (αsm-actine, COL1A1, COL1A2, COL3A1, COL5A2, COL6A1, TIMP-1, PAI-1, etc …) participant ainsi à leur régulation transcriptionnelle (Verrecchia et al., 2001), (Massague, 2000) ***(Figure 4)***.

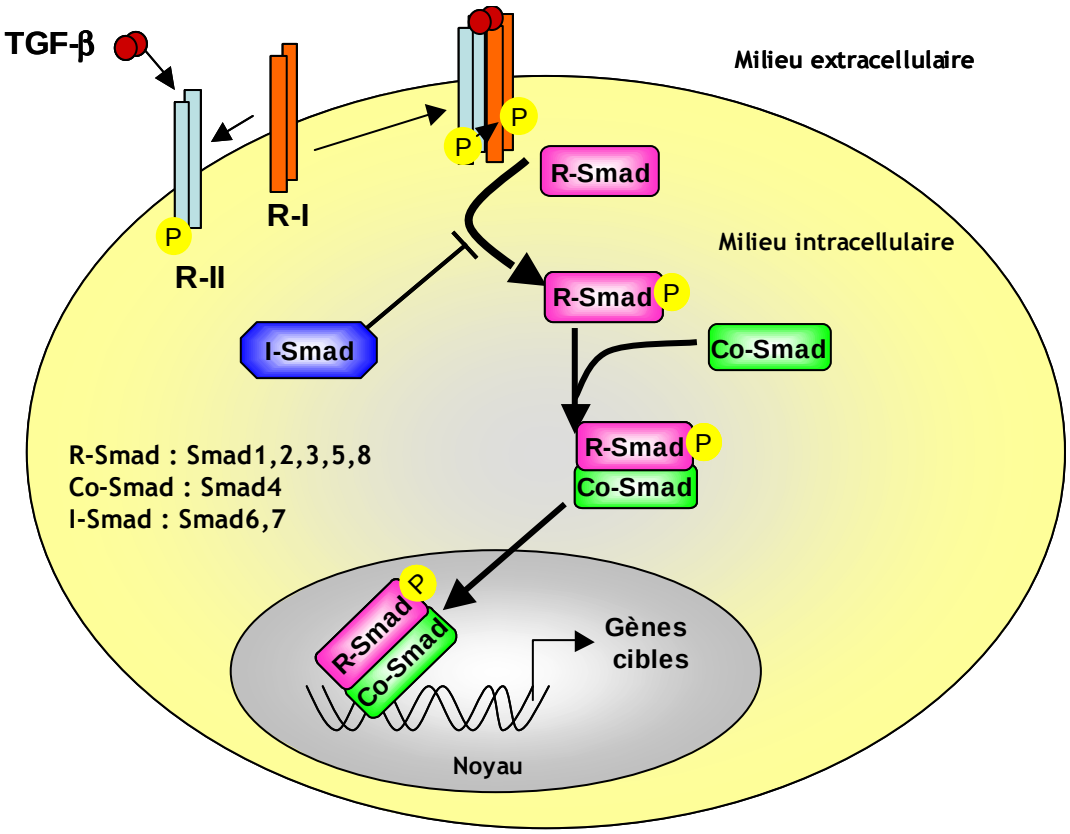

Figure 4 : Représentation simplifiée de la voie de signalisation TGF-β/SMAD
Après activation de sa forme latente, le TGF-β se lie au récepteur de type II (RII) possédant une activité kinase. La phosphorylation de RII par la liaison de son ligand va provoquer son hétérodimérisation avec le récepteur de type I (RI) et entraîner la phosphorylation de ce dernier. Les R-SMADs phosphorylées par le R-I activé vont s' hétérodimériser avec une co-SMAD et ces complexes hétérodimériques vont migrer dans le noyau cellulaire et activer la transcription de gènes cibles. Des Smads inhibitrices (I-Smads) sont capables d'inhiber la voie de signalisation par plusieurs mécanismes dont l'inhibition de la phosphorylation des R-Smads.

D'autre part, des SMADs inibitrices (SMAD6 et 7) sont capables d'inhiber la voie de signalisation du TGF-β. Enfin la réponse transcriptionnelle finale sur un gène cible fait intervenir de nombreux co-activateurs comme p300, CBP, PCAF, GCN5 (Feng et al., 1998; Itoh et al., 2000; Kahata et al., 2004) et des co-répresseurs comme Ski, SnoN ou TGIF (Luo, 2004; Pessah et al., 2001; Seo et al., 2006) ***(Figure 5)***. Cette voie de transduction du signal par les protéines de la famille SMAD a longtemps été considérée comme une voie spécifique de régulation de l'expression des protéines de la matrice extracellulaire par le TGF-β. Cependant des résultats récents de la littérature démontrent que cette voie peut être activée par d'autres facteurs solubles comme l'angiotensine II ou l'endothéline I dans les cellules musculaires lisses vasculaires (Rodriguez-Vita et al., 2005; Rodriguez-Vita et al., 2005) et conduire à la surexpression du CTGF. Enfin, un autre facteur de croissance, le PDGF a été montré comme étant capable d'activer la voie de transduction SMAD dans des cellules étoilées hépatiques (Yoshida et al., 2005).

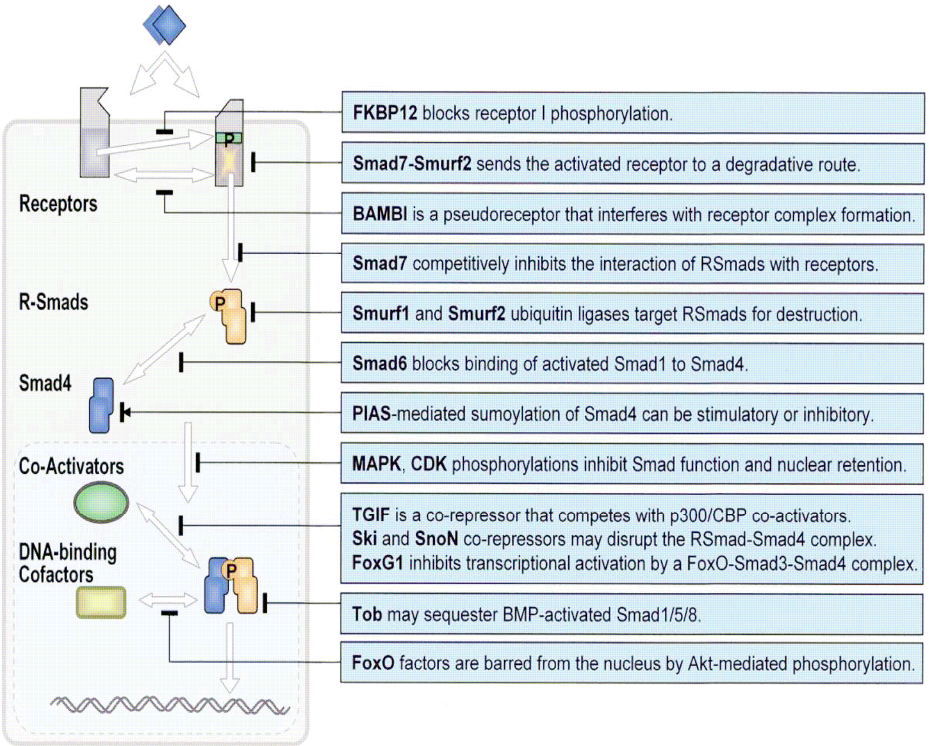

Figure 5 : Principaux mécanismes et acteurs moléculaires impliqués dans la régulation négative de la voie de signalisation Smad (D'après Massagué J *et al*, Genes & Dev, 2005)

5.2 TGF-β Médiateur moléculaire clef de la fibrose radio-induite.

Stimulée dans de nombreuses pathologies fibrotiques et notamment les fibroses radio-induites, cette cytokine est donc depuis longtemps considérée comme une cible thérapeutique anti-fibrosante (Martin et al., 2000). Une surexpresion du TGF-β a été décrite dans de nombreux modèles de fibrose radio-induite chez l'animal ainsi que chez l'homme notamment après des irradiations thérapeutiques pour des traitements de cancer du sein, du poumon ou du rectum. Par

exemple, le taux plasmatique de TGF-β1 a été montré comme une bonne mesure prédictive des lésions tardives chez des patients irradiés au niveau du thorax. Ainsi, les patients ayant un taux élevé de TGF-β1 plasmatique avant la radiothérapie présentent un risque accru de développer des séquelles tardives (Anscher et al., 1997). D'autre part, les entérites radiques intestinales tardives humaines (plusieurs années après l'irradiation) ne sont pas associées à une modification du taux tissulaire d'ARNm du TGF-β1. Cependant, l'analyse immuno-histochimique montre une expression accrue de TGF-β1 dans les vaisseaux ainsi que dans les myofibroblastes et la matrice extracellulaire (Vozenin-Brotons et al., 2003).

Parce qu'il est sécrété sous forme d'un complexe inactif latent, un pool potentiel important de TGF-β1 actif peut être mobilisé très rapidement indépendamment d'une induction transcriptionnelle. Les radiations ionisantes ont été décrites comme un des facteurs exogènes capables d'activer très rapidement le TGF-β et ceci à des faibles doses d'irradiation (0.1 Gy) (Ehrhart et al., 1997; Ewan et al., 2002). Bien que ce processus d'activation globale du TGF-β ait été décrit *in vivo* dans un modèle d'irradiation de glandes mammaires murines depuis plus de 10 ans par le groupe de Barcellos-Hoff (Barcellos-Hoff et al., 1994), cette même équipe en a décrypté récemment le mécanisme. Ainsi, ils ont montré que les espèces réactives de l'oxygène générées après irradiation pouvaient activer exclusivement l'isoforme TGF-β1 et ceci via l'oxydation d'une méthionine présente en position 253 du LAP (Jobling et al., 2006).

De nombreuses études expérimentales d'atteintes radio-induites au niveau pulmonaire, intestinal ou encore cutané sont associées à des surexpressions du TGF-β1. L'irradiation cutanée chez le porc est associée à une augmentation de l'expression des ARNm du TGF-β1 dès 6h après irradiation. *In vitro*, l'irradiation d'une lignée de kératinocytes a montré que la trans-activation radio-induite du promoteur du TGF-β1 était en partie AP-1-dépendante (Martin et al., 1997). D'autre part, 6 semaines après une irradiation cutanée de 30 Gy chez la souris, une augmentation de l'expression du TGF-β1 est observée par marquage immuno-histochimique (Flanders et al., 2002). Au niveau pulmonaire, une forte augmentation des ARNm a été décrite 1 à 14 jours post- irradiation chez la souris (Finkelstein et al., 1994; Rube et al., 2000) et une surexpression de la protéine TGF-β1 est observée de 6 semaines et jusqu'à 6 mois après irradiation chez le rat (Anscher et al., 2006; Vujaskovic et al., 2001). Dans un modèle d'irradiation abdominale à 10 Gy chez le rat, une augmentation des ARNm du TGF-β1 est décrite dans le muscle intestinal 24h après l'irradiation et dans l'intestin total à 3 et 7 jours (Linard et al., 2003; Strup-Perrot et al., 2006; Strup-Perrot et al., 2005). Dans un modèle d'irradiation localisée d'une anse d'intestin grêle à 21 Gy, une augmentation du taux d'ARNm du TGF-β1 est observée dès 24h, jusqu'à 2 semaines et même

jusqu'à 26 semaines post-irradiation (Wang et al., 2001). Dans ce même modèle, des expériences d'hybridation *in situ* et des marquages immunohistochimiques ont montré une augmentation de l'expression des ARNm et de l'immunoréactivité du TGF-β1 dans l'endothélium (Wang et al., 1998). L'ensemble de ces résultats montrent que, quel que soit l'organe irradié ou le modèle utilisé, la plupart des dommages radio-induits aux tissus sains sont associés à une surexpression du TGF-β1.

C'est la raison pour laquelle ce facteur de croissance constitue une cible thérapeutique de choix même si son rôle et les conséquences moléculaires de son action ne sont pas encore complètement élucidés, et plus particulièrement au niveau des différents types cellulaires. L'inhibition du TGF-β1 ou de la voie de transduction du signal TGF-β1 semble être une stratégie efficace mais son action pléiotropique et multi-fonctionnelle pourrait être un facteur limitant. Ainsi, la délétion du gène TGF-β1 chez la souris est létale (Kulkarni et al., 1993) et un blocage complet de son expression ou de son activité est accompagné d'effets secondaires délétères. Cependant des stratégies thérapeutiques chez l'animal ciblant le TGF-β ou la voie du TGF-β dans les lésions radio-induites ont été réalisées. Par exemple l'injection d'une protéine recombinante du TGF-βRII soluble a démontré une efficacité thérapeutique dans un modèle d'atteinte intestinale radio-induite chez la souris avec une diminution de la sévérité des lésions fibrotiques et une préservation de la surface muqueuse (Zheng et al., 2000). Récemment, l'injection d'un anticorps neutralisant anti-TGF-β permet de limiter efficacement la sévérité des dommages aigus et tardifs chez des rats irradiés à forte dose au niveau pulmonaire (Anscher et al., 2006). De manière intéressante, six semaines après une irradiation cutanée à forte dose de 30 à 50 Gy, l'équipe de Roberts a montré que les souris SMAD3 -/- sont protégées des dommages radio-induits avec réduction des lésions fibrotiques. Les lésions histologiques observées chez les souris SMAD3 -/- sont caractérisées par une augmentation de la re-épithélialisation et une baisse du nombre de mastocytes, de neutrophiles, de macrophages ou encore de myofibroblastes (Flanders et al., 2003; Flanders et al., 2002).

D'autres études montrant une efficacité thérapeutique chez l'animal et chez l'homme ont été décrites comme ayant une action anti-TGF-β "indirecte". Ainsi, l'activité anti-fibrosante *in vivo* de la superoxyde dismutase (SOD) a été montrée dans des modèles expérimentaux (Lefaix et al., 1996; Vujaskovic et al., 2002) et chez l'homme (Delanian et al., 1994). Des études mécanistiques réalisées par la suite ont permis de démontrer que la SOD était capable de reverser *in vitro* le phénotype de myofibroblastes de fibrose cutanée radio-induite par une action anti-TGF-β1 provoquant l'inhibition de l'expression de l'α-sma et de la chaîne α1 du collagène de type I. (Vozenin-Brotons et al., 2001). D'autre part, l'efficacité thérapeutique de l'association

pentoxifylline/vitamine E utilisée parfois en clinique (Delanian et al., 2003; Delanian et al., 2005) passe par une action anti-TGF-β1 (Lefaix et al., 1999). Le groupe de Vujaskovic a montré que l'efficacité thérapeutique du facteur de croissance KGF dans un modèle de fibrose radio-induite pulmonaire chez le rat était associée à une inhibition de l'expression de molécules clefs de la voie du TGF-β comme le TGF-β1 lui-même ou encore le TGFβ-RII et SMAD3 (Chen et al., 2004). La même année, l'efficacité anti-fibrosante dans un modèle d'irradiation localisée à forte dose de la patte a été décrite chez des souris traitées à l'halofuginone (Xavier et al., 2004). Cette molécule a démontré une efficacité thérapeutique, notamment par sa capacité à inhiber la synthèse des collagènes, dans des modèles de fibrose cutanée (McGaha et al., 2002), hépatique (Bruck et al., 2001) et pulmonaire (Nagler et al., 1996). Ainsi, dans ce modèle de fibrose radio-induite de la patte, les auteurs ont montré que l'halofuginone était capable de stimuler l'expression d'un répresseur de la voie TGF-β/SMAD, i.e. SMAD7, d'inhiber l'expression du TGFβ-RII et la phosphorylation de SMAD2 et SMAD3 (Xavier et al., 2004). D'autre part, l'équipe d'Hauer-Jensen qui travaille depuis plus de 20 ans sur la toxicité radio-induite intestinale en utilisant notamment un modèle élégant d'anse irradiée localisée chez le rat a publié plusieurs études utilisant des stratégies thérapeutiques anti-fibrosantes. Dans ce modèle expérimental, l'hirudine (un inhibiteur de la thrombine) (Wang et al., 2004), l'orazipone (un anti-inflammatoire immuno-modulateur) (Boerma et al., 2006), le clopidogrel (un inhibiteur d'adhésion plaquettaire) (Wang et al., 2002) ou encore l'octreotide (analogue de la somatostatine) (Wang et al., 2001) ont tous démontré une efficacité thérapeutique associée à une diminution de l'expression de TGF-β chez les animaux irradiés traités comparés aux irradiés non-traités. Enfin, des inhibiteurs d'enzyme de conversion de l'angiotensine ont montré des efficacités thérapeutiques par leur capacité à inhiber le TGF-β1 ou sa voie de signalisation dans des modèles de fibroses radio-induite rénales chez la rat (Brown et al., 2000; Oikawa et al., 1997).

L'ensemble de ces résultats démontre le rôle clef que joue le TGF-β1, sa voie de signalisation et probablement ses effecteurs sur les dommages radio-induits. D'autre part, le rôle clef de la voie de signalisation SMAD et plus particulièrement SMAD3 dans les lésions radio-induites *in vivo* a été démontré même si les mécanismes précis restent flous (Flanders, 2004; Flanders et al., 2003; Flanders et al., 2002). Bien entendu, les stratégies thérapeutiques visant spécifiquement le TGF-β restent difficile à mettre en place en clinique du fait de l'action pleïotropique de ce facteur de croissance. C'est pourquoi des stratégies visant à limiter son action soit en inhibant un des acteurs de la voie de signalisation SMAD ou encore un effecteur du TGF-β1 qui a un rôle démontré dans les lésions radio-induites pourraient constituer des pistes intéressantes. Dans ce contexte, une des questions posées dans ce travail de thèse sur laquelle nous reviendrons

plus tard sera de savoir si un des effecteurs du TGF-β, i.e. PAI-1, joue un rôle déterminant dans les dommages radio-induits aux tissus sains.

6. Le CTGF, autre facteur de croissance impliqué dans la fibrose radio-induite.

Le CTGF (ou CCN2) est un peptide de 38 kD appartenant à la famille des CCN (CYR61, CTGF et NOV) (Perbal, 2004). Initialement mis en évidence dans des cellules endothéliales ombilicales humaines (Bradham et al., 1991), le CTGF est exprimé par les fibroblastes, les chondrocytes, les cellules musculaires lisses vasculaires et certaines lignées tumorales (Brigstock, 2003). Il est impliqué dans la prolifération, la migration, l'adhésion cellulaire, la production de matrice extracellulaire et joue un rôle important dans le développement, la différenciation, l'angiogénèse et la cicatrisation (Moussad and Brigstock, 2000). Frazier *et al* ont monté que cette cytokine est capable de stimuler la prolifération fibroblastique et la formation du tissu de granulation (Frazier et al., 1996). D'autre part, une surexpression du CTGF (ARNm et/ou protéine) a été observée dans les plaques d'athérosclérose (Oemar et al., 1997) (Cicha et al., 2005) et dans les lésions fibrotiques de nombreux organes comme le foie, le poumon, le pancréas, le rein, l'intestin et la peau (Leask and Abraham, 2004) (Perbal, 2004). *In vitro* le CTGF est fortement stimulé par le TGF-β1 (Kucich et al., 2001) et un élément de réponse aux SMAD et une boîte TGFβ-RE dans le promoteur du CTGF permettent sa transactivation par le TGF-β1. Le mécanisme d'action du CTGF reste encore très mal connu. Il est capable de se lier au Low Density Lipoprotein related receptor (LRP), à des intégrines et des Heparan Sulfate Proteoglycans (HSPs). Cependant, il n'y a pas de mécanisme de transduction du signal du CTGF décrit à ce jour dans la littérature. Néanmoins, une étude a montré que le CTGF agit en synergie avec le TGF-β1 (Abreu et al., 2002). Ainsi, par son domaine riche en cystéines, le CTGF est capable de lier avec une faible affinité le TGF-β1 dans l'espace extracellulaire entraînant ainsi une augmentation de la liaison TGF-β1 au recepteur TGF-β RII. Par des techniques de « gene reporter », les auteurs ont montré que cette augmentation de liaison induit une stimulation de la transactivation de gènes cibles du TGF-β1 (Abreu et al., 2002). Le CTGF est sur-exprimé dans les entérites radio-induites humaines (Vozenin-Brotons et al., 2003) et cette expression se retrouve aux sites d'accumulation du collagène et des fibroblastes/myofibroblastes activés. Ces résultats suggèrent que le CTGF est un médiateur de la fibrogénèse radio-induite pouvant être responsable de l'activation cellulaire (transdifférenciation des myofibroblastes) et de l'accumulation de matrice extracellulaire. De plus, les cellules musculaires lisses et les myofibroblastes subépithéliaux isolés d'entérite radio-induite surexpriment

le CTGF de façon constitutive, ce qui suggère une implication du CTGF dans le maintien de la différenciation fibrogénique des cellules du mésenchyme intestinal (Bourgier et al., 2005; Haydont et al., 2005). Des études sont actuellement en cours au laboratoire pour déterminer le rôle précis du CTGF dans les lésions radio-induites et savoir s'il pourrait être une cible thérapeutique intéressante.

B L'endothélium vasculaire.

Les compartiments épithéliaux, mésenchymateux et vasculaires contribuent à l'initiation, la progression et le maintien des dommages radio-induits aux tissus sains. Dans ce travail de thèse, nous aborderons principalement le rôle du compartiment vasculaire. Dès 1968, Rubin et Casarett proposaient le concept d'un lien de causalité strict entre les dommages aigus vasculaires et les effets tardifs (Rubin and Casarett, 1968). Ce concept reposait principalement sur le caractère ubiquitaire du compartiment vasculaire et sur des observations histopathologiques.

1. Structure des vaisseaux sanguins.

Les vaisseaux sanguins sont constitués de trois tuniques distinctes, l'intima, la média et l'adventice ***(Figures 6 et 7)***.

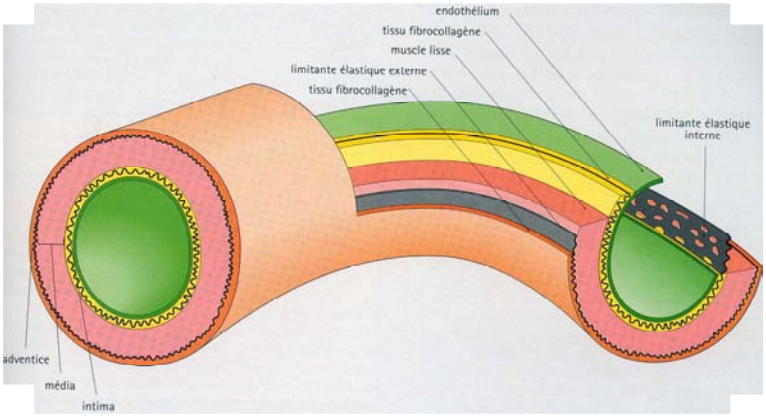

Figure 6 : Schéma d'une artère musculaire : La couche la plus importante de la paroi d'une artère musculaire est la média. Constituée de muscle lisse, elle s'individualise entre deux limitantes élastiques interne et externe. (D'après Stevens et Lowe, Histologie Humaine, Traduction Française par Pierre Validire 2002)

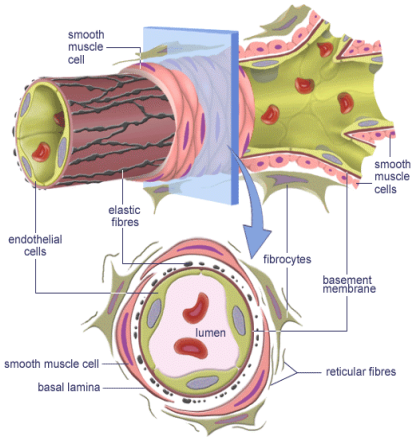

Figure 7 : Schéma d'une artériole : les artérioles sont de petits vaisseaux sanguins qui naissent d'une artère pour relier celle-ci à un capillaire. Elles sont formées d'une couche de cellules endothéliales reposant sur une lame basale formée principalement de fibres élastiques dont l'élastine. Sous cette lame basale se trouve une fine couche de cellules musculaires lisses vasculaires et des fibrocytes. Les fibrocytes ou pericytes sont des cellules mésenchymateuses quiescentes non différenciées associées aux petits vaisseaux sanguins capables de se différencier en fibroblastes, cellule musculaire lisses ou macrophages.

Selon le type de vaisseau, l'importance de ces trois tuniques varie, pouvant aller de la simple monocouche de cellules endothéliales (microvascularisation) à des vaisseaux très épais (artères). L'intima est constituée d'une monocouche de cellules endothéliales directement en contact avec le sang et d'une lame basale formée de collagènes et de fibres élastiques. Quelle que soit la taille du vaisseau, l'intima est toujours constituée d'une monocouche de cellules endothéliales et pour certaines artères élastiques très épaisses, elle peut contenir des cellules musculaires lisses vasculaires ou cellules myo-intimales. La média contient exclusivement des cellules musculaires lisses vasculaires et des constituants matriciels comme l'élastine ou des collagènes. La média est très développée dans les gros vaisseaux comme les artères et pratiquement inexistante dans les capillaires. L'adventice est la couche externe de la paroi vasculaire et est constitué principalement de collagènes et de fibroblastes notamment dans les veines. Dans l'adventice, on observe également des nerfs du système nerveux autonome permettant l'innervation du muscle lisse de la média.

Très schématiquement, en aval des grosses artères élastiques comme l'aorte, la carotide, la sous-clavière et l'artère rénale, on trouve les artères musculaires, dont la paroi devient proportionnellement plus musculaire et dont le diamètre diminue progressivement au fur et à mesure de leurs ramifications dans la profondeur des tissus jusqu'a devenir des artérioles. Aux artérioles composées d'une média de 1 à 4 couches de cellules musculaires lisses, font suite des vaisseaux encore plus petits composés d'une monocouche de cellules endothéliales ou capillaires. Par des anastomoses artério-veineuses présentes dans la microcirculation le sang passe des capillaires aux veinules, puis aux veines dont le diamètre augmente progressivement en se rapprochant du cœur.

2. L'endothélium vasculaire et ses fonctions.

Les cellules endothéliales tapissent la lumière des vaisseaux sanguins et sont impliquées dans la régulation de la plupart des fonctions physiologiques vasculaires ***(Figure 8)***. La majorité des cellules endothéliales sont présentes dans la micro-circulation et on estime que chaque cellule de l'organisme est présente à une distance maximum de quelques micromètres d'un capillaire sanguin. Ce réseau micro-vasculaire extrêmement dense permet l'approvisionnement des tissus en nutriments, hormones et gaz respiratoires mais participe également à l'élimination des déchets métaboliques et aux échanges de produits de sécrétion entre le sang et les fluides interstitiels.

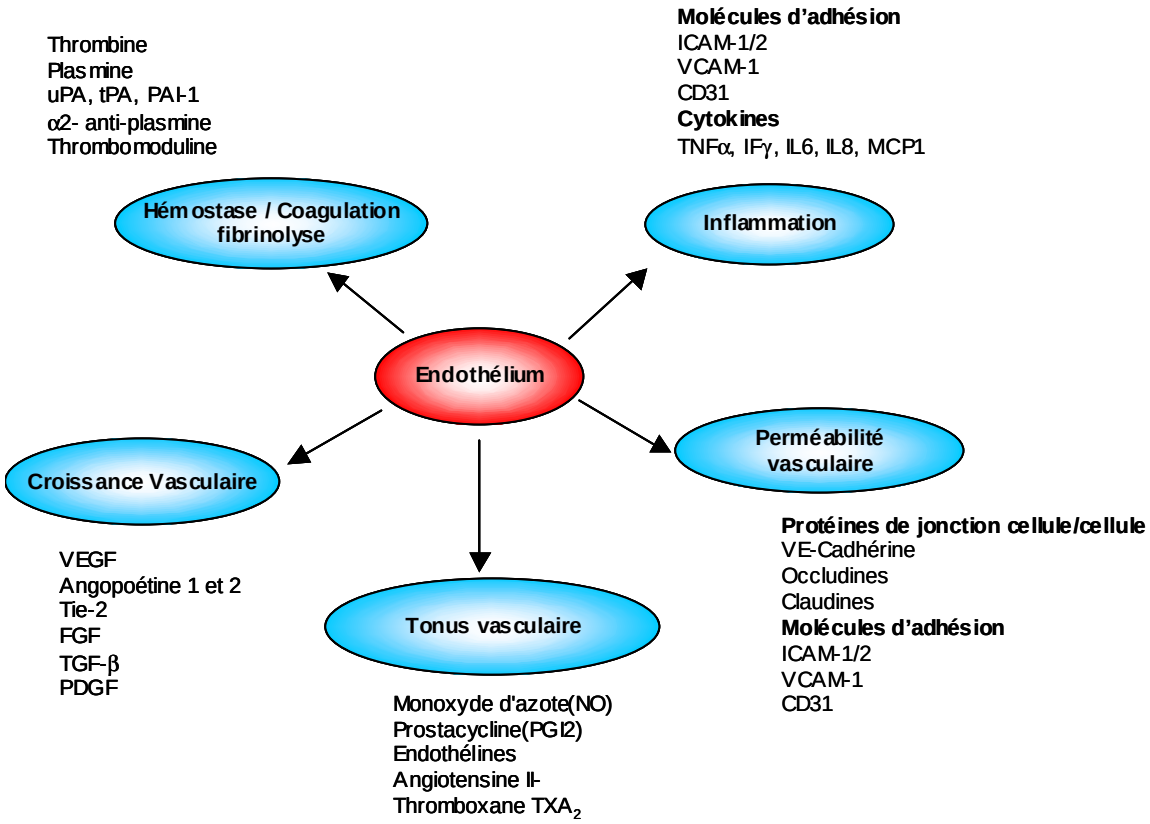

Figure 8 : Fonctions physiologiques de l'endothélium vasculaire et principaux acteurs moléculaires impliqués

La fonction première de l'endothélium est celle de barrière et de filtre sélectif entre les tissus sous jacents et la circulation sanguine et lymphatique. La perméabilité de l'endothélium est déterminante dans le maintien de l'homéostasie tissulaire et des modifications de cette perméabilité vasculaire peuvent entraîner l'extravasation des protéines sériques dans les tissus, conduire à un œdème périvasculaire et tissulaire et affecter la fonction de l'organe. Les jonctions entre cellules endothéliales régulent en grande partie la perméabilité vasculaire (Bazzoni and Dejana, 2004). Parmi elles, les jonctions serrées sont formées d'occludines et de claudines qui sont des protéines transmembranaires. Les jonctions communicantes sont elles formées principalement de connexines alors que les jonctions adhérentes sont formées de VE cadhérine (Vascular Endothelial Cadherin). L'organisation de ces jonctions varie bien entendu selon le type de vaisseau mais également en fonction de l'état physiologique et physiopathologique du tissu ou de l'organe.

La seconde fonction principale de l'endothélium est le maintien de l'homéostasie vasculaire. La cellule endothéliale est en contact direct avec le sang circulant et représente une surface non thrombogène ou thrombo-résistante par la production de substances anticoagulantes et antiagrégantes. Ainsi, l'endothélium joue un rôle essentiel dans l'hémostase et régule à la fois les

phénomènes de coagulation et de fibrinolyse. Nous reviendrons plus tard sur une description plus détaillée de ces mécanismes physiologiques en abordant le rôle de PAI-1.

D'autre part, l'endothélium joue un rôle clef dans la réponse inflammatoire et immunitaire en régulant le trafic de leucocytes entre le sang et les tissus. Pour cela, les cellules endothéliales sont capables de sécréter des chemo-attractants et d'exprimer des molécules d'adhésion (CAMs) jouant un rôle clef dans l'adhésion, le recrutement et la transmigration des cellules circulantes. Ainsi, VCAM -1 (Vascular Cell Adhesion Molecule-Type 1), ICAM-1 et ICAM-2 (Intercellular Adhesion Molecule-Type 1 et 2), PECAM-1 (Platelet Endothelial Cell Adhesion Molecule-Type 1) et les sélectines (E et P) sont parmi les molécules d'adhésion les plus importantes dans le recrutement des cellules circulantes au site lésé. D'autre part, les cellules endothéliales sont elles mêmes capables de sécréter des cytokines pro et anti-inflammatoires comme le TNFα, IFN-γ, IL6, IL8, MCP1 et participer activement au statut inflammatoire du tissu dans lequel elles se trouvent.

Les cellules endothéliales jouent également un rôle dans le contrôle du tonus vasculaire en sécrétant des molécules agissant sur les cellules musculaires lisses (CML) vasculaires. Ainsi, le rôle des cellules endothéliales dans les processus de vasoconstriction et vasodilatation s'effectue par la production ou le catabolisme de facteurs hémodynamiques locaux. L'endothéline, les prostaglandines, le thromboxane A2 et l'angiotensine II sont les principaux agents vasoconstricteurs alors que la prostacycline et le monoxyde d'azote (NO°) ont une action vasodilatatrice. Par exemple, principalement synthétisé par la NO-Synthase endothéliale, le NO° agit en augmentant la production de GMPc dans les CML vasculaires ce qui déclenche une action vasodilatatrice. D'autre part, la prostacycline, formée à partir de l'acide arachidonique exerce une fonction vasodilatatrice en augmentant la concentration d'AMPc dans les CML vasculaires. Le rôle de l'endothéline est lui plus subtil. L'endothéline joue un rôle vasoconstricteur sur les CML vasculaires mais également un rôle vasodilatateur en stimulant la production de NO° et de prostacycline par les cellules endothéliales. Enfin, également dérivés de l'acide arachidonique, les prostaglandines et le thromboxane A2 exercent une action vasoconstrictrice sur les CML vasculaires *via* des récepteurs couplés à la phospholipase C.

Enfin, les cellules endothéliales jouent un rôle déterminant dans l'angiogénèse. L'angiogénèse est un processus biologique complexe caractérisé par la formation de nouveaux capillaires à partir de vaisseaux pré-existants et ceci en conditions physiologiques ou physiopathologiques (Carmeliet, 2003). Le mécanisme angiogénique implique une série séquentielle et orchestrée d'évènements moléculaires et cellulaires incluant l'activation des cellules endothéliales par des facteurs de croissance, la dégradation et la synthèse de matrice extracellulaire, la migration et la prolifération des cellules endothéliales et des cellules musculaires lisses

vasculaires. Il existe de nombreuses molécules pro-angiogéniques identifiées et parmi elles, le Vascular endothelial Growth Factor (VEGF), le FGF-2, le PDGF ou encore l'angiopoïétine-1 ont été décrites comme jouant un rôle fondamental dans le processus d'angiogénèse .

3. Physiopathologie de l'endothélium vasculaire.

De nombreuses pathologies ou de réactions physiopathologiques sont associées à des perturbations de l'endothélium vasculaire. Le premier exemple est une lésion directe provoquée par une dénudation de l'endothélium. Dans ce cas, le contact direct du compartiment sanguin avec un support matriciel ou cellulaire non thrombo-résistant induit extrêmement rapidement une adhésion et une agrégation des plaquettes au site lésé (Reddick et al., 1982). Un dysfonctionnement des cellules endothéliales dans ce processus physiologique peut bien entendu entraîner des problèmes de cicatrisation liés à des perturbations des mécanismes d'hémostase, de coagulation et de fibrinolyse. D'autre part, sans qu'il y ait dénudation endothéliale, des dysfonctionnements dans la régulation extrêmement fine des molécules pro-thrombotiques et/ou anti-fibrinolytiques sont des facteurs pathologiques associés notamment aux problèmes de thrombose vasculaire (Rosenblum, 1997). Des variations de paramètres sanguins peuvent également contribuer à une dysfonction des cellules endothéliales. Par exemple, dans le cas du diabète, des taux élevés de glucose circulant modifient les propriétés de l'endothélium par différents mécanismes comme l'activation de la voie de la protéine C, des modifications d'expression génique ou encore par l'induction d'un stress oxydatif (Rask-Madsen and King, 2007). D'autre part, la cellule endothéliale participe activement à la dissémination métastatique des cellules cancéreuses en exprimant des récepteurs nécessaires à la transmigration des cellules tumorales dans les tissus.

Les pathologies vasculaires comme l'athérosclérose, l'hypertension, l'artériosclérose, les rétinopathies prolifératives, les ischémies vasculaires sont bien entendu associées à des modifications physiopathologiques de l'endothélium vasculaire. La dysfonction endothéliale est associée à des modifications fonctionnelles comme une stimulation de la vasoconstriction et une baisse de la thromborésistance ***(Figure 9)***.

Comme nous l'avons vu, en conditions normales, les cellules endothéliales jouent un rôle fondamental dans le maintien de l'homéostasie vasculaire. En conditions physiopathologiques suite à un stress (stress oxydant, dyslipidémie, hypertension, diabète), la baisse de production ou de biodisponibilité du NO° entraîne un déséquilibre entre les molécules vasodilatatrices et les vasoconstrictrices. Parallèlement à cette orientation vasculaire vers la vasoconstriction, la cellule

endothéliale activée synthétise des molécules de surface stimulant l'adhésion des leucocytes et des plaquettes et donc la formation de thrombus. D'autre part, la perte d'acteurs anti-mitogènes et la stimulation d'acteurs pro-mitogènes et pro-migratoires stimulent la prolifération et la migration de CML vasculaires dans l'intima.

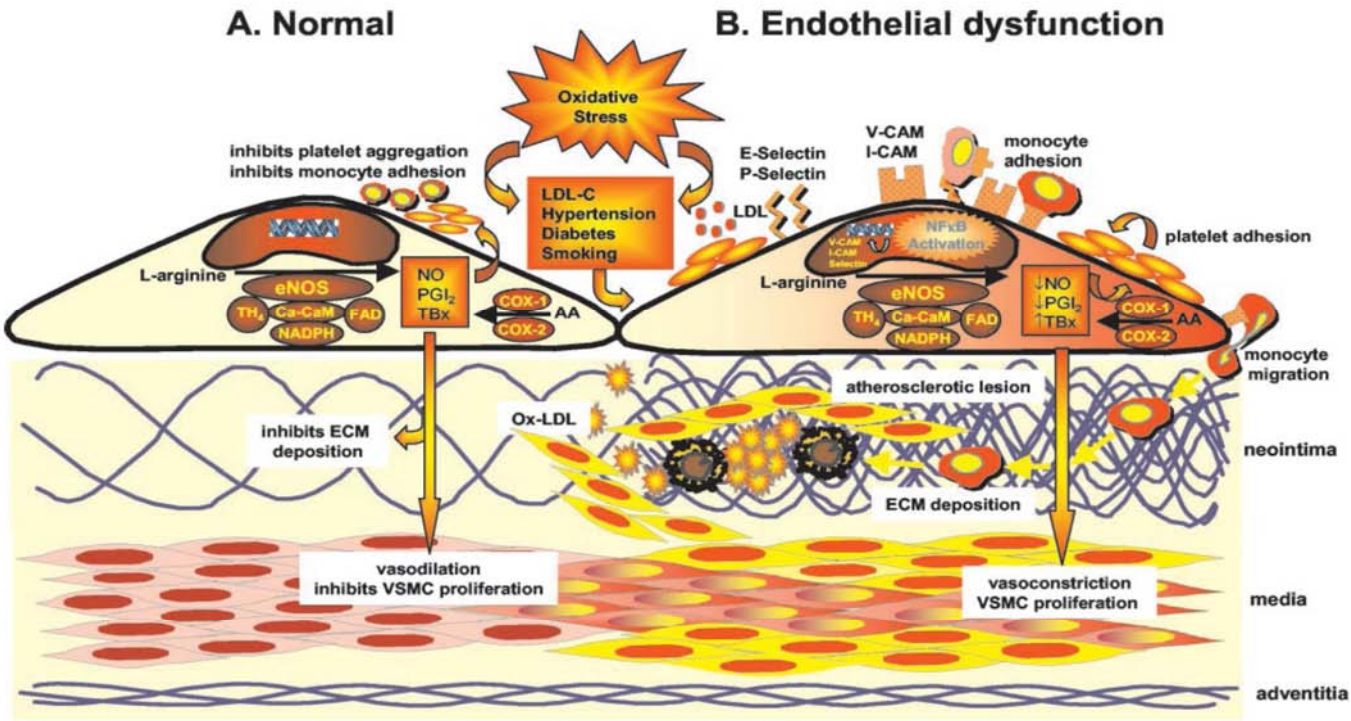

Figure 9 : Représentation schématique des dysfonctionnements de l'endothélium (D'après Melo LG *et al*. Arterioscler Thromb Vasc Biol. 2004).

4. Relation Cellules endothéliales / cellules musculaires lisses dans les dommages vasculaires.

Le concept d'un dialogue étroit entre les deux principaux types cellulaires constituant les vaisseaux que sont les cellules endothéliales et les cellules musculaires lisses est évoqué depuis de très nombreuses années. Bien entendu la proximité *in situ* des cellules endothéliales et des cellules musculaires lisses vasculaires a permis d'émettre l'hypothèse que chaque type cellulaire influence le phénotype et certaines fonctions de l'autre. L'émergence des approches *in vitro* permettant de cultiver ensemble deux types cellulaires, et d'étudier ainsi les signaux paracrines, a apporté des preuves expérimentales aux concepts initiaux. Il y a un peu plus d'une dizaine d'années, des études expérimentales de co-cultures grâce à l'utilisation d'inserts poreux ont commencé à être publiées dans la littérature. L'avantage de ces techniques de culture cellulaire comparées aux approches utilisant les milieux conditionnés est de pouvoir étudier de manière dynamique et cinétique l'influence d'un type cellulaire sur l'autre. Ainsi, Peiro et al. ont montré qu'en utilisant une

approche par milieu conditionné et par un système de co-culture les résultats obtenus sont très différents (Peiro et al., 1995). Ces auteurs ont ainsi montré qu'un milieu conditionné de cellules endothéliales (cellules endothéliales aortiques bovines) avait un effet stimulateur sur la prolifération de CML vasculaires de rats alors qu'au contraire, en co-culture, ces mêmes cellules endothéliales inhibent la prolifération des CML vasculaires. L'argument avancé concernant ces différences est qu'en système de co-culture les molécules de durée de vie très courte ou instables comme le monoxyde d'azote ou les prostaglandines peuvent agir alors qu'en milieu conditionné les effets paracrines de ces molécules instables sont perdus. Le rôle essentiel des cellules endothéliales dans le contrôle du tonus vasculaire et leur capacité à sécréter des agents vasoconstricteurs, vasodilatateurs et mitogènes a également été largement abordé. Ainsi, il a été montré que le monoxyde de carbone (CO), sécrété par les CML vasculaires, pouvait réguler la prolifération des CML vasculaires *via* l'inhibition par le CO de l'expression d'agents paracrines mitogènes (endothéline-1 et PDGF-B) sécrétés par les cellules endothéliales (Morita and Kourembanas, 1995).

Les cellules endothéliales sont constamment soumises *in vivo* à des forces de cisaillement exercées par le flux sanguin. L'adaptation vasculaire aux modifications hémodynamiques locales permet aux vaisseaux d'ajuster en permanence leur calibre et leur paroi afin de normaliser les contraintes de cisaillement imposées à l'endothélium. Ce contrôle fonctionnel et structural du vaisseau est assuré par de nombreux mécanismes autocrines et paracrines où le dialogue cellules endothéliales / CML vasculaires est déterminant. C'est dans ce contexte et en utilisant une approche par co-culture avec un système de flux que des équipes se sont intéressées au rôle des CML vasculaires dans le phénotype des cellules endothéliales induit par des forces de cisaillement. Ainsi, en conditions statiques, les CML vasculaires stimulent l'expression génique de ICAM-1, VCAM-1 et E-sélectine des cellules endothéliales. En conditions dynamiques, le flux imposé aux cellules endothéliales inhibe l'induction de l'expression des molécules d'adhésion endothéliales induite par les CML vasculaires (Chiu et al., 2003).

Dans des conditions physiopathologiques, l'importance des interactions cellules endothéliales/CML vasculaires a également été décrite. Ainsi il a été montré un rôle clef de l'adhésion des plaquettes aux cellules endothéliales durant les phases d'initiation de la formation de la plaque d'athérome (Massberg et al., 2002). Récemment, Tull et al ont montré que les plaquettes pouvaient adhérer aux cellules endothéliales co-cultivées en présence de CML vasculaires par un mécanisme dépendant de l'activation des cellules endothéliales par le TNF-α et le TGF-β1 (Tull et al., 2006). La même équipe avait démontré quelques années plus tôt, en utilisant les mêmes approches par co-cultures, que des CML vasculaires au phénotype sécrétoire avaient une capacité accrue à stimuler l'adhésion des leucocytes aux cellules endothéliales (Rainger and Nash, 2001).

L'ensemble de ces exemples démontre parfaitement l'importance du dialogue cellules endothéliales /CML vasculaires dans la fonction physiologique globale des vaisseaux et également son implication en conditions physiopathologiques. Un des objectifs de ce travail de thèse sera d'aborder cette question et plus précisément l'influence des cellules endothéliales sur le phénotype des CML vasculaires après irradiation.

C Effets des radiations ionisantes sur les cellules endothéliales.

Les radiations ionisantes ont de multiples effets sur l'endothélium qui contribuent à l'initiation et la progression des dommages radio-induits aux tissus sains. Parmi ces principaux effets, on distingue la mort cellulaire radio-induite, la perte de thromborésistance, l'activation endothéliale et la sécrétion de facteurs solubles comme des cytokines ou des facteurs de croissance ***(Figure 10)***.

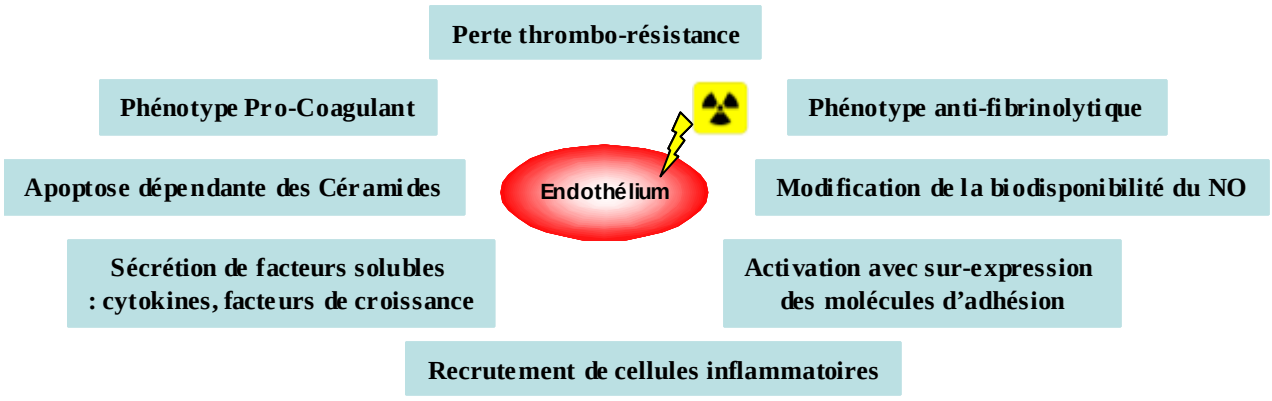

Figure 10 : Principaux effets des radiations ionisantes sur l'endothélium vasculaire

1. Apoptose des cellules endothéliales après irradiation.

La mort des cellules endothéliales est une étape clef dans la réaction tissulaire après irradiation. Les cellules endothéliales sont considérées comme radiosensibles comparé à d'autres types cellulaires comme notamment les cellules mésenchymateuses. Le rôle clef de l'apoptose endothéliale radio-induite reste encore aujourd'hui sujet à controverse. En 2001, l'équipe de R. Kolesnick montra que l'apoptose endothéliale était l'évènement initial et responsable du syndrome gastro-intestinal après une irradiation corps entier à 15Gy chez la souris (Paris et al., 2001). Ainsi, les souris déficientes en sphingomyélinase acide, chez lesquelles l'apoptose endothéliale radio-

induite est limitée, sont protégées du syndrome gastro-intestinal avec une augmentation de la fraction de cellules épithéliales souches survivantes. Ces résultats démontraient que la mort de l'endothélium était l'événement initiateur responsable de la mort des cellules clonogéniques épithéliales. Certains auteurs contestent ce concept et s'appuient sur le fait que l'apoptose des cellules épithéliales est observée pour des doses insuffisantes pour générer la mort des cellules endothéliales. Récemment, par l'utilisation d'un système d'irradiation par Boron-thérapie qui permet de délivrer une dose d'irradiation très forte spécifiquement au niveau de l'endothélium, une équipe a montré que l'irradiation à forte dose de l'endothélium vasculaire n'a pas d'effet sur la survie clonogénique des cellules souches intestinales (Schuller et al., 2006). Clairement, aujourd'hui il reste difficile de savoir si la mort radio-induite endothéliale est l'évènement initiateur des dommages tissulaires. Malgré ces résultats contradictoires, la participation de l'apoptose endothéliale dans les effets délétères tissulaires après irradiation fait toujours l'unanimité.

2. Activation de l'endothélium après irradiation.

L'activation de l'endothélium vasculaire après irradiation a été largement étudiée ces 15 dernières années à la fois *in vivo* et *in vitro*. Cette activation se traduit par une augmentation de l'expression de protéines d'adhésion qui participent au recrutement des cellules circulantes. Ainsi des augmentations de l'expression de VCAM-1, ICAM-1, PECAM-1, E- sélectine et P-sélectine ont été décrites *in vitro* sur des cellules endothéliales irradiées (Gaugler et al., 2004; Heckmann et al., 1998; Quarmby et al., 2000; Quarmby et al., 1999). Dans un modèle d'inflammation intestinale radio-induite, l'équipe de Panès a montré une augmentation de l'expression de ICAM-1 et VCAM-1 (Molla et al., 2003). *In vivo*, l'étude des cinétiques d'expression de ces deux molécules d'adhésion montre que ICAM-1 est sur-exprimée très rapidement après irradiation (24h) alors que la sur-expression de VCAM-1 est plus tardive (de 1 à 2 semaines). Cette surexpression précoce de ICAM-1 après irradiation à également été décrite dans des poumons de souris irradiées au niveau du thorax (Hallahan et al., 1996; Hallahan and Virudachalam, 1997). Le rôle clef de ces différentes protéines d'adhésion dans le recrutement des leucocytes dans diverses pathologies inflammatoires aiguës et chroniques est bien documenté. Concernant l'inflammation radio-induite, l'utilisation de souris ICAM-1 -/- montre que ces animaux ont une inflammation pulmonaire et intestinale moins importante que les souris sauvages démontrant l'implication de cette protéine dans le recrutement précoce des leucocytes dans le tissu irradié (Halalhan et al. 1997).

3. Interactions leucocytes ou plaquettes avec l'endothélium après irradiation.

Plusieurs études ont montré des modifications d'interactions entre les cellules circulantes et l'endothélium après irradiation. Ainsi, l'irradiation entraîne une augmentation des interactions plaquettes/endothélium à la fois *in vitro* et *in vivo* (Gaugler et al., 2004; Mouthon et al., 2003). Le rôle clef de PECAM-1 a été démontré *in vitro* dans l'adhésion et l'agrégation plaquettaire sur des cellules endothéliales irradiées (Gaugler et al., 2004). La stimulation radio-induite de l'adhésion des plaquettes aux cellules endothéliales est inhibée en présence d'un anticorps anti-PECAM-1. D'autre part, l'équipe de Panès a montré, *in vivo* par microscopie intravitale, une baisse de l'adhésion des leucocytes à l'endothélium chez des souris ICAM-1 -/- comparées aux souris sauvages 24h après une irradiation à 10 Gy en zone abdominale (Molla et al., 2003). Ces études démontrent que l'augmentation de l'expression des molécules d'adhésion par les cellules endothéliales après irradiation joue un rôle déterminant dans le recrutement des cellules circulantes et donc dans l'inflammation radio-induite des tissus.

4. Irradiation et activation du système de coagulation.

L'activation du système de coagulation fait partie des processus physiologiques stimulés après une irradiation. Ainsi, la perte de la thrombo-resistance endothéliale suite à un stress ionisant résulte de la stimulation de la fibrinogénèse et d'une baisse de la fibrinolyse associées à des surexpressions de facteur tissulaire TF (Tissue Factor) (Verheij et al., 1995), du facteur de von Willebrand (vWF) (Jahroudi et al., 1996; Verheij et al., 1997) et d'une baisse d'expression de prostacycline (Rubin et al., 1985) et de thrombomoduline (Zhou et al., 1992). Le rôle de la thrombine dans l'initiation des lésions radio-induites a été décrit car elle joue un rôle déterminant dans l'activation de la fibrinogénèse. De plus, la thrombine stimule la prolifération des fibroblastes dans les phases précoces suite à une irradiation pulmonaire chez le rat (Huang et al., 2001). Les cellules endothéliales irradiées ont une production accrue de thrombine associée à une perte de la thrombomoduline (Wang et al., 2002). De manière intéressante, outre son rôle pro-coagulant, la thrombine est capable de stimuler la synthèse de matrice extracellulaire (Chambers et al., 1998; Howell et al., 2001), la différenciation myofibroblastique (Bogatkevich et al., 2001) ainsi que la synthèse de CTGF (Chambers et al., 2000). Un traitement par un inhibiteur de la thrombine (Hirudin) a montré une bonne efficacité dans un modèle d'atteinte radio-induite intestinale chez le rat (Wang et al., 2004). Cependant le mécanisme d'action de la thrombine dans les phénomènes associés aux lésions radio-induites n'est pas encore clairement démontré.

Depuis plusieurs années, l'équipe de M. Hauer-Jensen s'intéresse notamment au système thrombomoduline-proteine C et son rôle dans les dommages radio-induits aux tissus sains (Hauer-Jensen et al., 2004). Le système TM-protéine C joue un rôle anticoagulant clef et une altération de ce processus est démontrée dans les dommages tissulaires radio-induits. Dans la plupart des vaisseaux sanguins, la thrombomoduline, qui est une glycoprotéine transmembranaire localisée à la surface luminale des cellules endothéliales, forme un complexe avec la thrombine et ainsi inhibe la conversion thrombine-dépendante du fibrinogène en fibrine. Ce complexe TM-Thrombine permet l'activation de la protéine C et au contraire limite l'expression des récepteurs à protéinases activés (PAR). Ainsi, des baisses d'expression de thrombomoduline sont associées, dans diverses pathologies, à une orientation de l'endothélium vers un phénotype pro-coagulant dû à l'action de la thrombine qui, non complexée à la thrombomoduline, exerce une action à la fois pro-coagulante, pro-inflammatoire et fibro-prolifération. L'équipe de M. Hauer-Jensen a montré, à la fois chez des patients irradiés et dans des modèles d'entéropathie radio-induite chez le rat, que l'irradiation provoque une baisse de l'expression de la thrombomoduline (Richter et al., 1997; Wang et al., 2002). Chez l'homme, cette baisse commence dès le début de la radiothérapie, et se perpétue jusqu'à la fin du traitement et même plusieurs mois après le traitement durant les phases de fibrose chronique (Richter et al., 1998). De la même manière, une baisse rapide et persistante d'expression de thrombomoduline est observée dans un modèle de fibrose intestinale radio-induite chez le rat associée à une activation chronique de PAR-1 dans les cellules musculaires lisses et particulièrement dans les vaisseaux pathologiques caractérisés par une sclérose vasculaire (Wang et al., 2002). Ce travail expérimental chez le rat permet d'appuyer le concept que des évènements précoces au niveau des cellules endothéliales contribuent à l'initiation et à la progression des dommages vasculaires et tissulaires radio-induits. Les mécanismes responsables de la baisse d'expression de la thrombomoduline sont liés à la fois aux effets directs de l'irradiation par la production d'espèces réactives de l'oxygène et par des effets indirects secondaires liés à l'inflammation radio-induite. Ainsi, l'Interleukine-1, le Tumor Necrosis–α et le TGF-β1 sont surexprimés après irradiation et ont été démontrés comme inhibant la transcription de la thrombomoduline (Lentz et al., 1991; Nawroth et al., 1986; Ohji et al., 1995).

5. Radiothérapie et pathologies vasculaires.

Les dommages vasculaires induits par une irradiation thérapeutique sont décrits depuis de nombreuses années (Fajardo and Stewart, 1970; Fajardo and Stewart, 1972). Ainsi l'irradiation en région thoracique pour le traitement de différents types de cancers est associée à l'apparition de pathologies cardio-vasculaires très graves comme des péricardites, des cardiomyopathies, des insuffisances coronaires et des problèmes d'arythmie cardiaque (Adams et al., 2003). L'atteinte vasculaire est considérée comme la cause majeure de la morbidité radio-induite à long terme chez les survivants de cancers traités par radiothérapie. Par exemple, une étude récente portant une population de 1926 enfants ayant survécu à 5 ans à une maladie de Hodgkin comparée à une population témoin de 3846 individus (enfants de mêmes parents) démontre que ces enfants ont un risque augmenté d'accident vasculaire cérébral probablement associé à des pathologies radio-induites des valves cardiaques ou des artères coronaires (Bowers et al., 2005). Après un traitement radiothérapeutique chez des patients souffrant de la maladie de Hodgkin (Bowers et al., 2005; Hull et al., 2003), de cancers du sein (Cuzick et al., 1994) ou encore de la tête et du cou (Dorresteijn et al., 2002), il y a une augmentation de l'incidence des pathologies vasculaires. Par exemple une étude sur 367 patients traités par radiothérapie pour des cancers de la tête et du cou montre une augmentation significative du risque d'accident vasculaire cérébral (Dorresteijn et al., 2002). Chez ces patients, il y a une augmentation globale de 5,6 fois du risque relatif d'accident vasculaire cérébral et, pour un suivi supérieur à 10 ans post-irradiation, ce risque relatif est de 10,1 fois. D'autre part, des études rétrospectives montrent une sténose et une réduction du flux sanguin associé à un épaississement de l'intima de carotides irradiées chez des patients traités par radiothérapie pour des cancers de la tête et du cou (Dorresteijn et al., 2005). Une méta-analyse sur 40 essais cliniques randomisés portant sur le bénéfice thérapeutique de la radiothérapie dans le traitement du cancer du sein (soit environ 20 000 patientes) montre une baisse de la mortalité chez les patientes traitées par radiothérapie. En revanche, l'effet favorable sur la mortalité du cancer du sein est contrebalancé, en partie, par une augmentation du taux de mortalité annuel liée à des accidents vasculaires probablement dus à l'irradiation des coronaires, des carotides ou d'autres artères principales (Early Breast Cancer Trialists' Collaborative Group. Lancet 2000).

Au niveau expérimental, plusieurs études montrent que l'irradiation localisée d'artères de lapins hypercholestérolémiques induit une accélération de la formation de plaques d'athérosclérose (Cottin et al., 2001). D'autre part, les radiations ionisantes accélèrent la formation des lésions aortiques chez des souris soumises à un régime pro-athérogène composé de 15 % de lipides et de 1.25 % de cholestérol (Tribble et al., 1999). Ces lésions athérosclerotiques sont deux fois moins

importantes chez des souris transgéniques sur-exprimant la superoxyde dismutase (CuZn-SOD) démontrant le rôle clef des espèces réactives de l'oxygène dans l'induction du processus pro-athérogène des radiations ionisantes (Tribble et al., 1999). Cependant, les mécanismes moléculaires et cellulaires impliqués dans les lésions vasculaires radio-induites restent encore très mal connus. Récemment l'équipe de Stewart a montré que l'irradiation accélère le développement des lésions d'athérosclérose chez des souris ApoE -/- après une dose unique de 14 Gy. L'analyse des lésions montre une forte implication des cellules inflammatoires et notamment des macrophages et des granulocytes et également une composition de plaques hémorragiques riches en dépôts de fibrine (Stewart et al., 2006).

6. Dommages vasculaires radio-induits.

Les lésions vasculaires radio-induites ont été observées très rapidement après la découverte des rayons X. Le concept selon lequel les dommages vasculaires et plus particulièrement la dysfonction ou la perte de l'endothélium contribuent à l'initiation, la progression et le maintien des dommages tissulaires radio-induits a été développé par Law et d'autres équipes dans les années 1980 (Law, 1981). Ces auteurs défendaient le concept selon lequel les dommages aux cellules endothéliales microvasculaires étaient la cause des lésions tardives dans les tissus irradiés. Leurs arguments principaux étaient les suivants : - la dysfonction endothéliale est très précoce et précède les changements histologiques observés pour les autres types cellulaires dans la plupart des tissus irradiés - le dépôt de fibrine et l'œdème périvasculaire précèdent la fibrose conjonctive et plus précisément l'accumulation de collagènes. Clairement les lésions vasculaires sont impliquées dans les lésions radiques aiguës et tardives. L'apoptose endothéliale, l'augmentation de la perméabilité vasculaire, l'activation cellulaire et le recrutement de cellules inflammatoires ainsi que l'activation du système de coagulation sont autant de phénomènes qui contribuent à l'induction et à la progression des dommages tissulaires radio-induits. Ces phénomènes sont observés très précocement après irradiation mais les lésions vasculaires plus tardives comme la fibrose et la réduction luminale génèrent également des zones d'hypoxie tissulaire qui contribuent et amplifient la cicatrisation pathologique radio-induite.

Au niveau morphologique les lésions vasculaires sont différentes en fonction de la taille des vaisseaux. La microvascularisation est considérée comme la plus radio-sensible où des ruptures et dilatations capillaires, l'hypertrophie et le détachement des cellules endothéliales de la lame basale, et des thromboses sont observés (Fajardo, 2005). Concernant les gros vaisseaux, il est très difficile de distinguer les lésions vasculaires d'athérosclérose « classiques » des lésions vasculaires radio-

induites. Ainsi, ces dernières se caractérisent par des fibroses vasculaires avec des réductions luminales, des dépôts excessifs de matrice extracellulaire au niveau de la média et de l'adventice, des hyperplasies néointimales et des formations de thrombus (Fajardo, 2005). Les similarités histologiques entre les lésions vasculaires radio-induites et les lésions d'athérosclérose laissent supposer que des mécanismes similaires d'initiation et de progression sont impliqués.

7. Rôle des dommages vasculaires dans les lésions tissulaires tardives radio-induites.

Le rôle du compartiment vasculaire dans les lésions tissulaires radio-induites est complexe. Par simplicité, nous distinguerons les effets précoces des effets à long terme de l'atteinte vasculaire afin de comprendre son implication dans la cinétique de la cicatrisation pathologique tissulaire radio-induite. Ces effets dépendent principalement de la radio-sensibilité de l'endothélium et de sa capacité à s'activer en réponse au stress ionisant. Les effets précoces sont principalement l'apoptose radio-induite, l'activation du recrutement des cellules circulantes et l'activation du système de coagulation par les cellules endothéliales. Les effets tardifs sont l'hypoxie tissulaire liée à l'ischémie vasculaire et la chronicité du dysfonctionnement de l'endothélium.

Comme nous venons de le voir, la déficience en thrombomoduline observée très rapidement après l'irradiation se perpétue de manière chronique à la fois chez le rat et chez l'homme (Richter et al., 1998; Wang et al., 2002). Ces résultats suggèrent que l'endothélium est dans un état pro-coagulant chronique qui peut contribuer à la persistance à long terme des effets délétères de l'irradiation. D'autre part, une étude *in vitro* montre que l'induction de l'expression de ICAM-1 dans les cellules endothéliales irradiées, associée à une augmentation de la capacité d'adhésion des neutrophiles, est conservée plus de 10 jours après l'irradiation suggérant un phénotype pro-inflammatoire des cellules endothéliales persistant dans le temps (Gaugler et al., 1997). Des résultats obtenus *in vivo* suggèrent que les dommages vasculaires contribuent aux fibroses radio-induites. Ainsi, dans un modèle de fibrose radio-induite pulmonaire chez le rat, Vujaskovic *et al.* ont montré une hypoxie importante associée à une fibrose sévère 6 mois après l'irradiation. Les auteurs suggèrent que cette hypoxie tissulaire est la conséquence des dommages aux cellules endothéliales, de l'œdème interstitiel et des dysfonctionnements de la vascularisation (Vujaskovic et al., 2001). Ces zones hypoxiques sont associées à une augmentation du dépôt matriciel de collagènes, de l'expression de facteurs pro-fibrosants (TGF-β1) et pro-angiogéniques (VEGF). Les résultats obtenus dans ce modèle de fibrose pulmonaire radio-induite n'ont pour l'instant pas été confirmés dans d'autres organes. Cependant, la réduction du réseau microvasculaire a été observée chez l'homme dans de nombreux organes irradiés comme le myocarde, le système nerveux central

ou la peau (Fajardo, 2005). Dans ce contexte, les sténoses vasculaires entraînant des zones d'hypoxie tissulaires sont imaginables mais n'ont pas été démontrées. L'hypoxie chronique et les phénomènes de ré-oxygénation associées sont démontrés comme étant favorables à la production d'espèces réactives de l'oxygène qui sont-elles mêmes délétères pour les tissus (Li and Jackson, 2002) .

D L'inhibiteur des activateurs du plasminogène de type I (PAI-1).

Comme nous l'avons vu, les lésions radio-induites sont associées à une surexpression du TGF-β1. Cependant, la difficulté de mettre en place des stratégies thérapeutiques visant à inhiber complètement ce facteur de croissance et les conséquences multiples possibles dues à son action pléiotropique reste un facteur limitant. C'est pourquoi, nous nous sommes intéressés au rôle d'une de ses cibles, l'inhibiteur des activateurs du plasminogène de type I. Comme nous allons le voir, PAI-1 joue un rôle clef dans l'homéostasie vasculaire par son action anti-fibrinolytique mais est également un facteur pro-fibrosant par sa capacité à limiter la dégradation matricielle.

1. PAI-1 : Généralités.

PAI-1 est une glycoprotéine d'un poids moléculaire de ~50 Kda, appartenant à la famille des « serine protease inhibitors » ou SERPINs. Alors que PAI-1 est très peu exprimé en conditions physiologiques, sa synthèse peut être stimulée dans certaines conditions pathologiques dans différents types cellulaires comme les cellules hépatiques, les cellules musculaires lisses, les adipocytes, les plaquettes, les cellules endothéliales, les cellules tumorales ou encore certaines cellules inflammatoires activées. La fonction majeure de PAI-1 est de s'opposer au système fibrinolytique en inhibant les activateur du plasminogène : « tissue type plasminogen activator » (t-PA) et « urokinase-type plasminogne activator » (u-PA). Comme nous allons le voir, par sa capacité à inhiber la formation de plasmine, PAI-1 joue un rôle déterminant dans l'homéostasie vasculaire en participant au contrôle de la coagulation intravasculaire et du remodelage matriciel. PAI-1 a de multiples rôles et il a été montré comme étant impliqué dans l'adhésion et la migration cellulaire (Czekay and Loskutoff, 2004). Impliqué dans de nombreuses pathologies vasculaires comme la thrombose, l'hyperplasie néo-intimale ou l'athérosclérose, PAI-1 participe également à l'angiogénèse, à la progression tumorale, à la dissémination métastatique, au syndrôme métabolique et aux fibroses (Alessi and Juhan-Vague, 2004; Binder et al., 2002; Kohler and Grant, 2000) ***(Figure 11)***.

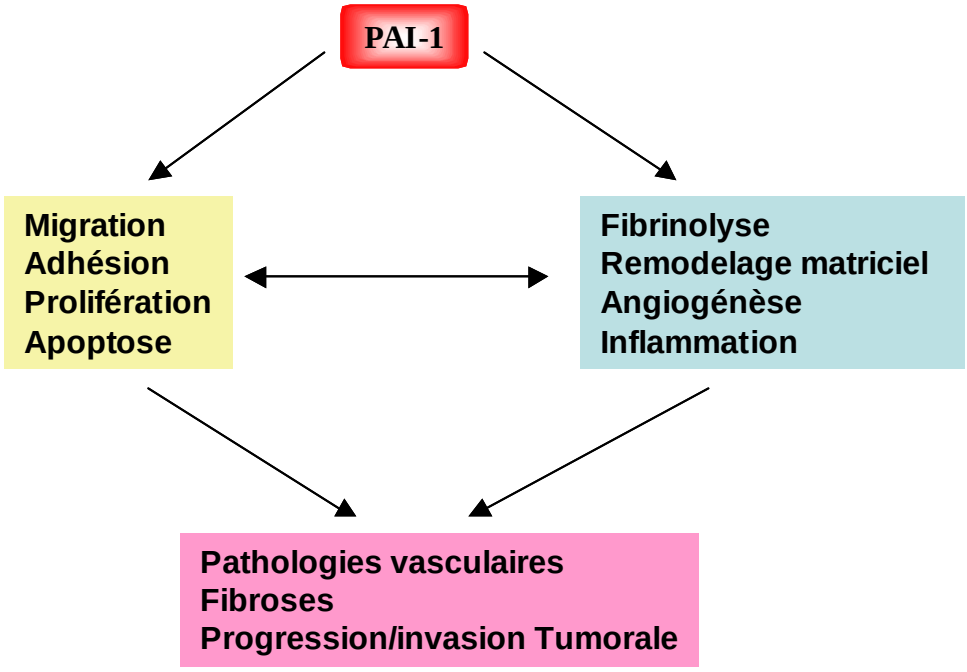

Figure 11 : Rôles de PAI-1 dans les grandes fonctions cellulaires et leurs implications dans les processus physiologiques et physiopathologiques

2. Régulation transcriptionnelle de PAI-1.

L'activité de PAI-1 est fortement régulée au niveau transcriptionnel. La transcription du gène de PAI-1 est activée par de nombreuses cytokines et facteurs de croissance et principalement, le TNFα, l'IL-1 et le TGF-β. D'autre part, l'insuline et le glucose stimulent la synthèse et la sécrétion de PAI-1 *in vitro* dans les cellules endothéliales. Le TGF-β est une cytokine clef capable de réguler la transcription de PAI-1. Des éléments de réponses aux SMADs sont identifiés dans le promoteur de PAI-1 en position –280, -580 et –730 et sont démontrés comme essentiels et suffisants à l'activation transcriptionnelle de PAI-1 dépendante du TGF-β (Dennler et al., 1998). D'autres éléments de réponse ont été identifiés comme impliqués dans la transcription de PAI-1 avec notamment des éléments de réponse à SP1, GRE (glucocorticoide response element), AP1, p53, NF-kB et HIF ***(Figure 12)***. Ainsi Fink et al ont identifié un élément de réponse à HIF en position -194 à -187 nécessaire et suffisant à l'induction transcriptionnelle de PAI-1 dans des cellules tumorales HEPG2 en conditions hypoxiques (Fink et al., 2002). D'autre part, un

polymorphisme correspondant à l'insertion (5G) ou à la délétion (4G) d'une guanine en position -675 du promoteur de PAI-1 a été identifié. Le polymorphisme 4G/4G chez l'homme est associé à des taux plasmatiques élevés de PAI-1 et ces individus ont un risque accru d'infarctus du myocarde comparé aux sujets 4G/5G et homozygotes 5G. De manière intéressante ce polymorphisme correspond à un élément de réponse au facteur de transcription NF-κB (Dawson et al., 1993). *In vitro*, les cellules homozygotes 4G ont une réponse à l'IL-1 via NF-κB beaucoup plus importante que les cellules homozygotes 5G (Dawson et al., 1993). Enfin un élément de réponse à p53 avait été identifié par l'équipe de Kunz en 1995 (Kunz et al., 1995) dont le rôle clef dans l'induction radio-induite de PAI-1 vient d'être démontré dans une lignée tumorale HEPG2 (Hageman et al., 2005).

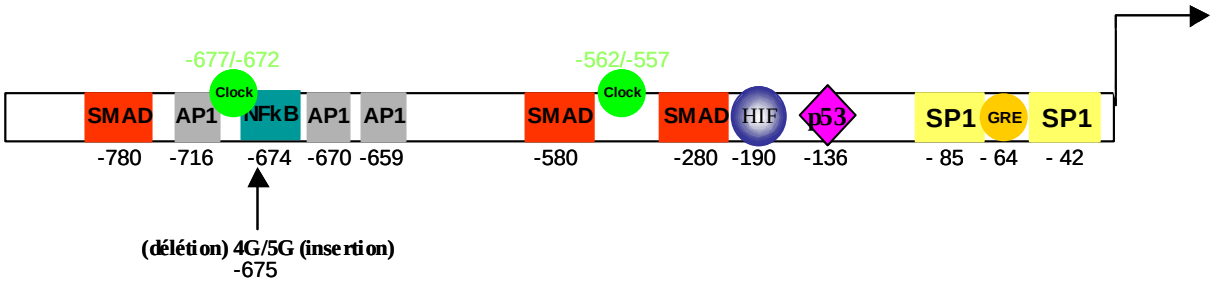

Figure 12 : Représentation schématique du promoteur de PAI-1

3. PAI-1 et la fibrinolyse.

L'hémostase et la coagulation.

Suite à une lésion tissulaire, une cascade d'évènements se déclenche pour stopper la perte du sang en obturant les vaisseaux sanguins lésés. Deux processus complémentaires sont impliqués : tout d'abord l'hémostase qui correspond à une action rapide pour obturer le vaisseau et la coagulation, phénomène plus lent mais plus régulé, permettant de boucher complètement la lésion et de solidifier le caillot formé ***(Figure 13).***

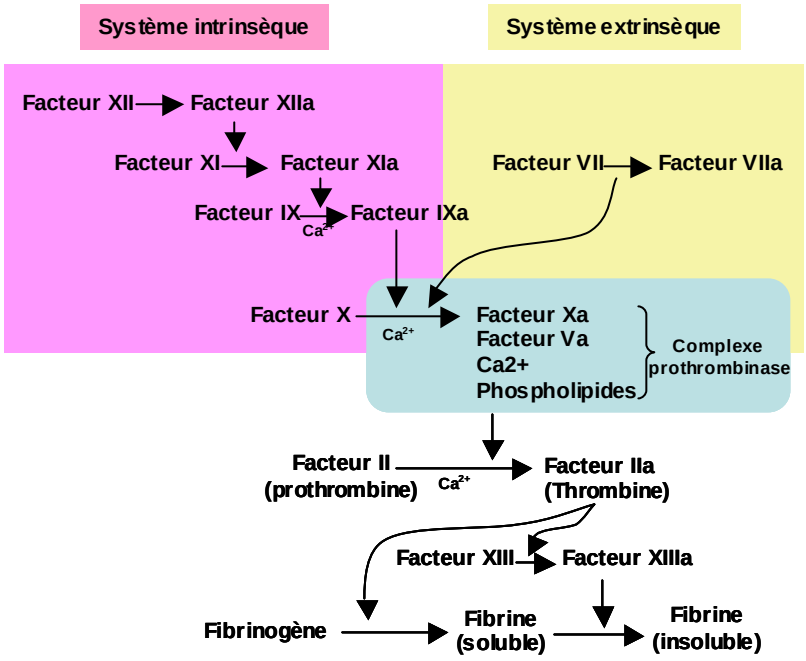

Figure 13 : Mécanisme d'activation du système de coagulation aboutissant à la formation de fibrine
(D'après Kohler HP et Gant PF, N Engl J Med, 2000)

La première réaction suite à la rupture d'un vaisseau est sa vasoconstriction. Ainsi, selon la nature du vaisseau et la pression sanguine, les parois du vaisseau peuvent se coller et limiter ou arrêter le saignement. Le phénomène d'agrégation plaquettaire joue le rôle principal dans le processus d'hémostase. Les plaquettes, au contact de la matrice extracellulaire sous jacente de l'endothélium ou du tissu conjonctif, s'agglutinent contre la lésion. Puis, d'autres plaquettes adhèrent aux précédentes permettant l'obturation complète de la lésion. Cette agrégation plaquettaire ou thrombus blanc stimule la sécrétion de plusieurs substances par les thrombocytes et les cellules vasculaires comme de l'ADP, de la sérotonine, d'un précurseur du thromboxane A2, du fibrinogène et du facteur V. Cette cascade de réactions aboutit à la formation d'un clou plaquettaire permettant de combler la lésion de la paroi vasculaire. Parallèlement à l'hémostase, la coagulation ou hémostase secondaire va permettre la consolidation du clou plaquettaire en mettant en place des cascades d'activation qui vont aboutir à la formation d'un caillot de sang. On distingue deux voies : - une intrinsèque, endogène, impliquant exclusivement des substances d'origine sanguine et qui nécessite le contact du sang avec une substance étrangère - une extrinsèque, exogène, faisant intervenir des facteurs tissulaires. Ces deux voies aboutissent à la formation de thrombine qui va permettre de transformer du fibrinogène plasmatique soluble en fibrine insoluble. La polymérisation de la fibrine va permettre la constitution d'une matrice où sont piégés les plaquettes et les globules rouges (thrombus rouge) et permettre l'arrêt définitif du saignement. Après la cicatrisation, le caillot est dissout par le mécanisme de fibrinolyse ***(Figure 14)***.

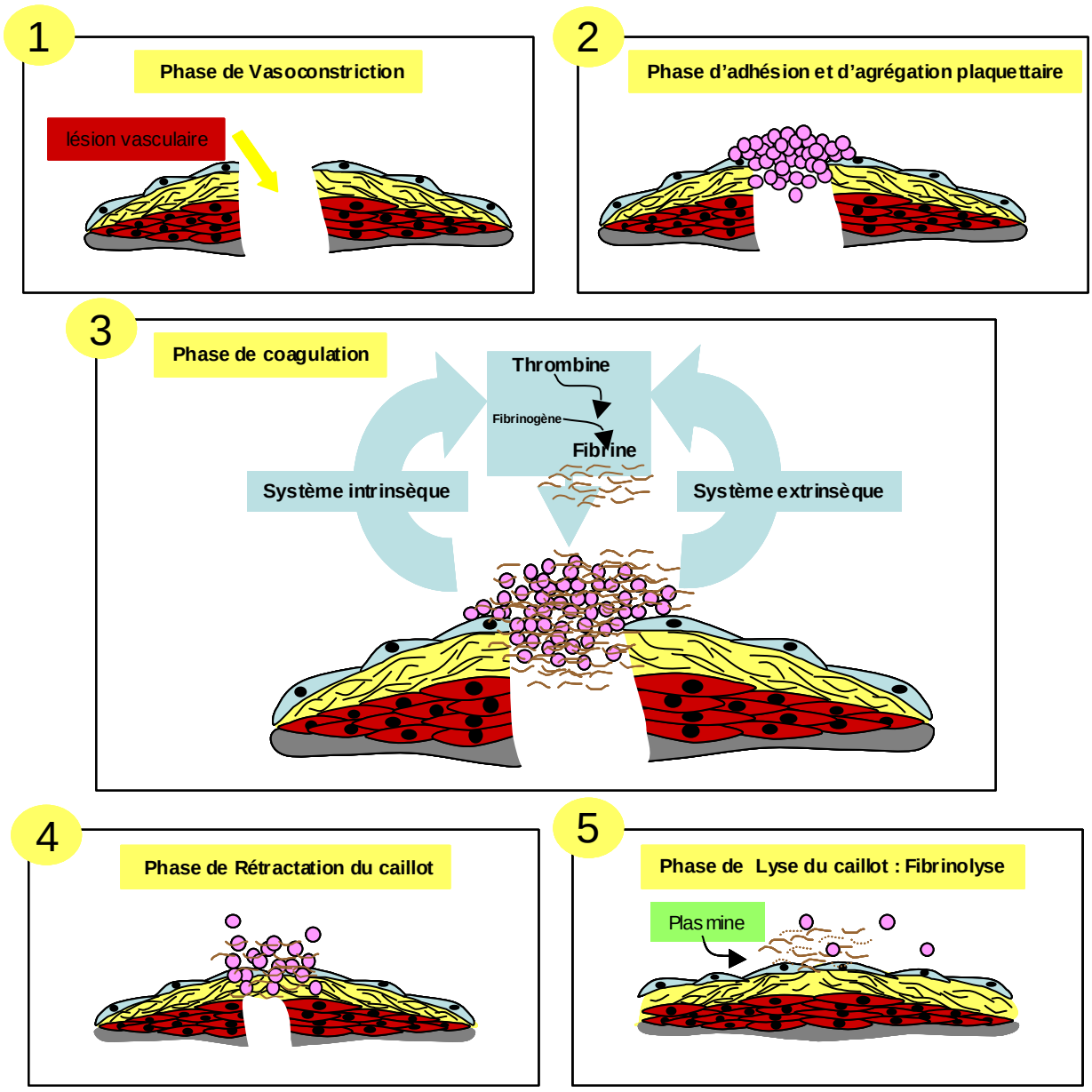

Figure 14 : Représentation du processus de coagulation et de fibrinolyse suite à une lésion vasculaire.

La fibrinolyse.

La fonction majeure de PAI-1 est de s'opposer à l'activation du plasminogène et au système fibrinolytique. Comme nous l'avons vu, la fibrine, issue de la transformation du fibrinogène sous l'action de la thrombine, est un constituant majeur du thrombus et elle est considérée comme un composant majeur des lésions vasculaires. Ainsi, le caillot de fibrine est une matrice propice à l'adhésion des plaquettes et des cellules inflammatoires. L'accumulation de fibrine au niveau de l'endothélium est régulée par une balance fine entre ses mécanismes de synthèse (thrombine dépendants) et ceux de sa dégradation (plasmine dépendants). Enzyme responsable de la

dégradation de la fibrine, la plasmine est synthétisée par le foie sous la forme d'un précurseur inactif, le plasminogène, qui est activé par les activateurs du plasminogène : t-PA et u-PA. L' α2-antiplasmine s'oppose à l'action de la plasmine à l'instar des inhibiteurs des activateurs du plasminogène. Ainsi, PAI-1 par son rôle inhibiteur de u-PA et t-PA limite la formation de plasmine et donc la dégradation de la fibrine **(Figure 15)**. Bien qu'il existe d'autes protéines capables d'inhiber uPA et tPA (PAI-2, neuroserpin), PAI-1 est considéré comme le plus rapide et spécifique inhibiteur des deux activateurs du plasminogène que sont tPA et uPA au niveau vasculaire et tissulaire. PAI-1 est ainsi capable de se lier à tPA et uPA formant un complexe stœchiométrique stable de rapport 1 :1 qui est alors éliminé de la circulation sanguine par le foie. La forme active de PAI-1 est instable avec une demie-vie d'environ 30 minutes qui est très fortement augmentée par sa liaison à la vitronectine.

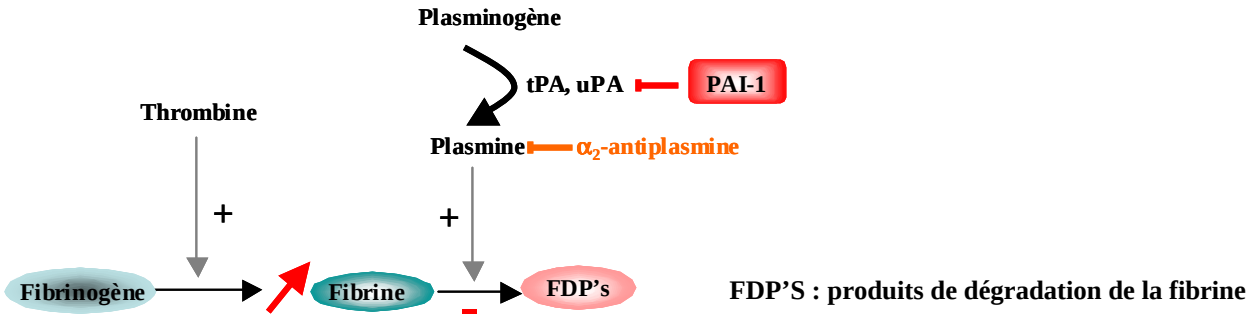

Figure 15 : Rôle de PAI-1 dans le processus de fibrinolyse

4. PAI-1 et les metalloproteinases matricielles.

La protéolyse matricielle est réalisée principalement par les métalloprotéinases matricielles (MMPs) qui sont des enzymes capables de dégrader la plupart des composants de la matrice extracellulaire (Newby, 2005). Cette famille d'enzyme est constituée des collagénases, des gélatinases, des stromélysines et des élastases. La plupart des MMPs sont sécrétées sous forme de pro-enzymes inactives (ou pro-MMP) mais il existe également des formes membranaires appelées membrane-type MMP (MT-MMP). Plusieurs mécanismes moléculaires sont impliqués dans la régulation de l'activité des MMPs. Tout d'abord, par l'action d'inhibiteurs endogènes comme les TIMPs (tissue inhibitor of metalloproteases), capables de se lier au site catalytique des MMPs et d'empêcher ainsi l'accessibilité à leur substrat. L'autre mécanisme clef impliqué dans la modulation de l'activité des MMPs est dépendant de l'action de la plasmine. Les MMPs sécrétées sous la forme

d'un zymogène latent sont activées par une protéolyse conduisant à la libération d'un pro-domaine et permettant l'accessibilité au site actif. La plasmine est capable d'activer la plupart des MMPs (MMP1, 3, 7, 9, 12) et ainsi PAI-1 est un inhibiteur indirect de la dégradation matricielle par sa capacité à limiter l'activation plasmine-dépendante des MMPs ***(Figure 16)***. D'autre part, la plasmine a également une action protéolytique propre sur différentes protéines matricielles comme la fibronectine, la laminine ou le collagène IV et est également capable d'inhiber les TIMPs. En résumé, PAI-1 en limitant la formation de plasmine joue un rôle pivot entre le système fibrinolytique et le système des metalloprotéinases.

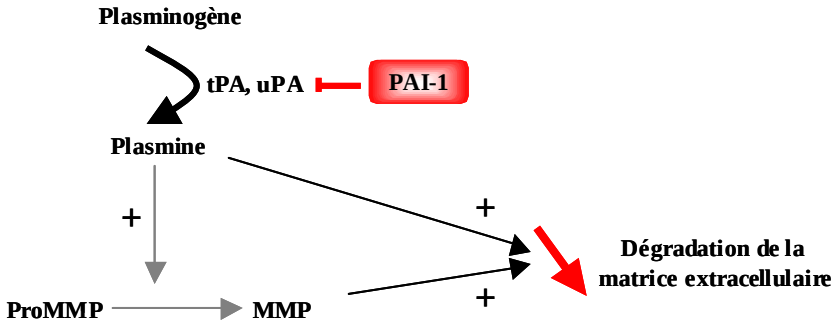

Figure 16 : Rôle de PAI-1 dans le processus de remodelage matriciel *via* l'activation des MMPs dépendante de la plasmine

5. PAI-1 et les pathologies vasculaires.

Un taux plasmatique élevé de PAI-1 chez l'homme est positivement corrélé au risque de développement de maladies cardio-vasculaires comme la sclérose vasculaire, la resténose, l'infarctus du myocarde et l'athérosclérose (Hamsten et al., 1987; Juhan-Vague et al., 1996; Kohler and Grant, 2000). D'autre part, il a été montré une association entre l'infarctus du myocarde et une augmentation du taux plasmatique de PAI-1. Ainsi, une augmentation importante du taux de PAI-1 24h après l'admission est observée chez les patients qui décèdent comparés à ceux qui survivent (Collet et al., 2003). De plus, des augmentations localisées de PAI-1 dans les plaques d'athéromes ont été décrites chez l'homme (Chomiki et al., 1994; Schneiderman et al., 1992) ainsi que dans la paroi artérielle de patients diabétiques (Pandolfi et al., 2001; Sobel et al., 1998).

Associé au risque de développer des pathologies cardiovasculaires, le syndrome métabolique est un ensemble de dysfonctions métaboliques prédisposant au développement et à la progression de l'athérosclérose. L'obésité viscérale, la résistance périphérique à l'insuline, l'hyperinsulinémie, les dyslipidémies (augmentation des VLDL et baisse des HDL) sont les principales caractéristiques du syndrome métabolique et les patients souffrant de ce syndrome ont un risque de développer

ultérieurement un diabète de type II (Despres and Lemieux, 2006). Le lien entre PAI-1 et le syndrome métabolique est établi avec une corrélation entre la sévérité du syndrome métabolique et des taux plasmatiques élevés de PAI-1 (Alessi and Juhan-Vague, 2006; Juhan-Vague and Alessi, 1997; Juhan-Vague et al., 1991).

De part son action anti-fibrinolytique déterminante, le rôle de PAI-1 dans le phénomène thrombotique a clairement été identifié. Une première étude avait montré que des taux élevés de PAI-1 contribuent au développement de thromboses veineuses en proportion avec le nombre de copies du gène de PAI-1 chez la souris (Erickson et al., 1990). Depuis, de nombreuses études expérimentales ont confirmé que PAI-1 favorise la thrombose vasculaire (Eitzman et al., 2000; Konstantinides et al., 2001). Récemment, Brogen *et al*, ont montré que les plaquettes synthétisent des quantités importantes de PAI-1 actif (Brogren et al., 2004). Ce pool plaquettaire de PAI-1 pourrait contribuer à la stabilisation du caillot sanguin. D'autre part, l'utilisation de souris chez lesquelles le gène de PAI-1 a été invalidé (Knockout PAI-1 ou PAI-1 -/-) (Carmeliet et al., 1993) a permis des avancées importantes dans la compréhension du rôle de cette protéine dans de nombreuses pathologies et mécanismes physiologiques et physiopathologiques dont la thrombose. Ainsi, la thrombose induite par une injection de Chlorure de fer (Farrehi et al., 1998) ou après injection d'endotoxine (Carmeliet et al., 1993) est réduite chez les souris PAI-1 -/- comparées aux souris sauvages. D'autre part, il a été montré que des souris transgéniques surexprimant de manière stable le gène humain de PAI-1 sous le contrôle du promoteur murin de la preendothéline-1 développent spontanément des thromboses artérielles coronaires conduisant à l'occlusion vasculaire (Eren et al., 2002). De plus, des résultats intéressants ont été publiés grâce au croisement de souris ApoE -/- qui développent spontanément de l'athérosclérose avec des souris PAI-1 -/-. Suite à une injection de Chlorure de fer, les souris ApoE-/-PAI-1-/- présentent un phénotype pro-thrombotique et des lésions d'hyperplasie néointimale moins sévères que les souris ApoE-/-PAI-1+/+ (Schafer et al., 2003; Zhu et al., 2001). Cependant, les études publiées sur le rôle de PAI-1 dans les lésions d'hyperplasie néointimale et l'athérosclérose sont contradictoires. Par exemple, par l'utilisation de souris ApoE -/- ou LDLr -/- croisées avec des souris PAI-1 -/- ou des souris transgéniques surexprimant PAI-1, Sjoland et al ont montré que, suite à un régime athérogène, la progression des lésions athérosclerotiques chez les souris prédisposées était indépendante du statut génétique de PAI-1 (Sjoland et al., 2000). D'autre part, l'équipe de Carmeliet a montré que les souris ApoE-/-PAI-1-/- présentaient des plaques plus importantes que les souris ApoE-/-PAI-1-/+ et ApoE-/-PAI-1+/+ suggérant un rôle athéroprotecteur de PAI-1. La même équipe avait déjà démontré par transfert de gène (adénovirus PAI-1) que, suite à une lésion vasculaire électrique ou mécanique, la déficience en PAI-1 accélère la cicatrisation vasculaire associée à des lésions néointimales avec

notamment une augmentation de la migration des CML vasculaires (Carmeliet et al., 1997). Le transfert de gène limite la migration des CML vasculaires et en conséquence la formation des lésions neointimales et la sténose luminale. L'ensemble des résultats contradictoires de la littérature démontre le rôle complexe que joue PAI-1 en fonction du modèle, du stress initial et de son rôle anti-fibrinolytique ou dans la migration cellulaire.

6. PAI-1 et la fibrose.

Comme mentionné précédemment, du fait de l'importance évidente de cette protéine dans ces pathologies vasculaires, un modèle de souris PAI-1 -/- a été généré en 1993 par P. Carmeliet (Carmeliet et al., 1993). Ces souris ont également été largement utilisées pour l'étude des pathologies fibrotiques. Ainsi, les souris knockout pour PAI-1 sont protégées contre l'apparition de certaines fibroses pulmonaires, rénales ou vasculaires après différents stress (chimiques ou mécaniques). Les souris PAI-1 -/- sont protégées de la fibrose pulmonaire induite par la bléomycine (Eitzman et al., 1996; Hattori et al., 2000) alors que les souris sur-exprimant PAI-1 développent une fibrose plus importante que les souris sauvages (Eitzman et al., 1996). Récemment, dans un modèle de stéatose hépatique induite par l'alcool, les souris PAI-1 -/- développent une stéatose moins sévère que les souris sauvages avec moins de nécrose, moins d'inflammation et moins d'accumulation de dépôt lipidique (Bergheim et al., 2006). D'autre part, la fibrose cardiaque est atténuée chez les souris PAI-1 -/- dans un modèle d'atteinte du myocarde après ligature de l'artère coronaire gauche (Takeshita et al., 2004). De la même manière, les souris déficientes en PAI-1 sont protégées de l'hypertension et de la fibrose vasculaire dans un modèle d'inhibition chronique de production de NO par ingestion dans l'eau de boisson d'un inhibiteur de NO-Synthase, le *N* $^{\omega}$-nitro L-arginine methyl ester (L-NAME) (Kaikita et al., 2001). De manière intéressante, le NO° est capable de réguler la signalisation de TGF-β dans les cellules endothéliales. Ainsi, les aortes de souris eNOS -/- présentent des taux de base augmentés de TGF-β1 et de collagène I associés à une augmentation de la phosphorylation des SMADs et de l'activité transcriptionelle SMAD-dépendante dans les cellules endothéliales (Saura et al., 2005).

Dans des pathologies rénales et notamment les fibroses rénales, le rôle de PAI-1 est largement documenté (Eddy and Fogo, 2006). Alors que PAI-1 n'est virtuellement pas exprimé ou très peu par un rein normal, de nombreuses pathologies humaines rénales sont associées à des surexpressions rénales de PAI-1 comme les néphropathies diabétiques (Paueksakon et al., 2002) les glomerulonéphrites (Hamano et al., 2002), les scléroses glomérulaires ou artérielles (Yamamoto et al., 1996) ou encore les micro-angiopathies thrombotiques (Xu et al., 1996). Au niveau

expérimental, les différents modèles animaux permettant de modéliser ces pathologies sont associés à des surexpressions rénales de PAI-1. De plus, l'utilisation des souris PAI-1 -/- dans ces modèles expérimentaux montre que la déficience en PAI-1 protège contre différentes lésions fibrotiques rénales comme la fibrose tubulo- interstitielle après obstruction urétérale (Oda et al., 2001) ou encore de la glomérulosclérose au cours d'une glomérulonéphrite anti-Thy-1 (Huang et al., 2003). Seule une étude a montré une sévérité plus importante des lésions fibrotiques chez les souris PAI-1 -/- comparées aux souris sauvages dans un modèle de glomérulonéphrite anti-membrane basale glomérulaire (Hertig et al., 2003). Enfin, de manière intéressante, les effets néphroprotecteurs des anti-hypertenseurs utilisés en clinique dans le traitement de nombreuses néphropathies comme les inhibiteurs de l'enzyme de conversion de l'angiotensine II ou les antagonistes des récepteurs à l'angiotensine II ont été montrés comme étant capables d'inhiber PAI-1 à la fois chez l'homme (Pahor et al., 2002) et dans des modèles expérimentaux (Ma et al., 2005; Oikawa et al., 1997). Enfin, l'inhibition de l'expression de PAI-1 fait partie des nombreux effets « non hypolipidémiants » des statines (Bourcier and Libby, 2000; Sakabe et al., 2004).

7. PAI-1 et la progression tumorale.

Le rôle de PAI-1 dans la progression tumorale a été activement étudié durant ces dix dernières années. En effet, plusieurs études cliniques montrent que l'expression de PAI-1 est un facteur de mauvais pronostic dans différents types de cancer comme les cancers du sein (Grondahl-Hansen et al., 1993), du poumon (Pappot et al., 2006), de l'œsophage (Sakakibara et al., 2004), de l'ovaire (Chambers et al., 1998), de la tête et du cou (Chin et al., 2005) ou encore les cancers gastriques (Heiss et al., 1995). D'autre part, des études expérimentales montrent que PAI-1 joue un rôle déterminant dans la progression tumorale *in vivo* chez la souris. L'implantation d'une lignée de kératinocytes malins (PVDA) chez des souris déficientes en PAI-1 a montré une très forte réduction de l'invasion et de l'angiogénèse tumorales comparée à la greffe effectuée sur des souris sauvages (Bajou et al., 1998). D'autre part, l'injection d'un adénovirus exprimant PAI-1 chez les souris PAI-1 -/- permet de restaurer l'invasion et l'angiogénèse tumorales. Une autre étude similaire a permis de confirmer ces résultats avec une inhibition totale de progression tumorale chez les souris PAI-1 -/- greffées avec un fibrosarcome murin (T241) (Gutierrez et al., 2000). Cependant, des résultats contradictoires montrent que la déficience ou la surexpression de PAI-1 chez des souris transgéniques ne modifie pas la croissance tumorale ou l'invasion métastatique pulmonaire après inoculation d'un mélanome murin B16 (Eitzman et al., 1996). Enfin, il a été montré que des cellules tumorales de prostate sur-exprimant PAI-1 ont une capacité métastatique réduite ainsi qu'une

réduction de la croissance tumorale et de l'angiogénèse tumorale associée (Soff et al., 1995). L'ensemble des données contradictoires de la littérature démontre le rôle complexe que joue PAI-1 dans la progression tumorale et met en lumière l'importance du taux d'expression de PAI-1 à la fois par les cellules de l'hôte mais également par les cellules tumorales. D'autre part, les relations étroites entre l'action de PAI-1 sur uPA et leurs mécanismes d'action dans la migration, l'invasion et la dissémination métastatique restent encore à éclaircir. Enfin, c'est vraisemblablement par son rôle pro et anti angiogénique dose dépendant (Devy et al., 2002) et par les grandes différences de modèles tumoraux utilisés que l'on peut expliquer les résultats contradictoires de la littérature. Cependant, bien que les mécanismes moléculaires précis liés à l'implication de PAI-1 dans la progression tumorale restent encore à élucider, les données expérimentales et les résultats cliniques suggèrent fortement que cette protéine pourrait être une cible potentielle de stratégie thérapeutique anti-cancéreuse.

8. PAI-1 et les rayonnements ionisants.

Le rôle de PAI-1 dans les dommages tissulaires radio-induits a été très peu étudié. Cependant, sans le démontrer, quelques résultats suggèrent un rôle clef de cette protéine dans les atteintes radio-induites. Ainsi, *in vivo*, dans un modèle de néphropathie radio-induite à 12 Gy chez le rat, une augmentation du taux d'ARNm de PAI-1 a été décrite (Brown et al., 2000; Oikawa et al., 1997). Dans ce même modèle, un traitement par des inhibiteurs de l'enzyme de conversion de l'angiotensine ou par des antagonistes du récepteur à l'angiotensine II a montré un bénéfice sur les dommages radio-induits concomitant à une atténuation de l'expression de PAI-1 (Brown et al., 2000; Oikawa et al., 1997). D'autre part, après une irradiation abdominale de 10 Gy chez le rat, une augmentation de l'expression de PAI-1 au niveau de l'intestin (Strup-Perrot et al., 2005) et du côlon (Strup-Perrot et al., 2006) est observée 3 jours après irradiation. Enfin, il a été montré une augmentation de l'expression de PAI-1 par une approche globale par cDNA array sur des tissus de patients souffrant d'entérite radique (Vozenin-Brotons et al., 2004)

In vitro, l'irradiation de cellules mésangiales de rats entraîne une augmentation dose-dépendante (de 0,5 à 20 Gy) du taux d'ARNm de PAI-1 (Zhao et al., 1999). D'autre part, l'équipe de Robbins a démontré *in vitro* que l'irradiation d'une lignée de cellules épithéliales rénales de rat (NRK52E) stimule l'expression de PAI-1 proportionnellement à l'augmentation de production d'espèces réactives de l'oxygène (ROS) et que cette surexpression est inhibée en présence d'un anti-radicalaire comme le N-acetylcystéine (NAC) (Zhao et al., 2000; Zhao et al., 2001). D'autre

part, la surexpression de la catalase par une stratégie adénovirale supprime, *in vitro*, la surexpression radio-induite de PAI-1 dans les cellules NRK52E (Zhao et al., 2001).

Enfin récemment le mécanisme de surexpression de PAI-1 après irradiation a été étudié dans une lignée tumorale. Ce travail montre une synergie entre la voie du TGF-β et la protéine p53 dans la régulation transcriptionnelle de PAI-1 après irradiation (Hageman et al., 2005). De plus, par des techniques de gene reporter et des modifications par mutagénèse dirigée de l'élément de réponse putatif à p53 présent dans le promoteur de PAI-1, les auteurs ont montré que cet élément de réponse est nécessaire à l'induction radio-induite de PAI-1 dans une lignée tumorale HEPG2.

9. Rôles potentiels de PAI-1 dans les dommages radio-induits.

Comme nous l'avons vu, PAI-1 joue un rôle pivot dans les mécanismes de fibrinolyse et de remodelage de la matrice extracellulaire. De manière intéressante, des modifications de l'activité des MMPs dans les dommages radio-induits ont été décrites. Ainsi, chez des patients souffrant d'entérite radique, une stimulation à la fois des acteurs impliqués dans la synthèse de matrice et ceux impliqués dans sa dégradation a été observée (Strup-Perrot et al., 2004). Cependant, l'équilibre synthèse /dégradation penche en faveur d'une accumulation de matrice extracellulaire. De la même manière, chez des patients traités pour un cancer de la prostate, une augmentation de l'activité de dégradation matricielle et plus précisément des activités gélatinolytiques des MMP 2 et 9 a été décrite dans la muqueuse rectale présente dans le champ d'irradiation (Hovdenak et al., 2002). Ces résultats montrent que les processus de synthèse et remodelage matriciel sont régulés après irradiation et suggèrent qu'ils contribuent à la réponse tissulaire aux rayonnements ionisants. Les mécanismes moléculaires ne sont pas connus et l'on peut supposer que PAI-1 pourrait avoir un rôle déterminant dans ce processus ***(Figure 17)***. D'autre part, comme nous l'avons vu, l'irradiation induit un changement phénotypique de l'endothélium avec notamment la perte de thromborésistance due à la fois à l'activation du système de coagulation mais également à une diminution de la fibrinolyse (Hauer-Jensen et al., 2004). Enfin, le fait que les souris génétiquement déficientes en PAI-1 soient protégées contre l'apparition de fibroses dans divers modèles nous a permis d'émettre l'hypothèse que cette protéine pouvait avoir une implication active dans les dommages aux tissus sains après irradiation. Un des objectifs de ce travail de thèse a été de savoir si PAI-1 joue un rôle déterminant dans les dommages radio-induits et d'apporter la preuve de principe qu'il pourrait constituer une cible thérapeutique potentielle.

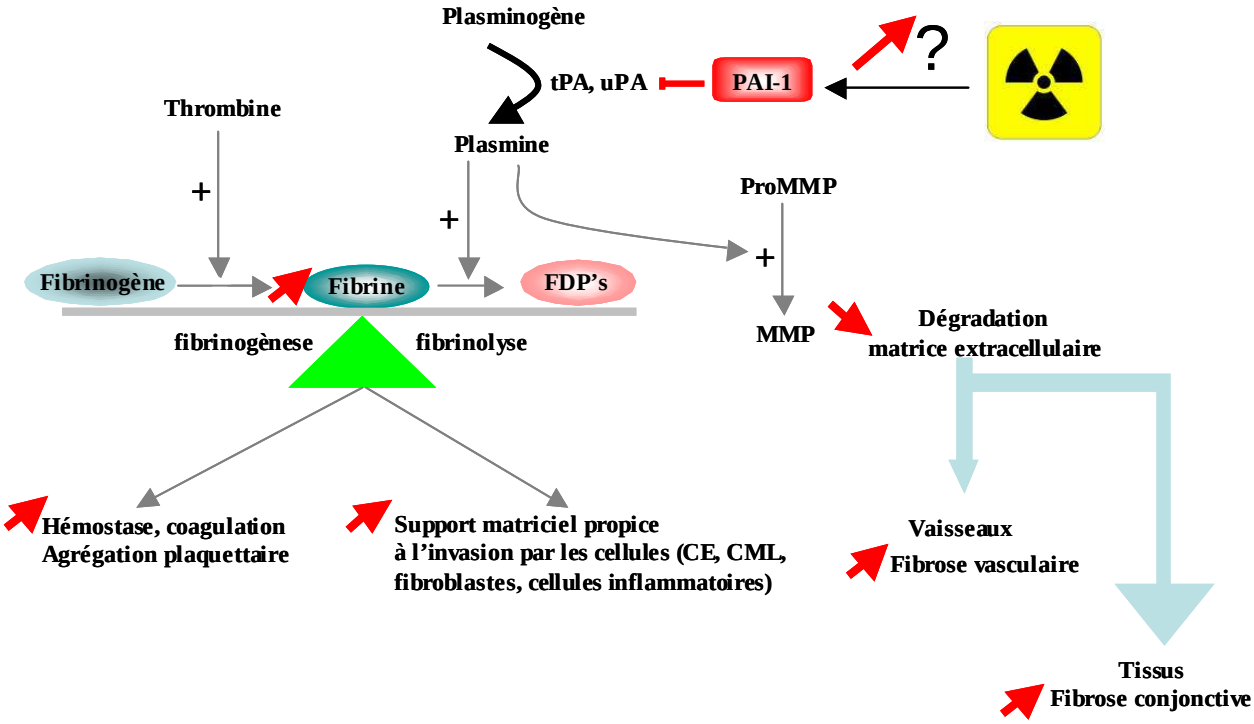

Figure 17 : Conséquences potentielles d'une modification de l'expression de PAI-1 (ex : cas d'une surexpression) sur les mécanismes impliqués dans les dommages radio-induits aux tissus sains

II Objectifs et Résultats

Objectifs de la thèse :

Ce travail de thèse repose sur le concept qu'un événement initial provoqué au niveau de l'endothélium suite à un stress par des rayonnements ionisants va contribuer à l'initiation et à la progression des lésions radio-induites. Dans ce contexte, deux questions ont été abordées :

- **La première question concerne la mise en évidence d'un dialogue entre les cellules endothéliales et les cellules musculaires lisses vasculaires**. Le premier objectif a été de caractériser de manière précise les lésions vasculaires radio-induites chez l'homme. Pour cela une étude rétrospective a été entreprise sur des prélèvements de rectum de patients traités par radiothérapie pré-opératoire dans le cadre d'un traitement d'un adénocarcinome du rectum. Dans un second temps, des modèles de co-culture ont été mis en place afin de savoir si les cellules endothéliales pouvaient influencer le phénotype des cellules musculaires lisses vasculaires. Enfin, les mécanismes moléculaires impliqués dans les signaux émis par les cellules endothéliales et influençant le phénotype des cellules musculaires lisses vasculaires ont été étudiés.

- **La seconde question concerne le rôle de PAI-1 dans les atteintes tissulaires radio-induites**. Bien que quelques résultats de la littérature suggèrent que PAI-1 pourrait jouer un rôle dans les atteintes radio-induites, aucune démonstration ne prouve qu'il ait un rôle déterminant. Comme nous l'avons vu, la synthèse de PAI-1 peut-être stimulée dans certaines conditions pathologiques. Il joue un rôle clef dans de nombreux processus comme le contrôle de la fibrinolyse ou encore le remodelage de la matrice extracellulaire. D'autre part le TGF-β1, facteur de croissance déterminant dans les dommages radio-induits, est capable de stimuler l'expression de PAI-1. Ainsi, partant du fait que la fibrinolyse et le remodelage matriciel participent à l'initiation et la progression des dommages radio-induits et que le TGF-β est un puissant stimulateur de PAI-1, nous avons voulu déterminer si PAI-1 joue un rôle dans les lésions radio-induites. Pour répondre à cette question, un modèle d'entéropathie radio-induite chez la souris a été mis en place et nous avons étudié la réponse tissulaire de souris génétiquement déficientes en PAI-1 et de souris sauvages. Parallèlement, des marquages immnunohistochimiques de PAI-1 ont été réalisés sur des tissus de patients traités par radiothérapie. Enfin, la compréhension des mécanismes moléculaires impliqués dans la régulation l'expression de PAI-1 dans des cellules endothéliales après irradiation a été entreprise.

Résumé Article 1 : Influence des cellules endothéliales sur le phénotype des cellules musculaires lisses après irradiation. Implication dans les dommages vasculaires radio-induits.

Objectifs

La compréhension des mécanismes physiopathologiques et moléculaires impliqués dans la pathogenèse des dommages radio-induits est déterminante dans l'optique de mettre en place des stratégies thérapeutiques visant à protéger les tissus sains afin d'optimiser les traitements des cancers par radiothérapie. Les lésions vasculaires sont des complications sévères observées au niveau des tissus sains après radiothérapie. L'irradiation des vaisseaux sanguins compromet les fonctions essentielles de la vascularisation nécessaires au maintien de l'homéostasie tissulaire. Les mécanismes précis impliqués dans les différentes phases du développement des lésions radio-induites restent flous. Les dommages vasculaires pourraient être impliqués dans l'initiation, accélérer et/ou exacerber les atteintes tissulaires radio-induites. Si l'activation radio-induite des cellules endothéliales est relativement bien décrite, très peu de données existent sur les modifications des relations entre cellules endothéliales et cellules musculaires lisses vasculaires après irradiation. L'hypothèse de ce travail est que les lésions vasculaires radio-induites pourraient résulter des dysfonctionnements des relations entre les cellules endothéliales (CE) et les cellules musculaires lisses vasculaires (CMLV).

Résultats

Dans un premier temps, une caractérisation des lésions vasculaires sur des résections de rectums irradiés à 45Gy et prélevés 4 à 6 semaines après la fin de la radiothérapie (38 patients) a été réalisée. Cette étude chez les patients a permis de caractériser de manière précise les lésions vasculaires radio-induites i.e. épaississement des vaisseaux, hypertrophie, dystrophie, fibrose péri-vasculaire, et hyperplasie néo-intimale. D'autre part, à l'aide de marquages immuno- histochimiques (α-sma, calponin, PCNA, Collagènes I et II) nous avons démontré l'importance de la prolifération, de la migration et de la différenciation fibrogénique des CMLV dans les lésions vasculaires radio-induites chez l'homme.

Dans un second temps, des expériences de co-culture ont été réalisées afin de déterminer si l'irradiation de cellules endothéliales pouvait entraîner la libération de facteurs solubles capables de modifier le phénotype de cellules musculaires lisses vasculaires humaines. La prolifération, la

migration et la différenciation ont été étudiées dans les CMLV en présence de CE (irradiation des CE ou des deux types cellulaires). D'autre part, les mécanismes moléculaires impliqués dans la différenciation fibrogénique des CMLV ont été étudiés.

En présence de CE irradiées, la prolifération des CMLV est stimulée ainsi que leur capacité à migrer dans une zone lésée. Par RT- PCR en temps réel et western blot nous avons montré que les taux des ARNm et des protéines CTGF, PAI-1, COL1A2 et COL3A1 sont stimulés dans les CMLV après exposition avec les CE irradiées. La sécrétion du TGF-β1 est stimulée dans les CE irradiées. De plus, les résultats de marquages immunocytochimiques (SMAD3 et SMAD4) et des expériences de « gene reporter dual luciferase» (Transfection plasmide CAGAX9-Luciférase) montrent que l'irradiation des CE entraîne la translocation nucléaire des SMADs et stimule la transcription SMAD-dépendante dans les CMLV. D'autre part, dans les CMLV transfectées avec des siRNA SMAD3 ou incubées avec un anticorps neutralisant anti-TGFβ-RII, le phénotype fibrogénique des CMLV induit par les cellules endothéliales irradiées est inhibé. Ces résultats montrent que les CE stimulent, par des effets paracrines, la différenciation fibrogénique des CMLV après irradiation par un mécanisme TGFβ/SMAD3 dépendant. Enfin, des marquages immuno-histochimiques chez les patients montrent que les lésions vasculaires radio-induites sont associées à une augmentation de l'immunoréactivité du TGF-β dans l'endothélium et de la forme phosphoryllée des SMADs 2 et 3 dans les CMLV. Ces résultats *in vivo* viennent appuyer la pertinence des résultats obtenus *in vitro*.

Conclusion

L'ensemble de ces résultats montre que les CE stimulent la prolifération, la migration et la différenciation fibrogénique des CMLV après irradiation. La voie TGFβ/SMAD est impliquée dans les mécanismes moléculaires liés aux modifications phénotypiques observées.

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Vascular Biology, Atherosclerosis and Endothelium Biology

Influence of Endothelial Cells on Vascular Smooth Muscle Cells Phenotype after Irradiation

Implication in Radiation-Induced Vascular Damages

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Damage to vessels is one of the most common effects of therapeutic irradiation on normal tissues. We undertook a study in patients treated with preoperative radiotherapy and demonstrated *in vivo* the importance of proliferation, migration, and fibrogenic phenotype of vascular smooth muscle cells (VSMCs) in radiation-induced vascular damage. These lesions may result from imbalance in the cross talk between endothelial cells (ECs) and VSMCs. Using co-culture models, we examined whether ECs influence proliferation, migration, and fibrogenic phenotype of VSMCs. In the presence of irradiated ECs, proliferation and migration of VSMCs were increased. Moreover, expressions of α-smooth muscle actin, connective tissue growth factor, plasminogen activator inhibitor type 1, heat shock protein 27, and collagen type III, alpha 1 were up-regulated in VSMCs exposed to irradiated ECs. Secretion of transforming growth factor (TGF)-β1 was increased after irradiation of ECs, and irradiated ECs activated the Smad pathway in VSMCs by inducing Smad3/4 nuclear translocation and Smad-dependent promoter activation. Using small interferring RNA targeting Smad3 and a TGFβ-RII neutralizing antibody, we demonstrate that a TGF-β1/TGF-β-RII/Smad3 pathway is involved in the fibrogenic phenotype of VSMCs induced by irradiated ECs. In conclusion, we show the importance of proliferation, migration, and fibrogenic phenotype of VSMCs in patients. Moreover, we demonstrate *in vitro* that ECs influence these fundamental mechanisms involved in radiation-induced vascular damages. ***(Am J Pathol 2006, 169:1484–1495; DOI: 10.2353/ajpath.2006.060116)***

About half of people with cancer are treated with radiation therapy either alone or in combination with other types of cancer treatments. However, normal tissue toxicity still remains a dose-limiting factor in clinical radiation therapy.[1] Vascular injury is one of the most common effects of radiotherapy on normal tissues. Damage to blood vessels and subsequent hypoxia and ischemia are known to contribute to severe tissue injury such as fibrosis and/or necrosis. Vascular fibrosis after radiotherapy contributes to severe normal tissue damage and, in some cases, may be a vital prognosis in patients.[2]

The endothelium is known to play a critical role in radiation-induced vascular injury. Irradiated endothelial cells (ECs) acquire a proinflammatory, procoagulant, and prothrombotic phenotype. Up-regulation of endothelial cell adhesion molecules such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1,[3,4] P-selectin,[5] and platelet-endothelial cell adhesion molecule-1[6] after irradiation leads to the increase of leukocyte/EC interactions and leukocyte transmigration. Moreover, irradiation increases the interactions of platelets with the endothelium[7]and decreases expression of the anticoagulant thrombomodulin.[8]

If radiation-induced EC activation and changes in the physiological properties of the vascular endothelium have been well documented, less is known about

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Table 1. Semiquantitative Histopathological Scoring System

Mucosa	Normal: 0
	Moderately abnormal with slight inflammation: 1
	Markedly abnormal with strong inflammation: 2
	Severely abnormal with severe inflammation and loss of epithelium (ulceration): 3
Muscularis mucosa	Normal: 0
	Markedly abnormal with slight matrix deposition and/or slight dystrophy: 1
	Severely abnormal with strong matrix deposition and/or severe dystrophy: 2
Submucosa	Normal: 0
	Moderately abnormal with inflammation: 1
	Moderately abnormal with edema: 1
	Markedly abnormal with slight matrix deposition: 2
	Severely abnormal with fibrosis: 3
Submucosal vessel	Normal: 0
	Markedly abnormal with slight matrix deposition and/or slight dystrophy: 1
	Severely abnormal with strong matrix deposition and/or severe dystrophy: 2
Muscularis propria	Normal: 0
	Markedly abnormal with slight matrix deposition and/or dystrophy: 1
	Severely abnormal with strong matrix deposition and/or dystrophy: 2
Mesentery	Normal: 0
	Markedly abnormal with slight matrix deposition: 1
	Severely abnormal with fibrosis: 2
Mesenteric vessel	Normal: 0
	Markedly abnormal with slight matrix deposition and/or slight dystrophy: 1
	Severely abnormal with strong matrix deposition and/or severe dystrophy: 2
Serosa	Normal: 0
	Markedly abnormal with slight matrix deposition: 1
	Severely abnormal with fibrosis: 2

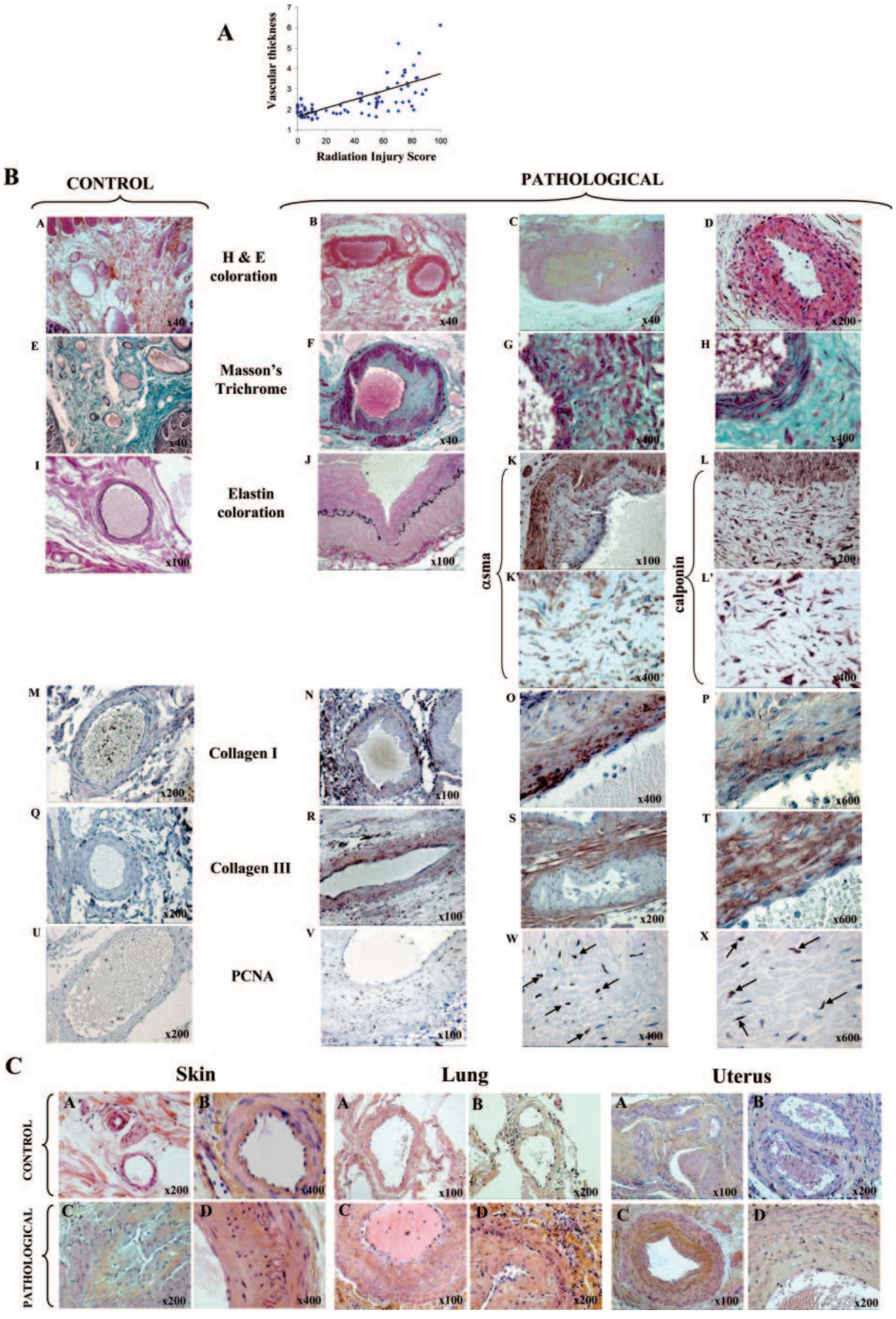
Histopathologic scoring of radiation injury in each compartment (mucosa, muscularis mucosa, submucosa, muscularis propria, serosa, and mesentery, as well as submucosal and mesenteric vessels) were assessed independently by two authors in a blinded manner. The individual abnormalities were assessed as normal (score = 0) or abnormal, graded according to severity (0 to 2 or 0 to 3). Finally, for every slide, the sum of the scores for each parameter in all compartments (the retrieved sum to 100) constitutes the radiation injury score.

dysfunction of the entire vessel wall. Cell-cell communications play a fundamental role in vascular remodeling after injury. ECs, inflammatory cells, and vascular smooth muscle cells (VSMCs) are involved in the pathogenesis of vascular diseases. The interactions between ECs and VSMCs are known to play a key role in the structure and function of the vessel. VSMC migration, proliferation, and differentiation are critical processes involved in vascular injury observed in vascular pathologies such as atherosclerosis,[9] intimal hyperplasia, and hypertension. If there is still debate as to whether or not radiation-induced vascular lesions are similar to those cited above, parallels may be drawn to offer some clues to the comprehension of vascular radiation damage. It has been shown that fibrogenic cytokines and growth factors are involved in mechanisms of vascular fibrosis, ie, migration and proliferation of smooth muscle cells, the increase of collagen expression, and the alteration of matrix remodeling. However, molecular mechanisms involved in radiation-induced vascular fibrosis are still unclear and may also result from imbalance in the cross talk between ECs and VSMCs. First, we performed a retrospective study in patients treated with preoperative radiotherapy for rectal cancer to analyze radiation-induced vascular damages. The second purpose of our work was to study, in an EC-VSMC co-culture model, the influence of paracrine factors released by ECs on VSMC proliferation, migration, and fibrogenic phenotype after irradiation. Moreover, molecular mechanisms involved in the fibrogenic phenotype of VSMCs in the presence of ECs were investigated.

Materials and Methods

Patients, Radiation Injury Score, Morphometric Analyses, and Immunohistology

Thirty-eight patients treated for rectal adenocarcinoma with preoperative radiotherapy (45 Gy; 2 or 1.8 Gy by fraction) were included in this study. Tumors were surgically resected 5 to 7 weeks after treatment. For each patient, specimens of normal tissue were taken in the irradiated field adjacent to the tumor and from microscopically normal mucosa distant from the tumor. Slides were colored by Meyer's hemalum, Masson's trichrome, and elastin coloration (Varehoeff-Van Gieson). Radiation injury scores determined by a pathologist (J.-C.S.) were measured in all patients (at least two slides per patient, normal and pathological). We first identified the distinct compartments in each slide, ie, mucosa, muscularis mucosa, submucosa, muscularis propria, serosa, and mesentery. Then, we scored in these different compartments several alterations that contribute to the global features of radiation-induced intestinal damages. The individual abnormalities were assessed as normal or abnormal, ranked according to severity as described in Table 1. For every slide, a score in each compartment was determined for parameters described in Table 1. Finally, for one slide the sum of the scores for each parameter in all compartments (the retrieved sum to 100) constituted the radiation injury score. Radiation injury score and morphometric measurements were determined independently by two authors, and discrepancies were resolved in conference. Vessel wall thickness was determined by the ratio


A
Vascular thickness
Radiation Injury Score
B
CONTROL
PATHOLOGICAL
H & E coloration
Masson's Trichrome
Elastin coloration
αsma
calponin
Collagen I
Collagen III
PCNA
C
Skin
Lung
Uterus
CONTROL
PATHOLOGICAL

between luminal surface and outer surface (between 15 to 25 vessels by section) using an imaging analysis system interfaced with the Visiolab 2000 software (Biocom, Les Ulis, France). For immunohistochemistry, 5-μm sections were used to immunolocalize α-smooth muscle actin (α-SMA; Sigma, St. Quentin Fallavie, France), calponin (DAKO, Glostrup, Denmark), proliferating cell nuclear antigen (PCNA; DAKO), collagen I and collagen III (Sigma), transforming growth factor-βTGF-β (R&D Systems, Minneapolis, MN), and phospho-(ser433/435) Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA). Biotinylated rabbit anti-mouse IgG and streptavidin/biotinylated-peroxidase kit (DAKO) were used before revelation by Vector NovaRED substrate kit (Bio-Valley, Marne la Vallée, France) and counterstained with hematoxylin. Moreover, radiation-induced vascular damages were studied in irradiated lung (55 to 60 Gy), uterus (60 Gy), and skin (50 Gy). Three patients for each organ were included. Specimens of normal tissue samples were taken in an irradiated field and from normal tissue at a large distance from the tumor. Slides were colored by Meyer's hemalum.

Cell Culture and Irradiation

ECs (dermal human microvascular endothelial cells) and VSMCs (aortic human smooth muscle cells) were purchased from Cambrex (Verviers, Belgium) and cultured respectively in EGM-2 MV and SmGM-2 culture mediums. ECs were grown in transwell (Falcon 0.4-μm PET cell culture inserts; Becton Dickinson Labware, Le Pont de Claix, France). Cells were irradiated with a ^{137}Cs source (IBL 637, dose rate 1 Gy min^{-1}).

Proliferation and Flow Cytometry

Viable cells were counted using trypan blue exclusion method and a standard hemacytometer. Cell cycle analyses were performed by flow cytometry. In brief, trypsinized cells were fixed with ice-cold 70% ethanol, treated with 0.01 mg/ml RNase A and 50 μg/ml propidium iodide, and analyzed using a FACSort flow cytometer (Becton Dickinson).

In Vitro *Migration Assay*

VSMC migration was determined by the scratch injury model.[10] VSMCs were fixed and colored with a methanol solution containing 3% paraformaldehyde and 0.25% crystal violet from 1 to 4 days after irradiation. To integrate radiation-induced cell death, the quantitative analysis of the migration index was calculated by the ratio between the density of migrating cells in the center of the scratched zone and in a size-matched area of the unscratched region. The contribution of VSMC proliferation was assessed by cycling cell labeling (Ki-67, see below).

RNA Isolation and Reverse Transcription (RT) Real-Time Polymerase Chain Reaction (PCR)

Total RNA was prepared with the total RNA isolation kit (Rneasy Mini Kit; Qiagen, Valencia, CA). Total RNA quantification and integrity was analyzed using Agilent 2100 Bioanalyzer, and 1 μg of RNA was used for RT with SuperScript II (Invitrogen Life Technologies, Carlsbad, CA) and random hexamer to generate first strand cDNA. The following primers were used (F, forward; R, reverse): CTGF (F, 5′-TGTGTGACGAGCCCAAGGA-3′; R, 5′-TCTGGGCCAAACGTGTCTTC-3′; 5′-carboxyfluorescein-CTGCCCTCGCGGCTTACCGA-3′), PAI-1 (F, 5′-GCACAACCCCACAGGAACAG-3′; R, 5′-GTCCCAGATGAAGGCGTCTTT-3′), HSP27 (F, 5′-AGGATGGCGTGGTGGAGAT-3′; R, 5′-GTGTATTTCCGCGTGAAGCA-3′), COL3A1 (F, 5′-CCAATCCTTTGAATGTTCCACGG-3′; R, 5′-CCATTCCCCAGTGTGTTTCGTGC-3′), COL1A2 (F, 5′-TGAAAACATCCCAGCCAAGAA-3′; R, 5′-AAACTGGCTGCCAGCATTG-3′), SMAD3 (F, 5′-CGAGCCCCAGAGCAATATTC-3′; R, 5′-CTGTGGTTCATCTGGTGGTCACT-3′), and α-SMA (gene expression assay Hs00426835-g1; Applied Biosystems, Foster City, CA). Thermal cycling conditions were 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on an ABI PRISM 7700 Sequence detection system (Applied Biosystems). Significant PCR fluorescent signals were normalized to a PCR fluorescent signal obtained from the housekeeping gene GAPDH (Pre-developed Taqman Assay; Applied Biosystems) for each sample. Relative mRNA quantitation was performed by using the comparative $\Delta\Delta C_T$ method.

Immunocytochemistry

Cells were grown on glass coverslips and fixed for 30 minutes with 0.5% paraformaldehyde. After permeabilization and saturation, cells were incubated overnight with primary antibodies anti-Ki-67 (DAKO), anti-Smad3 (Zymed Laboratories, South San Francisco, CA), and anti-Smad4 (Santa Cruz Biotechnology). Cells were then incubated with a goat anti-mouse or rabbit IgG tagged with Alexa Fluor 488 (Molecular Probes), rinsed, and incubated in RNase A/propidium iodide solution. Cells were analyzed on Bio-Rad MRC 1024 ES confocal imaging system (Bio-Rad, Hercules, CA).

Figure 1. Characterization of radiation-induced vascular damages. **A:** Radiation injury score and vessels morphometric measurements (the ratio between luminal surface and outer surface) were performed in tissues from 38 patients treated by radiotherapy for rectal adenocarcinoma. Values of radiation injury score for every point constitute the sum (the retrieved sum to 100) of the score of every parameter observed in each compartment for one slide. **B:** Representative microscopic images from control (**A**, **E**, **I**, **M**, **Q**, **U**) and irradiated (**B–D**, **F–H**, **J–L**, **N–P**, **R–T**, **V–X**) submucosal vessels: H&E coloration (**A–D**), Masson's trichrome (**E–H**), elastin coloration (**I–J**); and immunolabeling of α-SMA (**K–K′**), calponin (**L–L′**), collagen I (**M–P**), collagen III (**Q–T**), and PCNA (**U–X**, **arrows** indicate some PCNA-positive cells) are shown. **C:** Representative microscopic images from control lung, uterus, and skin (**A**, **B**), and irradiated (**C–D**) tissue are shown (Meyer's hemalum coloration).

Transient Transfection and Reporter Gene Assay

VSMCs were transiently cotransfected with (CAGA)9-Lux reporter and pRL-TK plasmids using FuGENE 6 (Roche Diagnostics, Meylan, France) as transfection reagent. Cells extracts were prepared for the Dual-Luciferase reporter assay system according the manufacturer's instructions (Promega, Charbonnières, France). Relative luciferase activity was measured using a Mithras luminometer (Berthold Technologies, Bad Wildbad, Germany).

Western Blot Analysis

The following protein-specific primary antibodies were used: anti-α-SMA (Sigma), anti-HSP27 (Stressgen Biotechnologies, Victoria, BC, Canada), anti-PAI-1 (Novocastra Laboratories Ltd., Newcastle, UK), anti-CTGF (R&D Systems), and anti-glyceraldehyde-3-phosphate dehydrogenase (Biodesign, Saco, ME). Proteins were separated by SDS-polyacrylamide gel electrophoresis before transfer onto nitrocellulose membranes. The membranes were blotted with primary antibodies followed by incubation with secondary antibody HRP-conjugated (Amersham, Orsay, France). Blots were developed using the enhanced chemiluminescence method (Amersham). Membranes were then dehybridized and reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase antibody to detect glyceraldehyde-3-phosphate dehydrogenase expression as control loading.

TGF-β1 Enzyme-Linked Immunosorbent Assay (ELISA) Assay

TGF-β1 production in the supernatants of ECs and VSMCs was determined by ELISA assay (Promega) with (total form) and without (active form) acid treatment according to the manufacturer's instructions.

RNA Interference

The sequence of small interferring RNAs (siRNAs) designed to specifically target Smad3 is 5′-ACCUAUCCCCGAAUCCGAUdTdT-3′. The efficiency of silencing was determined by RT real-time PCR using specific primers and Western blot (anti-Smad3; Zymed Laboratories).

Statistical Analyses

Data are given as mean ± SEM. Statistical analyses were performed by analysis of variance or Student's *t*-test with a level of significance of $P < 0.05$.

Results

Characterization of Radiation-Induced Vascular Damages in Patients Treated with Radiotherapy

We undertook a retrospective study in 38 patients treated with preoperative radiotherapy for rectal cancer. Radiation injury score was determined as well as vessels morphometric measurements. Radiation-induced tissue damage was appreciated by a semiquantitative histopathological scoring system (Table 1) of mucosal injury, submucosal edema and inflammation, dystrophy, and extracellular matrix remodeling in the submucosa, muscularis mucosa, muscularis propria, serosa, and mesentery. A correlation between vascular thickening and radiation injury score was observed ($P < 0.001$, $n = 83$ slides, Figure 1A). Radiotherapy treatment is associated with several kinds of vascular damage: vascular dystrophy and hypertrophy (Figure 1B, B–D), vascular and perivascular fibrosis (Figure 1B, F–H), and intimal hyperplasia associated with luminal narrowing (Figure 1B, C,F,J). Immunolabeling of collagen I and collagen III revealed a strong increase of immunoreactivity in vessels from pathological (Figure 1B, N–P, R–T) compared with normal tissues (Figure 1B, M,Q). PCNA labeling (Figure 1B, U–X) showed proliferation of VSMC in hypertrophic vessels (Figure 1B, V) compared with normal (Figure 1B, U). In areas of neointimal hyperplasia, α-SMA (Figure 1B, K,K′), calponin (Figure 1B, L,L′), and PCNA-positive cells (Figure 1B, V–X) in a rich collagen matrix (Figure 1B, F,P) are also observed demonstrating migration and proliferation of VSMC. Interestingly, vascular dystrophy, hypertrophy, and intimal hyperplasia were observed in irradiated lung, uterus, and skin, illustrating that radiation-induced vascular damage is not organ-dependent (Figure 1C).

Irradiated ECs Induce VSMC Proliferation

The effect of ECs on VSMC proliferation after irradiation was investigated using cell counting and cell cycling distribution analyses (Figure 2). Interestingly, proliferation of VSMCs decreased in presence of ECs, irradiated or not. However, the number of nonirradiated VSMCs was higher in presence of irradiated ECs compared with nonirradiated ECs (Figure 2, A–B). Irradiation inhibits the proliferation of VSMCs, and this effect is decreased in presence of irradiated ECs. Analyses of cell cycle distribution showed that irradiation induces a classic G_1 arrest in VSMCs, which was not affected by the presence of ECs, irradiated or not. Twenty-four hours after irradiation, irradiated ECs increased the percentage of nonirradiated VSMCs in S phase compared with VSMCs alone or VSMCs with nonirradiated ECs. Moreover, 24 to 72 hours after irradiation, the number of irradiated VSMCs in S phase increased in presence of ECs, and this effect was more pronounced in presence of irradiated ECs (Figure 2C).

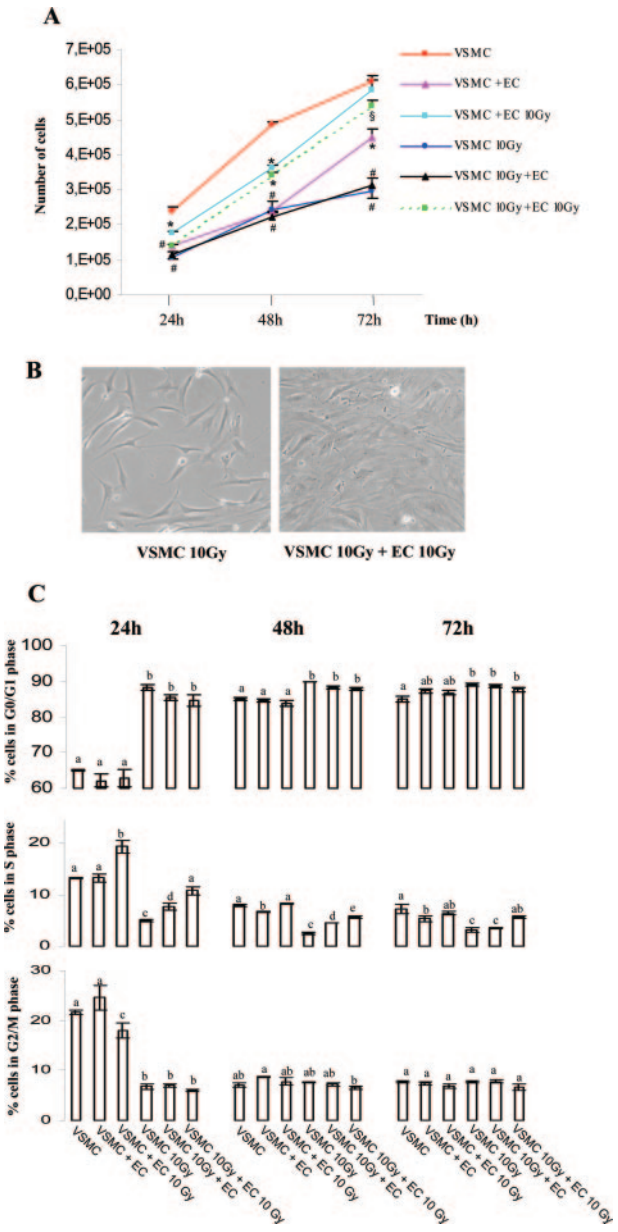


Figure 2. Irradiated ECs influence cell cycle progression and VSMC proliferation. In all experiments, 50% confluent VSMCs were serum-starved for 24 hours before co-culture and irradiation. VSMCs were changed with complete culture medium, and Transwell-containing confluent ECs were incubated with VSMCs and then irradiated. **A:** Proliferation of VSMCs was determined by cell counting. Data are the mean ± SEM of three experiments realized in triplicate or quadruplicate. * or # or $^{\S}P < 0.05$ versus VSMCs; values with different symbols are statistically different. **B:** Representative cultures obtained 2 days after irradiation are shown. **C:** Cell cycle distribution was determined by propidium iodide staining. Data are the mean ± SEM, and for each time, values with different footnote letters are statistically different from each other ($P < 0.05$).

Irradiated ECs Induce VSMC Migration after Irradiation

Irradiation did not influence the ability of VSMCs to colonize a wounded area (Figure 3). Moreover, migration index was increased in VSMCs in the presence of irradiated ECs. Migration of irradiated VSMCs was stimulated in the presence of ECs, and this effect was further improved in the presence of irradiated ECs.

Irradiated ECs Induce VSMC Fibrogenic Phenotype

We next analyzed the ability of irradiated ECs to affect the fibrogenic phenotype of VSMCs. *In vivo*, both cell types are irradiated, so we therefore performed co-culture of irradiated ECs in the presence of irradiated VSMCs at the same dose (2 or 10 Gy; Figure 4A). In the presence of irradiated ECs, mRNA expression of CTGF, PAI-1, collagen type I, alpha 2 (COL1A2), and COL3A1 increased in irradiated VSMCs. Variations at protein levels were confirmed by Western blot for α-SMA, CTGF, PAI-1, and heat shock protein 27 (HSP27). To be sure that in this case we observed paracrine effects of ECs and not direct effects of irradiation, VSMCs were irradiated alone (Figure 4B). In the absence of ECs, irradiation decreases the mRNA and protein levels of α-SMA. In contrast, the other target genes were unaffected. These results suggest that changes in VSMC phenotype observed in the presence of ECs were not due to direct radiation effects and that irradiated ECs produced paracrine factors that subsequently induced VSMC fibrogenic phenotype. To confirm the paracrine effects of ECs, irradiated ECs were cultured in the presence of nonirradiated VSMCs. As shown in Figure 4C, expression of α-SMA, CTGF, PAI-1, HSP27, and COL3A1 increased in VSMCs exposed to irradiated ECs.

Role of TGF-β/SMAD Pathway in VSMC Fibrogenic Phenotype Induced by Irradiated ECs

TGF-β1 Secretion Is Increased in ECs but not in VSMCs after Irradiation

TGF-β1 is a well-known growth factor involved in VSMC fibrogenic phenotype. TGF-β1 secretion was measured in supernatants of ECs and VSMCs by ELISA assay. Interestingly, secretion of total and active forms of TGF-β1 increased after irradiation of ECs (Figure 5). No difference in the secretion of TGF-β1 was observed in supernatant of irradiated VSMCs.

Irradiated ECs Activate Smad Pathway in VSMCs

Immunofluorescence labeling of Smad3 and Smad4 in VSMCs was performed to determine whether ECs activate the Smad pathway (Figure 6A). In 24-hour serum-starved VSMCs, Smad3 and Smad4 were localized in the cytosol. In the presence of irradiated ECs, Smad3 and Smad4 were translocated to the nucleus of irradiated and nonirradiated VSMCs. Transient transfection of VSMCs with the $(CAGA)_9$Lux vector showed that irradiated ECs activate Smad-dependent gene transcription in VSMCs (Figure 6B).

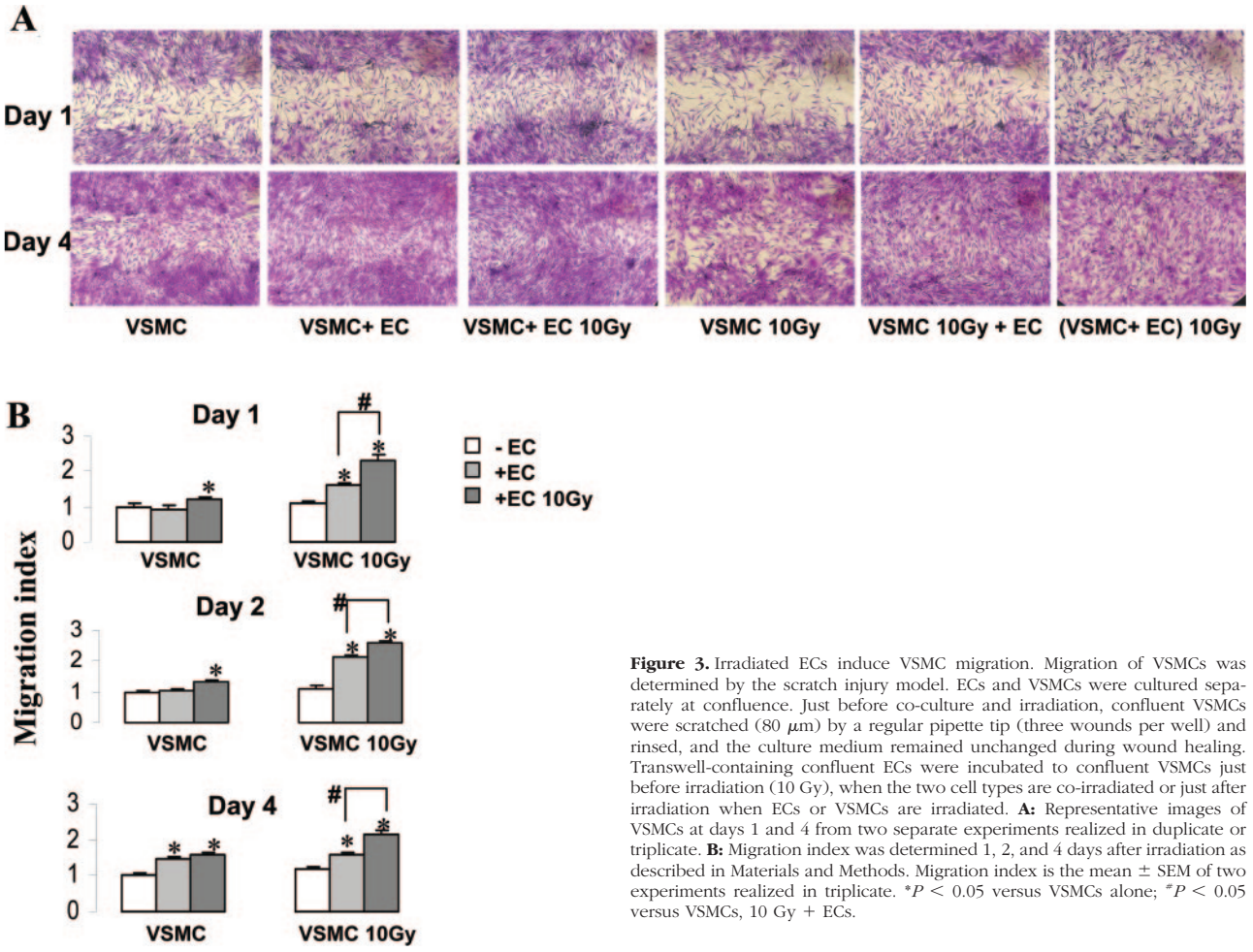


Figure 3. Irradiated ECs induce VSMC migration. Migration of VSMCs was determined by the scratch injury model. ECs and VSMCs were cultured separately at confluence. Just before co-culture and irradiation, confluent VSMCs were scratched (80 μm) by a regular pipette tip (three wounds per well) and rinsed, and the culture medium remained unchanged during wound healing. Transwell-containing confluent ECs were incubated to confluent VSMCs just before irradiation (10 Gy), when the two cell types are co-irradiated or just after irradiation when ECs or VSMCs are irradiated. **A:** Representative images of VSMCs at days 1 and 4 from two separate experiments realized in duplicate or triplicate. **B:** Migration index was determined 1, 2, and 4 days after irradiation as described in Materials and Methods. Migration index is the mean ± SEM of two experiments realized in triplicate. $^{*}P < 0.05$ versus VSMCs alone; $^{\#}P < 0.05$ versus VSMCs, 10 Gy + ECs.

Irradiated ECs Induce Fibrogenic Phenotype of VSMCs by a Smad-Dependent Pathway

Knock-down of Smad3 in VSMCs was performed to investigate the role of this protein in fibrogenic phenotype of VSMCs induced by irradiated ECs. Twenty-four hours after Smad3 siRNA transfection in VSMCs, Smad3 mRNA and protein levels decreased by 80 and 90%, respectively (Figure 7A). In the presence of irradiated ECs, α-SMA, HSP27, CTGF, PAI-1, COL1A2, and COL3A1 mRNA levels decreased in siRNA (si)-Smad3-transfected irradiated VSMCs compared with control-irradiated VSMCs (Figure 7B).

Fibrogenic Phenotype of VSMCs Induced by Irradiated ECs Involves a TGF-β-Dependent Pathway

To inhibit the TGF-β1 pathway, VSMCs were incubated with a neutralizing antibody directed against TGF-β-RII. The efficiency of TGF-β-RII neutralizing antibody was investigated by its ability to affect Smad nuclear translocation. Twenty-four hours after irradiation, the translocation of Smad3 and Smad4 induced by irradiated ECs decreased in the presence of TGF-β-RII antibody (Figure 8A). Next, the fibrogenic phenotype of nonstarved irradiated VSMCs in the presence of irradiated ECs (10 Gy) was investigated (Figure 8B). Results showed that the expressions of α-SMA, PAI-1, COL1A2, and COL3A1 decreased in the presence of TGF-β-RII antibody, demonstrating that a TGF-β1/TGF-β-RII mechanism is involved in the fibrogenic phenotype of VSMCs induced by ECs.

Radiation-Induced Vascular Damages Are Associated with Overexpression of TGF-β and Phospho-Smad 2/3

To support *in vitro* results, we investigated whether radiation-induced vascular damages are associated with overexpression of TGF-β and P-Smad 2/3 in patients treated by radiotherapy (Figure 9). Immunohistochemical staining showed that TGF-β expression increased in irradiated rectum and, in particular, in endothelium. Moreover, a strong increase of P-Smad 2/3 in VSMCs was observed in pathological vessels compared with normal vessels. These *in vivo* results demonstrate the physiological relevance of an up-regulation of TGF-β expression in endothelium and an activation of Smad signaling in VSMCs.

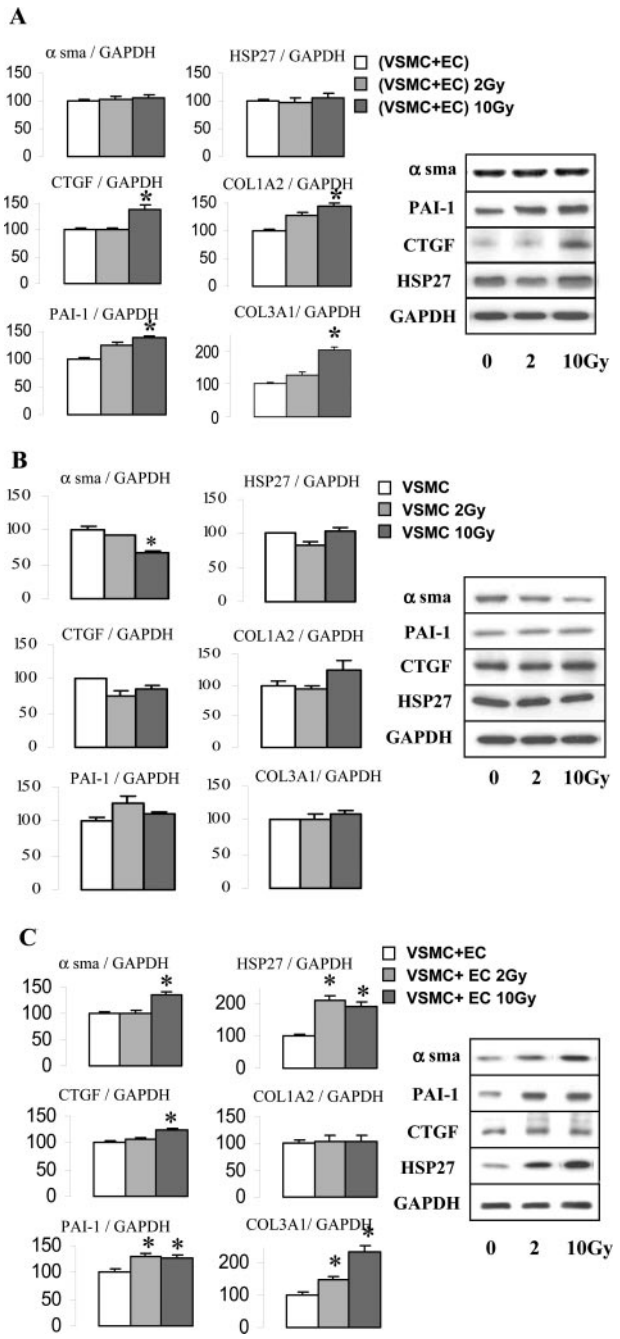


Figure 4. Irradiated ECs induce a VSMC fibrogenic phenotype. ECs and VSMCs were cultured separately at confluence and settled together at the moment of co-culture and irradiation (2 or 10 Gy). Fibrogenic phenotype of VSMCs was investigated by real-time PCR (24 hours after irradiation) and Western blot (48 hours after irradiation). **A:** Co-culture of irradiated ECs in the presence of irradiated VSMCs at the same dose. **B:** VSMCs irradiated alone to investigate direct radiation effects. **C:** Co-culture of irradiated ECs in the presence of nonirradiated VSMCs. Representative Western blots. Data are the mean ± SEM of two to four independent experiments realized in duplicate or triplicate. $^*P < 0.05$ versus control.

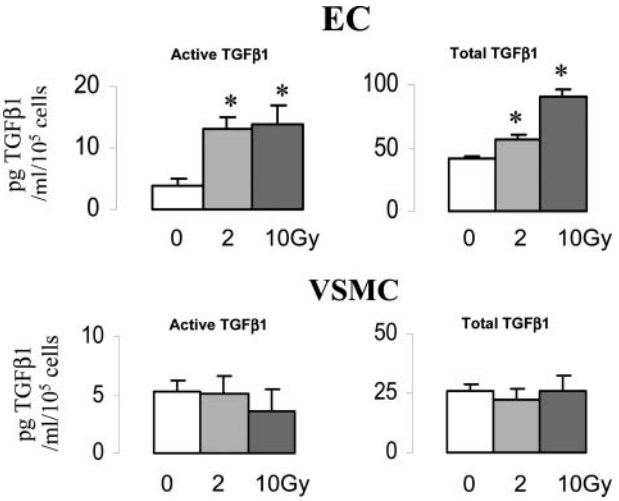


Figure 5. Irradiation increases TGF-β1 secretion in ECs. Total and active TGF-β1 contents were determined by ELISA assay in EC and VSMC supernatants 24 hours after 2 or 10 Gy irradiation. Data are the mean ± SEM of three experiments realized in triplicate. $^*P < 0.05$ versus control.

Discussion

We demonstrate here the importance of proliferation, migration, and fibrogenic phenotype of VSMCs in patients treated with radiotherapy. This study shows that ECs influence these fundamental mechanisms involved in the initiation and progression of vascular damages. The main results of this work are that, *in vitro*, ECs promote VSMC proliferation, migration, and fibrogenic phenotype after irradiation.

The vascular wound healing process is characterized by the proliferative response of VSMCs after injury. Deregulation of VSMC proliferation contributes to the restenotic lesion, atherosclerosis, vascular hypertrophy, and vascular remodeling after hypertension.[11] Our results obtained in patients underline the importance of proliferation of VSMCs in radiation-induced vascular damages after radiotherapy. We first showed that nonirradiated ECs have an antiproliferative effect on VSMCs. This is in line with Peiró et al[12] who previously demonstrated that bovine aortic endothelial cells inhibit proliferation of rat VSMCs in a co-culture model. Moreover, our results show that irradiated ECs can stimulate proliferation of VSMCs and/or that irradiated ECs fail to inhibit VSMC proliferation. This is in contrast with de Crom et al[13] who showed that a very high-dose radiation (40 Gy) of ECs did not affect the proliferation of VSMCs. The strong differences in radiation dose ranges could explain this discrepancy. Cell cycle analyses revealed that irradiated ECs influence cell cycle progression of VSMCs. We can postulate that at the same time ECs produce growth promoters and growth inhibitors that may modulate VSMC growth, ie, platelet-derived growth factor, vascular endothelial growth factor, or basic fibroblast growth factor, but also molecules with short half-lives such as nitric oxide. Nitric oxide inhibits VSMC proliferation by altering the activation of CDK2 and the expression of cyclin A.[14] In an interesting way, a lack of endothelial nitric-oxide synthase was observed in irradiated human cervical arteries from patients treated by radiotherapy for neck cancer[15]and in the rabbit ear central artery 2 weeks after an irradiation of 45 Gy,[16] whereas an up-regulation of endothelial nitric-oxide synthase was described in bovine aortic endothelial cells.[10] Further studies are needed to understand molecular mechanisms involved in the control of VSMC prolif-

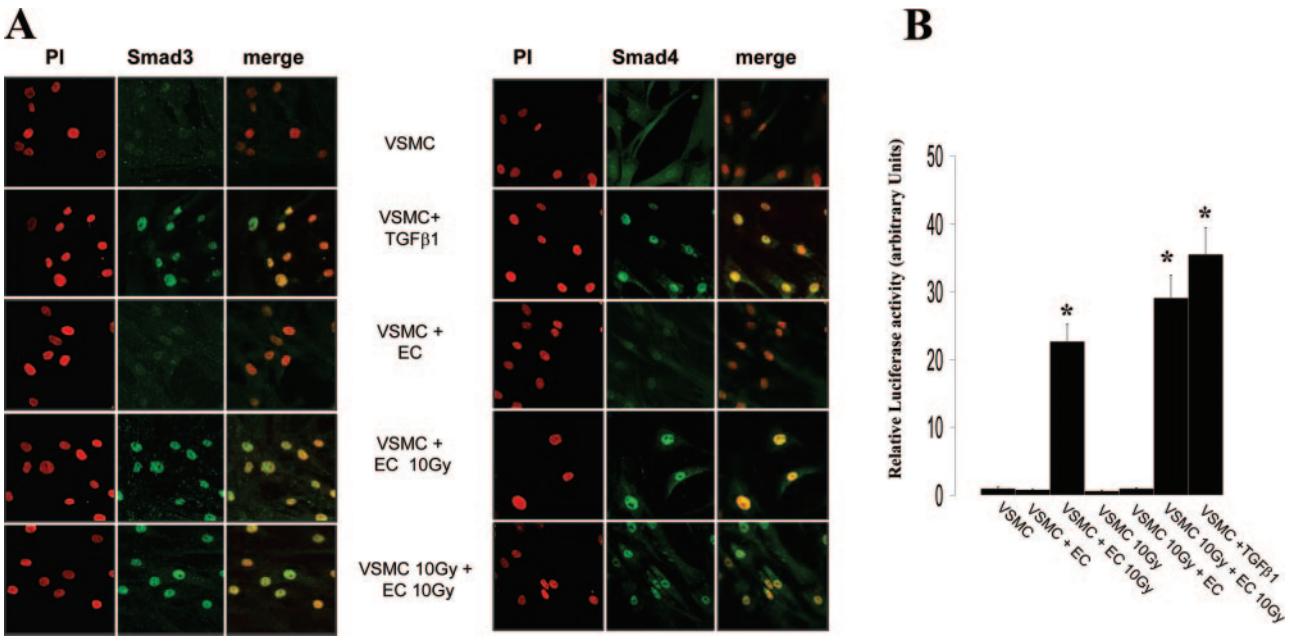


Figure 6. Irradiated ECs activate Smad pathway in VSMCs. **A:** ECs induce the nuclear translocation of Smad3 and Smad4 in VSMCs after irradiation. VSMCs and ECs were cultured separately at confluence. VSMCs were incubated in serum-free medium for 24 hours before experiment. Just before co-culture and irradiation (10Gy), ECs were changed in complete medium and VSMCs with serum-free medium. Twenty-four hours after irradiation, Smad3 and Smad4 nuclear translocation in VSMCs was followed by immunofluorescence and examined by confocal microscopy. Representative immunostainings of three independent observations are shown, as well as staining of VSMCs treated with 10 ng/ml TGF-β1 for 1 hour. **B:** ECs induce a Smad-dependent transcription in VSMCs. VSMCs (50% confluent) were transiently cotransfected in complete medium with (CAGA)9-Lux reporter (1 μg) and pRL-TK (0.2 μg) plasmids using FuGENE 6 (Roche Diagnostics) as transfection reagent (3 μl/1.2 μg of DNA). Twenty-four hours after transfection, VSMCs were changed with serum-free medium then incubated with confluent ECs. Relative luciferase activity (ratio Firefly/Renilla) was measured 24 hours after co-culture and irradiation. Transfection efficiency (about 40%) was estimated using pEGFP-N1 vector (Clontech, Mountain View, CA). VSMCs treated by 3 ng/ml TGF-β1 for 24 hours are shown. Data are the mean ± SEM ($n = 6$) *$P < 0.05$ versus VSMCs alone.

eration by ECs after irradiation and nitric oxide pathway could be an attractive target.

We also found that ECs induce a fibrogenic phenotype in VSMCs, which overexpressed CTGF, PAI-1, and fibrillar collagens. In support to this view, collagen I- and collagen III-positive stainings were markedly increased in vascular adventitia of patients treated for rectal adenocarcinoma by radiotherapy, suggesting that VSMCs have a higher capacity to secrete collagen *in vivo*. CTGF, a member of the CCN family,[17] is a fibrogenic cytokine, considered as a mediator of profibrotic effects of TGF-β1, especially the overproduction extracellular matrix. CTGF activates TGF-β1 signal transduction by enhancing the ability of TGF-β1 binding to its receptors at low concentrations of TGF-β1.[18] High levels of CTGF were found in fibrotic lesions in various organs such as liver, lung, skin, and kidney,[19] as well as in atherosclerotic plaques.[20] We found that CTGF is overexpressed in VSMCs exposed to irradiated ECs. CTGF up-regulation in VSMCs was also observed in vessels of the submucosa and the subserosa in bowels of patients who developed radiation enteritis.[21] In an interesting way, CTGF was described as also implied in the migration of VSMCs,[22] suggesting that CTGF could have a role in the capacity of VSMCs to migrate and probably in the formation of neointimal hyperplasia. VSMC migration in radiation-induced vascular lesion is supported by intimal hyperplasia in submucosal blood vessels from patients treated by radiotherapy for rectal cancer and was also present in

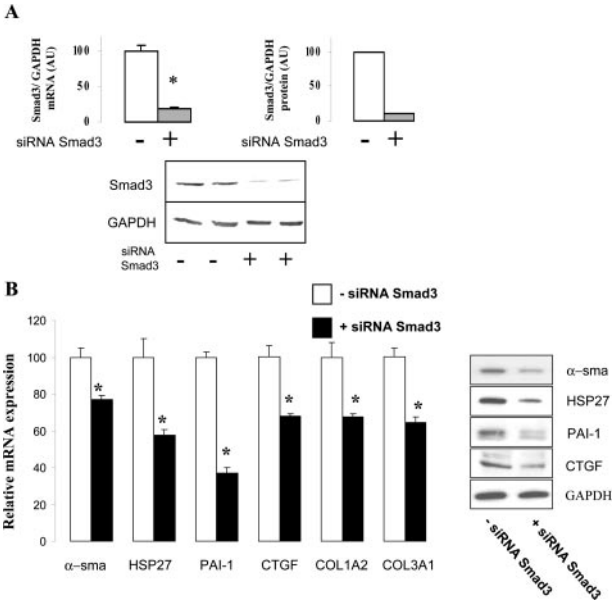


Figure 7. Smad3 is involved in fibrogenic phenotype of VSMCs induced by ECs after irradiation of both cell types. VSMCs were transfected with cytofectin (1 μg/ml) and 100 nmol/L siRNAs targeting Smad3. **A:** The silencing efficiency was determined by real-time PCR and Western blot. **B:** VSMCs and ECs were cultured separately at confluence. VSMCs were transfected 24 hours before co-culture and irradiation of both cell types (10 Gy). Just before irradiation, ECs were changed in complete medium and VSMCs with complete medium ± siRNA Smad3 transfection solution. Fibrogenic phenotype of VSMCs was investigated by real-time PCR (24 hours after irradiation) and Western blot (48 hours after irradiation). Data are the mean ± SEM of two experiments realized in triplicate. *$P < 0.05$ versus 10 Gy irradiated cells without siRNA Smad3. Representative Western blots are shown with irradiation of both cell types at 10 Gy ± siRNA Smad3.

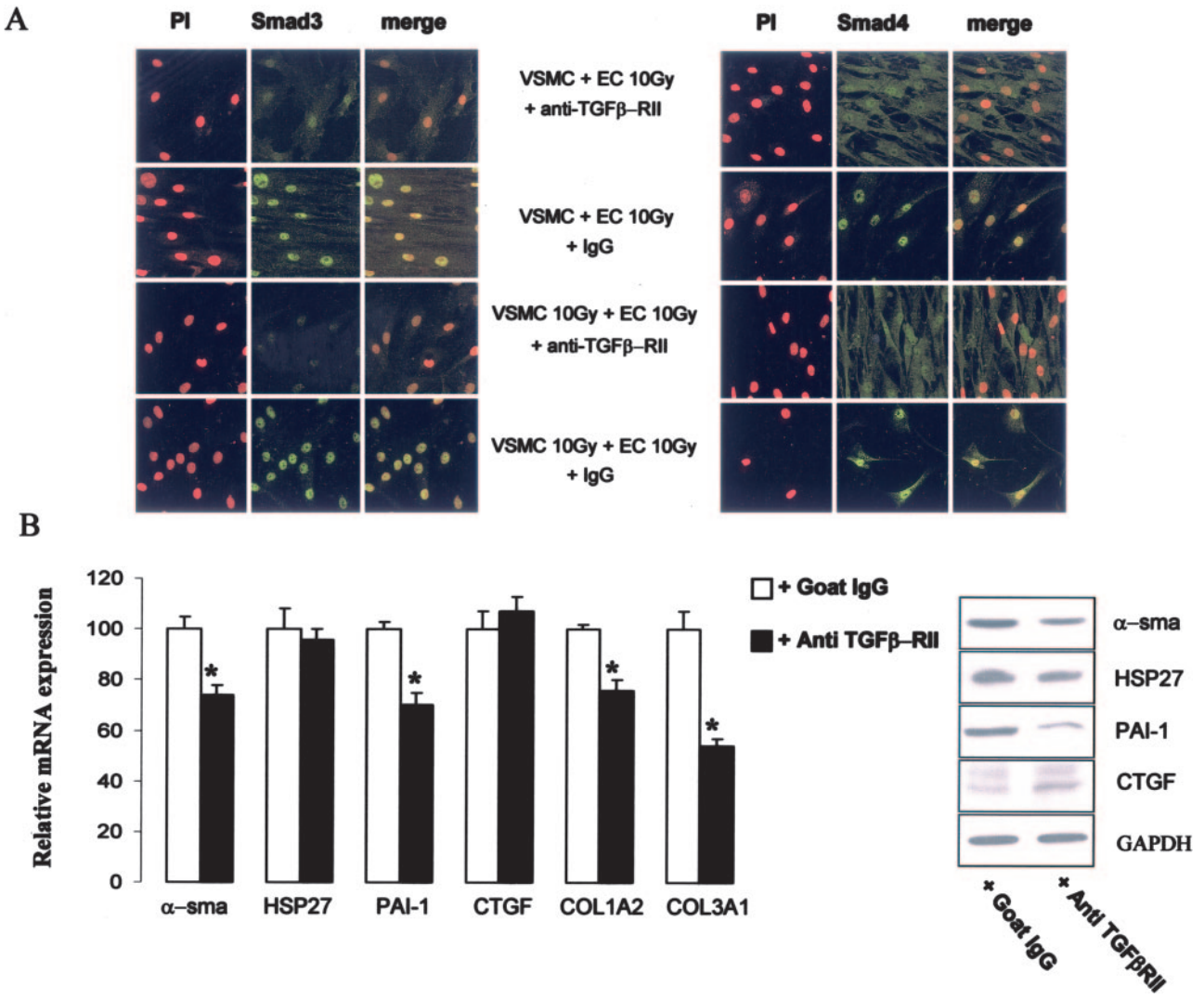


Figure 8. TGFβ-RII is involved in fibrogenic phenotype of VSMCs induced by ECs after irradiation of both cell types. VSMCs and ECs were cultured separately at confluence. **A:** VSMCs were serum-starved during 24 hours and preincubated 2 hours before co-culture and irradiation of both cell types (10 Gy) with a goat anti-human TGFβ-RII neutralizing antibody (10 μg/ml in serum-free medium) or normal goat IgG (10 μg/ml). Just before irradiation, ECs were changed in complete medium and VSMCs with serum-free medium. Twenty-four hours after irradiation, Smad3 and Smad4 nuclear translocations in VSMCs were analyzed by immunofluorescent staining. **B:** VSMCs were preincubated 2 hours before co-culture and irradiation of both cell types (10 Gy) with a goat anti-human TGFβ-RII or normal goat IgG (10 μg/ml). Just before irradiation, ECs and VSMCs were changed in complete medium. Fibrogenic phenotype of VSMCs was investigated by real-time PCR (24 hours after irradiation) and Western blot (48 hours after irradiation). Data are the mean ± SEM. $^*P < 0.05$ versus 10 Gy-irradiated cells with normal IgG. Representative Western blots are shown with co-irradiation of both cell types at 10 Gy ± goat anti-human TGFβ-RII.

irradiated lung, uterus, and skin. Intimal hyperplasia occurs in atherosclerosis, hypertension, and after vascular injury, and VSMC proliferation and migration are critical processes implicated in this vascular lesion.[23] Using the scratch injury model, we demonstrated that VSMC migration was increased in presence of irradiated ECs. Immunohistochemistry and immunolabeling revealed that intimal thickness was characterized by an extracellular matrix containing α-SMA and calponin-positive cells, demonstrating the presence of VSMCs in area of intimal hyperplasia. Moreover, we demonstrated that irradiated ECs induce the overexpression of PAI-1 in VSMCs. The two major functions of PAI-1 are to impair fibrinolysis and to affect matrix degradation by inhibiting the plasmin-dependent activation of matrix metalloproteases. PAI-1 gene expression is up-regulated in macrophages and smooth muscle cells in human atherosclerotic lesions,[24] and increased expression of PAI-1 in the artery wall promotes neointima growth after balloon injury.[25] Interestingly, overexpression of PAI-1 was described in radiation-induced nephrosclerosis[26] and in human radiation enteritis.[27] Further investigations are needed to understand the role of PAI-1 in radiation-induced vascular damages.

In various organs such as skin, intestine, lung, and kidney, TGF-β1 is considered as a key factor involved in radiation fibrosis,[28] mediating collagen synthesis and playing a crucial role in fibroblast differentiation into myofibroblast.[29] Our study shows that the increase of TGF-β1 secretion in ECs after irradiation may act as a paracrine factor influencing the fibrogenic phenotype of VSMCs. Moreover, up-regulation of TGF-β was ob-

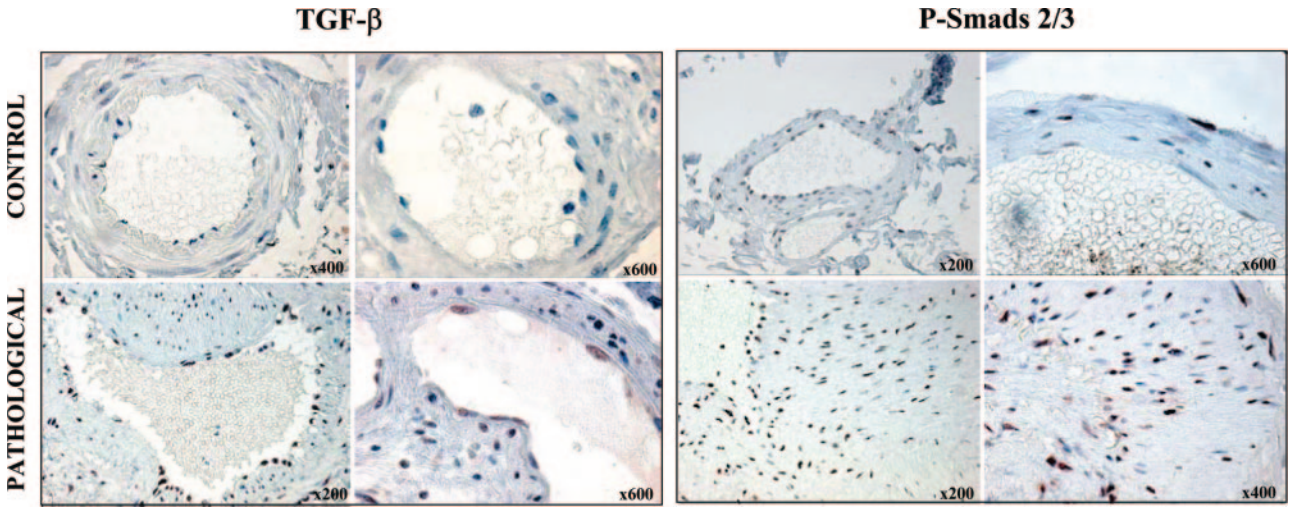


Figure 9. Overexpression of TGF-β and P-Smad 2/3 in radiation-induced vascular lesions. Immunohistochemical stainings of TGF-β and P-Smad 2/3 were performed in tissues from patients treated by radiotherapy for rectum adenocarcinoma. Representative microscopic images from control and irradiated submucosal vessels are shown.

served in endothelium in vascular lesions from patients treated with radiotherapy. This is in line with Wang et al who demonstrated in an *in vivo* model of radiation enteropathy in rats an increase of TGF-β1 mRNA and protein expression in ECs.[30] TGF-β signal transduction is initiated by ligand-induced heterodimeric complex formation of TGFβ-RII with TGFβ-RI, two serine/threonine kinase receptors. Activation of TGFβ-RI by phosphorylation with the TGFβ-RII causes the phosphorylation and the nuclear translocation of Smads, which induces transcriptional activation of various genes.[31] *In vivo*, we found that radiation induced vascular damages were associated with a strong increase of P-Smad 2/3 in VSMCs. Interestingly, in hypertensive patients, it was shown recently that TGF-β/Smad2/3 signaling is activated in arteriosclerosis and in particular in VSMCs.[32] *In vitro*, we found that irradiated ECs activate the Smad pathway in VSMCs by inducing nuclear translocation of Smad3 and Smad4 and Smad-dependent promoter activation. These results strongly suggest that ECs induce Smad-dependent gene transcription in VSMCs after irradiation. This was supported by the fact that mRNA expressions of various profibrotic factors, which have Smad-responding elements in their promoters, were increased in VSMCs in the presence of irradiated ECs. To be sure that a Smad-dependent pathway was involved, the knockdown of Smad3 by RNA interference in VSMCs was realized. Smad3 silencing fully abolished the irradiated EC-induced up-regulation of target genes in VSMCs, demonstrating that Smad3 mediates the fibrogenic phenotype of VSMCs induced by irradiated ECs. Skin damages are reduced in Smad3 knockout mice following ionizing radiation exposure, suggesting that inhibition of Smad3 will be protective against radiation-induced tissue damage and fibrosis.[33] Our results might support the hypothesis that EC/VSMC cross talk via the Smad3 pathway may contribute to the initiation of vascular fibrosis. Moreover, neutralizing antibody directed against TGFβ-RII blunted α-SMA, PAI-1, and collagen up-regulation. All together, these results demonstrate that a TGFβ1/TGFβ-RII/Smad3 pathway is involved in the fibrogenic phenotype of VSMCs induced by irradiated ECs. We showed in our study that CTGF and HSP27 up-regulation in VSMCs exposed to ECs is Smad3-dependent. Interestingly, in the presence of TGFβ-RII neutralizing antibody, increases of CTGF and HSP27 remained unchanged, suggesting that a Smad3-dependent/TGFβ1-independent pathway may be involved. The Smad pathway can also be activated by a TGFβ-independent mechanism. Indeed, it was recently shown that angiotensin II activates the Smad pathway in rat VSMCs via AT1.[34] Nuclear translocation of Smad, phosphorylation of Smad2, DNA-binding activity, and Smad-dependent gene transcription were increased in VSMCs exposed to angiotensin II. Moreover, it has been shown that angiotensin II via AT1 receptors[34] and endothelin-1 via the ET_A receptor[35] increase the expression of CTGF in VSMCs independently of TGF-β. Further experiments are needed to know whether angiotensin II or other factors may have a role in the fibrogenic phenotype of VSMCs induced by ECs.

In conclusion, this is the first study which shows that the cross talk between ECs and VSMCs can initiate molecular mechanisms involved in radiation-induced vascular damages. ECs increase VSMC proliferation and migration after irradiation. The Smad3-dependent pathway is involved in the fibrogenic phenotype of VSMCs induced by ECs. These data contribute to the knowledge of the normal tissue response after irradiation and especially on the role of endothelial cells and Smad pathway in radiation-induced vascular damages. Future research is needed to determine molecular mechanisms involved in normal tissue toxicity to develop therapeutics strategies to prevent the severity of normal tissue injury without compromising, and even improving, tumor control.

Acknowledgment

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Résumé Article 2 : Rôle essentiel de l'inhibiteur des activateurs du plasminogène de type 1 (PAI-1) dans les dommages tissulaires radio-induits.

Objectifs

La toxicité radio-induite aux tissus sains est un facteur limitant dans le traitement des tumeurs par radiothérapie. La compréhension des mécanismes physiopathologiques et moléculaires impliqués dans la pathogenèse des dommages radio-induits est déterminante dans l'optique de mettre en place des stratégies thérapeutiques visant à protéger les tissus sains. Stimulé dans de nombreuses fibroses radio-induites, le TGF-β1, sa voie de signalisation par les protéines SMAD et ses effecteurs sont depuis longtemps considérés comme des cibles thérapeutiques potentielles pouvant limiter les lésions radio-induites. Parmi les effecteurs du TGF-β1, nous nous sommes intéressés au rôle de PAI-1. La fonction majeure de PAI-1 est de s'opposer au système fibrinolytique en inhibant les activateurs du plasminogène. Ainsi, par sa capacité à inhiber la formation de plasmine, PAI-1 joue un rôle déterminant dans l'homéostasie vasculaire en participant au contrôle de la coagulation intravasculaire. D'autre part, PAI-1 est un inhibiteur de la dégradation matricielle par sa capacité à limiter l'activation des metalloprotéinases dépendante de la plasmine. L'objectif de ce travail est de déterminer si PAI-1 joue un rôle essentiel dans les dommages radio-induits aux tissus sains.

Résultats

Dans un modèle d'irradiation d'une anse intestinale extériorisée, nous avons suivi l'état clinique ainsi que les lésions histologiques 2 et 6 semaines après exposition à 19Gy, chez des souris génétiquement déficientes en PAI-1 (PAI-1 -/-) et des souris sauvages (Wt). Les résultats montrent que 2 semaines après irradiation, la survie des souris Wt (n=55) est de 42 % alors que celle des souris PAI-1 -/- (n=32) est de 75 %. La différence est maintenue jusqu'à 6 semaines après irradiation. Le score d'atteinte tissulaire aux deux temps d'étude est moins sévère (- 35%) chez les souris PAI-1 -/- irradiées comparées aux Wt irradiées. D'autre part, la fonctionnalité intestinale est moins altérée chez les souris PAI-1 -/- 3 jours après irradiation. Enfin, par microscopie intravitale, nous avons montré que l'irradiation induit une augmentation des interactions des leucocytes et des plaquettes avec l'endothélium chez les souris Wt mais pas chez les souris PAI-1 -/-. De manière intéressante, nous avons observé une forte surexpression de PAI-1 dans l'endothélium vasculaire sur des tissus de rectum de patients traités par radiothérapie (n=25). *In vitro*, l'expression (ARNm et

protéine) et la sécrétion de PAI-1 est stimulée après irradiation de cellules endothéliales (CE). Par des techniques de « gene reporter » et western blot, nous avons montré que la voie de signalisation SMAD est stimulée après irradiation dans les CE. D'autre part, par western-blot, co-immunoprécipitation et expériences d'interférence ARN, nous avons montré que l'induction radio-induite de PAI-1 dans les CE est dépendante à la fois de Smad3 et du gène suppresseur de tumeur p53.

Conclusion

L'ensemble de ces résultats démontre le rôle essentiel de PAI-1 dans les dommages radio-induits aux tissus sains et en font une cible thérapeutique potentielle prometteuse. De plus, nos résultats montrent qu'une coopération entre Smad3 et p53 est impliquée dans l'induction de l'expression de PAI-1 dans les cellules endothéliales après irradiation.

Essential role of plasminogen activator inhibitor type-1 in radiation-induced normal tissue damages

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Short title : PAI-1 in radiation-induced tissue damages

Abstract

Early and late normal-tissue toxicity is a critical clinical problem following radiotherapy. TGF-β1 is a key mediator involved in radiation-induced damages and we hypothesised that the TGF-β1 target gene PAI-1 is an essential mediator of early and late normal-tissue toxicity. In a model of radiation enteropathy, we showed that PAI-1 knockout mice are protected against radiation-induced damages with an increase of the survival and a better intestinal functionality compared to wild type (Wt) mice. Radiation injury is attenuated in irradiated PAI-1 -/- mice compared to irradiated Wt mice and irradiation increases blood cells-endothelial interactions in Wt and not in PAI-1 -/- mice. *In vivo,* PAI-1 is up-regulated in endothelium in radiation-induced normal tissue damages in mice as well as in patients treated with radiotherapy. *In vitro,* irradiation increases PAI-1 expression in endothelial cells (EC). Moreover, we demonstrate that a p53/Smad3 pathway is involved in radiation-induced PAI-1 expression in EC. This study shows that PAI-1 plays a critical role in radiation induced damages and suggests that PAI-1 is an attractive target to prevent or reduce the side effects of radiation therapy.

Key Words : PAI-1, radiation, endothelial cells, SMAD

Introduction

More than half of cancer are treated with radiation therapy alone or in combination with surgery and/or chemotherapy . The goal of radiotherapy is to deliver enough ionizing radiation dose to destroy the tumor while minimizing the dose that reaches the surrounding normal tissue. Nevertheless, radiation-induced normal tissue injury is a dose-limiting factor in the treatment of cancer with radiotherapy (1-3). Early and late side effects not only limit radiation dose escalation but also might affect the patient's quality of life. The knowledge of normal-tissue radiobiology is required to determine molecular mechanisms involved in normal tissue pathogenic pathway in order to identify therapeutics target and develop strategies to prevent and /or reduce side effects of radiotherapy.

Radiation-induced fibrosis is a common late effect of radiation therapy. Early phases of radiation-induced fibrogenesis are characterised by an orchestrated wound-healing response initiated by some events that include activation of coagulation system, inflammation, mucosal repair, granulation tissue formation and extra cellular matrix remodelling (4). This complex and integrated response involves a large number of molecular pathways activated by pro and anti inflammatory cytokines, chemokines and growth factors. Among these factors, TGF-β1 is consider as a key fibrogenic cytokine involved in radiation-induced normal tissue damages (5). TGF-β1 up-regulates expression of several molecule that could contribute to acute and late radiation damages. Among them, we hypothesized that plasminogen activator inhibitor type-1 (PAI-1) is a critical mediator that triggers pathway leading in acute and late normal tissue lesion after irradiation. PAI-1 belongs to the family of serine protease inhibitors and is the main inhibitor of fibrinolysis. Both plasminogen activators (u-PA and t-PA) convert plasminogen to plasmin that degrades insoluble fibrin. PAI-1 inhibits u-PA and t-PA and thus reduces plasmin generation (6). PAI-1 not only reduces fibrinolysis but also plays a role in extracellular matrix remodelling by reducing plasmin-dependent matrix metalloproteinases (MMP) activation (7).

In pathological conditions, PAI-1 is produced by a variety of cell types such as hepatocytes, adipocytes, smooth muscle cells, platelets and especially endothelial cells. The endothelium is known to play a critical role in radiation-induced injury. Irradiated endothelial cells (EC) acquire a pro-inflammatory, a pro-coagulant and a pro-thrombotic phenotype. Up-regulation of endothelial cell adhesion molecules after irradiation leads to the increase leukocyte/EC interactions and leukocyte transmigration (8-11). Moreover, irradiation increases the interactions of platelets (12) with the endothelium and decreases expression of the anti-coagulant thrombomodulin (13). This loss of vascular thrombo-resistance of endothelium following radiation is a result of an increased fibrinogenesis and a decreased fibrinolysis (14). In this context, the role of PAI-1 in radiation induced damages could be crucial. Ionizing radiation increases PAI-1 expression in rat mesangial cells, NRK52E cells and HEPG2 cells (15-18). Moreover, PAI-1 overexpression has been described in radiation-induced nephrosclerosis (19, 20) and in human radiation enteritis (21) suggesting a role of PAI-1 in radiation-induced normal tissues damages.

In this study, we analysed consequences of PAI-1 genetic deficiency on the normal tissue response to radiation exposure. Moreover, immunolabelling of PAI-1 was performed in tissues from patients treated with radiotherapy. Finally, molecular mechanisms involved in PAI-1 expression after irradiation in endothelial cells were investigated.

Results

PAI-1 -/- mice are protected against radiation-induced normal tissue damages.

To investigate the effect of ionizing radiation on normal tissue *in vivo,* we used a model of radiation-induced enteropathy in mice which consists in exposing a jejunal segment from wild type and PAI-1-/- mice to a single dose of 19Gy. The survival of mice was followed and radiation injury score was determined two and six weeks after irradiation. The survival of PAI-1-/- mice is increased compared to Wt mice following radiation exposure (Figure 1A). Two weeks after irradiation 75 % of PAI-1-/- mice are alive compared to 41 % for Wt mice and this difference remains the same after six weeks (Figure 1B). The totality of the intestinal segment was assessed for histology and radiation injury was determined using a radiation injury scoring system. Irradiation causes significant radiation injury in Wt and PAI-1 -/- mice (Figure 2A). However, acute and late radiation injury is more severe in Wt mice compared to PAI-1 -/- mice irradiated (+ 35% at two weeks and + 45% at 6 weeks, $p<0.05$). At two weeks, we observed a better epithelial cover and a reduced inflammation in mice deficient for PAI-1 compared to Wt mice (Figure 2B). Moreover, at 6 weeks, a reduction of fibrosis was observed in PAI-1 -/- (Figure 2, C-D). We also observed a strong remodelling of extracellular matrix in Wt mice associated with a pronounced intestinal wall fibrosis mainly in muscularis propria infiltrated with collagen and a subserosal fibrosis (Figure 2D). In order to confirm if the attenuation of radiation injury in PAI-1 -/- mice is related to a better intestinal functionality, we measured the capacity of irradiated tissues to respond to electrical field and exogenous carbachol stimulations. This measure is classically used to evaluate intestinal integrity following irradiation (22). We chose to test the intestinal functionality before the separation the survey curves between irradiated Wt and PAI-1 -/- mice i.e. 3 days after irradiation. No differences concerning basal parameters are observed between sham Wt mice and PAI-1 -/- mice. The functionality of the intestine is reduced in both type mice after irradiation but severity of the alteration is more pronounced in Wt mice (Figure 3). The functionality decreases of

83 % in Wt mice and 62 % in PAI-1 -/- mice following electric stimulation and decreases of 92 % in Wt mice and 72 % in PAI-1 -/- mice following carbachol stimulation. In order to measure that exteriorization have no effect on basal parameters, two groups without jejunal exteriorization were assessed (Wt and PAI-1 -/- n=10 per group) and no differences is observed between these groups or compare to the sham groups (data not show).

PAI-1 expression is increased *in vivo* after irradiation

PAI-1 mRNA expression was measured in order to know if the strong alteration of intestinal functionality observed rapidly after irradiation is associated with modification of PAI-1 expression. PAI-1 mRNA level is increased 4-fold ($p<0.05$) in Wt irradiated mice compared Wt sham mice (Figure 4A). vWF and PAI-1 immunolabellings show that PAI-1 overexpression is observed in endothelial cells from mesenteric vessels (Figure 4B : A,C,E F,H, I) and mucosal microcirculation (Figure 4B : B,D,G,J). These results suggest that endothelial PAI-1 up-regulation contributes to radiation-induced damages.

Irradiation increases blood cells/endothelial cells interactions in Wt mice and not in PAI-1 -/- mice.

Increased leukocytes/platelets interaction with endothelium was shown after irradiation. We hypothesized that PAI-1 antifibrinolytic activity could be crucial in this physiological response following radiation. We investigate blood cells-endothelial interactions in Wt and PAI-1 -/- mice in order to confirm this hypothesis. To avoid the putative effect of chirurgy in the model of exteriorized jejunal segment, leukocytes/platelets-endothelium interactions using intraviatal microscopy was performed 24 hours after an abdominal irradiation of 15 Gy. Leukocytes and platelets velocities rolling as well as number of rolling platelets and number of temporary and definitive stops of platelets were determined. Because any statistical differences were observed for all parameters measured between non-irradiated Wt and PAI-/-, we choose to present results as percent of each parameters for each strain except for definitive stops of platelets. Moreover, blood

numeration (leukocytes and platelets) was determined in non irradiated Wt and PAI-1 -/- mice and no differences was observed (data not shown). Irradiation decreases the rolling velocity of leukocytes and platelets in PAI-1 +/+ mice but not in PAI-1 -/- mice (Figure 5, A-C). Moreover a strong increase in the number of rolling platelets and the number of temporary and definitive stops of platelets was observed in irradiated PAI-1 +/+ mice compared to non irradiated PAI-1 +/+ (Figure 5C). Interestingly, irradiation have no effects on rolling velocity of platelets as well as others parameters of platelet adhesion in PAI-1 -/- mice.

Radiation-induced normal tissue damages in humans are associated with overexpression of PAI-1 in vascular endothelium

To demonstrate physiological significance of our results, we undertook a retrospective study in patients. Radiation injury score and immunohistochemical staining of PAI-1 were performed in tissues from patients treated by radiotherapy for rectum adenocarcinoma. Radiation-induced normal tissues damages are characterised by a strong expression of PAI-1 in endothelial cells (Figure 6, A-B) suggesting that the PAI-1 endothelial pool play an important role in normal tissue damages after irradiation.

In vitro, irradiation increases PAI-1 expression and secretion in EC

PAI-1 mRNA expression was investigated by real time PCR in two types of endothelial cells. PAI-1 mRNA level is increased in HUVEC and HMVEC 24h and 48h after 2 or 10Gy irradiation (Figure 7A). Moreover, irradiation increases the protein level and the secretion of PAI-1 in HMVEC (Figure 7, B-C). Similar results were observed in HUVEC (Data not show).

Irradiation activates the Smad pathway in EC.

TGF-β/Smad pathway plays a key role in the control of PAI-1 expression in various cell types under different conditions. Here we provide evidence that irradiation activates Smad pathway in EC. Smad-dependent transcription was performed using TGF-β/Smad responsive reporter $CAGA_9$-lux and results showed that irradiation activates Smad-dependent transcription in EC (Figure 8A).

Moreover, irradiation rapidly increases phosphorylation of endogenous Smad2 and Smad3 (Figure 8B). We confirmed this effect in EC which overexpressed Smad2 or Smad3 using Flag-tagged Smad2 and Smad3 expression vectors (Figure 8C). Nuclear translocation of Smad2 ,3 and 4 was followed by immunoblotting using Myc-tagged versions of Smads. Nuclear translocation of Smad2, Smad3 and Smad4 as well as phosphorylated Smad 2 and 3 are stimulated 2 h after irradiation (Figure 8D). However, 24h hours after irradiation, we observed an increased of nuclear expression of Smad3 and Smad4 but not Smad2 suggesting different effects of Smad2 and 3 on radiation-induced Smad target genes in EC. To support *in vitro* results and to demonstrate *in vivo* relevance of our observations, immnunolabelling of activated Smad2/3 was performed in tissues from patients treated with radiotherapy (Figure 8E). We observed an increased expression of phospho-Smad2/3 in endothelium in pathological tissues compare to control tissues suggesting that, *in vivo,* Smad pathway is involved in PAI-1 overexpression in EC.

Radiation-induced PAI-1 expression involves Smad pathway

The wild type human PAI-1 promoter contains three Smad responsive element (CAGA) at positions –730, -580 and –280 (23). To provide evidence that Smad pathway is involved in radiation-induced PAI-1 transcription, HUVEC were transfected with Wt human PAI-1-luc reporter or human PAI-1-luc reporter with mutation of the three CAGA boxes (Δb123-PAI-1 Luc) (23). Irradiation stimulates luciferase activity in EC transfected with Wt-PAI-1-luc but not with Δb123-PAI-1 Luc demonstrating that at least one CAGA box is involved in radiation-induced PAI-1 transcription (Figure 9A). These results suggest the stimulation of PAI-1 transcription after irradiation is Smad dependent. To investigate the role of Smad in radiation-induced PAI-1 transcription, EC were co-transfected with Flag-tagged Smad2, Smad3 or Smad7 and Wt-PAI-1-luc luciferase activity was measured. In EC overexpressing SMAD7, the radiation-induced PAI-1 transcription is inhibited demonstrating that this inhibitory Smad abolish the mechanism (Figure 9B). Moreover, overexpression of Smad3 but not Smad2 strongly stimulates the radiation-induced

Wt-PAI-1-luc luciferase activity showing that Smad3 influence the radiation-induced PAI-1 transcription (Figure 9B). To confirm these results, PAI-1 immunoblotting was performed in EC transfected with Myc-Smad2 or Myc-Smad3. PAI-1 expression is increased in EC overexpressing Smad3 (Figure 9C) and when we co-expressed Flag-Smad2 and Myc-Smad4 or Flag-Smad3 and Myc-Smad4, only the combination of Smad3/Smad4 leads to an increase in the radiation–induced PAI-1 expression (Figure 9D). Because Smad3 but not Smad2 is involved in PAI-1 expression after irradiation, we next examined whether irradiation induces the ability to Smad3 to bind Smad4. Co-immunoprecipitation experiments show that irradiation stimulates the formation of Smad3/smad4 complexes (Figure 9E). All together, these results demonstrate that Smad pathway is involved in radiation-induced PAI-1 expression in EC and revealed that Smad3 and not Smad2 plays a crucial role in this mechanism.

Smad3 and p53 are necessary for radiation-induced PAI-1 expression.

Ionizing radiation leads to double-strand DNA breaks and the tumor suppressor p53 is one of the key proteins involved in radiation cellular response which coordinates apoptosis, cell cycle progression and mechanisms of DNA repair. Recently, Hageman *et al* showed that radiation-induced PAI-1 transcription in tumor cells is p53 mediated (15). We hypothesised that p53 is involved in PAI-1 expression in EC after irradiation. As expected, irradiation of EC stimulates expression and nuclear translocation of p53 (Figures 10A-B). To investigate the transcriptional cooperation between p53 and Smads, HUVEC were transfected with Myc-Smad3 or Myc-Smad4 and co-immunoprecipitation experiments were performed. Smad3 but not Smad4 co-immunoprecipitates with p53 after irradiation suggesting that a Smad3/p53 transcriptional cooperation could activate PAI-1 transcription (Figure 10C). To prove that Smad3 and p53 were involved, we decided to knockdown their expression using siRNA. For Smad3, the efficiency of silencing was determined by reporter gene assay and western blot. In presence of siRNA Smad3, the TGF-β1 induced $(CAGA)_9$-Luc luciferase activity is inhibited and western-blot show a strong

decrease of Smad3 expression (Figures 10, D-E). Moreover, p53 expression is strongly decreased in HUVEC transfected with siRNA p53 (Figure 10F). Next, the effect of Smad3 or p53 silencing on radiation-induced PAI-1 transcription and expression was studied. The radiation-induced Wt-PAI-1-luc luciferase activity is abolished in presence of siRNA Smad3 or siRNA p53 (Figure 10G). This effect was confirmed by western-blot and demonstrate that the silencing of smad3 and p53 leads to the inhibition of the radiation-induced PAI-1 expression in EC (Figure 10H).

Discussion

In this report, we demonstrate for the first time the major role of PAI-1 in radiation-induced normal tissues damages. These data supply the proof of concept that PAI-1 may represent a molecular target to limit injury following radiotherapy. Moreover, investigation of molecular mechanisms involved in radiation-induced PAI-1 expression in EC revealed a new role of Smad pathway in the pathogenesis of radiation-induced damages.

In a model of radiation enteropathy in mice, we clearly demonstrate that PAI-1 plays a crucial role in radiation-induced intestinal lesion following a high single dose of radiation. More than fifty percent of wild type mice died in the next ten days following radiation as prevoioully described (24). PAI-1 knockout mice are protected against radiation-induced damages with a very higher proportion of surviving mice and a radiation injury score less severe compared to irradiated Wt mice. These results show that genetic deficiency of PAI-1 protect against radiation-induced damages and suggest an early deleterious effect of PAI-1 in Wt mice. PAI-1 mRNA expression is rapidly induced in irradiated intestinal segment from wild type mice and we showed that the functionality of the intestine 3 days after irradiation is more severely affected in Wt mice compared to PAI-1 -/- mice. Moreover, PAI-1 up-regulation is observed in endothelium. Radiation causes loss of vascular thromboresistance due partly to a decreased fibrinolysis (25). PAI-1 limits fibrinolysis and we hypothesized that PAI-1 contribute to the acute response of endothelium to ionising radiation. We show that radiation decreases the rolling velocity of leucocytes and platelets only in Wt mice. Moreover, radiation enhances platelet adhesion in Wt mice but not in PAI-1 -/- mice. These results show that genetic deficiency of PAI-1 limit the interaction between blood cells and endothelium following radiation and strongly suggest that PAI-1 play a crucial role in the vascular response to ionising radiation. PAI-1 deficiency in human is associated with abnormal bleeding (26) and PAI-1 knockout mice are less likely to develop venous thrombi (27) while mice overexpressing a human transgene develop spontaneous thromboses (28). Our results suggest that

radiation-induced hypo-fibrinolysis is abolished in PAI-1 -/- mice and consequently protect these mice against deleterious effects of the loss of trombo-resistance of the endothelium. However our results don't exclude a direct role of platelets. Indeed, the release of TGF-β from platelets at sites of vascular injury may induce PAI-1 expression in surrounding EC and thus inhibits fibrinolysis. Moreover, platelets express large amounts of PAI-1 (29) and radiation-induced platelet adhesion could contribute to stabilization of blood clots. Further investigations are needed to determine the precise role of PAI-I and discriminate the contribution of different pools of PAI-1 (*i.e.* platelets, endothelium) in radiation-induced injury.

We observed a reduction of fibrosis in PAI-1 -/- 6 weeks after irradiation suggesting that early effects could contribute to delayed effects of radiation injury. Our results support the concept of "consequential late effects" defined as late radiation damage occurs as a direct consequence of the severity of acute damage (2). A large number of strategies in experimental models showed that the reduction of late effects is associated with an improvement of acute effects (30-33). So, in a clinical point of view, our results suggest that pharmacological strategies aimed to inhibit PAI-1 activity could prevent acute and late effects of radiation-induced normal tissues damages. PAI-1 is up-regulated in fibrotic diseases including hepatic, pulmonary or renal fibrosis and PAI-1 knockout mice are protected against fibrosis in various model (34-37). Here we demonstrate that radiation-induced fibrosis is less severe in PAI-1 -/- suggesting that PAI-1 inhibition could be a good anti-fibrotic strategy in different models of radiation-induced fibrosis. By competing with endogenous PAI-1, a mutant non-inhibitory PAI-1 was described to reduce glomerulosclerosis (38) and a pharmacological PAI-1 inhibitor protect mice against long-term nitric oxide synthase inhibition induced thrombosis (39). The efficiency of pharmacological inhibition of PAI-1 in radiation-induced normal tissues damages is an attractive perspective of our study.

TGF-β1 is a key fibrogenic cytokine involved in radiation-induced normal tissue damages (5) and is a strong inductor of PAI-1 expression. TGF-β1 transduces the signal by inducing

heteromeric complexes of typeI and typeII receptors with intrinsic serine/threonine kinase activity (40). Activated type I receptor kinases phosphorylate Receptor-regulated Smads (R-Smads) at their extreme C-terminal serine residues and phosphorylated R-Smads form heteromeric complexes with common-partner (Co)-Smad, *i.e.* Smad4 (41). Smad complexes accumulate in the nucleus and control the expression of target genes. We show for the first time that PAI-1 is overexpressed in the endothelium of pathological tissue from patients treated with radiotherapy. Interestingly, radiation-induced damages in patients were associated with a increase of phosphorylated -Smad 2/3 in endothelium suggesting that Smad pathway is involved in the overexpression of PAI-1 in EC *in vivo*. *In vitro,* TGF-β1 secretion is increased in EC after irradiation (42) and an upregulation of TGF-β in endothelium was observed in radiation-induced damages in rats (43) and in patients treated with radiotherapy (42). All together, this results suggest that radiation-induced TGF-β in endothelium can activate Smad pathway via an autocrine loop. *In vitro* experiments were performed to determine molecular mechanism involved in radiation-induced PAI-1 expression in human endothelial cells. PAI-1 expression is increased in EC following radiation and we show that irradiation activates Smad pathway in EC. While both Smad2 and Smad3 mediate signals from TGF-β, a large number of evidence demonstrates that they have different and non-redundant functions. The transcriptional mechanism is different and while Smad3 bind DNA directly through its MH1 domain, Smad2 activates transcription of target genes indirectly through other DNA binding proteins (41). In this study, we demonstrate that radiation-induced PAI-1 transcription in EC is Smad3-dependent. These results confirm and support the concept that Smad3-targeted genes are critical mediators of radiation-induced damages in normal tissues. Our group showed recently that a Smad3 pathway is involved in the fibrogenic phenotype of vascular smooth muscle cells induced by irradiated EC (42) and interestingly, Flanders *et al* showed that Smad3 null mice are less susceptible to radiation-induced injury (44, 45). However it is not clear whether the putative

Smad3 genetic deficiency is associated with alteration of PAI-1 expression following radiation and experiments to answer this issue need to be performed.

Crosstalk between Smad pathway and other signalling pathways have been previously described (46). Our results demonstrate that p53 is necessary for radiation-induced PAI-1 expression in EC. The tumor suppressor p53 is one of the most frequently mutated genes found in human cancers. p53 plays a key role in the cell response to ionizing radiation and the activation of p53 in response to DNA damages allows the preservation of genomic stability by controlling mechanisms of DNA repair, cell cycle progression and apoptosis (47). However, it is now clear that p53 play a key role in other physiologicals and pathophysiologicals conditions by controlling the expression of various genes. Our study reveals that p53 could contribute to radiation-induced normal tissues toxicity by inducing PAI-1 expression in endothelial cells in cooperation with Smad3. PAI-1 was described as a direct target of p53 (48) in tumor cells and a study using a DNA microarray technology showed that PAI-1 is a p53 regulated gene following ionizing radiation (49). Radiation increases PAI-1 expression in rat mesangial cells (16), NRK52E cells (18) and recently Hageman et al showed that mutation of a p53 binding element in the PAI-1 promoter abolished the radiation-induced PAI-1 transcription in HEPG2 cells (15). Radiation and TGF-β cooperate in the radiation-induced PAI-1 transcription suggesting that a p53/Smad pathway is involved in this mechanism. The crosstalk between p53 and Smad pathway is involved in embryonic development in the frog (50, 51) and Cordenonsi *et al* showed that a specific cooperation and physical interaction of Smads with p53 play a key role in embryogenesis (50). They showed that p53 is a DNA-binding Smad transcriptional partner that physically bind to R-Smads to controle the activation of promoters that contained both p53 and Smad binding elements. Our results show that irradiation induces the physical interaction between p53 and Smad3 in EC and knockdown experiments demonstrate that both Smad3 and p53 are necessary for the radiation-induced PAI-1 expression. To our knowledge, this is the first report which demonstrates that a

p53/Smad3 pathway is involved the response of normal cells to ionizing radiation. Our results also suggest that p53 could contribute to radiation-induced normal tissues damages by inducing the expression of PAI-1 in endothelial cells and subsequently contribute to the loss of thromboresistance of endothelium. Further investigations are needed to understand the precise role of p53/PAI-1 connexion in radiation-induced normal tissues damages.

In conclusion, our study shows for the first time that PAI-1 plays a critical role in radiation-induced damages in normal tissues. These data contribute to the knowledge of the normal tissue response after irradiation and especially on the role of Smad3/p53 pathway in radiation-induced PAI-1 expression in endothelium. Our results indicate PAI-1 may represent a therapeutic target in order to prevent and/or reduce side effects of radiotherapy.

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Methods

Animals and irradiation procedure

Experiments were conducted in compliance with legal regulations in France for animal experimentation (European act # 2001-486, June 6 2001), and standard operating procedures and protocols were approved by the ethics committee for animal experimentation of the Institute for Radioprotection and Nuclear Safety. Wild type C57Bl/6J and PAI-1 -/- mice (52) (Charles River Laboratories) were maintained on a 12-hour light-dark cycle. They were given a standard chow diet and had free access to water. Mice were anaesthetized by inhalation of 5% isoflurane and maintained under anaesthesia with 1.7% isoflurane during radiation exposure. Animals first underwent laparotomy and a 2 to 3cm-long jejunal segment (10cm from the ileocoecal valve) was exteriorised and exposed to a single dose of 19Gy gamma irradiation (Co^{60} source, 4000 Ci, dose rate 1.4Gy/min). The animal, except the exteriorised intestine, was entirely protected by appropriated lead screens. During irradiation, abdominal organs were covered with sterile saline solution-moistened gauze. Sham irradiation was performed by maintaining the jejunal segment exteriorised without radiation exposure. After radiation exposure or sham-irradiation, the exposed segment was returned to the abdominal cavity and peritoneum/abdominal muscles and skin were separately closed with interrupted sutures. After surgery mice were kept in standard cages and had free access to chow and water.

Histology and Immunohistochemistry

The totality of the intestinal segment was assessed for histology. Longitudinal sections of the intestine were fixed in 4% formaldehyde solution and embedded in paraffin. Slides were stained by haematoxylin-eosin-safran and Sirius red colorations. Radiation injury was determined score using a radiation injury scoring system described and validated by Hauer-jensen's group including mucosal ulcerations, epithelial atypia, thickening of serosa, vascular sclerosis, fibrosis of the intestinal wall, ileitis cystica profunda and lymph congestion (43). The individual abnormalities

were graded according to severity (0 to 3) and the sum of the scores for each parameter constitutes the radiation injury score. It was determined independently by two authors in a blinded manner and discrepancies were resolved in conference. For immunohistochemistry, frozen sections from Wt mice were fixed with acetone and von Willebrand factor (vWF) and PAI-1 labellings were performed using specific antibodies (rabbit anti-human vWF, Dakocytomation ; rabbit anti-mouse PAI-1, Abcam). Slides were then incubated with goat anti-rabbit IgG tagged with Alexa Fluor 488 (Molecular Probes), rinsed, and counterstained with DAPI (Vector).

Ussing chamber experiments

Ussing chamber experiments were used to evaluate intestinal functionality in wild type and PAI-1-/- mice exposed to a localised dose 19Gy. Tissue samples were rinsed with saline (0.9% NaCl) and mounted in Ussing chambers with 0.126 cm^2 aperture (Corning Costar Corporation). Tissues were bathed with a modified Krebs buffer containing (in mM) 115.0 NaCl, 8.0 KCl, 2.0 KH_2PO_4, 2.4 $MgCl_2$, 1.3 $CaCl_2$, 25.0 $NaHCO_3$, 37°C, pH 7.4, and gassed with 95% O_2/5% CO_2. The serosal buffer contained 10.0 mM glucose, while the mucosal buffer 10.0 mM mannitol. Tissue responses to electrical field stimulation (EFS) and carbachol were measured by clamping the potential difference to 0 mV, under short circuit current (Isc) conditions with a voltage-clamp apparatus (DVC-1000, World Precision Instruments). EFS (100 V, pulse duration of 500µS, total stimulation time of 3 s, frequency of 35 Hz) was applied with a dual impedance stimulator (Harvard Instruments). Carbachol (final concentration 5.10^{-5} M), was added on the serosal side (added volume 20µl). Each tissue was submitted to 35Hz EFS and subsequent carbachol stimulation.

Intravital microscopy

Intravital microscopy was used to quantify rolling and adhesion of leukocytes and platelets in the mesenteric venules from wild type C57Bl/6J and PAI-1 -/- mice after an abdominal single exposure of 15 Gy. Sham-irradiated mice were handled like irradiated mice but were not exposed to the radiation source. 24 H after irradiation, mice were anesthetized by intraperitoneal injection

of 2.5% tribromoethanol and leukocytes and platelets were stained by intravenous administration of 10.4% Rhodamin 6G (62.5 µl/mouse, Sigma). The mesentery was gently exteriorized through a midline abdominal incision and was placed in a 5% gelatin coated Plexiglas chamber for observation of the mesenteric microcirculation. 100-250 µm diameter venules were selected for direct observation of leukocyte- and platelets -endothelium interactions as previously decribed (12). Venules were filmed for 5 to 10 minutes and video recording was resumed for another venule (up to four vessels were analyzed for each mouse). Quantification of blood cells-endothelial interactions (rolling and adhesion) was made using the software Histolab 4.3.6 (Microvision Instruments). A platelet was considered to be adherent when it was stationary on the endothelium for more than 2 minutes. Leukocyte and platelet-endothelial interactions were analyzed within a square of 50X50µm² and quantifications were normalized thereafter per 0.01 mm²/min.

Cell culture and irradiation

HMVEC (Dermal human microvascular endothelial cells) and HUVEC (human umbilical endothelial cells) were purchased from Cambrex and cultured in EGM-2 MV culture medium. Cells were irradiated with a ^{137}Cs source (IBL 637, dose rate 1 Gy. min $^{-1}$).

RNA isolation and RT real time PCR

Total RNA was prepared with the total RNA isolation kit (Rneasy Mini Kit, Qiagen). Total RNA quantification and integrity was analysed using Agilent 2100 bioanalyzer and 1 µg of RNA was used for RT with SuperScript II (Invitrogen Life Technologies) and random hexamer to generate first strand cDNA. The following primers were used : human PAI-1 (F 5' GCA CAA CCC CAC AGG AAC AG 3', R 5' GTC CCA GAT GAA GGC GTC TTT 3'. Thermal cycling conditions were 10 min at 95°C followed by 40 cycles of 95°C for 15s and 60°C for 1 min on an ABI PRISM 7700 Sequence detection system (Applied Biosystems). Significant PCR fluorescent signals were normalized to a PCR fluorescent signal obtained from the housekeeping gene GAPDH or (Pre-

developed Taqman Assay, Applied Biosystems) for each sample. Relative mRNA quantitation was performed by using the comparative $\Delta\Delta C_T$ method.

Plasmids

Expression vectors for 6xMyc-Smad2, 6xMyc-Smad3, 6xMyc-Smad4, Flag-Smad2, Flag-mad3, Flag-Smad7, (CAGA)9-Lux, WtPAI-1 Luc, Δ123-PAI-1 Luc were previously described (23, 53, 54).

Reporter Gene assay

HUVEC were transiently co-transfected with expression vectors reporter and pRL-TK plasmids using FuGENE (Roche Diagnostics) as transfection reagent. Cells extracts were prepared for the Dual-Luciferase reporter assay system according the manufacturer's instructions (Promega). Relative luciferase activity was measured using a Mithras luminometer (Berthold Technologies).

Protein extraction

Cells were lysed at 4°C in RIPA buffer (10 mM Tris, pH 8.8, 150 mM NaCl, 1 mM EDTA, 1 % sodium deoxycholate, 1 mM sodium vanadate, 0.1 % SDS, 1 % Igepal and protease inhibitor cocktail). Cell lysate was centrifuged at 13500 rpm 15 min and supernatant constitute total protein. Furthermore, nuclear protein extracts were prepared using the method of Schreiber (55).

Immunoblotting

The following protein-specific primary antibodies were used: anti–PAI-1 (Novocastra Laboratories Ltd) anti-Smad2, anti-Smad3 (Zymed Laboratories) , anti-Smad4 (B-8) , anti-p53 (DO1), anti-Myc (9E10) (Santa Cruz Biotechnology), anti-Phospho Smad3, anti-Phospho Smad2 (Cell Signalling Technology), anti-FlagM2 (Sigma), and anti-GAPDH (Biodesign). Proteins were separated by SDS-PAGE electrophoresis before transfer onto nitrocellulose membranes. The membranes were blotted with primary antibodies followed by incubation with secondary antibody HRP-conjugated (Amersham). Blots were developed using the enhanced chemiluminescence method (Amersham).

Membranes were then deshybridised and reprobed with anti-GAPDH antibody to detect GAPDH expression as control loading.

Immunoprecipitation

Cell lysates were subjected to immunoprecipitation with appropriate antibodies overnight followed by adsorption to Sepharose protein G for 1h. Immunoprecipitates were resolved by immunoblotting as descrived above.

PAI-1 ELISA assay

PAI-1 production in the supernatants of EC was determined by ELISA assay (American Diagnostica) according to the manufacturer's instructions.

RNA interference

The sequence of siRNAs designed to specifically target Smad3 is 5'-ACCUAUCCCCGAAUCCGAUdTdT- 3' (42). SiRNA targeting human p53 were purchased from Santa Cruz Biotechnology.

Patients, radiation injury score and immunohistology

Twenty Five patients treated for rectal adenocarcima with preoperative radiotherapy (45Gy; 2 or 1.8 Gy by fraction) were included in this study. Tumors were surgically resected 5 to 7 weeks post-treatment. For each patient, specimens of normal tissue were taken in the irradiated field adjacent to the tumor and from microscopically normal mucosa distant from the tumor. Slides were colored by haematoxylin-eosin-safran coloration and radiation injury score was determined independently by two authors as previously described in details (42). For immunohistochemistry, 5µm sections were used to immunolocalize PAI-1 (Novocastra Laboratories Ltd) and phospho-(ser433/435) Smad2/3 (Santa Cruz Biotechnology). Biotinylated rabbit anti-mouse IgG and Streptavidin/biotynilated-peroxydase kit (Dakocytomation) were used before revelation by NovaRED substrate kit (Vector) and counterstained with hematoxylin.

Statistical analyses

Data are given as mean ± SEM. Statistical analyses were performed by ANOVA or Student's t test with a level of significance of $P<0.05$. For intravital microscopy analyses, statistical significance was assessed by Mann-Whitney Rank Sum Test. Mouse survival curves were calculated with the Kaplan-Meier method and compared using the log rank test.

Figure legends

Figure 1 : Survival of PAI-1 -/- mice is increased following 19 Gy intestinal irradiation.

A) Kaplan-Meier analyses represents the percent survival of PAI-1 +/+ and PAI-1-/- mice during 14 days after irradiation. P< 0.005 vs the three others groups (Log Rank Test). **B)** Percent survival of PAI-1 +/+ and PAI-1-/- mice six weeks after irradiation. P< 0.005 vs PAI-1 +/+ 19 Gy (Log Rank Test)

Figure 2: Acute and late radiation enteropathy were reduced in PAI-1 -/- mice.

A jejunal segment from PAI-1 +/+ and PAI-1-/- mice was exposed to a single dose of 19Gy. Two and six weeks after irradiation, the totality of the intestinal segment was assessed for histology and Radiation injury was determined using a radiation injury scoring system **A)** Radiation injury score was measured 2 and 6 weeks after irradiation, n=12 mice per group, **P*<0.05 *vs* PAI-1 +/+ Sham mice, # *P*<0.05 *vs* PAI-1 +/+ irradiated mice. **B)** Representative microscopique alterations obtained in PAI-1 +/+ and PAI-1 -/- mice are shown (original magnification x100). Slides were stained with haematoxylin-eosin-safran coloration. **C)** Representative macroscopique features obtained in PAI-1 +/+ and PAI-1 -/- mice obtained 6 weeks after irradiation are shown. **D)** Representative microscopique alterations obtained in PAI-1 +/+ and PAI-1 -/- mice obtained 6 weeks after irradiation are shown (original magnification x100). Slides were stained by Sirius red coloration.

Figure 3 : Acute Intestinal functionality is better in PAI-1 -/- compared to PAI-1 +/+ mice after irradiation.

Percent inhibition of *I*sc in response to **A)** electrical field stimulation (35 Hz) or **B)** exogenous carbachol stimulation in irradiated tissues from PAI-1 +/+ and PAI-1 -/- mice 3 days after irradiation. Results are means ± SEM with n=13 to 17 mice per group, **P*<0.05 *vs* Sham mice, # *P*<0.05 *vs* PAI-1 +/+ irradiated mice.

Figure 4 : PAI-1 expression is increased *in vivo* after irradiation

A) PAI-1 mRNA expression was measured 3 days after irradiation (n=10 mice per group *p<0.05). **B)** Ten-micron frozen sections of PAI-1 +/+ mice intestine (3 days after irradiation) were stained with antibodies against vWF or PAI-1 (green) and counterstained with DAPI (blue). Representative images (X200 original magnification) are shown and arrows indicate vessels.

Figure 5 : Irradiation increases blood cells-endothelial interactions in PAI-1+/+ and not in PAI-1 -/- mice.

Intravital microscopy was used to quantify rolling and adhesion of leukocytes and platelets in the mesenteric venules from PAI-1 +/+ and PAI-1 -/- mice (n=6 mice per group) after an abdominal single exposure of 15 Gy. The mesentery was exteriorized and blood cells-endothelial interactions (rolling and adhesion) was quantified. 24h after irradiation **A)** Rolling velocity of leukocytes was measured in PAI-1 +/+ and PAI-1 -/- mice. **B)** Representative images from 3 irradiated PAI-1 +/+ (Top) and 3 PAI-1 -/- mice (Bottom) are shown. The black arrows indicate leukocyte and the white arrows indicate platelets. **C)** Parameters platelets adhesion were determined. Results are means ± SEM. Statistical significance was assessed by Mann-Whitney Rank Sum Test. * P<0.05, ** P<0.001

Figure 6 : Radiation-induced normal tissue damages in human is associated with overexpression of PAI-1 in endothelium

A) Radiation injury score was determined in 25 patients treated by radiotherapy for rectum adenocarcinoma. For each patient, specimens of tissue were taken in the irradiated field adjacent to the tumor (pathological) and from microscopically normal mucosa distant from the tumor (control). Immunohistochemical staining of PAI-1 was performed and slides were separated according to the positivity of PA1-1 expression in endothelium. **B)** Representative microscopic images from control and pathological submucosal vessels are shown.

Figure 7 : Irradiation increases PAI-1 expression and secretion in EC

A) PAI-1 expresssion was investigated by real time PCR in HUVEC and HMVEC 24h and 48h after 2 or 10Gy irradiation. Data are the mean ± SEM of 4 independent experiments realized in duplicate or triplicate. **B)** PAI-1 protein expresssion was measured by western-blot in HUVEC and HMVEC. Representative western-blot of 3 independent experiments are shown. **C)** PAI-1 content was determined by ELISA assay in HMVEC supernatants 24 hours after 2 or 10Gy irradiation Data are the mean ± SEM of 3 experiments realized in triplicate. **P*<0.05 *vs* control

Figure 8 : Irradiation activates Smad pathway in EC

A) HUVEC were transiently co-transfected in complete medium with (CAGA)9-Luc reporter (1µg) and pRL-TK (0.2µg) plasmids using FuGENE as transfection reagent. 48h after transfection, culture medium was changed and cells were irradiated. Relative luciferase activity (ratio Firefly/Renilla) was measured 24h after irradiation. HUVEC treated with 5 ng/mL of TGF-β1 for 24 hours is shown. Data are the mean ± SEM of 3 independent experiments (n=6 by experiment). **P*<0.05 *vs* control. **B)** Phospho-Smad2 and phospho-Smad3 protein expression was measured by western-blot in HUVEC 2h after irradiation. **C)** HUVEC were transfected with Flag-Smad2 or Flag-Smad3 expression vector 48h before irradiation and the phosphorylation of Smad2 and Smad3 protein expression was measured by western-blot 2h after irradiation. **D)** HUVEC were transfected with the indicated expression vector 48h before irradiation and nuclear expression of Smad2, Smad3, Smad4, phospho-Smad2 and phospho-Smad3 was measured by western-blot 2h and 24 h after irradiation. HUVEC treated with 10 ng/mL of TGF-β1 for 2h or 24 h hours is shown. **E)** Immunohistochemical staining of Phospho-Smad2/3 was performed in tissues from 10 patients treated by radiotherapy for rectum adenocarcinoma. Representative microscopic images from control and irradiated submucosal vessels are shown.

Figure 9 : Radiation-induced PAI-1 expression involves Smad pathway.

A) HUVEC were transiently co-transfected in complete medium with Wt PAI-1-Luc reporter or CAGA box-mutated PAI-1 Luc reporter (Δb123-PAI-1 Luc) and pRL-TK plasmids using FuGENE as transfection reagent. 48h after transfection, culture medium was changed and cells were irradiated. Relative luciferase activity (ratio Firefly/Renilla) was measured 24h after irradiation. HUVEC treated by 5 ng/mL of TGF-β1 for 24 hours is shown. Data are the mean ± SEM of 3 independant experiments (n=6 by experiment). **P*<0.05 *vs* control. **B)** HUVEC were transiently co-transfected with indicated expression vector and Wt PAI-1-Luc reporter plasmids . Relative luciferase activity was measured 24h after irradiation. **C)** HUVEC were transfected with indicated expression vector and PAI-1 protein expression was measured by western-blot 24h after irradiation. HUVEC treated by 10 ng/mL of TGF-β1 for 24 hours is shown. **D)** HUVEC were transiently co-transfected with Myc-Smad4 and Flag Smad2 or Flag Smad3 and PAI-1 protein expression was measured by western-blot 24h after irradiation. **E)** HUVEC were transiently co-transfected with indicated expression vector. 24h after irradiation cell lysates were immunoprecipitated (IP) with anti-Flag antibody and immunoblotted (IB) with anti-Myc. HUVEC treated by 10 ng/mL of TGF-β1 for 24 hours is shown.

Figure 10 : Radiation-induced PAI-1 expression involves Smad3 and p53.

A) p53 expression in HUVEC was measured by western-blot 24h after irradiation. **B)** Nuclear expression of p53 in HUVEC was measured by western-blot 2h and 24 h after irradiation. HUVEC treated by 10 ng/mL of TGF-β1 for 2h or 24 hours is shown. **C)** HUVEC were transfected with Myc-Smad3 or Myc-Smad4. 2h and 24 h after irradiation. The association of p53 with Smad3 or Smad4 was analysed by blotting the anti-Myc immunoprecipitate with anti-p53. **D)** HUVEC were transiently transfected with (CAGA)9-Luc reporter and pRL-TK plasmids in absence or presence of siRNA Smad3. Relative luciferase activity was measured 24h after irradiation. HUVEC treated by 5 ng/mL of TGF-β1 for 24 hours is shown. **E-F)** HUVEC were transfected with siRNA Smad3

or siRNA p53 and the silencing efficiency was determined by western-blot. **G)** HUVEC were transiently co-transfected with Wt PAI-1-Luc reporter and pRL-TK plasmids in presence or absence of siRNA Smad3 or siRNA p53. 48h after transfection, culture medium was changed and cells were irradiated. Relative luciferase activity was measured 24h after irradiation. **H)** HUVEC were transfected with siRNA Smad3 or siRNA p53 24h before irradiation. PAI-1 expression was measured by western-blot 24h after irradiation.

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Figure 1

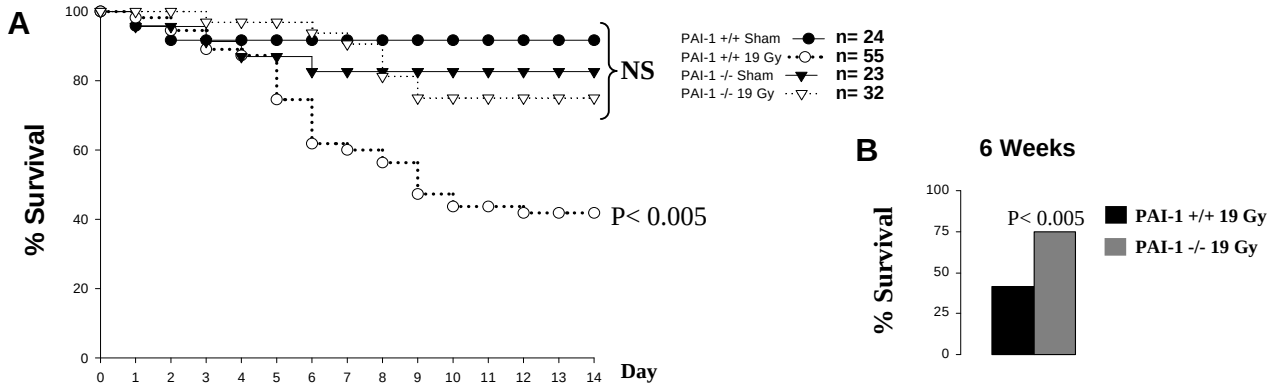

A
% Survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day
PAI-1 +/+ Sham n= 24
PAI-1 +/+ 19 Gy n= 55
PAI-1 -/- Sham n= 23
PAI-1 -/- 19 Gy n= 32
NS
P< 0.005
B
6 Weeks
% Survival
100
75
50
25
0
P< 0.005
PAI-1 +/+ 19 Gy
PAI-1 -/- 19 Gy

Figure 2

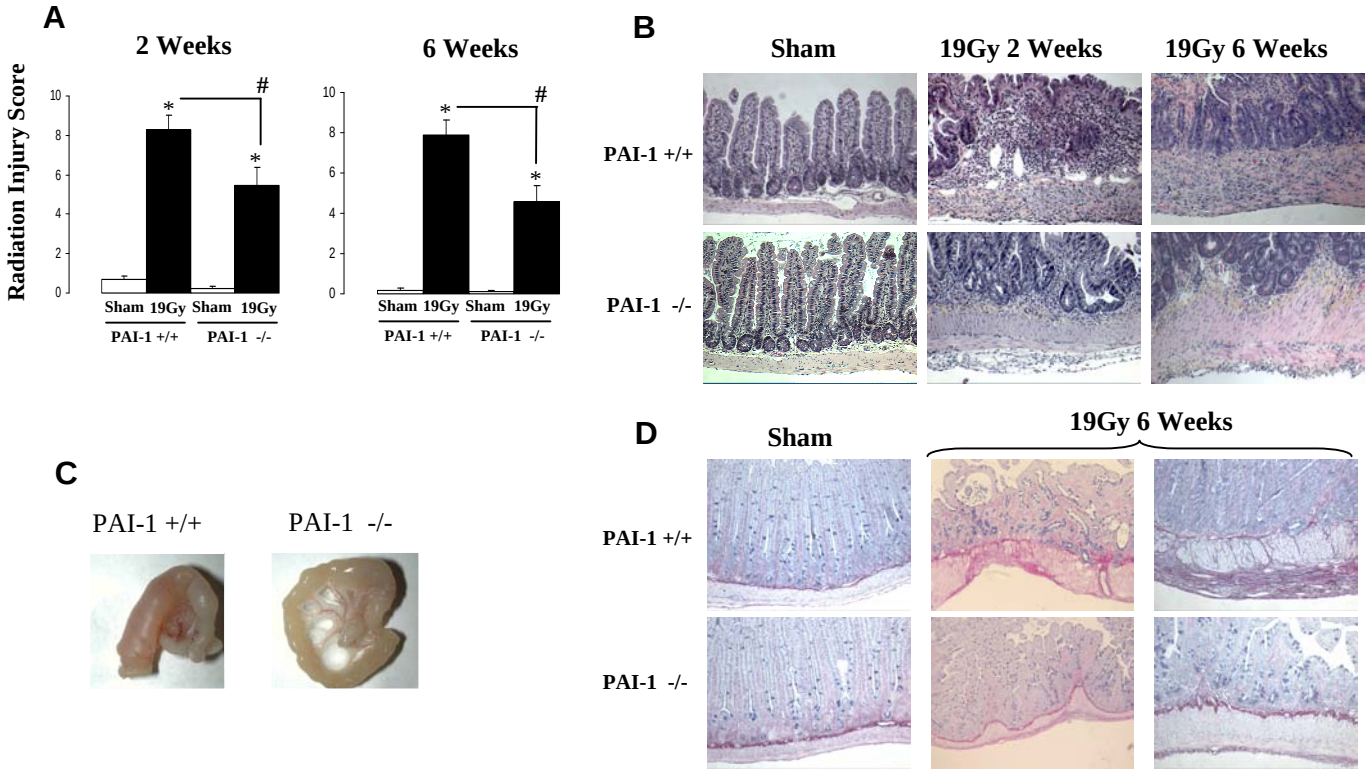

A
2 Weeks
6 Weeks
Radiation Injury Score
10
8
6
4
2
0
*
#
Sham 19Gy Sham 19Gy
PAI-1 +/+
PAI-1 -/-
B
Sham
19Gy 2 Weeks
19Gy 6 Weeks
PAI-1 +/+
PAI-1 -/-
C
PAI-1 +/+
PAI-1 -/-
D
Sham
19Gy 6 Weeks
PAI-1 +/+
PAI-1 -/-

Figure 3

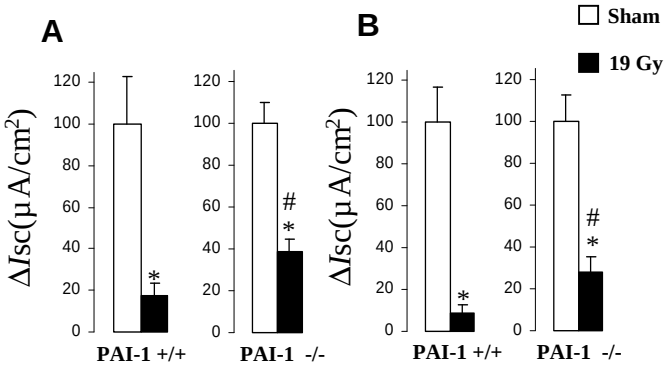


Figure 4

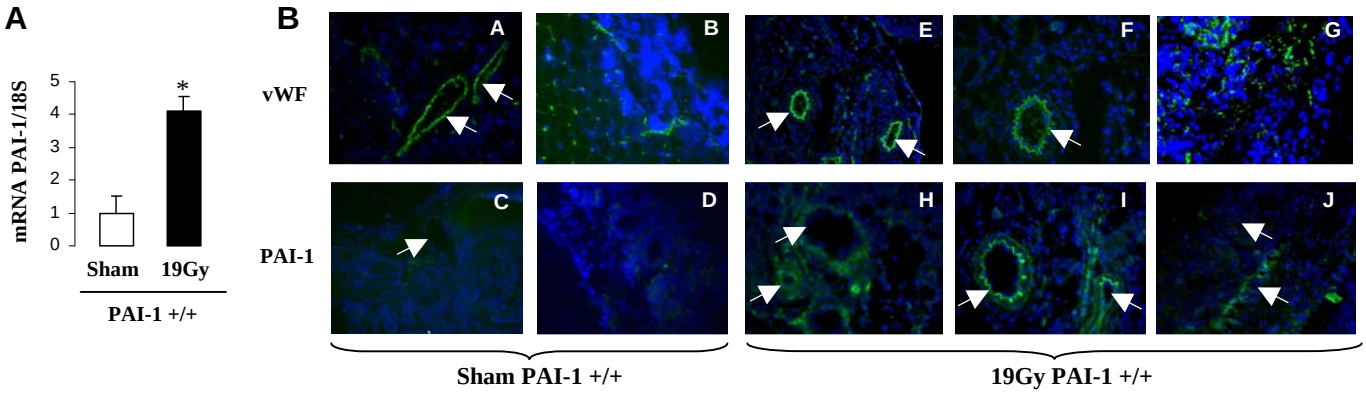

A
mRNA PAI-1/18S
5
4
3
2
1
0
*
Sham
19Gy
PAI-1 +/+
B
vWF
PAI-1
A
B
E
F
G
C
D
H
I
J
Sham PAI-1 +/+
19Gy PAI-1 +/+

Figure 5

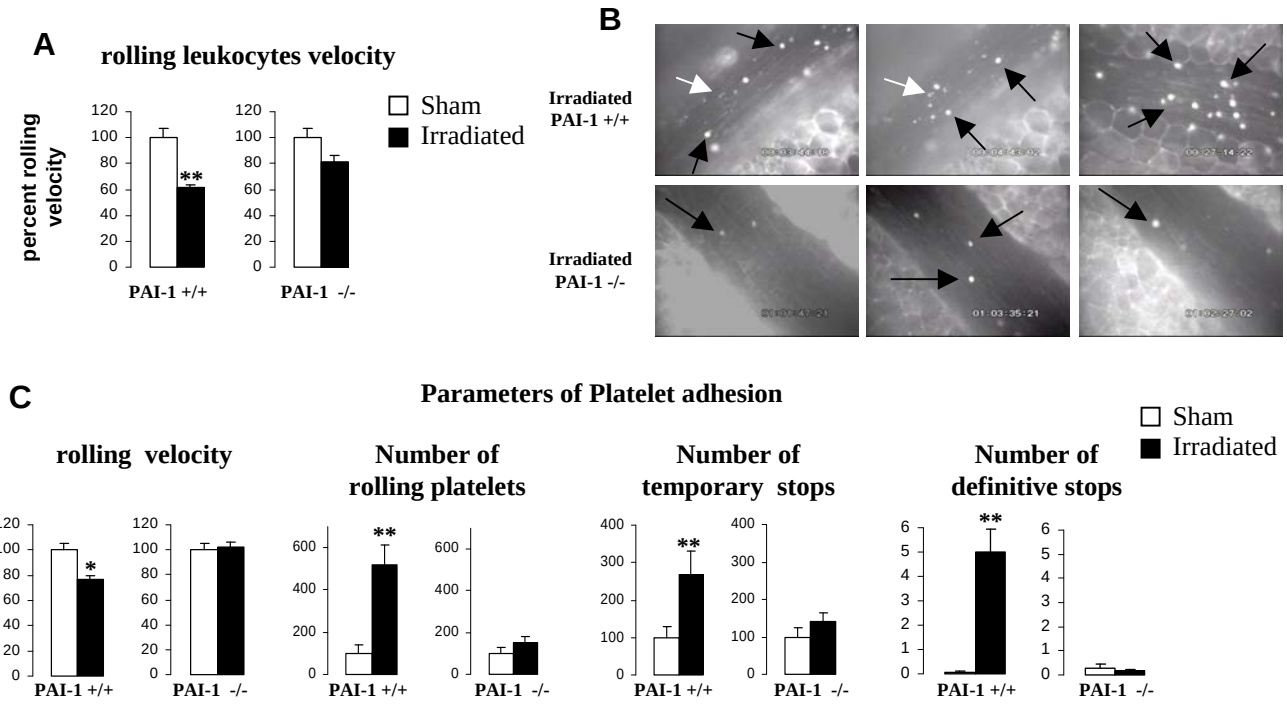

A
rolling leukocytes velocity
percent rolling velocity
120
100
80
60
40
20
0
**
PAI-1 +/+
PAI-1 -/-
Sham
Irradiated
B
Irradiated PAI-1 +/+
Irradiated PAI-1 -/-
C
Parameters of Platelet adhesion
Sham
Irradiated
rolling velocity
*
Number of rolling platelets
600
400
200
0
**
Number of temporary stops
400
300
200
100
0
**
Number of definitive stops
6
5
4
3
2
1
0
**
PAI-1 +/+
PAI-1 -/-

Figure 6

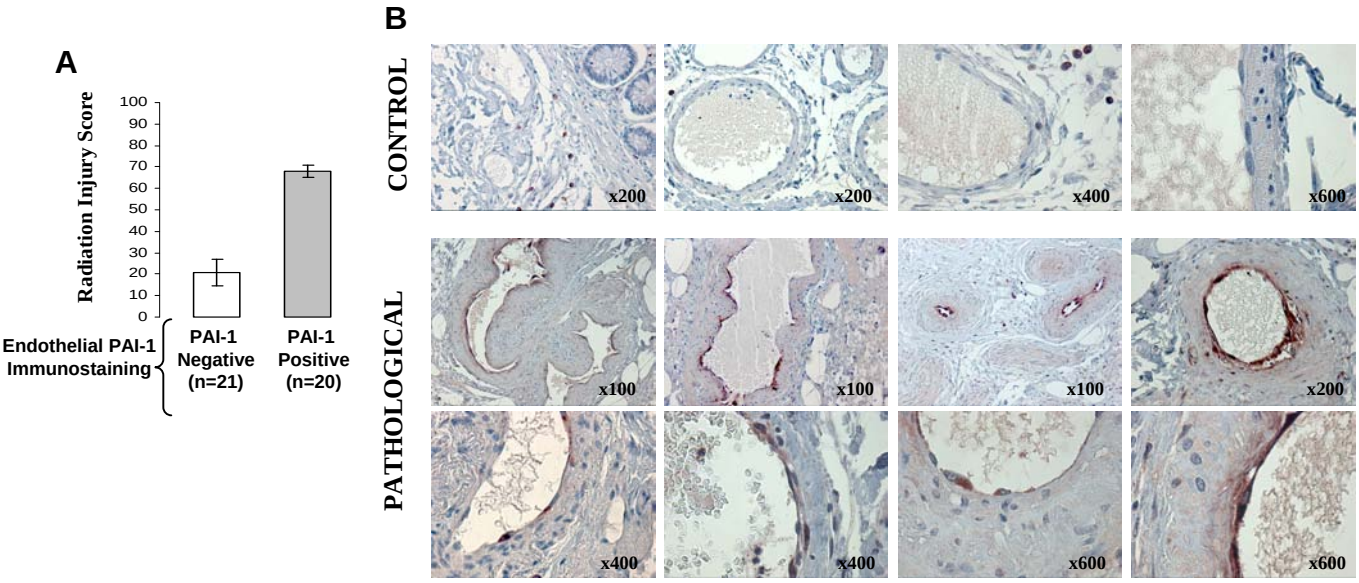

A
Radiation Injury Score
100
90
80
70
60
50
40
30
20
10
0
Endothelial PAI-1 Immunostaining
PAI-1 Negative (n=21)
PAI-1 Positive (n=20)
B
CONTROL
PATHOLOGICAL
x200
x200
x400
x600
x100
x100
x100
x200
x400
x400
x600
x600

Figure 7

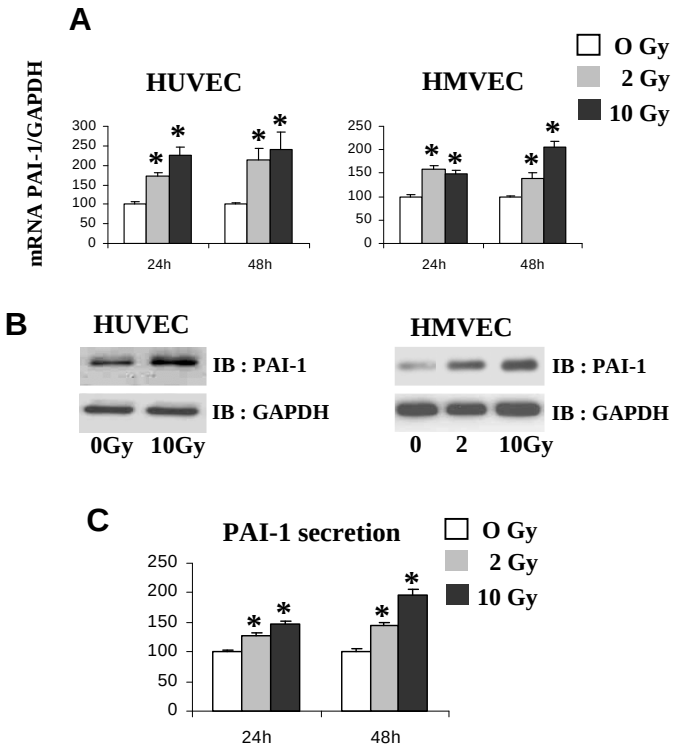

A
mRNA PAI-1/GAPDH
HUVEC
HMVEC
O Gy
2 Gy
10 Gy
300
250
200
150
100
50
0
24h
48h
B
HUVEC
HMVEC
IB : PAI-1
IB : GAPDH
0Gy 10Gy
0 2 10Gy
C
PAI-1 secretion
O Gy
2 Gy
10 Gy
250
200
150
100
50
0
24h
48h

Figure 8

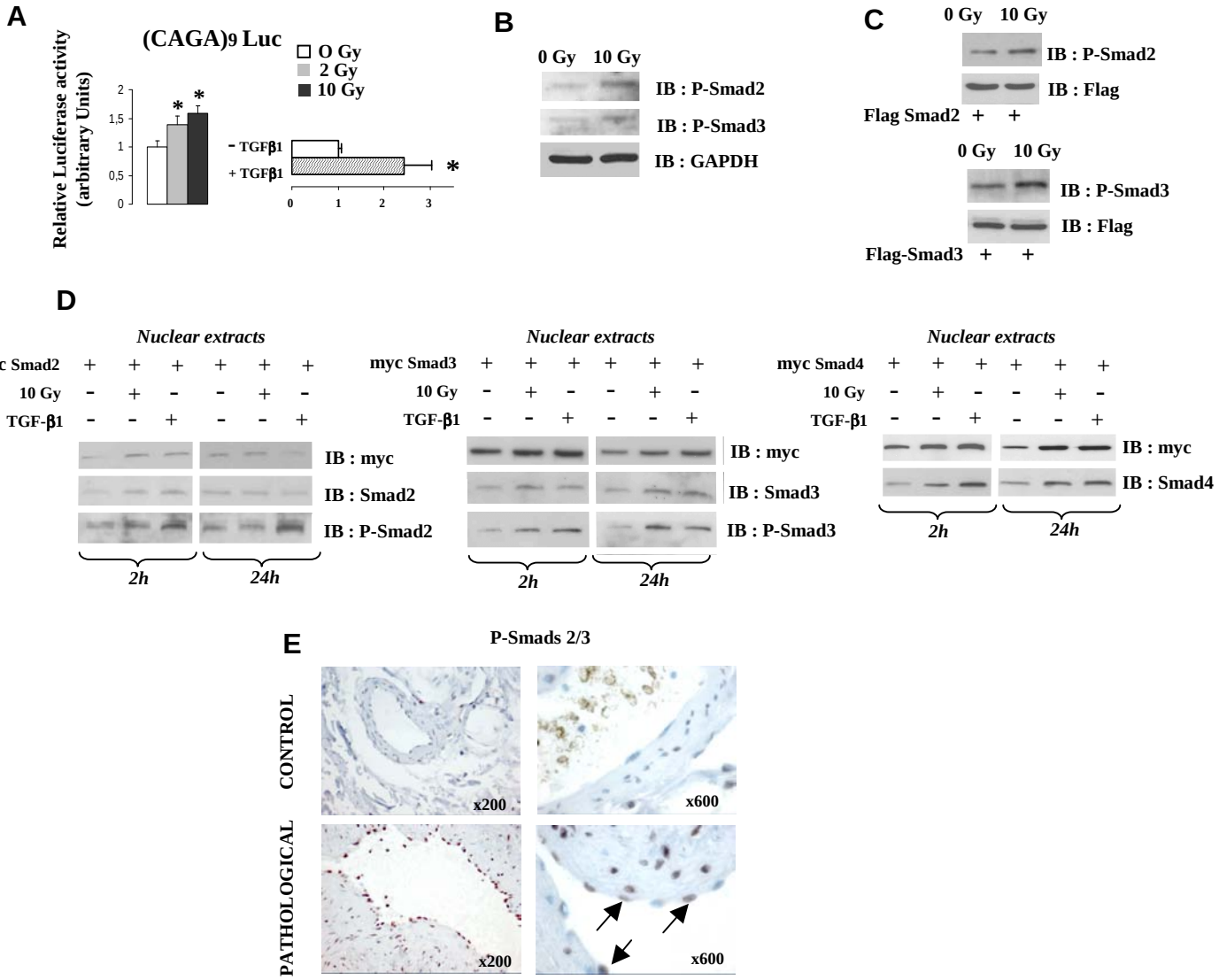

A
(CAGA)9 Luc
O Gy
2 Gy
10 Gy
Relative Luciferase activity (arbitrary Units)
- TGFβ1
+ TGFβ1
B
0 Gy 10 Gy
IB : P-Smad2
IB : P-Smad3
IB : GAPDH
C
0 Gy 10 Gy
IB : P-Smad2
IB : Flag
Flag Smad2 + +
0 Gy 10 Gy
IB : P-Smad3
IB : Flag
Flag-Smad3 + +
D
Nuclear extracts
myc Smad2
10 Gy
TGF-β1
IB : myc
IB : Smad2
IB : P-Smad2
2h
24h
Nuclear extracts
myc Smad3
10 Gy
TGF-β1
IB : myc
IB : Smad3
IB : P-Smad3
2h
24h
Nuclear extracts
myc Smad4
10 Gy
TGF-β1
IB : myc
IB : Smad4
2h
24h
E
P-Smads 2/3
CONTROL
PATHOLOGICAL
x200
x600
x200
x600

Figure 9

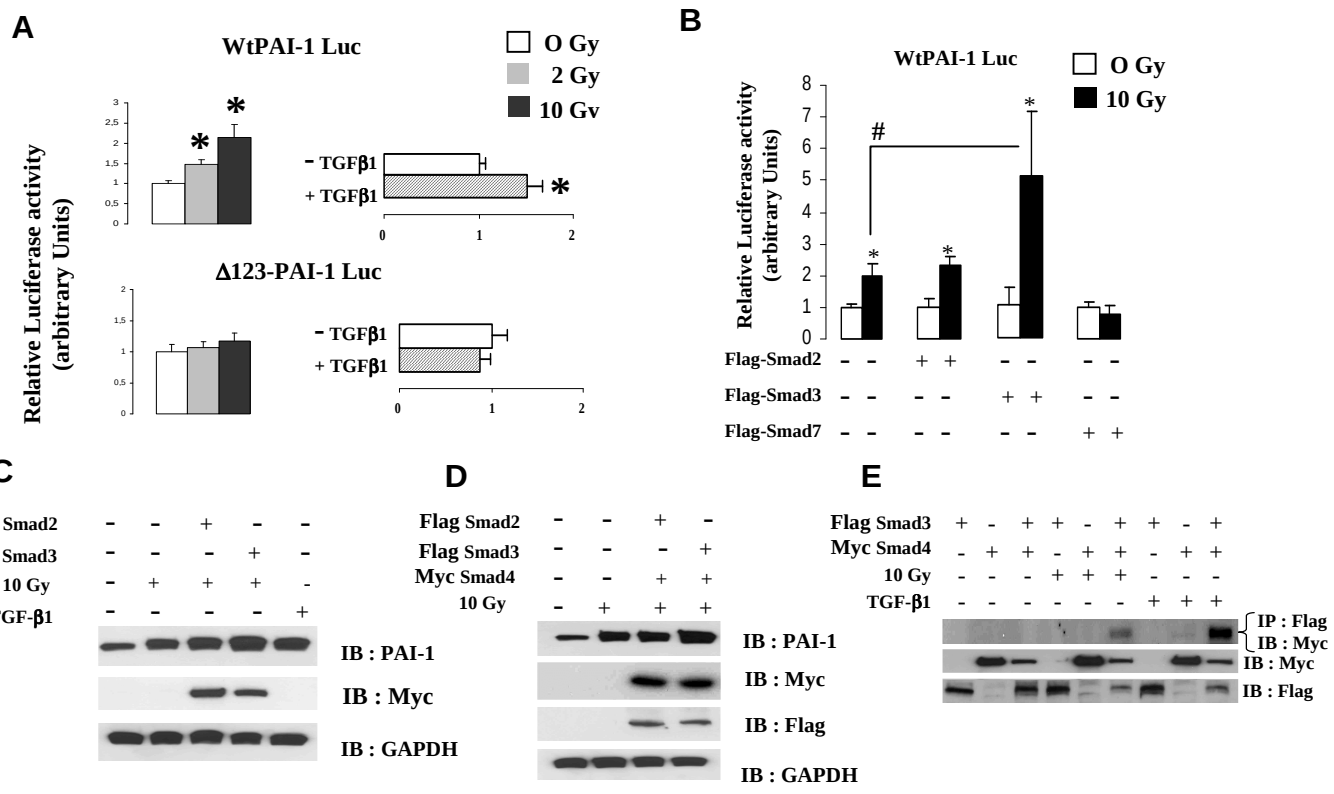
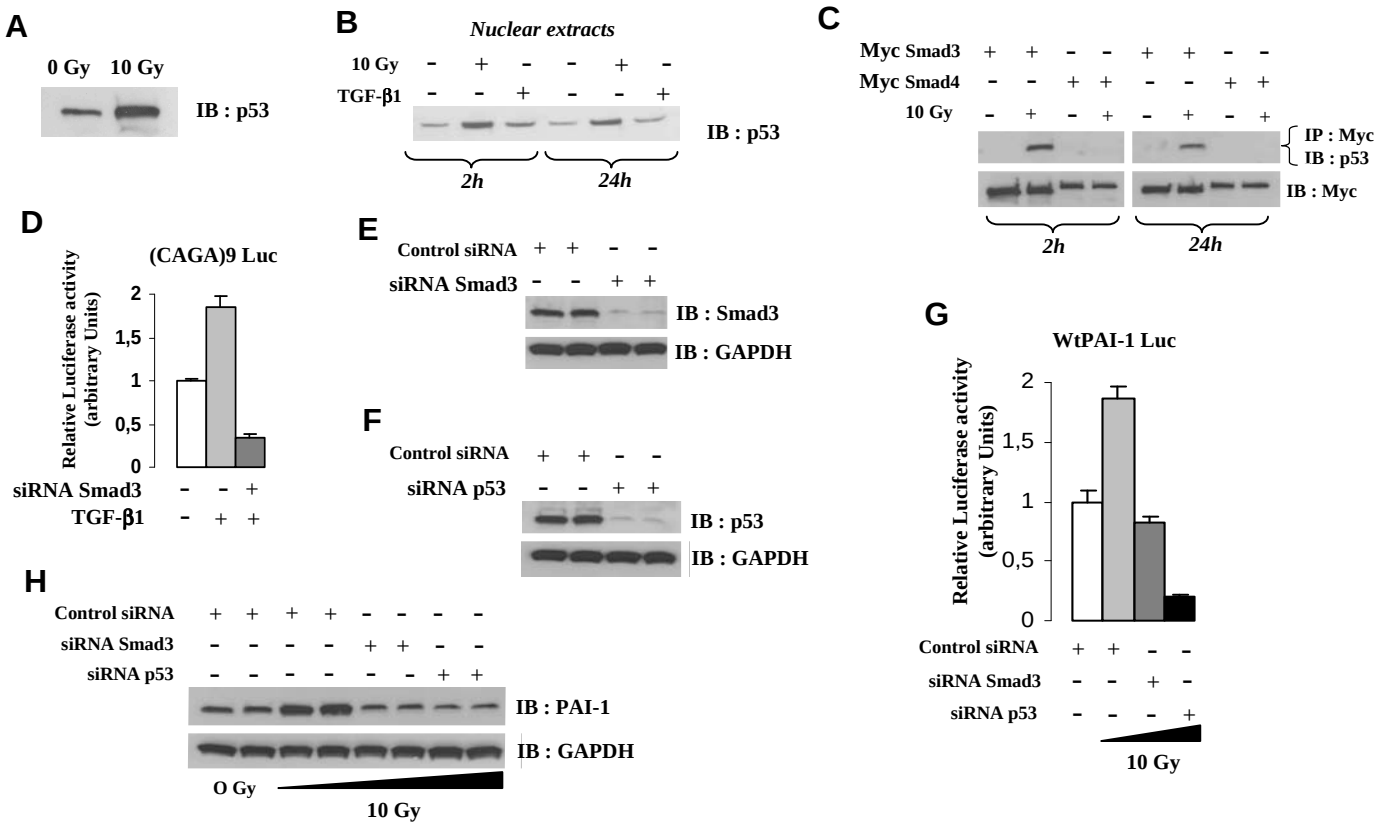

A
WtPAI-1 Luc
O Gy
2 Gy
10 Gv
Relative Luciferase activity (arbitrary Units)
- TGFβ1
+ TGFβ1
Δ123-PAI-1 Luc
B
WtPAI-1 Luc
O Gy
10 Gy
Relative Luciferase activity (arbitrary Units)
Flag-Smad2
Flag-Smad3
Flag-Smad7
C
Myc Smad2
Myc Smad3
10 Gy
TGF-β1
IB : PAI-1
IB : Myc
IB : GAPDH
D
Flag Smad2
Flag Smad3
Myc Smad4
10 Gy
IB : PAI-1
IB : Myc
IB : Flag
IB : GAPDH
E
Flag Smad3
Myc Smad4
10 Gy
TGF-β1
IP : Flag
IB : Myc
IB : Myc
IB : Flag

Figure 10


A
0 Gy 10 Gy
IB : p53
B
Nuclear extracts
10 Gy
TGF-β1
IB : p53
2h
24h
C
Myc Smad3
Myc Smad4
10 Gy
IP : Myc
IB : p53
IB : Myc
2h
24h
D
(CAGA)9 Luc
Relative Luciferase activity (arbitrary Units)
siRNA Smad3
TGF-β1
E
Control siRNA
siRNA Smad3
IB : Smad3
IB : GAPDH
F
Control siRNA
siRNA p53
IB : p53
IB : GAPDH
G
WtPAI-1 Luc
Relative Luciferase activity (arbitrary Units)
Control siRNA
siRNA Smad3
siRNA p53
10 Gy
H
Control siRNA
siRNA Smad3
siRNA p53
IB : PAI-1
IB : GAPDH
O Gy
10 Gy

III Discussion générale et perspectives

Le nombre de personnes atteintes de cancer augmente chaque année. Parmi ces plusieurs centaines de milliers de patients, plus de la moitié sont traités par radiothérapie seule ou en combinaison avec d'autres traitements comme la chirurgie ou la chimiothérapie. Ainsi, on estime par exemple à 180 000 en France, 1.5 million en Europe et 1 million aux Etats-Unis le nombre de personnes traitées par radiothérapie chaque année. L'efficacité des traitements par rayonnements ionisants vient de leurs capacités à générer des lésions létales aux cellules principalement par l'induction de dommages à l'ADN. Dans le domaine de la radiobiologie, beaucoup d'efforts sont fournis pour décrypter les mécanismes de radiosensibilité ou de radiorésistance des cellules tumorales. Dans le domaine de l'oncologie, l'étude de ces mécanismes permet de trouver de nouvelles cibles moléculaires et de tester des nouvelles drogues afin d'optimiser l'efficacité anti-tumorale des associations radio-chimiothérapeutiques et, plus précisément, de trouver des radio-sensibilisants des cellules cancéreuses. Une orientation complémentaire de ces recherches vise à comprendre les mécanismes associés aux effets délétères de l'irradiation sur les tissus sains. En effet, l'amélioration des traitements anticancéreux génèrent logiquement une augmentation de la survie des patients et donc une augmentation de la probabilité de développer des séquelles tardives (Andreyev, 2005). L'objectif ultime de ces recherches est de mettre en place des stratégies visant à protéger ou limiter les atteintes radio-induites aux tissus sains sans compromettre et même dans l'idéal d'améliorer l'efficacité curative des traitements des tumeurs par radiothérapie.

Récemment Bentzen a discuté de l'importance et de la nécessité d'intensifier les études de recherche fondamentale ciblées sur la compréhension des mécanismes moléculaires associés aux dommages radio-induits aux tissus sains (Bentzen, 2006). En effet, malgré quelques résultats pré-cliniques et cliniques démontrant une certaine efficacité thérapeutique, il n'existe pas aujourd'hui de stratégie thérapeutique consensuelle visant à protéger le tissu sain irradié dans le cadre d'un traitement d'un cancer par radiothérapie. Pour l'instant les efforts se sont essentiellement portés sur l'optimisation des protocoles radiothérapeutiques eux-mêmes ainsi que sur l'amélioration des techniques de radiothérapie qui visent à limiter notamment la dose ou le volume de tissu sain irradié. C'est pourquoi, en complément de ces avancées dans le domaine de la radiothérapie, il convient de s'attacher à décrypter les bases moléculaires impliquées dans les séquences des événements associés aux lésions radio-induites des tissus sains. Dans ce domaine, les orientations de recherche sont principalement de deux types : - des études visant à comprendre les mécanismes initiaux des dommages radio-induits et de développer des stratégies visant à prévenir l'apparition

des effets tardifs des radiothérapies comme les fibroses radiques - des études visant à comprendre les mécanismes impliqués dans le maintien des lésions tardives et de développer des stratégies de réversion de la fibrose établie. Clairement, les stratégies préventives constituent un intérêt et un impact clinique très important et la mise au point de telles stratégies représente un intérêt croissant pour les cliniciens.

Comme nous l'avons vu dans l'introduction, les compartiments vasculaires, mésenchymateux, épithéliaux ou encore les réactions inflammatoires et immunitaires participent à l'initiation et à la chronicité des dommages. Depuis de nombreuses années, le rôle du compartiment vasculaire est systématiquement décrit dans les revues spécialisées comme étant une composante déterminante et prometteuse en terme de stratégie de recherche (Denham and Hauer-Jensen, 2002). Cependant les études publiées dans le domaine restent rares et concernent 5 à 10 publications par an. C'est la raison pour laquelle nous nous sommes principalement attachés à éclaircir les mécanismes impliqués dans l'initiation des lésions radio-induites aux tissus sains et notamment le rôle du compartiment vasculaire. **L'objectif initial de ce travail de thèse était de contribuer à l'avancement de la compréhension du rôle de l'endothélium dans les dommages vasculaires et tissulaires radio-induits et d'identifier de nouvelles cibles thérapeutiques potentielles.**

Les lésions vasculaires chez l'homme sont associées à la prolifération, à la migration et à un phénotype pro-fibrosant des cellules musculaires lisses vasculaires.

Dans chaque réaction tissulaire suite à un stress, le compartiment vasculaire et plus précisément la cellule endothéliale joue un rôle déterminant. Ainsi, l'apoptose endothéliale, l'augmentation de la perméabilité vasculaire, l'activation cellulaire et le recrutement de cellules inflammatoires ainsi que l'activation du système de coagulation participent à l'initiation et la progression des lésions vasculaires et tissulaires radio-induites. Comme nous l'avons vu dans l'introduction, le dialogue entre les cellules endothéliales et les cellules musculaires lisses vasculaires est un élément essentiel de l'initiation des dommages vasculaires dans les pathologies comme l'athérosclérose, l'hyperplasie néointimale ou l'hypertension. Suite à une irradiation, le rôle des relations cellules endothéliales/CML vasculaires n'a quasiment pas été abordé jusqu'à présent. Seule une étude *in vitro,* utilisant des modèles de co-culture, montre que l'irradiation des cellules endothéliales à une très forte dose unique de 40 Gy n'influence pas la prolifération des CML vasculaires (de Crom et al., 2001). Cependant, des études à des doses d'irradiation plus relevantes méritaient d'être entreprises afin de confirmer ces résultats. Bien que les lésions microscopiques des

vaisseaux soient connues depuis longtemps et relativement bien décrites, il n'existe pas dans la littérature des études visant à caractériser au niveau moléculaire les lésions vasculaires radio-induites.

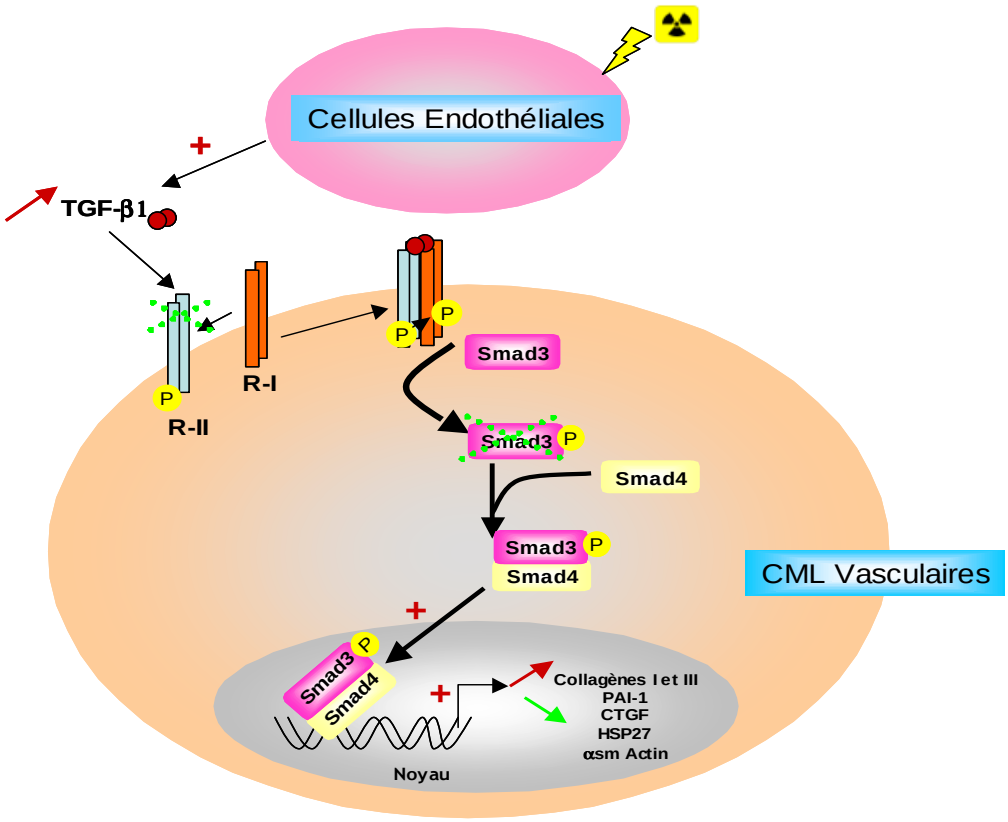
La connexion du laboratoire avec des cliniciens radiothérapeutes de l'Institut Gustave Roussy de Villejuif nous a permis de réaliser une étude rétrospective afin d'affiner la description des lésions vasculaires radio-induites chez l'homme. La première contrainte était de choisir des lésions relativement homogènes en terme de dose d'irradiation et d'apparition des lésions après la radiothérapie. Pour cela, nous avons choisi d'étudier les lésions vasculaires observées après radiothérapie chez des patients atteints d'adénocarcinome du rectum. Le traitement conventionnel à l'Institut Gustave Roussy pour ce type de cancer est l'association radiothérapie (45 Gy) / chimiothérapie (5-FU) suivi par une résection chirurgicale environ 6 semaines après la dernière dose d'irradiation. Une quarantaine de patients sont inclus dans cette étude et, pour chaque patient, un fragment tissulaire en zone irradiée proche de la tumeur et un fragment en zone macroscopiquement saine à distance de la tumeur ont été sélectionnées par un médecin pathologiste pour les analyses histologiques et immuno-histochimiques.

Comme décrit par Fajardo, nous avons identifié les lésions vasculaires qui se caractérisent principalement par des hypertrophies et dystrophies vasculaires sévères, de la fibrose vasculaire et péri-vasculaire, ainsi que des lésions d'hyperplasie néo-intimale (Fajardo, 2005). D'autre part, une forte corrélation a pu être mise en évidence entre l'épaississement vasculaire et le score d'atteinte radio-induite globale du tissu incluant tous les compartiments. Ceci montre d'une part que les lésions vasculaires sont une composante de l'atteinte tissulaire globale et suggère que la sévérité de l'atteinte vasculaire pourrait constituer un facteur déterminant dans la réponse tissulaire aux rayonnements. Les marquages immuno-histochimiques de l'α-sma, de la calponine, du PCNA, et des collagènes fibrillaires I et III ont permis de confirmer plusieurs hypothèses émises dans la littérature. Ainsi, nous avons montré que les lésions vasculaires étaient associées à une stimulation de la prolifération des cellules musculaires lisses. D'autre part, la surexpression des collagènes I et III, à la fois dans les parois vasculaires et dans les zones périvasculaires ont permis de mettre en évidence le phénotype pro-fibrosant de ces cellules. Enfin, nous avons clairement identifié les cellules musculaires lisses vasculaires dans les zones d'hyperplasie néo-intimale suggérant fortement que la migration et la prolifération de ces cellules participent à ce type de lésion radio-induite. De manière intéressante, même si l'évènement initiateur est différent, on peut observer des processus qui semblent similaires à ceux décrits dans les lésions vasculaires associées à d'autres pathologies comme l'athérosclérose. Dans ce contexte, nous avons émis l'hypothèse que les cellules endothéliales avaient un rôle prépondérant et nous avons mis en place des stratégies expérimentales

afin de savoir si ces cellules étaient capables d'influencer la prolifération, la migration et le phénotype pro-fibrosant des CML vasculaires après irradiation.

Les cellules endothéliales irradiées stimulent, in vitro, le phénotype pro-fibrosant des CML vasculaires par un mécanisme dépendant de la voie TGF-β/SMAD.

Les expériences de co-culture nous ont permis de répondre à cette question et nous avons montré que les cellules endothéliales irradiées stimulaient *in vitro*, la migration, la prolifération et le phénotype fibrogénique des CML vasculaires. Ces résultats mettent en lumière les nombreuses hypothèses concernant des signaux paracrines pouvant participer à l'influence d'un type cellulaire sur le phénotype d'un autre type cellulaire. Nos résultats montrent que des signaux paracrines provenant de cellules endothéliales irradiées influencent des fonctions des CML vasculaires impliquées dans les lésions vasculaires radio-induites. D'autre part, un nouveau mécanisme d'action du TGF-β1 et de la voie de signalisation SMAD dans les lésions radio-induites a été mis en évidence. En effet, nos résultats montrent qu'en présence de cellules endothéliales irradiées, il y a une activation de la transcription dépendante d'un élément minimal-consensus de réponse aux SMADs ainsi qu'une stimulation de la translocation nucléaire des SMADs dans les CML vasculaires. L'utilisation d'un siRNA SMAD3 et d'un anticorps neutralisant anti-TGFβ-RII démontre l'implication de la voie de signalisation TGF-β/SMAD dans l'induction du phénotype fibrogénique des CML vasculaires induit par les cellules endothéliales irradiées.



Effets de l'irradiation

Effets de l'inhibition par un anti-TGF-β RII ou un siRNA Smad3

Ces résultats ouvrent également de nouvelles perspectives de recherche. En effet, prenons comme exemple les résultats concernant le CTGF. Notre étude montre que le CTGF est sur-exprimé dans les CML vasculaires en présence de cellules endothéliales irradiées. En présence de siRNA SMAD3, cette induction est inhibée mais pas en présence de l'anticorps neutralisant anti-TGF-β RII. Ceci démontre que l'induction de l'expression du CTGF dans les CML vasculaires est dépendante de SMAD3 mais indépendante de la liaison du TGF-β1 au récepteur de type II. Nous avons montré que l'irradiation stimule la sécrétion du TGF-β1 dans les cellules endothéliales suggérant fortement que le TGF-β1 sécrété pouvait activer la voie SMAD dans les CML vasculaires. Cependant ces résultats n'excluent pas une potentielle implication d'autres facteurs paracrines pouvant stimuler l'expression du CTGF *via* l'activation de la voie SMAD. Ainsi les publications récentes par l'équipe de Rodriguez-Vita *et al* permettent de formuler plusieurs hypothèses et perspectives de recherche à nos travaux. En effet, ces auteurs ont montré que la translocation nucléaire des SMADs, la transcription SMAD-dépendante ainsi que la liaison des SMADs à l'ADN est stimulée dans des CML vasculaires de rat exposées à de l'angiotensine II (Rodriguez-Vita et al., 2005) . D'autre part, dans ce même modèle cellulaire, la même équipe a montré que le CTGF pouvait être induit indépendamment du TGF-β mais dépendamment de la voie SMAD soit sous l'action de l'angiotensine II *via* son récepteur de type 1 (AT1) ou de l'endothéline 1 *via* son récepteur de type A (ET_A) (Rodriguez-Vita et al., 2005; Rodriguez-Vita et al., 2005). A la lumière de nos résultats et de ceux obtenus par Rodriguez-Vita *et al*, une perspective à notre travail serait d'identifier les mécanismes SMAD-dépendants et indépendants du TGF-β impliqués dans l'induction du phénotype fibrogénique des CML vasculaires induit par les cellules endothéliales irradiées. Les cellules endothéliales synthétisent des grandes quantités d'angiotensine II et d'endothéline 1 et la première question serait de savoir si l'irradiation influence l'expression et/ou la sécrétion de ces deux molécules par les cellules endothéliales. De plus, à partir du même protocole que nous avons utilisé avec l'anticorps neutralisant anti-TGFβ-RII, il serait très intéressant de reproduire l'expérience soit avec un inhibiteur du récepteur à l'angiotensine AT1 comme le Losartan ou un antagoniste du récepteur aux endothélines comme le Bosantan.

D'autres perspectives intéressantes pourraient être envisagées. En effet, une des remarques redondantes à notre étude *in vitro* lors de sa présentation à différents congrès ou réunions de laboratoire était de la relier à un éventuel mécanisme impliqué *in vivo*. Nos résultats obtenus chez les patients traités par radiothérapie et qui montrent que les lésions vasculaires sont associées à une augmentation de l'expression du TGF-β dans l'endothélium et une augmentation de la forme phosphorylée des SMADs 2 et 3 dans les CML vasculaires viennent conforter et renforcer nos

résultats obtenus *in vitro*. Cependant ils n'apportent pas la preuve absolue que du TGF-β d'origine endothéliale peut activer la voie SMAD dans les CML vasculaires. Clairement, il n'y a pas de moyen simple et rapide de démontrer ce mécanisme *in vivo*. Seule l'utilisation de modèles complexes de souris transgéniques pourrait apporter des preuves expérimentales *in vivo* aux mécanismes moléculaires que nous avons observés *in vitro*. Ainsi, l'utilisation, s'il existait, d'un modèle de souris KO inductible pour le TGF-β dépendant d'un promoteur spécifique de l'endothélium pourrait constituer un outil de choix pour répondre à la question. Même si l'intérêt vis-à-vis des connaissances fondamentales de répondre à une telle question est indiscutable, on s'éloigne de la mise en place de nouvelles stratégies thérapeutiques. Aussi, sans répondre à cette question, nos résultats viennent renforcer des hypothèses et des perspectives déjà abordées par d'autres études.

En effet, nos résultats *in vitro* et *in vivo* montrent une activation de la voie SMAD au niveau du compartiment vasculaire après irradiation. Le groupe d'A. Roberts travaille depuis plusieurs années sur le rôle de SMAD3 dans différents mécanismes physiologiques et dans des pathologies comme les lésions radio-induites cutanées. L'utilisation de souris SMAD3 -/- a permis de répondre à la preuve de principe que ce facteur de transcription constituait une bonne cible thérapeutique dans ce type de dommages induits par une irradiation. Ainsi, après une irradiation cutanée à forte dose, les souris SMAD3 -/- sont protégées des dommages radio-induits avec une réduction des lésions fibrotiques associée à une augmentation de la re-épithélialisation et une baisse du nombre de mastocytes, de neutrophiles, de macrophages ou encore de myofibroblastes (Flanders et al., 2003; Flanders et al., 2002). Les atteintes vasculaires dans ce modèle n'ont pas été décrites. Nos résultats viennent compléter les connaissances concernant le rôle de SMAD3 après irradiation et nous pouvons émettre l'hypothèse que la protection des souris SMAD3 -/- après irradiation pourrait être due à l'altération des relations de signalisation entre les cellules endothéliales et les CML vasculaires. Une perspective intéressante à notre travail serait d'étudier dans un modèle d'atteinte vasculaire strict, comme par exemple l'irradiation de la carotide, la réaction post-irradiation de souris SMAD3 -/- ou de souris traitées avec un inhibiteur de SMAD3. D'autre part, nos résultats pourraient expliquer l'efficacité thérapeutique de certaines molécules comme l'halofuginone dont l'effet bénéfique est SMAD3 dépendant (Xavier et al., 2004).

L'utilisation d'inhibiteurs pharmacologiques de SMAD3 dans les dommage radio-induits aux tissus sains a été suggérée de l'équipe de Roberts suite aux travaux sur les souris SMAD3 -/-. Pour l'instant, une telle approche thérapeutique n'a pas été publiée même si elle représente un intérêt incontestable. A notre connaissance, il n'existe pas d'inhibiteur pharmacologique spécifique de SMAD3 mais certaines drogues sont capables d'inhiber son action. L'halofuginone en fait partie

et a montré son efficacité thérapeutique dans les dommages radio-induits (Xavier et al., 2004). Des essais cliniques ont montré une efficacité de l'halofuginone en application locale pour des pathologies de fibroses cutanées et les premiers essais de phase I avec une administration orale ont montré que ce produit était bien toléré avec cependant des problèmes gastrointestinaux à de fortes doses. D'autres petites molécules sont capables d'inhiber l'activité de SMAD3 indirectement. Ainsi les SB-431542 et SB-505124 qui sont des analogues d'inhibiteurs des p38MAPK sont capables d'inhiber la phosphorylation et la translocation de SMAD3 (DaCosta Byfield et al., 2004; Laping et al., 2002). Cependant, ces inhibiteurs ont une action également sur la voie MAPK induite par le TGF-β. Récemment, la naringenin, un analogue des flavonoides, a été montrée comme capable d'inhiber l'expression de SMAD3 sans moduler les autres acteurs de la voie comme SMAD2,4,7 ou encore les récepteurs I et II au TGF-β (Liu et al., 2006). Cependant, sa capacité à activer les voies PI3K et MAPK démontre sa non-spécificité et rend délicate son utilisation. Ainsi, l'absence d'inhibiteur pharmacologique spécifique de SMAD3 reste un problème pour vérifier la pertinence de cette cible thérapeutique et la venue sur le marché dans les années à venir de telles molécules sera d'un intérêt certain pour des études expérimentales visant à limiter les lésions radio-induites aux tissus sains. D'autre part, les avancées prometteuses réalisées avec l'utilisation de siRNA *in vivo* pourraient également permettre de répondre à cette question dans l'avenir.

Enfin, des résultats récents démontrant que la perte d'expression de co-represseurs de la voie Smad, i.e. Ski et SnoN, ou de SMAD7 dans un modèle de fibrose rénale chez la souris suggèrent que la perte d'expression de co-represseurs ou de SMAD inhibitrice est un mécanisme déterminant dans la progression de la fibrose dépendante du TGF-β (Fukasawa et al., 2004; Fukasawa et al., 2006). Ces résultats ouvrent de nouvelles perspectives et suggèrent que des stratégies visant à surexprimer des co-represseurs de la voie SMAD constituent de future stratégies anti-fibrosantes prometteuses (Huang et al., 2006). L'effet de l'irradiation sur les co-represseurs de la voie SMAD n'a jamais été étudié et l'investigation de l'éventuel rôle de ces co-répresseurs dans les dommages radio-induits représente une perspective intéressante et originale à nos travaux.

Les cellules endothéliales stimulent, in vitro, la prolifération et la migration des CML vasculaires après irradiation.

Nos expériences de co-culture ont également montré que les cellules endothéliales irradiées stimulaient la prolifération des CML vasculaires en influençant leur progression dans le cycle. L'irradiation des CML vasculaires entraîne un blocage classique des cellules en phase G1 du cycle

cellulaire. Les analyses de cycle montrent que les cellules endothéliales, irradiées ou non, stimulent le pourcentage de CML vasculaires en phase S à 24 et 48h après irradiation. Cependant, les résultats obtenus par comptage cellulaire montrent que seules les cellules endothéliales irradiées stimulent la prolifération des CML vasculaires. L'apparente incohérence de ces deux résultats mérite plusieurs remarques et ouvre de nouvelles perspectives. Les expériences de cycle cellulaire par incorporation d'iodure de propidium et analyse par cytométrie en flux permettent d'avoir une mesure du pourcentage de cellules à un temps t dans une phase particulière du cycle cellulaire. Cependant cette technique ne permet pas d'avoir d'information sur la vitesse de prolifération des cellules dans les différentes phases du cycle. Ainsi, nos résultats pris dans leur ensemble suggèrent que les CML vasculaires irradiées progressent plus rapidement dans le cycle en présence de cellules endothéliales irradiées qu'en présence de cellules endothéliales non-irradiées. De plus, seules les cellules endothéliales irradiées augmentent le pourcentage de CML vasculaires irradiées en phase S à 24 h. Ainsi, ces résultats suggèrent également des différences de sensibilité des CML vasculaires irradiées ou non vis-à-vis des facteurs paracrines produits par les cellules endothéliales irradiées.

Les mécanismes impliqués dans ce processus sont inconnus et des expériences complémentaires sont nécessaires afin de les identifier. Tout d'abord, une analyse plus approfondie concernant la prolifération des CML vasculaires en présence de cellules endothéliales irradiées mériterait d'être entreprise. Ainsi, des expériences de cinétique d'incorporation de thymidine tritiée pourraient donner des informations importantes quant à l'influence des cellules endothéliales sur la vitesse de prolifération des CML vasculaires. D'autre part, les facteurs paracrines sécrétés par les cellules endothéliales impliqués dans l'influence de la prolifération des CML vasculaires sont inconnus. Cependant plusieurs hypothèses peuvent être envisagées. Bien entendu, on peut tout d'abord penser à l'action de cytokines ou de facteurs de croissance ayant un rôle dans la progression des cellules dans le cycle cellulaire. Cependant on peut raisonnablement avoir la certitude que les cellules endothéliales irradiées ou non sécrètent à la fois des protéines mitogènes et anti-mitogènes. Par exemple, nos résultats montrent que la sécrétion du TGF-β est stimulée après irradiation des cellules endothéliales. Or, bien que des études initiales démontraient que le TGF-β pouvait stimuler la prolifération des CML (Battegay et al., 1990), il est maintenant admis que ce facteur de croissance inhibe la prolifération des CML vasculaires dans la plupart des conditions (Grainger, 2004; Grainger et al., 1994). Ceci pose deux questions : quel est l'effet du TGF-β1 sur la prolifération de CML vasculaires irradiées ? Y'a-t-il une sensibilité différente des CML vasculaires irradiées ou non vis-à-vis du TGF-β1 ou d'autres facteurs paracrines ? L'identification des molécules impliquées est bien évidemment difficile à appréhender. A mon sens, deux stratégies

différentes mais complémentaires sont envisageables. La première est de faire par analogie à ce que nous avons réalisé pour comprendre les mécanismes moléculaires impliqués dans le phénotype fibrogénique des CML vasculaires induit par les cellules endothéliales. A savoir, identifier les modulations d'expression des acteurs moléculaires impliqués dans la progression dans le cycle (ex cyclines, cyclines dépendantes kinases, p21 etc …). Des pistes d'identification pourraient alors être émises en fonction des modulations des acteurs moléculaires ciblés et des connaissances dans la littérature de facteurs solubles capables d'en moduler l'expression. La seconde stratégie serait, non pas de partir des effets sur les CML vasculaires, mais de mesurer par une approche protéomique les produits de sécrétion libérés par les cellules endothéliales après irradiation. Ainsi, la connaissance du protéome sécrété par les cellules endothéliales après irradiation serait une perspective intéressante à notre travail et pourrait permettre d'avoir des pistes d'identification concernant les facteurs protéiques solubles impliqués, et ceci non seulement vis-à-vis de la prolifération mais également de la migration et du phénotype fibrogénique.

Les approches par co-culture comme nous l'avons mentionné dans l'introduction permettent d'étudier de manière dynamique et cinétique l'influence d'un type cellulaire sur l'autre impliquant l'action de molécules à durée de vie très courte ou instables comme les gaz. Ainsi, nos résultats obtenus sur les cellules non irradiées viennent appuyer ceux de Peiro et al qui ont montré qu'en co-culture, les cellules endothéliales inhibent la prolifération des CML vasculaires (Peiro et al., 1995). Les auteurs avaient évoqué la piste de l'action du NO° dont on sait qu'il a une forte action anti-mitogène sur les CML vasculaires *via* sa capacité à inhiber l'expression de la cycline A et l'activation de CDK2 (Guo et al., 1998; Ishida et al., 1997; Tanner et al., 2000). Des résultats contradictoires sont décrits dans la littérature concernant l'effet de l'irradiation sur la production de NO. Ainsi, *in vitro*, il a été décrit une stimulation de l'expression de la NO synthase endothéliale (eNOS) après irradiation de cellules endothéliales aortiques bovines (Sonveaux et al., 2003). D'autre part, une baisse de l'expression de la eNOS a été décrite dans l'endothélium à la fois chez l'homme au niveau de l'artère cervicale suite à une radiothérapie pour un cancer du cou (Sugihara et al., 1999) et chez le lapin au niveau de l'artère de l'oreille 2 semaines après une irradiation de 45 Gy (Zhang et al., 2003). Dans notre modèle, des études seraient nécessaires pour savoir si le NO° joue, ou non, un rôle dans la capacité des cellules endothéliales irradiées à influencer la prolifération des CML vasculaires. Cependant, des résultats obtenus au laboratoire par western-blot montrent que l'irradiation diminue de manière importante l'expression de la eNOS dans les mêmes cellules endothéliales humaines que nous avons utilisées en co-culture (Claire Squiban, observations personnelles). Il reste à définir si cette baisse de l'expression de eNOS est corrélée à une diminution de la production de NO° mais ces résultats permettent de formuler l'hypothèse que

les cellules endothéliales irradiées auraient perdu leur capacité à inhiber la prolifération des CML vasculaires par une diminution de production de NO°.

Enfin notre étude montre également que les cellules endothéliales irradiées stimulent la capacité des CML vasculaires à recoloniser une zone lésée. Les marquages immuno-histochimiques de l'α-sma, de la calponine chez les patients démontrent la présence de CML vasculaires dans les lésions d'hyperplasie néo-intimale. Ainsi, par comparaison avec le même type de lésion observée dans d'autre pathologies comme l'hypertension et l'athérosclérose et l'implication démontrée de la migration des CML vasculaires dans ces pathologies, nos résultats *in vivo* suggèrent que la migration des CML vasculaire est une composante des lésions d'hyperplasie néo-intimales induites par l'irradiation. En effet, la migration des CML vasculaires joue un rôle clef dans l'initiation et la progression de ce type de lésions vasculaires (Newby and Zaltsman, 2000). Les mécanismes moléculaires impliquent des chemo-attractants synthétisés par les plaquettes, la migration et la prolifération des CML vasculaires facilitées par l'action des MMPs.

De manière intéressante, nous avons vu que ce type de lésion est observé dans d'autres organes suite à une radiothérapie et notamment le poumon, la peau et l'utérus. Cette notion d'implication de la migration des CML vasculaires n'a, à notre connaissance, jamais été étudié ni même évoqué dans la littérature. Certes nos résultats effleurent la question mais apportent la preuve de principe de l'intérêt d'effectuer des études supplémentaires pour comprendre les mécanismes et les acteurs moléculaires impliqués dans la migration des CML vasculaires après irradiation.

En conclusion de cette première partie, notre étude a permis de caractériser et de mettre en évidence l'importance des relations cellules endothéliales/CML vasculaires dans les lésions vasculaires induites par l'irradiation. D'autre part, un nouveau mécanisme d'action du TGF-β et de la voie SMAD a pu être mis en évidence. Ceci vient conforter la pertinence de cibler le TGF-β, la voie SMAD ou un des gènes cibles de cette voie pour limiter les dommages radio-induits. C'est précisément sur une des cibles reconnues du TGF-β1 dépendant des SMADs que nous avons axé la deuxième partie de ce travail.

PAI-1 est un acteur moléculaire clef des lésions radio-induites aux tissus sains.

Ainsi, nous nous sommes intéressés au rôle de PAI-1 et nous avons voulu déterminer si cette protéine pouvait représenter une cible thérapeutique capable de prévenir et/ou de limiter les dommages radio-induits. Plusieurs arguments de la littérature permettaient de formuler l'hypothèse

d'une participation active de cette protéine. Le premier est que PAI-1 est un des effecteurs du TGF-β1 et que l'implication de ce facteur de croissance dans les dommages radio-induits est aujourd'hui une certitude (Bentzen, 2006; Martin et al., 2000). Le second est que PAI-1 est stimulé *in vitro* par l'irradiation et qu'une surexpression a été décrite dans un modèle de fibrose rénale radio-induite et également au niveau intestinal quelques jours après irradiation (Brown et al., 2000; Oikawa et al., 1997; Strup-Perrot et al., 2006; Strup-Perrot et al., 2005). De plus, une meilleure capacité de cicatrisation dans un modèle d'atteinte cutanée a été démontrée chez les souris PAI-1 -/- (Chan et al., 2001). Enfin, le rôle de PAI-1 dans différents modèles de fibrose et notamment des fibroses rénales, hépatiques, pulmonaires et vasculaires a été démontré dans la littérature (Eitzman et al., 1996; Eitzman et al., 2000; Huang et al., 2003).

La stratégie expérimentale pour répondre à cette question a été développée par une triple approche : - une approche *in vivo* en utilisant des souris génétiquement déficientes en PAI-1 - une approche *ex vivo* avec des marquages immuno-histochimiques de PAI-1 sur des tissus de patients ayant été traités par radiothérapie - une approche *in vitro* afin de décrypter les mécanismes moléculaires impliqués dans la régulation radio-induite de PAI-1 dans des cellules normales et plus précisément dans des cellules endothéliales.

L'utilisation de souris PAI-1 -/- imposait tout d'abord de mettre en place un modèle d'atteinte radio-induite pertinent chez la souris. Le recul du laboratoire sur les dommages aux tissus sains au niveau intestinal portait exclusivement sur des modèles animaux réalisés chez le rat (Francois et al., 2003). Notre objectif initial était de déterminer si la déficience en PAI-1 pouvait avoir une conséquence sur les effets aigus mais également sur les effets plus tardifs de l'irradiation. Dans ce contexte, il était nécessaire d'irradier à une forte dose pour pouvoir provoquer des effets tardifs et particulièrement des lésions fibrotiques mais ceci imposait de limiter le volume d'irradiation. Par exemple, des irradiations abdominales chez la souris C57Bl/6J à des doses de 15 à 20 Gy sont létales dans les quelques jours suivant l'irradiation (17 Gy abdominale, DL100 à 9 jours) et des doses plus faibles permettent de maintenir les souris en vie mais ne provoquent que des effets aigus et pas de lésions tardives. C'est pourquoi notre choix s'est porté sur un modèle d'irradiation localisée d'une anse grêle après extériorisation utilisé chez le rat (Francois et al., 2003) qui permet d'irradier localement à forte dose une petite partie de l'intestin sans entraîner de mort précoce. La transposition de ce modèle à la souris a déjà été publiée une fois par l'équipe d'Hauer-Jensen et c'est sur la base de cet article que nous avons mis en place le modèle (Zheng et al., 2000). Bien entendu, la principale difficulté de ce modèle est liée à l'obligation de réaliser une chirurgie chez la souris mais il permet de provoquer des effets aigus et tardifs et correspondait à notre objectif scientifique initial.

Très brièvement, ce modèle consiste, sous anesthésie gazeuse, à extérioriser une anse jéjunale après laparotomie, d'irradier cette anse à 19 Gy grâce à une source de cobalt 60 puis de la replacer dans la cavité abdominale avant de faire des sutures musculaires et cutanées. Une irradiation localisée à 19 Gy chez des souris C57Bl/6J sauvages provoque cependant une importante mortalité. Dans notre étude, 58 % (n=55) des souris sauvages sont mortes dans les 12 jours suivant l'irradiation ce qui est cohérant avec l'article de Zheng *et al* qui mentionnait un taux de survie d'environ 50 % (Zheng et al., 2000). Le premier résultat déterminant de notre étude est que la survie des souris PAI-1 - /- est significativement augmentée dès le quatrième jour comparée aux souris sauvages. Deux semaines après l'irradiation, 75 % des souris irradiées PAI-1 -/- sont vivantes contre 42 % pour les souris sauvages et cette différence est maintenue jusqu'à 6 semaines. Ces résultats démontrent que la déficience en PAI-1 confère aux souris une protection contre les dommages radio-induits et par conséquent suggèrent fortement que PAI-1 joue un rôle délétère dans la réaction tissulaire suite à un stress par rayonnements ionisants. Les résultats des scores histologiques d'atteintes radio-induites établis à 2 semaines et 6 semaines post-irradiation montrent des lésions moins sévères chez les souris PAI-1 -/-, confirmant l'effet « radioprotecteur » de la déficience en PAI-1. D'autre part, l'analyse des courbes de survie montre que les effets létaux radio-induits chez les souris sauvages s'expriment dans les dix premiers jours suivant l'irradiation démontrant l'implication d'évènements précoces. Ainsi, nous avons choisi d'étudier la fonctionnalité intestinale 3 jours après l'irradiation c'est à dire avant que les deux courbes de survie des souris irradiées ne se séparent. Bien que l'irradiation affecte de manière importante la fonctionnalité des deux lignées de souris, la sévérité de cette atteinte fonctionnelle est moins sévère chez les souris déficientes en PAI-1 comparées aux souris sauvages. Ainsi, dans un modèle d'entéropathie radio-induite, l'ensemble de ces résultats démontre clairement le rôle de PAI-1 dans les lésions radio-induites aux tissus sains. Ceci apporte la preuve de principe qu'un traitement pharmacologique inhibant l'activité de PAI-1 constituerait une excellente cible thérapeutique.

Dans ce contexte, la perspective de tester un inhibiteur de PAI-1 est la suite logique de nos travaux. Dans d'autres modèles, une telle stratégie a déjà été décrite dans la littérature. L'utilisation d'un mutant de PAI-1 non actif, capable d'entrer en compétition avec le pool de PAI-1 endogène, a montrée son efficacité dans un modèle de glomérulonéphrite chez le rat (Huang et al., 2003). Il existe un seul inhibiteur pharmacologique de PAI-1 potentiellement disponible et décrit dans la littérature. Le Tiplaxtinin ou PAI-039 est un inhibiteur pharmacologique de PAI-1 (Elokdah et al., 2004) ayant démontré une efficacité dans quelques modèles expérimentaux. Par exemple, dans un modèle de pathologie veino-occlusive suite à une inhibition chronique du NO chez la souris, le Tiplaxtinin permet d'inhiber les thromboses veineuses hépatiques (Smith et al., 2006). De plus, le

Tiplaxtinin accélère la fibrinolyse dans un modèle de thrombose coronaire chez le chien (Hennan et al., 2005). D'autre part, dans un modèle de fibrose cardiaque induite par l'angiotensine II chez la souris, un traitement par le Tiplaxtinin ne permet pas de limiter l'hypertrophie ventriculaire ni la fibrose cardiaque mais permet d'atténuer le remodelage matriciel au niveau de l'aorte (Weisberg et al., 2005). Enfin, cette molécule a également démontré sa capacité à réduire la différenciation adipocytaire et prévenir l'obésité chez des souris soumise à un régime riche en graisses (Crandall et al., 2006). Nous avons proposé et obtenu un contrat de collaboration avec le laboratoire pharmaceutique produisant cette molécule. Un traitement pharmacologique sur notre modèle d'atteinte radio-induite sera réalisé en 2007 afin de confirmer la pertinence d'une telle inhibition pour limiter les dommages radio-induits aux tissus sains. Une clause de confidentialité ne nous permet pas de détailler le projet scientifique proposé.

Plusieurs hypothèses concernant les mécanismes d'action de PAI-1 dans les dommages radio-induits ont été émises dans l'introduction. La fibrinolyse et le remodelage de la matrice extracellulaire ont été démontrés comme participant aux processus d'initiation des lésions ainsi qu'aux phases de cicatrisation pathologique radio-induite (Hauer-Jensen et al., 2004; Henderson et al., 1983; Hovdenak et al., 2002; Strup-Perrot et al., 2004). L'irradiation induit un changement phénotypique de l'endothélium avec notamment la perte de thromborésistance due à la fois à l'activation du système de coagulation mais également à une diminution de la fibrinolyse (Henderson et al., 1983; Wang et al., 2002). Ainsi, nous avons émis l'hypothèse que la déficience en PAI-1 pouvait être associée à des modifications des propriétés d'activation de l'endothélium vasculaire après irradiation. Pour répondre à cette question, nous avons choisi de mesurer par microscopie intravitale chez les souris PAI-1 -/- les interactions entre les cellules circulantes et l'endothélium. La laparotomie et une manipulation de l'intestin inhérentes au modèle d'anse extériorisée peuvent influencer les paramètres étudiés. C'est pourquoi, nous avons choisi un modèle d'irradiation abdominale sans chirurgie en conservant une dose forte d'irradiation i.e. 15 Gy. D'autre part, les études précédentes démontrant que l'irradiation entraîne une augmentation des interactions plaquettes/endothélium et leucocytes/endothélium à la fois *in vitro* et *in vivo* prouvent que ces évènements s'initient très rapidement (Gaugler et al., 2005; Mouthon et al., 2003; Panes et al., 1995). Ainsi, les interactions entre les leucocytes et l'endothélium ainsi que plusieurs paramètres d'adhésion plaquettaire ont été mesurés par microscopie intravitale chez les souris KO pour PAI-1 et les souris sauvages 24h après une irradiation abdominale de 15 Gy. Les résultats montrent que chez les souris sauvages, l'irradiation entraîne une augmentation des interactions leucocytes/endothélium *in vivo* avec une diminution de 39% de la vitesse de rolling des leucocytes à la surface des vaisseaux. Ces résultats confirment les travaux publiés dans la littérature obtenus *in*

vitro sur des cellules endothéliales humaines (Vereycken-Holler et al., 2002) ou *in vivo* chez la souris (Johnson et al., 2004; Molla et al., 2003) ou le rat (Molla et al., 1999). Chez les souris PAI-1 -/-, l'irradiation entraîne une diminution non significative de 18% de la vitesse de rolling des leucocytes à la surface des vaisseaux. Les résultats obtenus sur les paramètres d'adhésion plaquettaire confirment la différence entre les deux lignées de souris vis-à-vis de l'irradiation. En effet, chez les souris sauvages, l'irradiation entraîne une augmentation des interactions plaquettes/endothélium avec une diminution de 25% de la vitesse de rolling des plaquettes ainsi qu'une augmentation du nombre de plaquettes adhérentes associées à une forte augmentation des arrêts temporaires et définitifs. L'irradiation n'a pas d'effet significatif sur l'ensemble de ces paramètres chez les souris PAI-1 -/-. Ces résultats montrent, par conséquent, que les interactions entre les cellules circulantes et l'endothélium après irradiation sont inhibées en cas de déficience en PAI-1. Ceci démontre que PAI-1 joue un rôle clef dans l'augmentation radio-induite des interactions leucocytes-plaquettes/endothélium et suggère que la protection des souris PAI-1 -/- vis-à-vis de l'irradiation pourrait en partie être liée à un défaut de ces processus précoces initiés dans l'endothélium vasculaire. Concernant l'absence d'interactions plaquettes/endothélium après irradiation chez les souris PAI-1 -/-, nous pouvons supposer que l'augmentation de la fibrinolyse démontrée chez ces souris (Kawasaki et al., 2000) contribue à ce phénomène . Ainsi, l'absence de PAI-1 entraîne une augmentation du taux de plasmine, une dégradation plus rapide du pool de fibrine formé localement et empêche ainsi les plaquettes d'adhérer à l'endothélium. A la lumière de ces résultats, une question se pose : quelles sont les participations respectives des pools de PAI-1 des plaquettes et de l'endothélium dans les effets que nous observons ? En effet, nos résultats permettent de démontrer que l'absence de PAI-1 entraîne une inhibition des interactions et suggèrent fortement que le pool de PAI-1 intravasculaire joue un rôle clef. Cependant nous ne pouvons pas exclure également l'éventuel effet de la déficience en PAI-1 des plaquettes chez les souris PAI-1 -/-. Un travail récent montre que les plaquettes synthétisent des quantités importantes de PAI-1 actif qui pourraient contribuer à la stabilisation du caillot sanguin (Brogren et al., 2004). Ceci ouvre des perspectives intéressantes à notre travail. Une approche *in vitro* utilisant la microscopie intravitale pourrait permettre de répondre à la question suivante : Est-ce la déficience en PAI-1 de l'endothélium et/ou celle des plaquettes qui explique l'absence d'effet de l'irradiation sur l'adhésion plaquettaire ? Par exemple, nous pourrions étudier *in vitro*, l'adhésion de plaquettes de souris PAI-1 -/- sur des cellules endothéliales normales et inversement l'adhésion de plaquettes normales sur un endothélium déficient en PAI-1.

D'autre part, une autre perspective intéressante à notre travail serait d'aborder l'implication des acteurs du remodelage matriciel. En effet, PAI-1 joue un rôle important dans les mécanismes de

dégradation de la matrice extracellulaire et une de nos hypothèses initiales était que les souris PAI-1 -/- seraient potentiellement protégées de la fibrose radio-induite par une augmentation de l'activité des MMPs. Le fait que, 6 semaines après irradiation, les lésions fibrotiques soient plus importantes chez les souris sauvages que chez les souris PAI-1 -/- vient conforter notre hypothèse. Une étude ciblée sur l'implication des acteurs du remodelage matriciel est donc une perspective logique à notre travail. Ainsi, des mesures d'activité des MMPs par zymographie *in situ* pourraient apporter des informations primordiales.

Enfin, le fait que les souris PAI-1 -/- soient protégées contre les dommages radio-induits suggère que la déplétion cellulaire radio-induite chez ces souris est moins importante. En effet, nous pouvons supposer que des modifications de radio-sensibilité chez ces souris contribuent à l'effet radio-protecteur observé. Des résultats récents montrent que des cellules endothéliales de souris PAI-1 -/- ont une capacité de prolifération supérieure aux cellules endothéliales de souris sauvages (Ploplis et al., 2004). De plus, il a été démontré que les cellules endothéliales de souris PAI-1 -/- sont caractérisées par une activation de signaux de survie et une baisse d'expression de facteurs proapoptotiques (Balsara et al., 2006). En effet, une activation de la voie PI3K-Akt avec une hyperactivation de Akt associée à une inactivation de PTEN est observée dans les cellules endothéliales déficientes en PAI-1. Cette activation de signaux de survie est également liée à une inhibition de l'expression de caspase 3 (Balsara et al., 2006). Ainsi, ces auteurs suggérent que les cellules endothéliales PAI-1 -/- sont résistantes aux signaux apoptotiques.

A la lumière de ces résultats plusieurs questions se posent : L'apoptose radio-induite est-elle réduite chez les souris PAI-1 -/- ? L'endothélium de souris PA1-1 -/- est-il résistant à l'apoptose radio-induite ? La sévérité moins importante des lésions radio-induites intestinales est-elle due à une différence de radio-sensibilité endothéliale associée à la déficience en PAI-1 ?

L'expression de PAI-1 est stimulée dans les cellules endothéliales in vivo et in vitro.

Une de nos préoccupations constantes pendant ce travail de thèse a été de compléter nos approches expérimentales *in vitro* et *in vivo* chez l'animal par des analyses sur des échantillons de patients traités par radiothérapie. Ainsi, nous avons réalisé des marquages immunohistochimiques de PAI-1 sur les biopsies de rectums irradiés obtenus par notre collaboration avec l'Institut Gustave Roussy. Les lésions radio-induites 6 semaines après une irradiation thérapeutique de 45Gy sont associées à une augmentation de l'expression de PAI-1 dans les vaisseaux et plus particulièrement dans les cellules endothéliales. Ces résultats suggèrent que PAI-1 pourrait contribuer à l'initiation et à la progression des dommages radio-induits aux tissus sains chez l'homme et viennent renforcer la

pertinence de notre démonstration obtenue chez la souris. Cette analyse faite sur les rectums irradiés pose la question de savoir si cette observation est spécifique de cet organe ou non. C'est pourquoi, nous avons initié un recrutement d'autres tissus irradiés dans le cadre de traitements par radiothérapie comme la peau, l'utérus ou encore le poumon dans l'objectif de réaliser en 2007 les marquages immunohistochimiques de PAI-1. De la même manière, au niveau expérimental, il serait pertinent d'étudier *in vivo* l'effet de l'irradiation chez les souris PAI-1 -/- dans d'autres modèles d'atteinte radio-induite. Ainsi, à l'instar de nos résultats obtenus dans le modèle d'entéropathie radio-induite, une perspective intéressante à nos travaux serait de savoir si les souris PAI-1 -/- sont également protégées de la fibrose radio-induite pulmonaire et cutanée.

A la suite de ces résultats obtenus chez les patients, nous avons voulu connaître l'effet de l'irradiation sur l'expression de PAI-1 dans les cellules endothéliales. L'irradiation stimule l'expression de PAI-1 avec à la fois une augmentation du taux d'ARNm et de l'expression cellulaire et sécrétée de la protéine. Nos résultats obtenus sur deux types de cellules endothéliales humaines (HUVEC et HMVEC) confirment ceux décrits sur des cellules tubulaires épithéliales ou mésangiales rénales de rat (Zhao et al., 1999; Zhao et al., 2000) ainsi que ceux obtenus dans une lignée tumorale HepG2 (Hageman et al., 2005). Pour aller plus loin, nous avons développé une stratégie expérimentale afin de comprendre les mécanismes moléculaires impliqués dans la régulation radio-induite de PAI-1 dans les cellules endothéliales.

Mécanismes moléculaires impliqués dans la régulation de l'expression radio-induite de PAI-1 dans les cellules endothéliales : Rôles de la voie SMAD et de la protéine p53.

Dans la première partie de ce travail, nous avons montré que, chez les patients traités par radiothérapie, les lésions vasculaires sont associées à une augmentation de l'expression du TGF-β dans l'endothélium et une augmentation de la forme phosphorylée des SMADs 2 et 3 dans les CML vasculaires (Milliat et al., 2006). D'autre part, l'analyse immunohistochimique montre également une augmentation de la forme activée des SMADs 2 et 3 dans l'endothélium. De plus, *in vitro*, l'irradiation de cellules endothéliales stimule la sécrétion de TGF-β1 et nous avons démontré une action paracrine influençant le phénotype fibrogénique des CML vasculaires par la voie SMAD. De la même manière nous pouvions légitimement émettre l'hypothèse d'une action autocrine du TGF-β1 sur les cellules endothéliales et nous avons donc étudié l'implication de la voie SMAD dans la régulation radio-induite de PAI-1 dans les cellules endothéliales. Les résultats montrent que la voie SMAD est activée après irradiation des cellules endothéliales avec une stimulation de la

phosphorylation des SMADs 2 et 3 ainsi qu'une stimulation de la translocation nucléaire des SMADs 2, 3, 4. De plus, il y a une activation de la transcription SMAD-dépendante ainsi que celle du promoteur de PAI-1 après irradiation. En revanche, lorsque les 3 éléments de réponse aux SMADs sont mutés, aucune induction transcriptionnelle n'est observée. Ceci démontre qu'au moins une des trois boîtes SMAD est nécessaire à la stimulation de PAI-1 après irradiation et suggère fortement que la voie SMAD est impliquée dans ce mécanisme. D'autre part, nos résultats montrent que l'irradiation stimule la formation de complexes SMAD3/SMAD4 mais pas de complexes SMAD2/SMAD4 suggérant un rôle différent des SMADs 2 et 3 dans l'activation radio-induite de PAI-1. Ceci a été confirmé par le fait que la surexpression de SMAD3 stimule la transcription radio-induite de PAI-1 alors que la surexpression de SMAD2 n'a pas d'effet. Le fait que ces deux R-SMADs aient des fonctions différentes dans l'induction radio-induite de PAI-1 confirme la non-redondance fonctionnelle de ces deux facteurs de transcription démontrée dans d'autres études.

Pour aller plus loin dans la compréhension des mécanismes moléculaires impliqués, nous avons voulu déterminer l'éventuelle coopération entre la voie SMAD et d'autres voies de signalisation dans l'induction radio-induite de PAI-1. Le travail récent publié par Hageman *et al* sur la coopération entre le TGF-β et la protéine p53 dans la stimulation de la transcription de PAI-1 dans des cellules tumorales a ainsi ouvert des hypothèses intéressantes (Hageman et al., 2005).

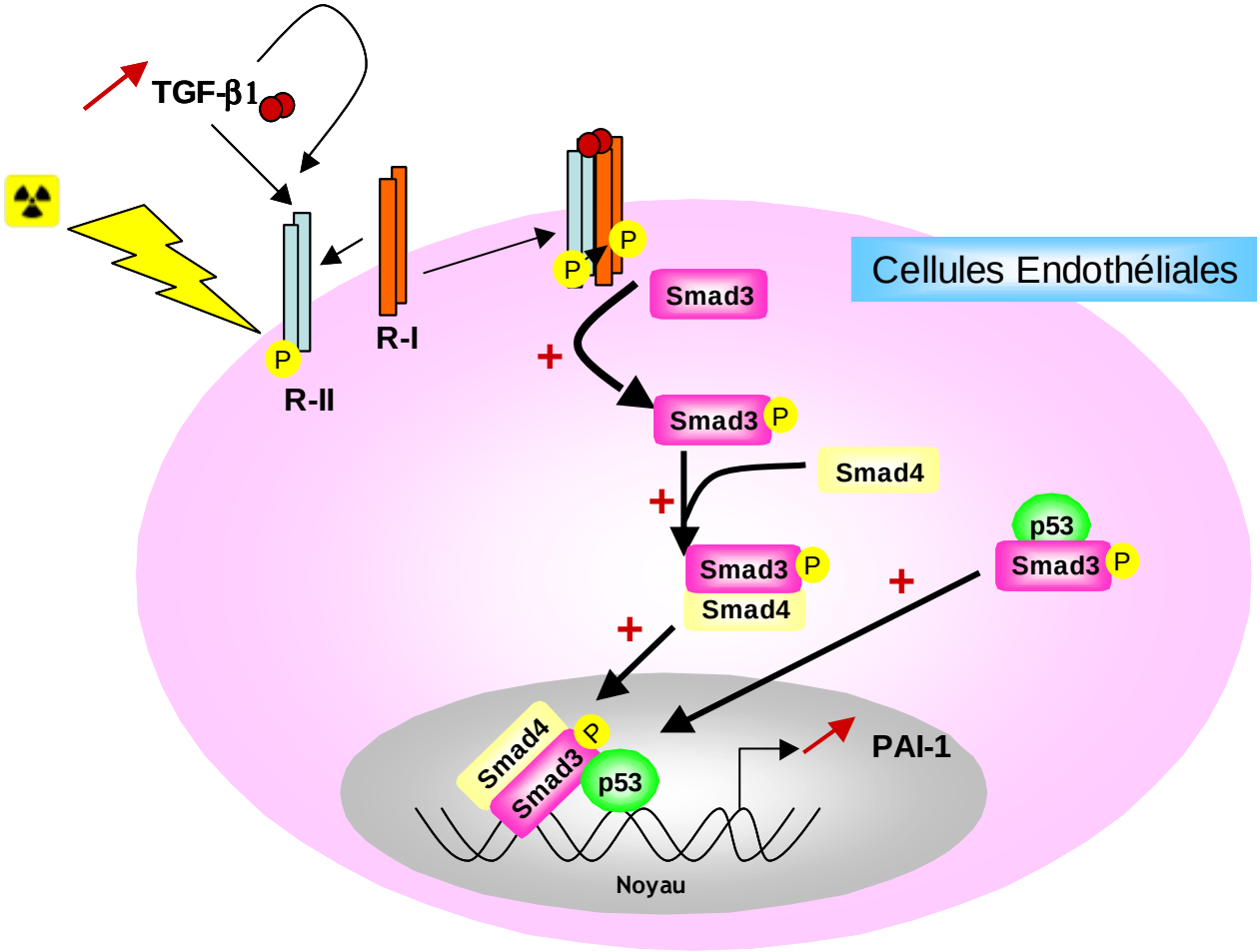
Le gène p53 est un gène suppresseur de tumeur muté dans de nombreuses tumeurs humaines (Sigal and Rotter, 2000). En réponse à différents stress et particulièrement après une irradiation par rayonnements ionisants, p53 est activée, migre du cytoplasme au noyau et agit alors comme facteur de transcription. p53 est capable d'activer un très grand nombre de gènes impliqués dans l'arrêt du cycle cellulaire, l'apoptose ou encore la réparation de l'ADN (Gudkov and Komarova, 2003). Cependant une étude génomique sur différentes cellules tumorales soumises à différents stress (Chlorure de Zinc, UV ou irradiation γ) a permis de mettre en évidence différents clusters de gènes activés ou réprimés par p53 (Zhao et al., 2000). Ainsi, ces données récentes de la littérature montrent que p53 a de nombreuses cibles moléculaires impliquées dans plusieurs fonctions comme l'adhésion et la migration cellulaire, le remodelage matriciel, la formation du cytosquelette ou encore l'angiogénèse (Zhao et al., 2000). Un travail initial de Kunz *et al.* avait montré que PAI-1 était une cible de p53 (Kunz et al., 1995) et, dans cette approche génomique de Zhao et al, PAI-1 est identifié comme une cible de p53 après irradiation. Récemment, Hageman *et al* ont démontré que la stimulation radio-induite de PAI-1 est fortement augmentée quand des cellules HepG2 irradiées sont traitées avec du TGF-β. Ces résultats démontrent un effet synergique de l'irradiation et du

TGF-β et suggérent une coopération entre des voies de signalisation activées par les rayonnements ionisants et ce facteur de croissance (Hageman et al., 2005). Ainsi, la mutation de l'élément de réponse à p53 présent dans le promoteur de PAI-1 entraîne la perte d'induction de PAI-1 en réponse à l'irradiation en présence et en absence de TGF-β exogène. De plus, l'implication de p53 a été confirmée par le fait que, dans des cellules tumorales p53 -/-, l'induction radio-induite de PAI-1 n'était pas observée. L'ensemble des ces résultats publiés par Hageman *et al* démontre donc que p53 est nécessaire à l'activation transcriptionnelle de PAI-1 après irradiation dans des cellules HepG2 et suggére une coopération entre la voie SMAD et p53. Cependant ce travail posait également un certain nombre de question : Est-ce que ce mécanisme décrit sur une lignée tumoarle est également valable sur des cellules normales et, notamment sur des cellules endothéliales ? Quelles sont les éventuelles SMADs impliquées dans ce mécanisme ? Par quel mécanisme moléculaire p53 et la voie SMAD contrôlent l'expression radio-induite de PAI-1 ?

Nos résultats démontrent que **1-** l'irradiation stimule la transcription de PAI-1 dans les cellules endothéliales **2-** un des trois éléments de réponse aux Smads est nécessaire à cette stimulation **3-** l'irradiation entraîne la translocation nucléaire des SMADs et de p53 dans les cellules endothéliales **4-** l'irradiation entraîne la formation de complexes SMAD3/SMAD4 et la surexpression de SMAD3 stimule la transcription radio-induite de PAI-1 **5-** l'irradiation stimule la formation de complexes p53/SMAD3 et l'inhibition par interférence ARN de l'une de ces deux protéines abolit complètement la transcription radio-induite de PAI-1.

La coopération entre p53 et la voie SMAD est impliquée dans l'embryogénèse chez la grenouille (Cordenonsi et al., 2003; Takebayashi-Suzuki et al., 2003). Un travail publié par Cordenosi et al a apporté des éléments essentiels quant aux mécanismes moléculaires impliqués (Cordenonsi et al., 2003). Ainsi ces auteurs ont démontré que p53 s'associe physiquement aux R-SMADs (ie SMAD2 et SMAD3) mais pas à SMAD4 en réponse à l'activine dans des cellules HepG2. Les R-SMADs sont formées d'un domaine C-terminal nommé MH1 (Mad Homology 1) et d'un domaine N-terminal nommé MH2 (Mad Homology 2) séparés par une région Linker (Massague et al., 2005). La formation des complexes R-SMADs et Co-SMADs est assurée par les domaines MH2. Ces mêmes domaines MH2 sont impliqués dans le recrutement de co-represseurs et de co-activateurs. Le domaine MH1 des R-SMADs (sauf SMAD2) peut se lier directement à l'ADN au niveau des éléments de réponse aux SMADs présents dans les promoteurs des gènes. Ainsi Cordenonsi *et al* ont montré que p53 s'associe physiquement à SMAD2 et SMAD3 par leurs domaines MH1 mais pas par le domaine MH2. De plus, l'interaction de p53 au domaine MH1 de SMAD3 n'affecte la liaison du mutant SMAD3-MH1 à l'ADN (Cordenonsi et al., 2003). A partir de l'ensemble de ces résultats ces auteurs ont démontré que p53 est un partenaire transcriptionnel

des SMADs qui s'associe physiquement à SMAD2 et SMAD3 pour stimuler la transcription des gènes cibles contenant dans leur promoteur des éléments de réponse aux SMADs et à p53 (ex : PAI-1, p21 ou encore MMP2). Bien entendu dans cette étude l'inducteur n'était pas l'irradiation mais les auteurs avaient suggéré l'intérêt d'explorer les effets d'agents provoquant des dommages à l'ADN (Cordenonsi et al., 2003; Dupont et al., 2004). Nos résultats viennent confirmer cette hypothèse en démontrant qu'en réponse à un stress par rayonnements ionisants dans des cellules humaines, une coopération entre p53 et SMAD3 est impliquée dans l'induction d'un gène cible contenant dans son promoteur des boîtes SMADs et p53 (i.e. PAI-1).



Effets de l'irradiation

Plusieurs perspectives découlent de ces travaux pour compléter la compréhension des mécanismes moléculaires impliqués dans l'induction transcriptionnelle de PAI-1.

Tout d'abord : Quel est ou quels sont les éléments de réponses aux Smads présent dans le promoteur réellement nécessaires à l'induction radio-induite de PAI-1 ? Des constructions avec les différentes mutations sur chaque boîte SMAD (–280, -580 et –730) et des expériences de Gene Reporter permettraient de répondre à cette question.

Quel est l'état de phosphorylation de p53 et son éventuelle implication dans la formation des complexes p53/SMAD3 ? Un travail récent a démontré que la phosphorylation de p53 au niveau de la serine 15 est nécessaire à l'induction de PAI-1 par un carcinogène (N-Methyl-N'-nitro-N-nitrosoguanidine) dans des cellules murines NIH3T3 (Parra et al., 2001). L'irradiation entraîne la phosphorylation de nombreuses sérines (Ser^{6}, Ser^{15}, Ser^{20}, Ser^{37}, Ser^{46} et Ser^{315}) (Saito et al., 2003) et l'identification des résidus phosphorylés de p53 et de leurs rôles dans la formation des complexes avec le domaine MH1 de SMAD3 n'a jamais été déterminée.

D'autre part, une étude par ChIP-Chip (Technique d'immunoprécipitation de la chromatine associée à une hybridation sur une puce ADN constituée de différentes région promotrices) permettant d'identifier les protéines liées à certaines régions du promoteur de PAI-1 et notamment la région –300 –100 qui contient une boîte SMAD et une boîte p53 permettrait d'identifier les complexes transcriptionnels fonctionnels impliqués.

Nos résultats mettent en lumière un nouveau rôle de p53 dans la réponse aux rayonnements ionisants et de son éventuelle implication dans les dommages radio-induits aux tissus sains. Le rôle de p53 dans les effets secondaires de la radiothérapie a déjà été abordé par l'équipe de Gudkov il y a quelques années et les travaux publiés par cette équipe ont donnée lieu à certains concepts très provocateurs (Gudkov and Komarova, 2003; Komarov et al., 1999; Komarova and Gudkov, 2000). Ainsi, une étude avait démontré que des souris traitées avec un inhibiteur chimique de p53 étaient protégées contre des doses létales d'irradiation (Komarov et al., 1999). Sur la base de ces résultats les auteurs ont suggéré que, dans un contexte tumoral où les cellules cancéreuses sont mutées ou inactivée pour p53, une stratégie thérapeutique visant à inhiber l'activité de p53 permettrait de limiter l'apoptose radio-induite des cellules normales présentes dans le champ d'irradiation et donc de limiter « spécifiquement » les dommages aux tissus sains sans compromettre la réponse tumorale. Clairement ce concept provocateur peut paraître séduisant mais dangereux car il est bien connu que l'inactivation de p53 peut conduire à la transformation néoplasique. Ainsi, même si inhiber p53 afin de limiter l'induction radio-induite de PAI-1 dans les cellules endothéliales pourrait éventuellement avoir un effet bénéfique sur la réponse des tissus sains, cette stratégie semble toutefois incompatible avec les stratégies anti-tumorales. Cependant nos résultats suggèrent que p53 contribue aux effets délétères des rayonnements ionisants sur les tissus sains par son implication dans l'induction radio-induite d'un acteur clef de la fibrinolyse. A notre connaissance, la connexion entre p53 et fibrinolyse n'a jamais été évoquée dans la littérature. Cependant, nos résultats et ceux de Kunz et al démontrant que p53 stimule la transcription de PAI-1 et au contraire inhibe celle de uPA et tPA dans des lignées tumorales (Kunz et al., 1995) ouvrent de nouvelles perspectives. En

effet, si cette démonstration était vérifiée dans des cellules endothéliales, on pourrait alors supposer que p53 jouerait un rôle clef dans l'orientation du phénotype anti-fibrinolytique de l'endothélium après irradiation.

En conclusion, l'ensemble de ce travail de thèse contribue à l'avancement des connaissances fondamentales concernant les dommages radio-induits aux tissus sains. La compréhension des mécanismes moléculaires impliqués à la fois dans l'influence des cellules endothéliales dans le phénotype des cellules musculaires lisses vasculaires après irradiation et également dans la régulation de l'expression radio-induite de PAI-1 dans les cellules endothéliales a permis de mettre en évidence des nouveaux rôles de la voie TGF-β/SMAD.

Nos travaux confirment l'intérêt d'inhiber la voie du TGF-β pour limiter les lésions radio-induites et en particulier de développer des inhibiteurs pharmacologiques de SMAD3. De plus, nous avons démontré que la déficience génétique en PAI-1 protège des dommages radio-induits. Ces résultats apportent la preuve de principe de l'implication de PAI-1 et ainsi de l'intérêt d'évaluer l'effet d'un inhibiteur de PAI-1 dans la prévention ou la réduction des effets secondaires des radiothérapies.

La difficulté de mettre en place des stratégies thérapeutiques visant à limiter les dommages radio-induits aux tissus sains est souvent liée au risque éventuel de protéger la tumeur. Ainsi, en parallèle à nos travaux sur les tissus sains, l'étude du rôle de PAI-1 dans la réponse tumorale à l'irradiation a commencé à être entreprise. L'objectif sera d'évaluer, *in vivo,* la compatibilité de notre stratégie de protection ciblée sur les tissus sains avec l'objectif curatif des traitements des cancers par la radiothérapie.

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V Annexes

Les articles présentés en annexes auxquels j'ai collaboré concernent l'étude des effets secondaires des radiothérapies et plus précisément les fibroses radio-induites intestinales chez l'homme.

François A, **Milliat F**, Vozenin-Brotons MC. Bowel injury with pelvic radiotherapy. **Radiation Physics and Chemistry 72 (2005) 399-407**

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Bowel injury associated with pelvic radiotherapy

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Abstract

Radiation therapists have to deal with the difficulty to give an efficient radiation dose to the tumor without generating unacceptable normal tissue injury. Acute reactions are experienced in most of the patients and are characterized by diarrhea resulting from intestinal mucosal injury. In some cases, intestinal wall fibrosis may develop, with hazard of occlusion syndrome. The only therapeutic recourse consists of surgical resection of the injured bowel.

Keywords: Radiation therapy; Intestinal toxicity; Complications

1. General introduction

Radiotherapy (RT) is dedicated to deliver a sufficient radiation dose to the tumor to avoid cancer recurrence. Tumor sterilization would be quite easy to obtain if the tolerance of the normal tissues located in the irradiation field did not limit the total radiation dose that can be delivered. Treatment efficacy is thus governed by an optimal compromise between tumor control and normal tissue damage, i.e., the risk/benefit ratio (Denham and Hauer-Jensen, 2002). Fractionated RT decreases the probability of receiving a critically high dose for the mobile parts of the intestine. However, this probability becomes important for fixed intestinal segments, such as the terminal ileum, the colon, and the rectum. These are the portions of the gastrointestinal tract the most often involved in radiation injury following pelvic tumor treatment, notably cervical, endometrial, and prostate cancers. Stem cells from the small and large intestine have different radiation sensitivities (Potten and Booth, 1997). The 5% risk of delayed complications at 5 years is thus reached for different dose ranges, i.e., 45–50 Gy for the small bowel and 60–65 Gy for the colon and the rectum (Cohen and Creditor, 1983). Normal tissue injury has long been divided according to clinical symptoms in independent acute and chronic phases (respectively, inflammatory and fibrosis phases) (Denham et al., 2001). Recent studies on the mechanisms of radiation fibrosis now tend to suggest a probable *continuum* between the steps of initiation, development, and persistence of radiation lesions, and open new therapeutic opportunities.

2. Clinical manifestations of pelvic RT

The diverse manifestations of intestinal complications of RT may develop insidiously, are often progressive, and may be lethal. Radiological changes in the bowel and their clinical expression may mimic those seen in other intestinal pathologies, such as Crohn's or celiac diseases. This renders the diagnosis difficult, especially when there is an important asymptomatic latent period

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between cancer treatment and the expression of clinical symptoms.

Acute radiation enteritis affects most patients who receive abdominal or pelvic RT. The early gastrointestinal symptoms are expressed during or immediately after therapy and include nausea, vomiting, abdominal pain, bacterial infection, and diarrhea, essentially related to intestinal mucosal disruption (Nussbaum et al., 1993; Nejdfors et al., 2000). Management of the acute radiation sickness is generally an association of symptomatic treatment and supportive care. The acute syndrome is often self-limiting and symptoms resolve in a few weeks following the completion of RT.

Acute symptoms may be succeeded by a chronic deteriorating course of disease often requiring surgery. Late effects may become manifest after latent periods of months to years. The most frequent chronic symptoms of intestinal radiation injury are recurrence of diarrhea and alternative constipation, with often severe and colicky abdominal pain accompanied by nausea and vomiting. These symptoms are generally associated with a considerable weight loss indicating severe intestinal functional abnormalities added to mechanical impossibilities to ensure normal transit. Intestinal wall thickening and luminal narrowing impair peristalsis and progressive stenosis may lead to complete intestinal occlusion. Moreover, severe ulceration and tissue necrosis may perforate the intestinal wall and create entero-cutaneous or entero-enteric fistulae. The etiology of functional perturbations is less known and may implicate both motility and intestinal transport disorders.

3. Predisposing factors to intestinal complications

The key factor in the risk of intestinal radiation injury is the irradiation itself: high doses (generally in excess of 45 Gy), high dose per fraction, and short treatment times are particularly implicated (Denham et al., 2000). Concomitant, previous or subsequent chemotherapy increases the risk, as well as intestinal ischemia or intestinal adhesions created by previous pelvic/abdominal surgery. Clinical evidences indicate that several systemic diseases increase the risk for both acute and late radiation damages. Pathologies such as diabetes mellitus, collagen vascular disease, hypertension, and inflammatory bowel disease share similar characteristics of vascular injury and are generally considered as contraindications for RT, especially during the active phase of the disease (for review, see Chon and Loeffler, 2002).

The response of normal tissues to radiation exposure is also under genetic influence. The determination of individual radiosensitivities may allow dose escalation to radioresistant patients without concomitant normal tissue injury (Gatti, 2001). Even if they seem to remain controversial (Slonina et al., 2000; Russell and Begg, 2002; Dikomey et al., 2003), in vitro assays of individual radiation sensitivities on lymphocytes or fibroblasts are under investigation in several laboratories (Barber et al., 2000; West et al., 2001; Ozsahin et al., 2003). The discrepancies encountered in correlation between in vitro radiation sensitivities and patient outcome are quite inconsistent but may have several explanations, such as the organ considered, the toxicity criteria, the time point of cell collection, and so forth. Other investigations have been done on punctual genetic mutations associated with high radiation sensitivity, such as Ataxia Telangiectasia or the Nijmegen breakage syndrome (Neubauer et al., 2002). Finally and based on the observation that different murine strains show variable predisposition to develop radiation rectal or pulmonary fibrosis (Skwarchuk and Travis, 1998; Haston et al., 2002), attempts have been done to establish genetic profiles to identify a range of candidate genes susceptible to be informative on individual patient radiation sensitivity. In the study from Andreassen et al. (2003), single nucleotide polymorphisms in four candidate genes have been correlated to increased risk of radiation-induced normal tissue reactions (*TGFB1*, *SOD2*, *XRCC3*, and *XRCC1*). Models based on multiple genetic markers seem to date the more relevant tools to predict individual radiation sensitivity.

4. Physiopathology of radiation intestinal toxicity

4.1. The intestinal mucosa: an acute-responding compartment

The bordering epithelium is composed of differentiated cells involved in the transport of intestinal nutrients, water and electrolyte movements, mucus secretion, immune defence or in the modulation of intestinal function. These differentiated cells originate from the proliferating compartment located in the base of the crypts of Lieberkuhn. The epithelium is thus continuously renewed and its structural and functional integrity is governed by the proliferating capacity of primitive cells. Radiation exposure affects crypt cell proliferation and mucosal injury occurs as soon as daughter cells no longer replace the extruded differentiated ones (Potten et al., 1983; Potten, 1990). Acute intestinal radiation injury is characterized by crypt cell loss, villus atrophy, epithelial atypia with mucosal erosion and intestinal barrier breakdown. Numerous inflammatory changes are observed, with inflammatory cell infiltration in the surface epithelium, lamina propria and submucosa (Rubin and Casarett, 1968). Recently, Hovdenak et al. (2000) showed that radiation proctitis developed very early (2 weeks) during the course of

pelvic RT. Depending on the dose received, mucosal damage can range from minimal degenerative changes in the germinal layers to complete cell loss, severe ulceration, and mucosal necrosis. Since symptoms of acute radiation sickness tend to cease after the completion of RT, acute mucosal injury was thought to be transient. However, delayed radiation enteritis is now considered as a *continuum* between acute and chronic reactions. The concept of "consequential late effects" introduced by Peters et al. (1988) on the oral mucosa recognizes that late radiation damage may occur as a consequence of acute, non-healing ulcers and necrosis. Consequential late effects are observable in organs in which the acute responding compartment represents a barrier against mechanical or chemical injury such as oral or intestinal mucosa (for review, see: Dörr and Hendry, 2001). Severe acute intestinal barrier breakdown may result in additional damage to the underlying compartments such as connective and vascular tissues, both implicated in late reactions. The detailed review of radiation effects on the supportive tissues of the gastrointestinal tract by Powers and Gillette (1995) provided evidence for common observations following localized intestinal radiation exposure in various animal models. Briefly, intestinal fibrosis develops for doses of 20 Gy or more, and might be present in the absence of mucosal ulceration. Fibrosis is, however, more severe and is accompanied by inflammation and necrosis if mucosal ulceration occurs during the acute phase (Followill et al., 1993; Denham et al., 2000).

The direct link between the severity of acute reaction and the development of late damage has not been fully established but more and more clinical studies report evidence suggestive of consequential late effects. For example, in the following of 220 patients treated with external beam radiation therapy to the whole pelvis (40–44 Gy, 2 Gy per fraction) followed with high dose-rate intracavitary brachytherapy (7.2 Gy, 3 fractions) for cervical carcinoma, Wang et al. (1998) showed a correlation between the severity of acute diarrhea and the prevalence of radiation proctitis 4 years after treatment. The five-year proctitis-free rate did fall from 72% in patients with no obvious acute diarrhea to 29% in the severe diarrhea group. Evidence for a consequential effect has also been demonstrated in patients treated for prostate or endometrial cancer (Schultheiss et al., 1997; Weiss et al., 1999; O'Brien et al., 2002).

An interesting study by Denham et al. (1999a) on consequential late reactions in patients with head and neck cancer, suggests that both the severity and duration of acute confluent mucositis must be taken into account to be predictive for late effects. Concerning the bowel and especially the small intestine, the difficulty remains the objective evaluation of both acute and late effects (especially acute small intestinal mucosal ulceration), to investigate the existence of a causal relationship between acute and late reactions, as suggested by Denham et al. (1999b).

Radiation-induced loss of exchange surface and epithelial disorganization are associated with acute intestinal functional impairment, which has been described in numerous animal experiments (for review, see MacNaughton, 2000). Globally, the injured epithelium and subsequent loss of intestinal barrier function compromise the host defence mechanisms and expose the whole organism to life-threatening infections. The exact pathogenesis of such dysfunction remains unknown but the majority of patients suffer from post-irradiation acute but also chronic bowel dysfunction. Functional gastrointestinal toxicity results from different pathophysiological mechanisms essentially related to defaults in both intestinal transport and motility (Ludgate and Merrick, 1985; Yeoh et al., 1993; Husebye et al., 1994, 1995). Disturbances in intestinal transport have been well-described after total body or abdominal exposure in animal models, suggesting different mechanisms such as loss of functional mucosa (Quastler, 1956), but also changes in water and electrolyte transport, barrier properties and/or neuro-immune regulations (Geraci et al., 1987; Gunter-Smith, 1987; MacNaughton et al., 1994; Lebrun et al., 1998; François et al., 1998, 2000, 2002).

Less is known about functional injury in the late phases of radiation enteritis, and especially after localized intestinal injury. We investigated small intestinal function in the rat after X-radiation exposure of a scrotalized ileal loop (François et al., 2003). First results showed acute and chronic impairment of water and electrolyte transport capacity in irradiated as well as in uninjured intestinal segments, probably with repercussions as far as in the duodenum (François, unpublished data). Functional impairment in apparently unaffected bowels has also been observed in patients after RT for gynecological cancer. Altered migrating motor complexes in proximal gut were associated with malabsorption syndrome and clinical symptoms of intestinal pseudoobstruction (Husebye et al., 1994). Höckerfelt et al. (2002) described interesting changes in enteric nerve fiber densities in the human colon following abdominal RT. Clinical studies on the possible mechanisms of intestinal dysfunction following RT remain sparse and the study by Höckerfelt et al. (2002) demonstrates the necessity for further investigations. Radiation-induced dysmotility of the small and large intestine has been well described in dogs following single or fractionated total abdominal irradiation (Otterson et al., 1992a, b; Summers et al., 1992; Summers and Hayek, 1993). Several studies offer some mechanistic clues such as disturbances in neural, neuropeptidic and/or inflammatory influences (Krantis et al., 1996; Picard et al., 2002; Linard et al., 2003a, b). These experimental and clinical data both on bowel transport and motility suggest that

the whole gut should be considered in the management of functional side effects following pelvic RT. Unfortunately, there are few clinical studies dealing with intestinal transport capacity and/or motor activity, principally due to the time-consuming and sometimes invasive techniques of investigation. Data available on intestinal physiopathology during the acute and late phases of radiation enteropathy are essentially based on patients' symptoms. These studies are of course very informative but further animal experiments will be necessary to understand the mechanisms responsible for intestinal dysfunction for several reasons: (1) it is known that diarrhea, a symptom shared by both acute and late phases of intestinal radiation toxicity, may have different etiologies, such as malabsorption, increased water and electrolyte secretion or dysmotility, or even more complex phenomenon such as immune or enteric nervous influences (Sellin, 2001); (2) there may be discrepancies between clinical and pathological observations (Dörr et al., 2002); and (3) a non-negligible percentage of patients experience changes in bowel habits suggesting intestinal dysfunction and responsible for a decreased quality of life. These symptoms are not life threatening and thus not referenced as late complications, but treatment may be improved by the understanding of the mechanisms of radiation-induced intestinal dysfunction.

4.2. Vascular compartment

The vascular compartment is implicated in the processes leading to and/or perpetuating radiation fibrosis (Hasleton et al., 1985). Crypt stem cells were considered as the primary target cells responsible for acute radiation-induced intestinal injury. It is now becoming evident that a one-population-based response to radiation exposure is not sufficient to explain tissue structural damage observed after irradiation. Another aspect of acute mucosal radiation response is governed by the high radiation sensitivity of the vascular and microvascular compartments (Baker and Krochak, 1989). Radiation-induced endothelial apoptosis in the microvessels has been recently shown to be determinant in the acute loss of proliferating capacity of intestinal stem cells (Paris et al., 2001). Pharmacologic (via basic fibroblast growth factor) or genetic inhibition of endothelial cell apoptosis (deletion of the acid sphingomyelinase gene) prevented radiation-induced crypt damage and death from the gastrointestinal syndrome in mice. Even if several studies have to be performed to precisely define the respective roles for crypt stem cells and endothelial cells in acute gastrointestinal radiation injury, these results clearly show the complex interplay between different cell types and tissue compartments in the development of radiation enteritis. During the subsequent mucosal inflammatory response, increased vascular permeability is implicated in plasma protein extravasation, the activation of the coagulation system and the production of profibrosing mediators, suggesting a possible link between endothelial dysfunction and chronic radiation enteritis (Nguyen et al., 2000; Wang et al., 2002a).

Structurally, obliterative changes observed during the late phase of radiation enteropathy, with intimal fibrosis and vessel distortion due to matrix deposition which lead to tissue hypoxia; processes probably involved in impaired tissue healing and regeneration. Functionally, the chronicity of radiation lesions of the intestine may implicate an imbalance in the coagulation homeostasis and especially a reduction in endothelial thrombomodulin (involved in the anticoagulant pathway) in favor of pro-thrombotic processes and subsequent impaired intestinal tissue blood supply (Richter et al., 1997).

4.3. Mesenchymal compartment

Berthrong and Fajardo (1981) have precisely described the pathophysiology of chronic radiation injury to the intestine. Briefly, the prefibrotic phase is characterized by a chronic inflammation during which the endothelial cell is thought to play a determinant role. The phase of established fibrosis shows an accumulation of inflammatory cells and activated proliferating fibroblasts responsible for fibrogenic cytokine secretion and extracellular matrix deposition (Hauer-Jensen et al., 1983; Langberg et al., 1994; Followill and Travis, 1995; Skwarchuk and Travis, 1998). The real late phase is a poorly vascularized fibrosis, with few fibroblasts and is characterized by the densification of fibrotic tissue due to a continuing remodeling of the extracellular matrix. Necrosis may develop, with loss of substance and tissue death, more frequently in patients presenting genetic and/or co-morbidity predisposition factors such as chronic infections.

Activation of mesenchymal cells and extracellular matrix deposition is characteristic of post-traumatic tissue reaction. The chronic activation of resident mesenchymal cells was demonstrated in late radiation injury in skin and lung, and characterized as the phenotypic differentiation of resident fibrocytes into fibrosis myofibroblasts (Rodemann and Bamberg, 1995). At the molecular level, TGF-β1 is recognized as a primary fibrogenic cytokine promoting the initiation, development, and persistence of delayed radiation injury. Increased TGF-β1 expression has been observed in various organs following radiation exposure (Martin et al., 2000). Recently, a study in our laboratory on fibrogenic signals in patients with radiation enteritis provided the first evidence of connective tissue growth factor (CTGF) as an essential fibrogenic mediator in the persistence of delayed intestinal radiation injury

(Vozenin-Brotons et al., 2003). The general concept of the role played by TGF-β1 in radiation fibrosis suggests that this molecule is involved in the initiation and induction of intestinal radiation fibrosis rather than just the later phases (Hauer-Jensen et al., 1998). In the later stages, CTGF may act as a fibrogenic mediator independently from TGF-β1 and may be responsible for the maintenance of the fibrotic lesion. This sequence of events has been observed in other pathologies such as scleroderma (Holmes et al., 2001).

5. Therapeutic issues

When intestinal radiation fibrosis is established, conservative measures are limited to high protein, high calorie diet supplemented with vitamins and antispasmodics. When there is hazard of intestinal occlusion syndrome, surgical resection of injured segment remains to date the only therapeutic solution, with problems of healing of the anastomoses in cases of irradiated or inflamed margins (anastomotic leakage). Efforts have thus been directed towards some therapeutic tools on one hand to prevent late complications, by reducing the severity of acute reactions and, on the other hand, to reverse established radiation fibrosis by acting on molecular targets implicated in connective tissue remodeling.

5.1. Mucosal compartment

One approach to decrease complication rate associated with intestinal irradiation is to physically reduce the volume of normal tissue present in the irradiation field by techniques such as patient positioning (Gallagher et al., 1986), conformal RT or intensity-modulated RT (Tait et al., 1997; Mundt et al., 2003; Roeske et al., 2003). Imaging techniques and computer planning are used to localize precisely the tumor, minimizing the volume of normal irradiated tissues, and thus improving tumor control rate by dose escalation (Glatstein, 2001). However, these more aggressive schedules are associated with severe acute toxicity (Peters et al., 1988; Denham et al., 1996). Based on the concept of consequential late effects earlier evoked (Dörr and Hendry, 2001), it is now quite well established that the frequency and/or severity of late effects are at least in part influenced by the severity of acute intestinal reactions. One therapeutic direction may thus be to minimize acute mucosal injury during RT to limit the consequential part of the delayed adverse effects. One may, however, keep in mind that an eventual protective effect on cancer cells remains the main pitfall of several therapeutic strategies.

Three major directions may be mentioned: scavenging the reactive oxygen species (ROS) produced during the very acute normal tissue response to ionizing radiation (superoxide dismutase, amifostine), ensuring an essentially mechanical mucosal protection (sucralfate) or stimulate intestinal cell production (growth factors). Prophylactic treatment with selenium and/or vitamin E as well as gene transfer of human manganese superoxide dismutase reduced radiation-induced intestinal injury in rodents (Mutlu-Türkoğlu et al., 2000; Guo et al., 2003). Pretreatment with the ROS scavenger amifostine was able to reduce the RT-induced acute bladder and lower gastrointestinal toxicity in patients with pelvic malignancies (Athanassiou et al., 2003). The efficacy of such treatments on late effects is, however, still unknown. In clinical settings, the study published by Henriksson et al. (1992) demonstrating beneficial effects of orally administrated sucralfate on acute stool frequency and diarrhea, was followed by several studies which failed to demonstrate striking benefits on late complications and results remain controversial (O'Brien et al., 2002). A growing field of interest is the use of growth factors to prevent or ameliorate radiation-induced intestinal mucosal injury. The principle is to accelerate cell growth and differentiation to balance radiation-induced epithelial cell loss. Keratinocyte growth factor (KGF) seems to be the more relevant among the growth factors tested (for review, see: Farrell et al., 2002). Recombinant human KGF is in phase I clinical trial to prevent oral mucositis in patients receiving chemotherapy for metastatic colo-rectal cancers (Meropol et al., 2003). Another recently discovered intestinotrophic factor is glucagon-like peptide-II (GLP-2). Several studies have been performed demonstrating that GLP-2 helps intestinal recovery in several models of mucosal injury (for review, see L'Heureux and Brubaker, 2001). The beneficial effects of GLP-2 treatment on radiation-induced acute gastrointestinal syndrome in mice have been recently observed (Potten et al., 2003). Attempts to prevent bowel injury following localized small intestinal irradiation with GLP-2 administration are currently under investigation in our laboratory. Other approaches aiming to modify both acute and late reactions in different organs are based on the limitation of the inflammatory response via the administration of modulators of prostaglandin metabolism, COX-II inhibitors, inhibitors of angiotensin-1-converting enzyme, or angiotensin-2 receptor blockers (Yoon et al., 1994; Molteni et al., 2000, 2001). Finally, the somatostatin analogue octreotide was found to markedly ameliorate acute and chronic radiation injuries in the rat and provided evidence of the importance of consequential phenomenon in the development of late effects (Wang et al., 2001). Octreotide has also been shown to be efficient in the control of moderate-to-severe acute radiation-induced diarrhea in patients undergoing pelvic RT, with however no data actually available concerning late complications (Yavuz et al., 2002).

5.2. Vascular compartment

The prevention of bowel adverse effects by the use of antithrombotic factors seems to date to be the most promising strategy. Wang et al. (2002a) demonstrated that radiation enteropathy in the rat was associated with deficiency in microvascular thrombomodulin. Moreover, short-term inhibition of platelet aggregation ameliorated radiation-induced toxicity in rat small intestine (Wang et al., 2002b). The use of HMG CoA reductase inhibitors, such as statins, may increase the production of thrombomodulin and thus restore the tissue coagulation homeostasis. This has been shown in cultured human endothelial cells, where statin treatment increased cellular thrombomodulin activity (Shi et al., 2003). As far as the authors are concerned, no data are actually available in vivo on the possible therapeutic benefits of statin treatment following intestinal radiation exposure.

5.3. Mesenchymal compartment

Targeting the mesenchymal compartment to inhibit the constant extracellular matrix remodeling is the only therapeutic issue available to reverse established radiation fibrosis. The enzyme superoxide dismutase has been shown to have curative anti-fibrosing properties via its action on TGF-β1 (Vozenin-Brotons et al., 2001). The role of TGF-β1 in intestinal radiation fibrosis was confirmed by the amelioration of radiation enteropathy in mice by the in vivo administration of a recombinant soluble TGFβ type-II receptor (Zheng et al., 2000). The combination of pentoxifylline and tocopherol has been shown to induce radiation fibrosis regression in several reports, such as the one recently published on breast cancer patients (Delanian et al., 2003). Possibilities of fibrosis reversion are investigated in our laboratory by the study of cellular and molecular pathways implicated in the development of intestinal fibrosis (Vozenin-Brotons et al., 2003).

6. Conclusion

Radiation enteritis continues to be a limiting factor in the application of pelvic RT. Improvement in RT techniques and a constant research in the best schedules to limit normal tissue injury is supported by more and more laboratories interested in the side effects of radiation treatment (Hauer-Jensen et al., 2003). Although severe toxicities (intestinal obstruction and fistulae) are becoming less common today with the use of modern RT, many patients are confronted to gastrointestinal problems ranging from intermittent diarrhea to malabsorption, which may result in important patient distress. To limit the late sequels of RT is also part of the cancer treatment, and as reported by Pedersen et al. (1994), truly successful therapeutic outcome requires the patient alive, cured, and free of significant treatment-related morbidity.

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Inhibition of Rho kinase modulates radiation induced fibrogenic phenotype in intestinal smooth muscle cells through alteration of the cytoskeleton and connective tissue growth factor expression

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INTESTINAL MOTILITY

Inhibition of Rho kinase modulates radiation induced fibrogenic phenotype in intestinal smooth muscle cells through alteration of the cytoskeleton and connective tissue growth factor expression

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Background: Late radiation enteritis in humans is associated with accumulation of extracellular matrix and increased connective tissue growth factor (CTGF) expression that may involve intestinal muscular layers.
Aims: We investigated the molecular pathways involved in maintenance of radiation induced fibrosis by gene profiling and postulated that alteration of the Rho pathway could be associated with radiation induced fibrogenic signals and CTGF sustained expression.
Patients and methods: Ileal biopsies from individuals with and without radiation enteritis were analysed by cDNA array, and primary cultures of intestinal smooth muscle cells were established. Then, the effect of pharmacological inhibition of p160 Rho kinase, using Y-27632, was studied by real time reverse transcription-polymerase chain reaction, western blot, and electrophoretic mobility shift assay.
Results: Molecular profile analysis of late radiation enteritis showed alterations in expression of genes coding for the Rho proteins. To investigate further the involvement of the Rho pathway in intestinal radiation induced fibrosis, primary intestinal smooth muscle cells were isolated from radiation enteritis. They retained their fibrogenic differentiation in vitro, exhibited a typical cytoskeletal network, a high constitutive CTGF level, increased collagen secretory capacity, and altered expression of genes coding for the Rho family. Rho kinase blockade induced a simultaneous decrease in the number of actin stress fibres, α smooth muscle actin, and heat shock protein 27 levels. It also decreased CTGF levels, probably through nuclear factor κB inhibition, and caused decreased expression of the type I collagen gene.
Conclusion: This study is the first showing involvement of the Rho/Rho kinase pathway in radiation fibrosis and intestinal smooth muscle cell fibrogenic differentiation. It suggests that specific inhibition of Rho kinase may be a promising approach for the development of antifibrotic therapies.

Late intestinal toxicity is one of the most common complications of pelvic radiation therapy. It may occur several months to years after radiation therapy and may significantly alter the quality of life of cancer survivors. Radiation enteritis is characterised by severe transmural fibrosis associated with extracellular matrix remodelling.[1 2] Tissue stricture is responsible for loss of the compliant relationship between the mucosa and muscularis layers and the ensuing loss of intestinal function.

Intestinal function depends on both its transport capacity and its motility, which ensures peristalsis. The contraction process is mainly controlled by the enteric nervous system and is achieved by smooth muscle cells. The structural and also the functional role of intestinal smooth muscle cells in intestinal connective tissue homeostasis, repair, remodelling, and fibrosis is increasingly recognised.[3 4] During fibrogenesis, intestinal function is dramatically altered due to impaired motility[5] and excessive transmural deposition of collagen secreted by fibrosis activated subepithelial myofibroblasts and smooth muscle cells.[1] The fibrogenic phenotype of intestinal smooth muscle cells has been poorly investigated[6] but differential isoactin expression (α smooth muscle actin (α-sm actin) *v* γ smooth muscle actin (γ-sm actin)) has been found to be associated with synthetic or contractile smooth muscle cells in vitro.[7] In radiation enteritis, we found a high expression level of α-sm actin associated with increased collagen deposition and increased expression of the fibrogenic growth factor connective tissue growth factor (CTGF) in the muscularis propria.[1] This suggests that CTGF could be associated with radiation induced fibrogenic differentiation in intestinal smooth muscle cells. Thus understanding the mechanisms responsible for CTGF over-expression in intestinal smooth muscle cells may give new insights into the maintenance of radiation enteritis.

In the present study, we investigated regulation of CTGF gene expression in intestinal radiation induced fibrosis by cDNA array and found specific alteration of genes coding for proteins of the Rho family. Rho proteins belong to a family of small GTPases (RhoA, B, C, Rac-1, cdc 42) that control a wide range of cellular functions including cell adhesion, formation of stress fibres, and cellular contractility through reorganisation of actin based cytoskeletal structures.[8 9] Modulation of these cellular functions by Rho proteins largely depends on activation of their downstream effector, Rho kinase (ROCK).[10] Furthermore, Heusinger-Ribeiro *et al* showed that CTGF gene expression depends on the Rho signalling

Abbreviations: CTGF, connective tissue growth factor; α/γ-sm actin, α/γ smooth muscle actin; HSP, heat shock protein; ROCK, Rho kinase; N/RE SMC, normal/radiation enteritis smooth muscle cells; COL1A1, type I collagen alpha 1; MLCK, myosin light chain kinase; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; NFκB, nuclear factor κB; TNF-α, tumour necrosis factor α; TGF-β1, transforming growth factor β1

pathway during kidney fibrogenesis.[11] Thus we hypothesised that both overexpression of CTGF and appearance of an immature cytoskeleton in intestinal fibrosis activated smooth muscle cells may be regulated by the Rho/ROCK pathway. We analysed the involvement of the Rho/ROCK pathway in the regulation of CTGF gene expression and actin cytoskeleton using physiologically relevant primary cultures of intestinal smooth muscle cells from individuals with and without radiation enteritis, together with a specific inhibitor of ROCK, Y-27632.

PATIENTS AND METHODS

Tissue sampling and immunohistochemistry

Tissue sampling was performed as previously described[1] and patient characteristics are shown in table 1. Procurement of tissue samples received prior approval from our institution's ethics committee and was performed according to the guidelines of the French Medical Research Council. Immunostaining was performed on fixed paraffin embedded samples sectioned at 5 μm, using an automated immunostainer (Ventana Medical Systems, Illkirch, France) with the avidin-biotin-peroxidase complex method. Collagen deposition was assess by Sirius red staining and adjacent sections were incubated with antibodies against vimentin (1:50; Sigma, St Quentin Fallavier, France) and CTGF (1:100; a gift from AC de Gouville).

Cells, immmunofluorescence, and confocal laser microscopy

Primary intestinal smooth muscle cells were isolated from the muscularis propria by complete enzymatic digestion at 37℃ (0.2% type II collagenase and 0.1% soybean trypsin inhibitor), subcultured in SmGM2 (Cambrex, Emerainville, France), and used between P3 and P4. Three cell lines were isolated from normal ileal muscularis propria and two cells lines from fibrotic muscularis propria. Confluent monolayers of normal (N SMC) and fibrotic (RE SMC) smooth muscle cells were incubated with 10, 50, and 100 μM Y-27632 (Bioblock, Illkirch, France) and subsequently analysed. After fixation (0.5% paraformaldehyde) and permeabilisation (0.1% triton X-100), cells were incubated with phalloidin-FITC (Sigma) or with primary antibodies and FITC conjugated antibody, rinsed, and incubated in Rnase A/propidium iodide. Stained cells were imaged by laser scanning confocal microscopy.

Gene array analysis

Total RNA was extracted from tissue (n = 6 normal ileum and n = 6 radiation enteritis) and confluent cells (n = 3 N SMC and n = 2 RE SMC) by the method of Chomczynski and Sacchi,[12] quantified by absorption spectrometry, and treated with RNase free DNase (0.5 unit/μl) to remove contaminating genomic DNA. Atlas Human 1.2 (1176 genes + nine housekeeping genes) and Cell Interaction (265 genes + nine housekeeping genes) expression arrays from Clontech Laboratories (Ozyme, St Quentin en Yvelines, France) were used, as previously described.[13] (A list of all of the genes included in these two arrays as well as their functions can be found at www.clontech.com/atlas and is deposited in the GEO database (www.ncbi.nih.gov/geo) under GEO accession numbers GPL127 and GPL135.) Duplicate radiolabelled probes were generated from a single preparation of RNA. Hybridisation intensities were obtained using the Atlas Image 1.5 software, converted into ratios, and adjusted for background and housekeeping gene expression:

(Gene × intensity − background) / (average intensity for housekeeping gene − background)

Baseline gene expression was established by averaging the arrays obtained from six control samples; 25–35% variation in gene expression was observed in the control group. This allowed us to create a single ''normal composite array'' used to compare the set of normal samples with each radiation enteritis sample. A change in gene expression greater than twice that of the averaged control group was considered significant and data were used only when signal intensities were above background (that is, 50% or more).

mRNA expression analysis using quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Real time RT-PCR was performed as previously described.[1] CTGF FAM probe was purchased from PE Biosystems (Courtaboeuf, France). CTGF, 5′-TGT GTG ACG AGC CCA AGG A-3′ (forward) and 5′-TCT GGG CCA AAC GTG TCT TC-3′ (reverse); 5′-FAM, CTG CCC TCG CGG CTT ACC GA-3′; type I collagen alpha 1 (COL1A1), 5′-CCT CAA GGG CTC CAA CGA G-3′ (forward) and 5′-TCA ATG ACT GTC TTG CCC CA-3′ (reverse); γ-sm actin, 5′-GCC CTC AGT CAC TGG GAG-3′ (forward) and 5′-TGT GTG GCT GAG TGA GCT GG-3′ (reverse); RhoB, 5′-GTC CCA ATG TGC CCA TCA TC-3′ (forward) and 5′-CTG TGC GGA CAT BCT CGT C-3′ (reverse). Optimised PCR used the ABI PRISM 7700 detection system in the presence of 135 nM specific forward, reverse primers, and fluorogenic probe. Both water and genomic DNA controls were included to ensure specificity. The purity of each PCR product was checked by analysing the amplification plot and dissociation curves. Relative mRNA quantitation was performed using the comparative ΔΔCT method.

Procollagen type I secretion

Confluent cells were cultured for 24 hours under serum free conditions and procollagen type I secretion was determined using the Procollagen Type I C-Peptide EIA kit (Takara Biomedicals, Cambrex).

Western blot analysis

Expression of heat shock protein (HSP) 27 (SPA-800; Stressgen Biotechnologies, Victoria, BC, Canada), α-sm actin, RhoA (sc-418; Santa-Cruz), and CTGF (sc-14939, Santa-Cruz

Table 1 Characteristics of the patient population

Time after RT (months)	Tumour site	Age (y)	Sex	Treatment
Patients with ileal stricture (n = 6)				
1–3 months	Rectal cancer (n = 2)	44–68	M/F	n = 2: RT 45 Gy/S/CT
4–16 months	Gynaecological cancer (n = 3)	37–49	F	n = 1: S/RT 45 Gy/CT; n = 1: S/RT 45 Gy/CT/BT 10–15 Gy; n = 1: S/BT 60 Gy
75 months	Hodgkin disease (n = 1)	51	M	RT 40 Gy/CT
Patients with normal ileum (n = 6)	Colon adenocarcinoma	29–81	3M/3F	Non irradiated, Right hemicolectomy

RT, pelvic radiotherapy; BT, brachytherapy; CT, chemotherapy; S, surgery.

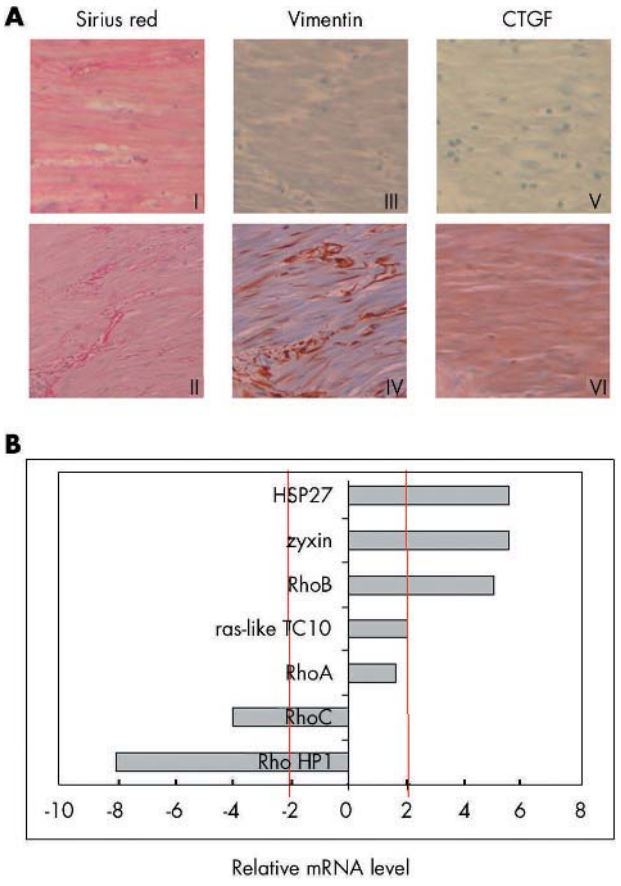

Figure 1 (A) Intestinal smooth muscle cells exhibited fibrogenic differentiation in vivo. In the muscularis propria, Sirius red staining showed collagen infiltration within smooth muscle bundles in radiation enteritis (II, ×200) versus normal bowel (I, ×200) that colocalised with vimentin positive cells (IV, ×200). Connective tissue growth factor (CTGF) immunostaining was negative in normal muscularis propria (V, ×200) whereas strong staining was observed in radiation enteritis (VI, ×200). (B) Gene array analysis revealed induction of genes coding for the Rho family and for actin polymerisation control in radiation enteritis samples (n = 6) compared with normal bowel samples (n = 6).

distributed by Tebu-Bio SA, Le Perray en Yvelines, France) were assessed by western blot analysis on total protein extracts from tissue or cells ($2–3\times10^6$) incubated or not with Y-27632 (10, 50, and 100 µM for 18 hours). Furthermore, nuclear and cytoplasmic protein extracts were prepared using the method of Schreiber and colleagues[14] from cells (1×10^6) incubated or not with Y-27632 (10 µM for 30 and 120 minutes) and sodium salicylate (25 mM for 45 minutes). Nuclear extracts were used in electrophoretic mobility shift assay (EMSA) experiments. Cytoplasmic extracts were used to measure IκB-α (sc-371; Santa-Cruz) and p65 (sc-8008; Santa-Cruz) protein levels by western blot. Proteins (5–15 µg) were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto a 0.45 µm nitrocellulose membrane. The membrane was incubated with the primary antibody, washed, and probed with the peroxidase labelled secondary antibody. Detection was achieved by enhanced chemiluminescence (ECL Amersham Pharmacia, Orsay, France). After dehybridisation, control loading was achieved by anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:2000; H86504M, Biodesign, Maine, USA). Densitometric analyses were performed using an image analyser (Biocom, Les Ulis, France) interfaced with the Phoretix image analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Electrophoretic mobility shift assay (EMSA)

PAGE purified double stranded oligodeoxynucleotides containing nuclear factor κB (NFκB) binding elements (5′-GAG GAA TGT CCC TGT TTG-3′) were 5′ end labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Life Technology, Cergy Pontoise, France). End labelled probes were purified using a G-50 column (Pharmacia, Saclay, France) and 1×10^5 cpm were incubated with 2–5 µg nuclear extract for 30 minutes at room temperature in a final volume of 20 µl containing 25 mM Tris HCl, pH 8, 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, and 1 µg/µl poly(dI-dC). For competition experiments, 10-fold excess cold competitor was added to the reaction mixture before incubation. Complexes were then resolved by 6% PAGE in 0.5× Tris-Borate-EDTA buffer. Gels were dried and complexes were visualised and quantified using an intensifying screen and a phosphorimager (Image Gauge software, FLA-3000, Fuji Ray Test, France).

Statistical analysis

All values are reported as mean (SEM). Data were analysed using one way ANOVA and the Student-Newman-Keuls test.

RESULTS

Intestinal smooth muscle cells exhibited fibrogenic differentiation in vivo

Fibrogenic differentiation of intestinal smooth muscle cells was investigated in radiation enteritis muscularis propria by histological staining of collagen and immunohistochemical detection of cytoskeleton markers (α-sm actin, vimentin, desmin) as well as CTGF expression. Compared with normal bowel, collagen infiltration was observed in radiation enteritis (fig 1A), associated with accumulation of vimentin positive cells (fig 1A). Strong CTGF immunoreactivity was also observed in the muscularis propria smooth muscle cells from radiation enteritis (fig 1A).

Genes coding for Rho family small GTPases and genes involved in actin polymerisation are altered in radiation enteritis samples

The global cDNA array approach revealed alterations in the expression profile of genes coding for intracellular signalling molecules of the Rho family. A significant and reproducible fivefold increase in RhoB gene expression was found in radiation enteritis samples (fig 1B) and confirmed by real time RT-PCR (×2.5, $p<0.05$). mRNA level of the gene coding for the ras-like protein TC10 reached a twofold increase whereas that of Rho HP1 and Rho C showed an eightfold and a fourfold decrease, respectively. Rho A mRNA level slightly increased in radiation enteritis samples (1.6-fold) but this difference was not confirmed at the protein level (data not shown). Expression of Cdc42 and Rac genes was not detected by cDNA array analysis nor were the genes coding for the LIM kinase and MLCK (myosin light chain kinase), which are involved in the control of actin polymerisation and act downstream of Rho. Conversely, gene expression of the actin filament assembly regulator zyxin and of the actin chaperone HSP27 significantly increased (5.5-fold) in radiation enteritis samples (fig 1B).

Primary smooth muscle cells isolated from radiation enteritis biopsies exhibit a fibrogenic phenotype

In order to study the molecular mechanisms involved in the maintenance of radiation induced fibrogenic differentiation in intestinal smooth muscle cells, primary cells were derived from normal (N SMC) and fibrotic (RE SMC) muscularis propria.

Primary N SMC exhibited a typical phenotype with a characteristic spindle shaped morphology and the presence of

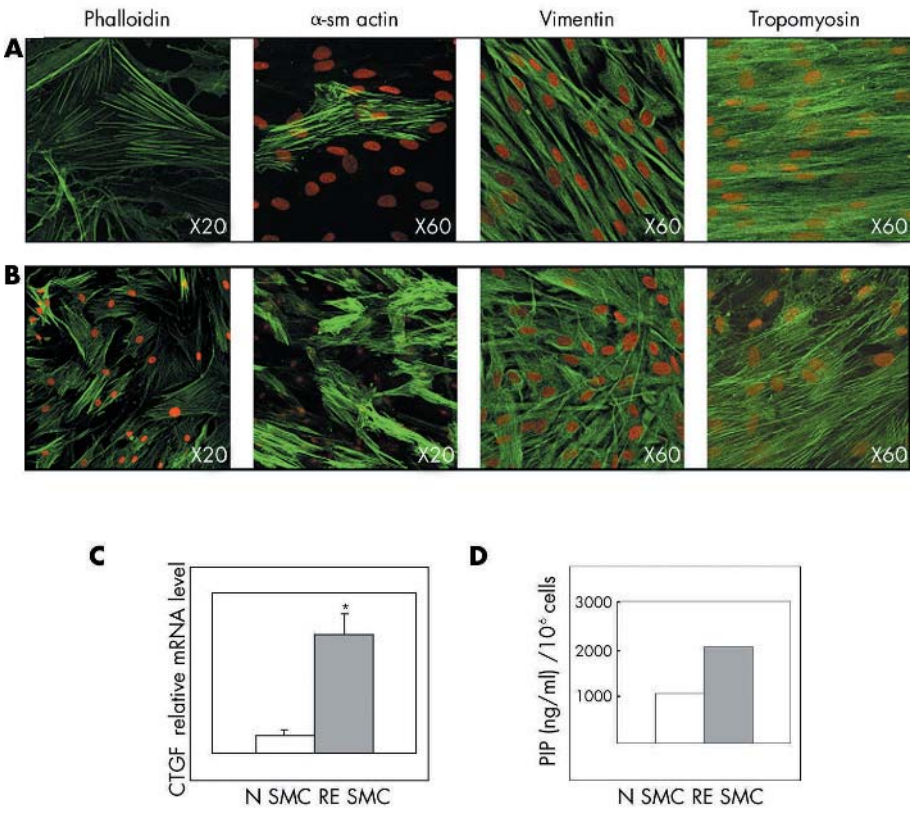

Figure 2 (A) Immunofluorescence experiments showed that normal smooth muscle cells (N SMC) exhibited typical intestinal differentiation markers in vitro. They were positive for vimentin and tropomyosin whereas only a few cells expressed α smooth muscle actin (α-sm actin). (B) Smooth muscle cells derived from radiation enteritis (RE SMC) showed greater densities of stress fibres and a strong constitutive α-sm actin protein expression. RE SMC (two cell lines) exhibited a prosecretory phenotype with a higher constitutive connective tissue growth factor (CTGF) mRNA level (experiments done in triplicate; C) and procollagen type I (PIP) secretion (experiments done in duplicate; D) than their normal counterpart (three cell lines). *$p<0.05$ compared with untreated N SMC.

actin stress fibres. At confluency, spontaneous retraction occurs and produces ''hill and valley'' pictures, as previously described.[7 15] RE SMC exhibited a more compact morphology and higher density of stress fibres than their normal counterparts (fig 2A and B, phalloidin). Cellular differentiation was assessed using the markers proposed by Graham and colleagues[16] and Brittingham and colleagues.[7] No differences were found between N and RE SMC regarding vimentin, tropomyosin protein expression (fig 2A, B), and γ-sm actin mRNA levels (data not shown) whereas high levels of α-sm actin were found in RE SMC, suggesting an immature and synthetic phenotype. Semi quantitative western blot analysis confirmed the high α-sm actin constitutive level in RE SMC that was barely detected in N SMC (see fig 4C, lane 0).

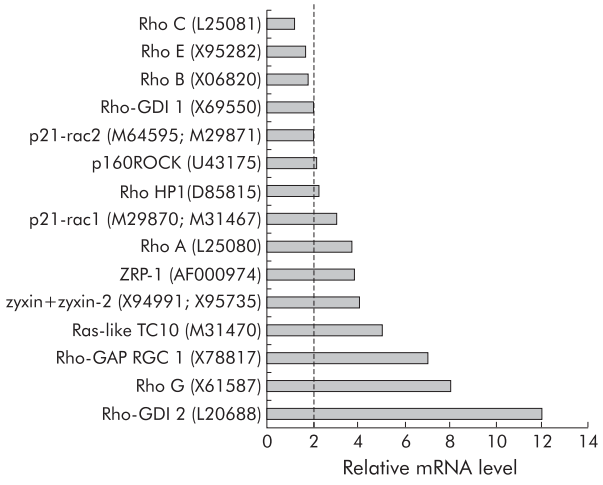

Figure 3 Gene array analysis revealed induction in the expression profile of genes coding for the Rho pathway in radiation enteritis smooth muscle cells (RE SMC) compared with normal smooth muscle cells (N SMC). Hybridisation intensities were obtained using the Atlas Image 1.5 software, converted into ratios, and adjusted for background and housekeeping gene expression. A ''normal composite array'' was established by averaging the three arrays obtained from the three N SMC lines and compared with the two RE SMC lines.

The synthetic phenotype of RE SMC was confirmed by the CTGF and type I procollagen study. Constitutive CTGF mRNA level was higher in RE SMC versus N SMC, as assessed by cDNA array analysis (×2.5) and real time RT-PCR (×7) (fig 2C). Furthermore, RE SMC secreted twofold more type I procollagen than their normal counterparts, as measured by ELISA (fig 2D).

The global cDNA array approach confirmed induction of genes coding for the Rho pathway in RE SMC (fig 3). Expression of genes coding for Rho A, B, C, and p21Rac increased, together with that of the gene coding for the p160 Rho kinase and for zyxin. A threefold increase in RhoB mRNA level in RE SMC versus N SMC was observed by real time RT-PCR analysis ($p<0.05$). Conversely, genes coding for the LIM kinase and MLCK were not detected, and HSP27 mRNA remained unchanged. Levels of endogenous Rho protein inhibitors however simultaneously increased (Rho GDI -1, -2, Rho E).

Rho kinase inhibition regulates the fibrogenic phenotype

To study the involvement of the Rho pathway in the maintenance of radiation induced fibrogenic differentiation, we used Y-27632, a pyrimidine derivative inhibitor of ROCK.

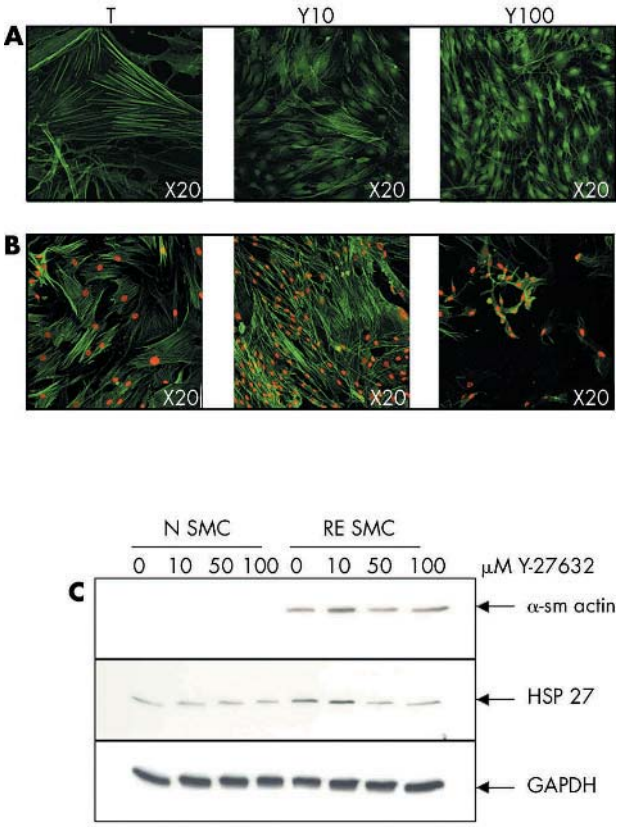

Figure 4 Alteration of actin stress fibre network by Rho kinase inhibition. F-actin was determined by FITC-phalloidin staining after Y-27632 incubation in normal smooth muscle cells (N SMC) (A) and radiation enteritis smooth muscle cells (RE SMC) (B). Rho kinase inhibition decreased heat shock protein (HSP)27 and α smooth muscle actin (α-sm actin) protein expression. (C) HSP27 and α-sm actin protein levels were assessed by western blot. Values were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels. The blot is representative of three independent experiments.

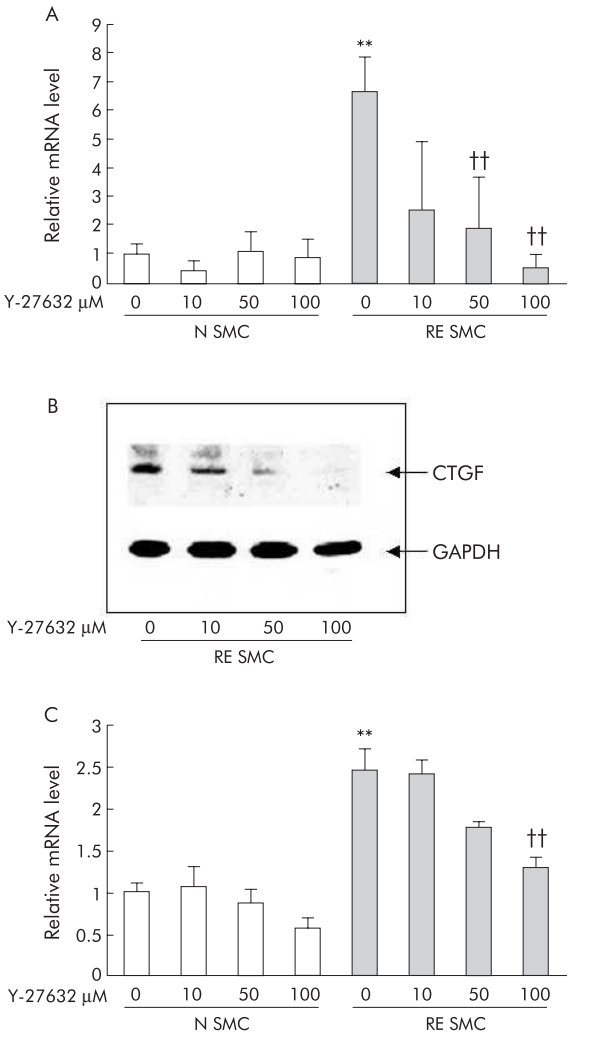

Figure 5 (A, B) Regulation of connective tissue growth factor (CTGF) expression by Rho kinase inhibition. (A) CTGF mRNA level was assessed by real time reverse transcription-polymerase chain reaction (RT-PCR). Values are mean (SEM); n = 4. **p<0.01 compared with untreated normal smooth muscle cells (N SMC); ††p<0.01 compared with untreated radiation enteritis smooth muscle cells (RE SMC). (B) CTGF protein level was assessed by western blot in RE SMC. The blot is representative of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (C) Regulation of type I collagen alpha 1 (COL1A1) expression by Rho kinase inhibition. COL1A1 mRNA levels were assessed by real time RT-PCR. Values are mean (SEM); n = 4. **p<0.01 compared with untreated N SMC; ††p<0.01 compared with untreated RE SMC.

Similar qualitative and quantitative modifications of the stress fibre network were observed after 18 and 24 hours of Y-27632 incubation, thus subsequent analyses were performed after 18 hours of incubation except for COL1A1 gene expression. With the smallest doses (10 and 50 µM Y-27632), the originally flat and confluent cells had assumed a more rounded morphology, and F-actin staining became sparse, especially in the central cell body. With the higher dose (100 µM Y-27632), cells were found to lack stress fibres and had a rounded morphology with very few cytoplasmic processes (fig 4A, B). In RE SMC, the morphological modifications induced by high doses of Y-27632 suggested apoptotic features and were associated with a dose dependent decrease in α-sm actin and HSP27 protein levels (fig 4B, C). Analysis of CTGF expression levels in RE SMC after incubation with Y-27632 showed a significant dose dependent decrease in CTGF mRNA to levels detected in untreated N SMC (fig 5A). This was further confirmed by western blot (fig 5B). In order to investigate the CTGF inhibition cascade further downstream, we studied COL1A1 gene expression and showed that COL1A1 mRNA levels decreased significantly in RE SMC after 24 hours of incubation with 100 µM Y-27632 (fig 5C). In N SMC, Y-27632 had no significant effect on α−sm actin or HSP27 protein expression or on CTGF or COL1A1 mRNA levels.

Rho kinase inhibition decrease NFκB DNA binding activity

Next we investigated the effect of ROCK inhibition on nuclear protein binding activity to NFκB consensus sequence located in the CTGF promoter. Incubation of cells with Y-27632 or sodium salicylate, an NFκB inhibitor, decreased NFκB DNA binding activity in RE SMC but not in N SMC (fig 6A). Western immunoblotting was used to determine whether inhibition of NFκB DNA binding activity occurs through stabilisation of the IκBα isotype. We found increased IκBα levels in cytoplasmic extracts of RE SMC treated with Y-27632 and sodium salicylate (fig 6B) which was not associated with increased levels of the p65 subunit in RE SMC (fig 6B).

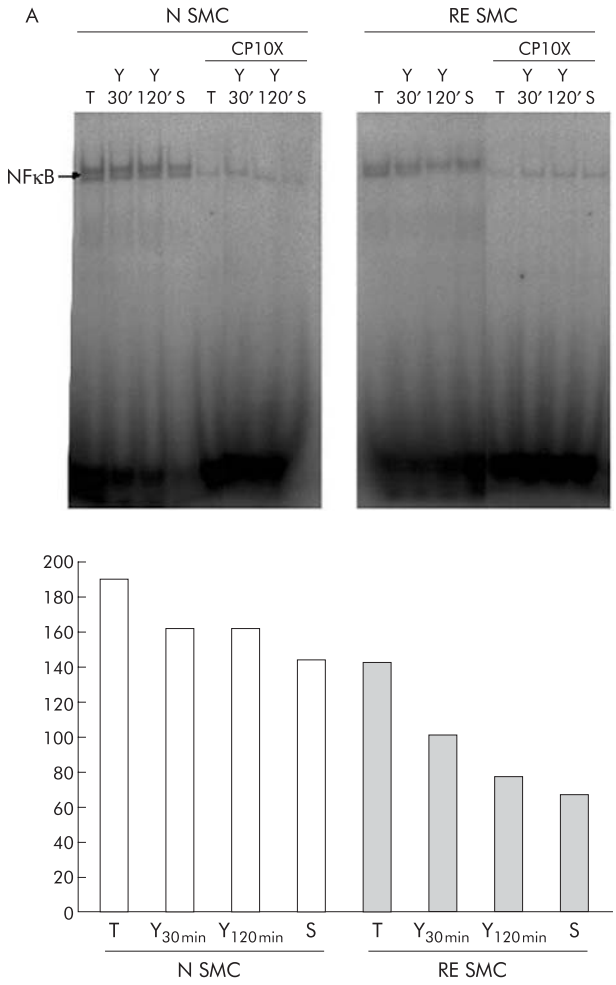

Figure 6 (A) Results of electrophoretic mobility shift assay (EMSA) protein binding to the nuclear factor κB (NFκB) consensus site after incubation with 10 μM Y-27632 (Y) for 30 minutes and 120 minutes, and with 25 mM sodium salicylate (S) for 45 minutes. Non-radioactive NFκB oligo (CPX10) blocked NFκB DNA binding. The blot is representative of two independent experiments. (B) Western blot analysis of IκBα and p65 in cytoplasmic extract of normal smooth muscle cells (N SMC) and radiation enteritis smooth muscle cells (RE SMC) incubated with Y-27632 (Y) and sodium salicylate (S). The blot is representative of two independent experiments.

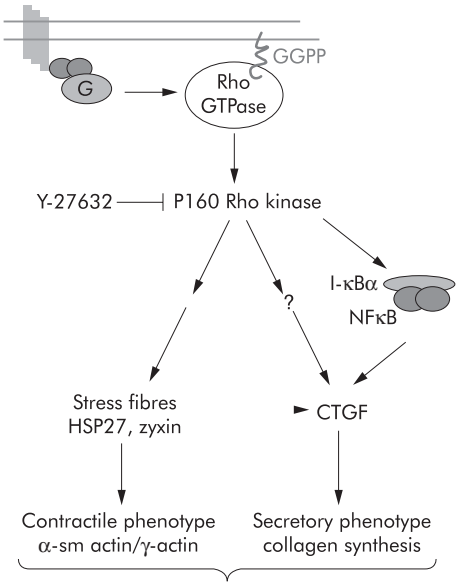

Figure 7 Chronic activation of the Rho/Rho kinase (ROCK) cascade in radiation enteritis is involved in intestinal smooth muscle cell differentiation towards an immature phenotype with altered prosecretory and contractile properties. CTGF, connective tissue growth factor; α/γ-sm actin, α/γ smooth muscle actin; HSP, heat shock protein; NFκB, nuclear factor κB.

DISCUSSION

The main finding of our study was that the small GTPase Rho/ROCK signalling pathway regulates the radiation induced fibrogenic programme. This conclusion was based on two observations: firstly, expression of the genes coding for proteins of the Rho/ROCK pathway was enhanced both in tissues and primary smooth muscle cells derived from radiation enteritis patients. Secondly, p160 ROCK blockade altered the actin network and decreased CTGF constitutive expression, most probably through inhibition of NFκB. Finally, CTGF inhibition led to decreased type I collagen synthesis. Our results suggest that p160 ROCK blockade tends to reverse fibrogenic differentiation in vitro, and provides new insight into the molecular mechanisms involved in maintenance of radiation induced fibrosis in the intestine (fig 7).

In an effort to characterise the cellular phenotype involved in maintenance of late radiation induced fibrosis, we developed a useful in vitro model of radiation fibrosis. Here we showed that primary smooth muscle cells derived either from normal or radiation enteritis samples retained their respective phenotype after isolation and prolonged culture, as previously described in other culture models.[17–20] Intestinal smooth muscle cells derived from radiation enteritis samples maintained an immature (α-sm actin expression and prominent stress fibres) and synthetic phenotype (procollagen and CTGF expression) in vitro. Furthermore, our ex vivo and in vitro studies showed concomitant enhanced expression of CTGF, Rho proteins, and p160 ROCK in smooth muscle cells isolated from radiation enteritis, suggesting that alteration of the Rho/ROCK pathway may be associated with the activation network involved in the maintenance of radiation induced fibrogenic differentiation.

In smooth muscle cells derived from radiation enteritis samples, inhibition of p160 ROCK using Y-27632[21] elicited disruption of the actin cytoskeleton and decreased expression of α-sm actin. Furthermore, we observed concomitant decreased expression of the actin chaperone HSP27, suggesting that regulation of cell morphology and stress fibre formation may be mediated by HSP27. Indeed, HSP27 has been proposed as a molecular link between the Rho signal transduction cascade and the cytoskeleton.[22 23] HSP27 is required for orientation of the cytoskeletal network composed of actin, tropomyosin, myosin, and caldesmon,[24] and acts in conjunction with zyxin to mediate actin assembly.

Regulation of the intracellular actin network in fibrosis activated smooth muscle cells may affect the mechanical tension within the tissue and modulate tissue stricture. Furthermore, regulation of the cytoskeleton organisation affects gene expression. Indeed, Goppelt-Struebe's group

recently found that changes in the microtubular and actin fibre network regulated CTGF expression in immortalised human renal fibroblasts.[25] They showed that inhibition of Rho mediated signalling using various pharmacological agents, including Y-27632, prevented upregulation of CTGF induced by microtubule disrupting agents. Our results extend these observations to cellular models that are physiologically relevant to intestinal fibrosis, as the modulation obtained after Y-27632 incubation reached significance only in cells derived from radiation enteritis. Our data further showed that inhibition of ROCK reversed the established phenotype (that is, sustained high expression of CTGF). These observations indicate that the Rho/ROCK pathway may be involved in sustained overexpression of CTGF in radiation induced fibrosis and that it may contribute to maintenance of the fibrogenic phenotype.

The molecular mechanisms involved in the Rho/ROCK dependent control of CTGF expression remain to be investigated but one attractive hypothesis concerns the transcription factor NFκB.[26] Segain and colleagues[27] recently demonstrated that blockade of ROCK with Y-27632 prevented production of proinflammatory cytokines (tumour necrosis factor α (TNF-α), interleukin 1β) through inhibition of IκB kinase and NFκB activation in Crohn's disease. As the CTGF promoter includes a NFκB consensus binding site,[28 29] we tested this hypothesis in our primary cells and found that incubation with Y-27632 inhibited NFκB DNA binding activity and induced cytosolic stabilisation of IκBα. This suggests that a regulatory cascade is activated after incubation with Y-27632: inhibition of p160 ROCK prevents activation of IκB kinase, which in turn stabilises IκBα, and inhibits NFκB nuclear translocation and CTGF transcriptional activation. This hypothesis seems consistent with the findings of Segain *et al* but does not concur with prior findings by Abraham and colleagues.[30] The latter showed that TNF-α suppresses transforming growth factor β1 (TGF-β1) induced CTGF expression and proposed that this inhibition may be directly or indirectly mediated by NFκB activation. These discrepancies could be explained by the fact that different cellular models were used (physiological model of fibrosis versus TGF-β1 stimulated cells) and different tissues were targeted. Further studies will however be necessary to fully define how NFκB acts on CTGF transcriptional activation in our model and to determine if NFκB modulation could occur specifically in cells isolated from radiation enteritis. CTGF is involved in maintenance of the fibrogenic phenotype and transactivation of genes coding for components of the extracellular membrane,[31] and as such its inhibition may be a promising novel antifibrotic strategy. In our model, the decrease in type I collagen mRNA levels observed after incubation with Y-27632 further supports this hypothesis. The precise mechanisms involved in maintenance of the fibrogenic phenotype are poorly known but alteration of the Rho pathway may be involved. In cells derived from radiation enteritis samples, we observed a concomitant increase in levels of RhoA and B and their physiological inhibitors, Rho E and Rho-GDI. Rho E inhibits Rho activity by direct binding to ROCK[32] whereas Rho-GDI acts by direct binding to the inactive form of Rho GDP.[9] Although expression of both Rho and Rho inhibitors is enhanced in radiation enteritis, the Rho/ROCK pathway seemed to be more active in cells derived from radiation enteritis samples. This suggests that endogenous control of Rho activity may contribute to maintenance of fibrogenic differentiation.

Taken together, these observations indicate that radiation induced fibrogenic differentiation of intestinal smooth muscle cells does not solely depend on local regulatory mediators but may also involve a genetic programme triggered by alteration of signal transduction pathways. Furthermore, these observations provide evidence that radiation induced fibrogenic differentiation can be modulated, thus opening new perspectives for antifibrotic therapies. Targeting the Rho/ROCK pathway may become a novel therapeutic approach to treat radiation fibrosis. Further studies will however be necessary to investigate the respective contribution of RhoA, B, C, Rac-1, and cdc42 in the fibrogenic phenotype and the effectiveness of inhibition of the Rho/ROCK signalling pathway in vivo.

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Conflict of interest: None declared.

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EDITOR'S QUIZ: GI SNAPSHOT

Robin Spiller, *Editor*

An unusual cause of upper gastrointestinal haemorrhage

Clinical presentation

A 59 year old man presented with melena. There was no history of non-steroidal anti-inflammatory drug use, peptic ulcer, or chronic liver disease. He had a history of iron deficiency anaemia for the past five years that required oral iron supplements intermittently. Previous oesophagogastroduodenoscopy and colonoscopy were negative.

Physical examination disclosed bluish vascular lesions on the upper trunk and undersurface of the tongue (fig 1A, 1B). Laboratory investigations revealed a haemoglobin level of 4.2 mg/dl and haematocrit of 15%, but normal international normalised ratio and platelet count. Oesophagogastroduodenoscopy and colonoscopy on an emergent basis were negative.

Question

What further investigation should be obtained to make a definitive diagnosis? What is the most likely diagnosis?

See page 373 for answer

This case is submitted by:

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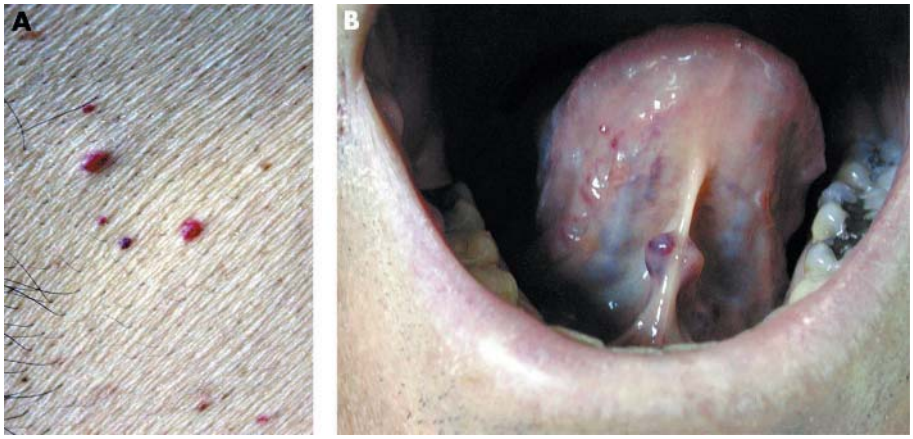

Figure 1 Physical examination of the patient revealed bluish vascular lesions on the upper trunk (A) and on the undersurface of the tongue (B).

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Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs, and TIMPs in late radiation enteritis

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Strup-Perrot, Carine, Denis Mathé, Christine Linard, Dominique Violot, Fabien Milliat, Agnès François, Jean Bourhis and Marie-Catherine Vozenin-Brotons. Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs, and TIMPs in late radiation enteritis. *Am J Physiol Gastrointest Liver Physiol* 287: G875–G885, 2004. First published June 3, 2004; 10.1152/ajpgi.00088.2004.—Radiation enteritis, a common complication of radiation therapy for abdominal and pelvic cancers, is characterized by severe transmural fibrosis associated with mesenchymal cell activation, tissue disorganization, and deposition of fibrillar collagen. To investigate the mechanisms involved in this pathological accumulation of extracellular matrix, we studied gene expression of matrix components along with that of genes involved in matrix remodeling, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs). Hybrid selection on high-density cDNA array, real-time RT-PCR, gelatin zymography and imunohistochemistry were used to characterize the mRNA expression profile, activity, and tissue location of extracellular matrix-related genes in radiation enteritis compared with healthy ileum. cDNA array analysis revealed a strong induction of genes coding for collagens I, III, IV, VI, and VIII, SPARC, and tenascin-C, extracellular-matrix degrading enzymes (MMP-1, -2, -3, -14, -18+19), and metalloproteinase inhibitors (TIMP-1, -2, plasminogen activator inhibitor-1) in radiation enteritis. This increase was correlated with the degree of infiltration of the mucosa by inflammatory cells, and the presence of differentiated mesenchymal cells in the submucosa and muscularis propria. Despite the fact that expression of collagens, MMPs, and TIMPs simultaneously increase, quantification of net collagen deposition shows an overall accumulation of collagen. Our results indicate that late radiation enteritis tissues are subjected to active process of fibrogenesis as well as fibrolysis, with a balance toward fibrogenesis. This demonstrates that established fibrotic tissue is not scarred fixed tissue but is subjected to a dynamic remodeling process.

fibrosis; radiation therapy; ileum; cDNA array; extracellular matrix

PELVIC RADIATION THERAPY IS frequently associated with normal intestinal tissue toxicity, which may result in the development of progressive fibrosis. During fibrogenesis, the compliant relationship between the mucosa and the submucosa is lost, which contributes to stricture formation, subsequent intestinal obstruction, and ultimate organ failure. The main feature of tissue fibrosis is excessive accumulation of abnormal and cross-linked collagen mainly composed of fibrillar and immature ECM components (8). The precise mechanisms underlying the dramatic deposition of connective tissue observed in tissue fibrosis remain unclear. However, failure to maintain homeostasis of the ECM and upsetting the balance between synthesis and degradation of ECM components may play an important role.

The synthesis of ECM components is regulated at the transcriptional, posttranscriptional, translational, and posttranslational levels. Collagen lysis is regulated by the balance between the activity of matrix metalloproteinases (MMPs) and that of their tissue inhibitors (TIMPs). MMPs consist of a family of at least 25 zinc-dependent proteases (3, 19, 40). The latter are classified according to their substrate specificity and structural features into gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, -10, -11), elastases (MMP-12), collagenases (MMP-1, -8, -13, -18), and membrane-type MMPs (MMP-14, -15, -16, -17). MMP activity is tightly controlled both at the transcriptional and the translational levels (19). Analysis of the control elements of the promoter region of MMP genes revealed common arrangements of the transcription factor binding sites. This specific promoter structure is thought to be required for the control of the tissue-specific expression of MMPs and to involve a functional cooperation between transcription factors of the AP-1 and Ets family (5). Most MMPs are secreted as zymogens and require proteolytic activation. In vivo activation of pro-MMPs is mostly mediated through the plasminogen-plasmin cascade and by MMPs themselves (18, 30). Another type of MMP activation, which has been reported for MMP-2, is through the membrane-type MMP-1 (MMP-14) (28). This process may be associated to fibrogenesis as MMP-2 degrades basement membrane type IV collagen (4), which is thought to facilitate the deposition of fibril-forming collagen. The third level of control of MMP activity is ensured by TIMPs, which are known to inhibit active MMPs at a stoichiometric ratio of 1:1 (38). Four subtypes of TIMPs (TIMP-1 to -4) have been identified so far (2). Whereas TIMP-1 inhibits a broad range of MMPs, TIMP-2 seems to specifically inhibit MMP-2.

The control of the ECM turnover during the wound healing process and fibrosis depends on a sharp balance between ECM synthesis and degradation, and involves cooperation among three groups of genes: ECM components, proteases, and pro-

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tease inhibitors. Recent global approaches, such as gene array analysis, allow an overall and integrated view of the regulation of ECM remodeling. This approach has been successfully applied to intestinal inflammatory and fibrotic disorders (34). In experimental models of T-cell-mediated intestinal injury, overexpression of MMPs (MMP-1, -3, -9) was found to be associated with a decrease in TIMP expression (26), whereas concomitant overexpression of MMPs (MMP-1, -2, -3, -14) and TIMP-1 was observed in inflamed mucosa of inflammatory bowel disease (IBD) samples (34). Overexpression of MMPs, however, globally exceeded that of TIMP-1, which led to a net increase in proteolytic activity in the inflamed mucosa. There are very little data on MMPs in relation to intestinal fibrosis and even less in relation to radiation-induced fibrosis, with most studies focusing on the relationship between MMPs and mucosal ulceration (11). However, conflicting theories have been proposed. The excessive accumulation of collagen may be the consequence of increased ECM synthesis associated with decreased ECM degradation. In skin radiation-induced fibrosis, Lafuma et al. (10) reported decreased activity of gelatinases associated with increased TIMP activity. Zhao et al. (42) likely reported that the increased expression of plasminogen activator inhibitor-1 (PAI-1) after exposure to ionizing radiation led to decreased ECM degradation and to collagen accumulation.

This study aimed at investigating the balance between fibrogenesis and fibrolysis during intestinal radiation-induced fibrosis. cDNA array analysis is a global approach that enabled us to simultaneously quantitate mRNA expression of ECM components, MMPs, TIMPs, and PAI-1 in bowel biopsies from patients with radiation enteritis. Changes in expression levels of MMPs and TIMPs were confirmed by real-time RT-PCR, and immunolocalization was used to characterize the cell types involved in the control of ECM remodeling.

MATERIALS AND METHODS

Tissue sampling. Twenty-two patients treated by surgery for intestinal occlusion caused by delayed radiation-induced enteritis entered the study. The patients characteristics are shown in Table 1. Histological and immunohistological studies were performed in 22 patients and tissue samples from six patients were frozen for subsequent mRNA and gelatin zymography studies. In most cases, the severity of the affliction did not allow resection of healthy intestine. Healthy ileum samples obtained from six patients without radiation enteritis, who underwent colon surgery, were used as controls. These control samples were free of malignancy and showed regular histology after hematoxylin and eosin staining. Collagen deposition was detected by Sirius Red staining. Procurement of tissue samples received prior approval from our institution's Ethics Committee and was performed according to the French Medical Research Council guidelines.

Gene array analysis. Total RNA was extracted from frozen tissue by the method of Chomczynski as already described (35), and quantified by absorption spectrometry. RNA was treated with RNase-free DNase (0.5 U/μl) to remove contaminating genomic DNA. RNA integrity was checked and PolyA RNA was purified from 20 μg of total RNA using the RNA Atlas Pur kit (Clontech, Ozyme, St. Quentin en Yveline, France). Radiolabeled cDNA was prepared according to Clontech's instructions and hybridized with the Cell Interaction and Atlas Human 1.2 arrays. A list of all the genes included in these two arrays as well as their functions can be found at www.clontech.com/atlas and is deposited into the Gene Expression Omnibus database (www.ncbi.nih.gov/geo) under GEO accession nos. GPL127 and GPL135. Phosphorimager intensifying screens were exposed to membranes and mRNA expression levels were determined by scanning the screen with a phosphorimager (Raytest; Fuji, Courbevoie, France). Analysis of differential mRNA expression was carried out by using the Atlas Image 1.5 software, and data were normalized with selected housekeeping genes (*HPRT, GAPDH, TUBA1, RPL13A, 40S ribosomal protein S9*) as already described (35). Signal intensities had to be significantly above background (i.e., 50% or more) to be considered. Only changes in the expression level greater than twofold the average control level were considered significant.

Determination of net collagen deposition in radiation enteritis. We used the method recently proposed by Sandler et al. (27) to assess net collagen deposition. Briefly, relative change in MMP activity in radiation enteritis was expressed as the ratio of the fold change in MMP mRNA to TIMP mRNA expression. The fold change in collagen mRNA in radiation enteritis was divided by this relative MMP activity: fold change in collagen/(fold change in MMP/fold change in TIMP). Values thus obtained reflected a tendency toward collagen deposition relative to steady state when >1 and toward matrix degradation when <1. Example: Type III collagen is a substrate for MMP-1, -3, and -14. MMP-1 and -3 are both inhibited by TIMP-1, and MMP-14 is inhibited by TIMP-2. The fold change of COL31A1 mRNA in radiation enteritis is 3.9; the fold changes of MMP-1, -3, and -14 mRNA in radiation enteritis are, respectively, 11.7, 19.8, and 2.3; the fold changes of TIMP-1 and -2 mRNA in radiation enteritis are, respectively, 5.4 and 2.5. Relative expressions of MMP-1/TIMP-1, MMP-3/TIMP-1, and MMP-14/TIMP-2 were, respectively, 2.1, 3.6, and 0.92. Collagen type 3 alpha 1 (COL3A1)/MMP-1:TIMP-1 = 1.8, COL3A1/MMP-3:TIMP-1 = 1.08, and COL3A1/MMP-14:TIMP-2 = 4.2.

Confirmation of differential gene expression. Two micrograms of total RNA were reverse transcribed with SuperScript II reverse tran-

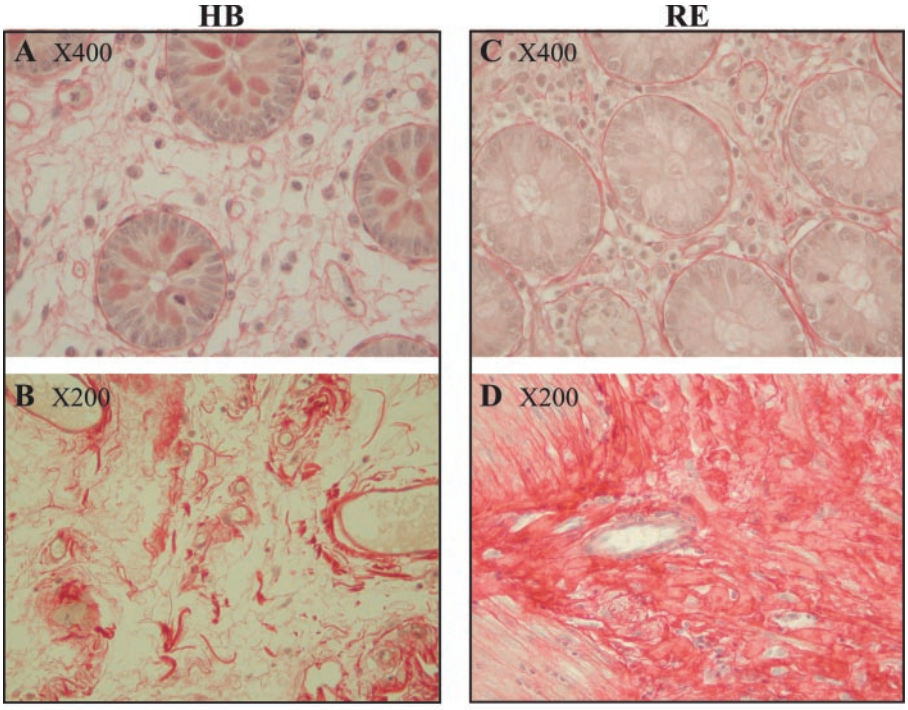
Table 1. *Characteristics of the patient population*

Time after RT, mo	Tumor Site	Age, yr	Sex	Treatment
Radiation enteritis (22 with ileal stricture)				
1–52	Rectum cancer (9)	44–69	5M/4F	RT 45 Gy/S/CT (7) RT 34–35 Gy/S/RT 15–25 Gy (2)
4–28	Gynecological cancer (12)	27–71	12F	S/RT 45 Gy/CT (4) S/RT 45 Gy/CT/BT 10–15 Gy (4) S/BT 60 Gy (2) BT 60 Gy/S/RT 45 Gy (1) RT 65 Gy (1)
75	Hodgkin's disease (1)	51	M	RT 40 Gy/CT
Healthy bowel (6)				
0	Colon adenocarcinoma	29–81	3M/3F	Nonirradiated Right hemicolectomy

RT, pelvic radiotherapy; BT, brachytherapy; CT, chemotherapy; S, surgery; Gy, gray; M, male; F, female. Number in parentheses is number of patients.

scriptase (Invitrogen, Cergy Pontoise, France) using random hexamers. Primers were generated with the Primer Express software (Applied Biosystems, Courtaboeuf, France) and were purchased from Invitrogen: collagen type I alpha 2 (*COL1A2*), 5′-CGCGGACTTTGTTGCTGCTTG-3′ (Forward); 5′-GGAAACCTTGAGGGCCTGGG-3′ (Reverse); *MMP-2*, 5′-CGCTCAGATCCGTGGTGAG-3′ (Forward); 5′-TTGTCACGTGGCGTCACAG-3′ (Reverse); *MMP-3*, 5′-CAAGCCCAGGTGTGGAGTTC-3′ (Forward); 5′-GGGTTTTGCTCCACTTCGG-3′ (Reverse); *MMP-14*, 5′-TGGACACGGAGAATTTTGTGC-3′ (Forward); 5′-ACCCCCATAAAGTTGCTGGAT-3′ (Reverse); *TIMP-1*, 5′-CACCCACAGACGGCCTTCT-3′ (Forward); 5′- CTTCTGGTGTCCGCACGAA-3′ (Reverse); *TIMP-2*, 5′-TGACTTCATCGTGCCCTGGG-3′ (Forward); 5′-CTGGACCAGTCGAAACCCTTGG-3′(Reverse). Optimized PCR used the ABI PRISM 7700 detection system in the presence of 135 nM specific forward and reverse primers for *COL1A2*, *MMP-2* and *-3*, *TIMP-2*, and 45 nM specific forward and reverse primers for *MMP-14* and *TIMP-1*. Both water and genomic DNA controls were included to ensure specificity. The purity of each PCR product was checked by analyzing the amplification plot and dissociation curves. Relative mRNA quantitation was performed by using the comparative $\Delta\Delta C_T$ method. Relative quantification in radiation enteritis $= 2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ is defined as the difference between the mean $\Delta C_{T(\text{radiation enteritis})}$ and the mean $\Delta C_{T(\text{healthy bowel})}$, and ΔC_T, the difference between the mean $C_{T(\text{COL1A2, MMPs, TIMPs})}$ and $C_{T(18S)}$-18S was used as endogenous control. Each sample was monitored for fluorescent dyes, and signals were regarded as significant if the fluorescence intensity exceeded 10-fold of the standard deviation of the baseline fluorescence, defined as threshold cycles (C_T). C_T were selected in the line in which all samples were in logarithmic phase.

Gelatin zymography. Frozen tissue samples were crushed to powder in liquid nitrogen, homogenized in a 50 mM Tris·HCl buffer (pH 7.6), containing 150 mM NaCl, 10 mM $CaCl_2$, 1% Triton X-100, and protease inhibitors (Sigma-Aldrich, St. Quentin Fallavier, France). Supernatants were collected, and protein concentration was determined by using the Lowry method. Gelatinase activity was assessed as follows: 1 mg/ml type A gelatin from porcine skin (Sigma-Aldrich) was copolymerized in 8% SDS-polyacrylamide gel, and was used as substrate. The samples were diluted 1:1 in sample buffer consisting of 62.5 mM Tris·HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.05% bromophenol blue. Four micrograms of each protein sample were separated by electrophoresis at a constant voltage of 100 V for 1–2 h at 4°C. The gel was then washed twice in 2.5% Triton X-100 and incubated overnight at 37°C in a buffer containing 50 mM Tris·HCl (pH 7.8), 5 mM $CaCl_2 \cdot 2H_2O$, 50 mM NaCl, 0.01% Brij 35, and 0.02% NaN_3. Gels were stained with 0.5% Coomassie blue in 25% isopropanol and 10% acetic acid for 60 min, and destained in a mixture containing 10% methanol and 10% acetic acid until the stacking gel was destained. Bands of gelatin lysis appear as clear zones countering a blue background. Densitometric analyses were performed by using an image analyzer (Biocom, Les Ulis, France) interfaced with the Phoretix image analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).



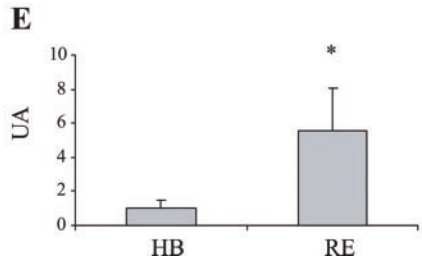


Fig. 1. Collagen staining by Sirius red in the mucosa (*A*) and the submucosa (*B*) of a healthy bowel (HB) sample and in the mucosa (*C*) and the submucosa (*D*) of a radiation enteritis sample. Gene expression of collagen type I alpha 2 chain (COL1A2), determined by real-time RT-PCR, was measured in radiation enteritis (RE) samples ($n = 6$) and compared with the expression in HB samples ($n = 6$). Values were normalized to 18S RNA and are means ± SE (*$P < 0.05$). UA, arbitrary unit.

Immunostaining. Four-micrometer-thick acid formaldehyde alcohol or Bouin-fixed paraffin-embedded sections were used to immunolocalize MMP-2 (1:150, 42–5D11), MMP-3 (1:75, SL-1 IIIC4), MMP-9 (1:3,000, 56–2A4), MMP-14 (1:5,000, 113–5B7), TIMP-1 (1:100, 102D1), and TIMP-2 (1:1,500, 67–4H11). Antibodies were purchased from Chemicon (Euromedex, Mundolscheim, France). These antibodies were described to recognize pro- and active forms of MMP without crossreacting with other MMPs and TIMPs. After dewaxing and rehydration, endogenous peroxidase activity was eliminated with 3% hydrogen peroxide in PBS. MMP-3 and TIMP-1 epitopes were unmasked in 10 mM citrate buffer (pH 6.0). To inhibit nonspecific staining, slides were incubated 10 min at room temperature with serum-free DAKO (Trappes, France) Protein Block and incubated overnight at 4°C with the primary antibody, diluted in DAKO Antibody Diluent. Slides were then rinsed in Tris·HCl/NaCl/Tween 20 (50 mM, 0.3 M, 0.1%, respectively). The primary antibody was detected by using the EnVision$^+$ anti-mouse horseradish peroxidase (DAKO) revealed by Vector NovaRED substrate kit (BioValley) and counterstained with IMeyer's hemalun. Known positive cases and negative controls (omission of the primary antibody and irrelevant mouse IgG1 incubation) were included in each run and were shown to be positive and negative, respectively. A semiquantitative analysis of MMP-2, -3, -9, and -14, and TIMP-1 and 2 was performed by using the following scoring system. Intensities of staining in epithelium, lamina propria, submucosa, vessels, muscularis propria, and serosa were assigned a score, where − represents no staining; +, weak staining; ++, mild staining; and +++, strong staining. Total score was the mean value obtained for each bowel layer.

Statistical analysis. For real-time RT-PCR and zymography, statistical differences between means of control group and radiation enteritis group were evaluated by using Student's *t*-test.

RESULTS

Histopathological study. Examination of hematoxylin and eosin-stained and Sirius Red-stained sections (Fig. 1, *A–D*) revealed common histological features in all radiation enteritis samples. Severe fibrosis affected the whole intestinal wall; transmural collagen accumulation was observed in the mucosa, submucosa, and muscularis propria. Real-time RT-PCR analysis showed that COL1A2 mRNA level increased in radiation enteritis (Fig. 1*E*).

Gene array analysis. A "normal" composite membrane, which included mRNAs expressed in all six control samples was generated and compared with membranes established for each radiation enteritis sample. Genes were selected when their expression level was altered by more than twofold compared with controls.

Levels of mRNA coding for the fibrillar collagen type I alpha 2 (COL1A2) and COL3A1 were increased by 3.8- and 5-fold in radiation enteritis (Fig. 2*A*; notice that collagen type I alpha 1 was not spotted on the array). Furthermore, the collagen I-to-collagen III ratio increased from 1.3 in control samples to 1.8 in radiation enteritis. The level of mRNA coding

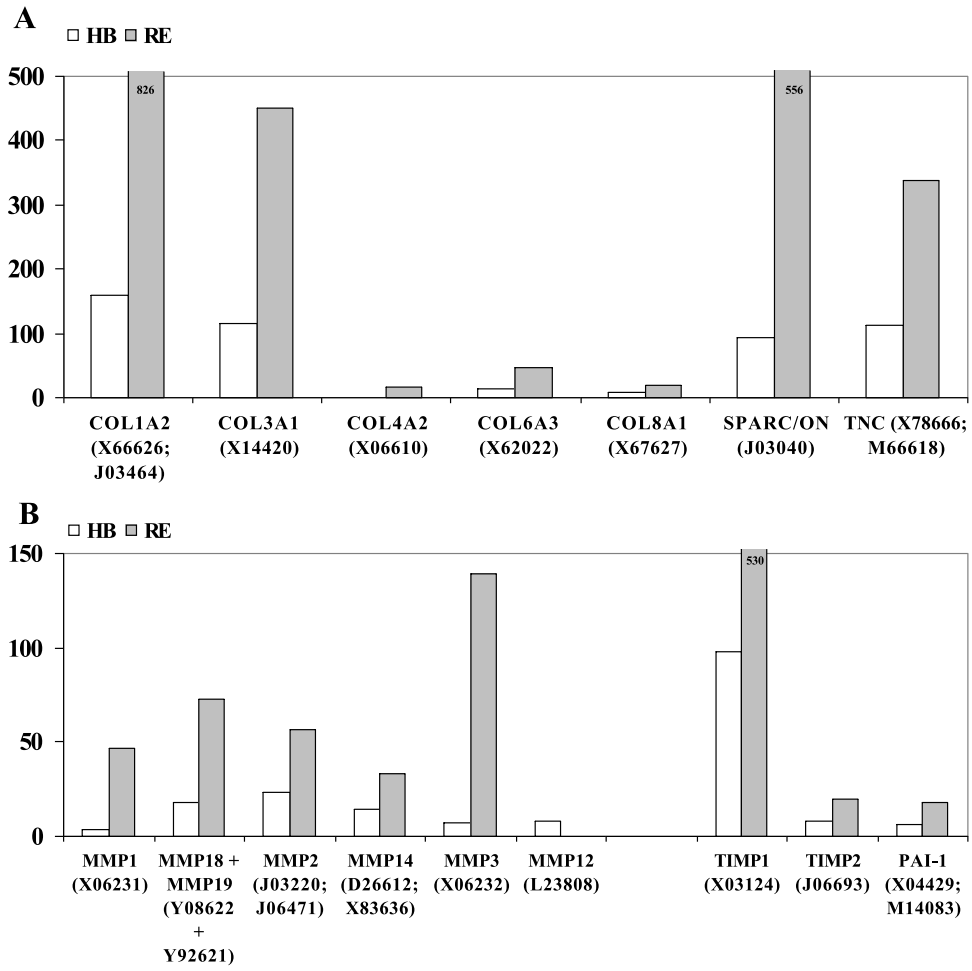


Fig. 2. Gene array analysis of extracellular components (*A*), matrix metalloproteinases (MMPs), tissue inhibitors of MMP (TIMPs), and plasminogen activator inhibitor-1 (PAI-1) genes (*B*) in HB samples ($n = 6$) and RE samples ($n = 6$). Hybridization intensities were obtained by using the Atlas Image 1.5 software, converted into ratios, and adjusted for background and housekeeping genes expression (Gene X intensity background)/(average intensity for all 6 housekeeping gene backgrounds). *Y*-axis includes name and accession no. of genes.

for the microfibrillar type VI alpha 3 collagen was increased by threefold, whereas mRNA coding for the alpha 1 and alpha 2 chains were, respectively, increased by 1.8 and 1.7 and were not included in the Fig. 2*A*, because they did not reach the cut-off value. The level of mRNA coding for the stromal component tenascin-C was also increased by threefold in radiation enteritis (Fig. 2*A*). These observations are consistent with the development of tensile ECM, which characterizes late radiation-induced fibrosis. Compositions of vascular and basement membrane ECM were also found to be altered in radiation enteritis. We observed two- and sixfold increases in type VIII alpha 1 collagen and Sparc mRNA levels, respectively. Collagen type IV alpha 2 (COL4A2) hybridization signal was below the background signal in healthy bowel sample, but was detectable in radiation enteritis samples (notice that COL4A3 and COL4A6 were also slightly increased but did not reach the cut-off value). Composition of basement membrane cDNA array analysis further showed that the expression level of interstitial collagenases (MMP-1 and MMP-18+19), gelatinase (MMP-2), membrane-type MMP (MMP-14), and stromelysin (MMP-3) increased by 2- to 19-fold in radiation enteritis (Fig. 2*B*), whereas hybridization signal of the macrophage-specific metalloelastase (MMP-12) was below the background signal in radiation enteritis but was detectable in healthy bowel samples. Both MMP-9 and -7 were not found to be differentially expressed. Levels of MMP inhibitors, TIMP-1, TIMP-2, and PAI-1 increased by five-, two-, and threefold in radiation enteritis, respectively (Fig. 2*B*).

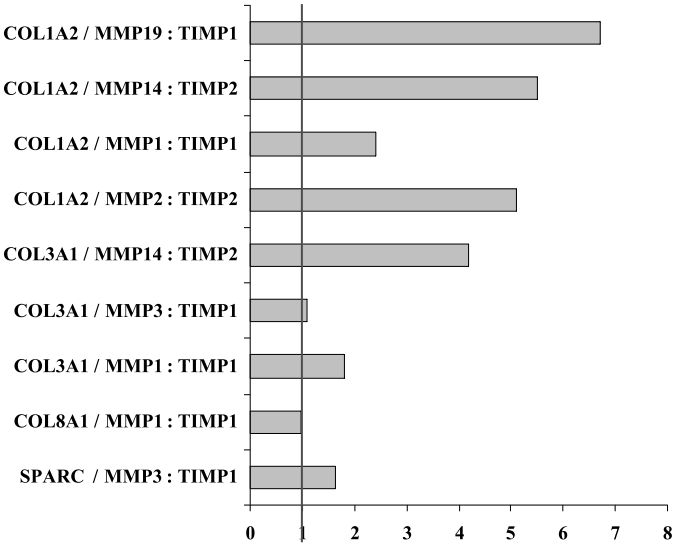


Fig. 3. Collagen-to-MMP-to-TIMP ratio in RE. Relative expression of MMP was calculated by determining the fold change of the MMP mRNA expression relative to its relevant inhibitor TIMP (i.e., fold change of MMP-1, -3, and -19 mRNA expression was divided by fold change TIMP-1 mRNA expression). Fold change of MMP-2 and -14 mRNA expression was divided by fold change TIMP-2 mRNA expression. The fold change for collagen mRNA was then divided by the fold change value for the relevant MMP/TIMP to yield the fold change in the ratio of collagen deposition to collagen degradation compared with the steady state.

Because the balance among collagen synthesis, collagen degradation by MMPs, and inhibition of MMPs by TIMPs regulates collagen deposition, we assessed net collagen deposition in radiation enteritis samples using the method developed by Sandler et al. (27). It is assumed that no net collagen deposition or degradation occurs in control samples. Thus the collagen/MMP:TIMP value was set to 1 for controls and used as a reference. Each collagen mRNA expression value was divided by the fold change value for the relevant MMP and TIMP (Fig. 3) and values above 1 are in favor of net collagen deposition. Results thus obtained suggest that net collagen deposition occurs in radiation enteritis despite the increase in MMP mRNA. Both the Sandler method (27)and Sirius Red spectrophotometric collagen assay (36) revealed a similar trend to net collagen deposition.

Gelatinase expression. Real-time RT-PCR analysis confirmed that MMP-2 and -14 (Fig. 4*I*) mRNA level increased in radiation enteritis. As regards MMPs, protein levels are thought to be well correlated with mRNA expression. Thus to investigate whether mRNA levels correlated with MMP activity, gelatin zymography was performed on whole tissue extracts. We found a strong MMP-2 activity (Fig. 4*II*) in radiation enteritis samples. Furthermore, despite the fact that MMP-9 mRNA induction did not reach the twofold cut-off value in the cDNA array analysis, we observed an increase in MMP-9 activity in zymography experiments although statistically nonsignificant due to interindividual variability (Fig. 4*II*), whereas real-time RT-PCR showed a fivefold increase of MMP-9 mRNA level (Fig. 4*I*).

Cellular localization of MMP-2, -9, and -14 was assessed by immunostaining. Representative immunohistochemical staining patterns in control and radiation enteritis samples are shown in Fig. 5. A strong increase in MMP-2 staining was found in each layer of the bowel in radiation enteritis (Fig. 5, *C–F*). In the mucosa, MMP-2 was detected at the apical end of epithelial cells, in α-sm actin positive subepithelial myofibroblasts, and inflammatory cells. Activated fibroblasts and leucocytes infiltrating the submucosa, as well as smooth muscle cells of the muscularis propria were also stained. Because MMP-14 is involved in the proteolytic activation of pro-MMP-2, and MMP-14 mRNA levels were found increased in radiation enteritis, we sought to determine the cell type involved in MMP-14 expression by immunolocalization (Fig. 5, *J–O*). A gradient of expression was observed along the crypt-villus axis: epithelial cells of the crypt were negative, whereas differentiated epithelial cells were strongly positive. MMP-14 was detected in the same cell types as those found positive for MMP-2 (i.e., subepithelial myofibroblasts, leucocytes, submucosal fibrosis myofibroblasts). Endothelial cells, however, appeared to be MMP-2 and -14 negative. An increased MMP-9 mRNA level was found in radiation enteritis, as shown by real-time RT-PCR experiments, and immunostaining provided evidence that MMP-9 protein was mainly expressed in leucocytes. MMP-9 staining was thus directly related to the degree of infiltration by inflammatory cells, which increased in radiation enteritis (Fig. 5, *H–I*).

Stromelysin expression. In our experimental conditions (10 ng of cDNA), MMP-3 mRNA was only detected in radiation enteritis samples, thus confirming the induction of MMP-3 mRNA observed by cDNA array analysis. MMP-3 immunostaining showed very few positive cells (mostly leucocytes) in the mucosa of control samples, whereas strong positive staining was observed in the mucosa and the submucosa of radiation enteritis samples. Epithelial cells and mucosal macrophages were hugely labeled, as well as submucosal inflammatory cells, fibrosis myofibroblasts, and endothelial cells (Fig. 6).

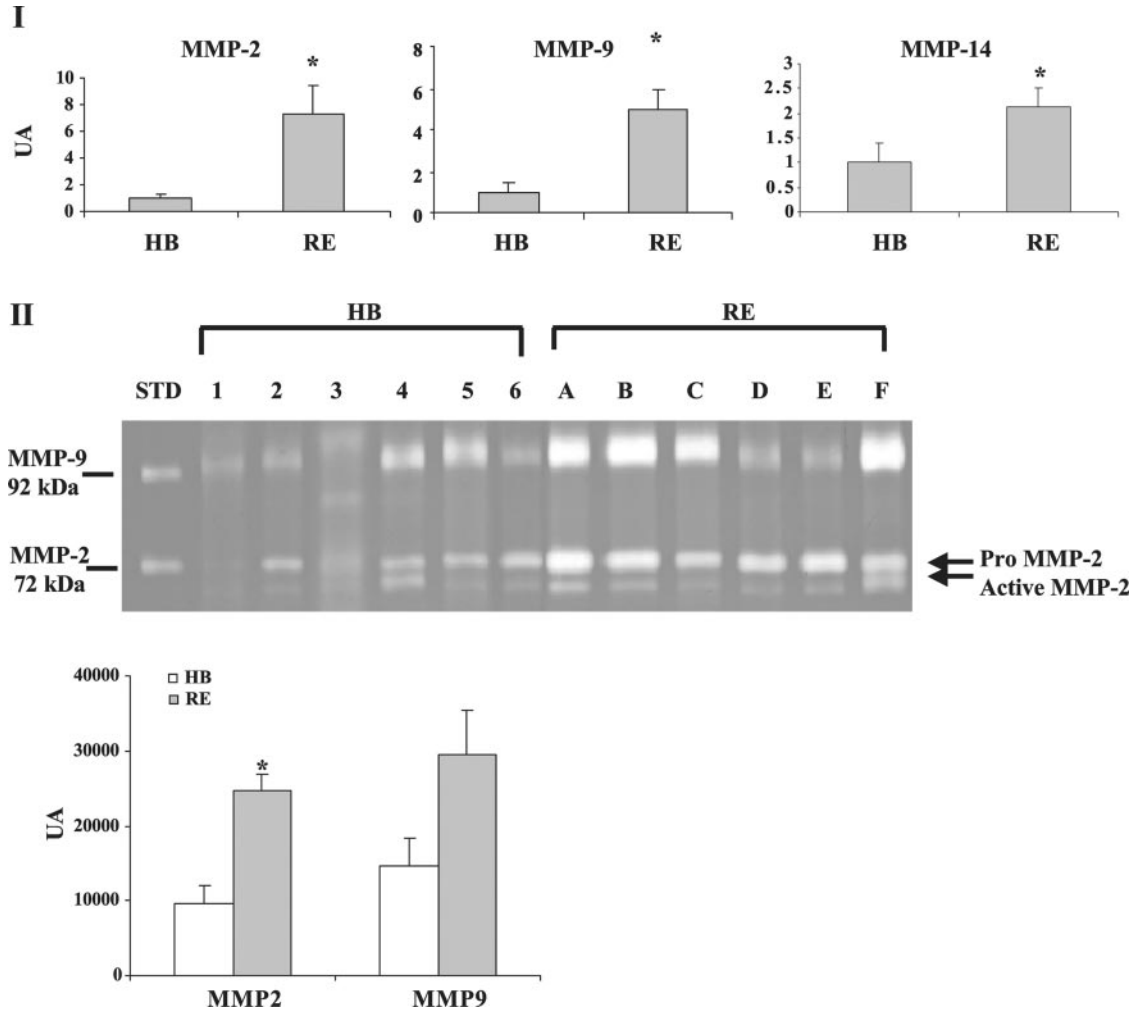


Fig. 4. *I*: gelatinases and MMP-14 mRNA levels in RE vs. HB. Gene expression of MMP-2, -9, and -14 determined by real-time RT-PCR was measured in RE samples ($n = 6$) and compared with the expression in HB samples ($n = 6$). Values were normalized to 18S RNA and are means ± SE (*$P < 0.05$). *II*: study of gelatinase activities in RE vs. HB by zymography. *Lane STD* shows MMP-2 and -9 standards. Gelatin zymography showed basal MMP-2 and -9 activities in HB specimens ($n = 6$; *lanes 1–6*) and a statistically significant increase of MMP-2 activity (*$P < 0.05$) in RE samples (n = 6; *lanes A–F*), whereas increased MMP-9 activity in RE samples was not found to be statistically significant due to heterogeneity between samples ($n = 6$; *lanes A–F*).

TIMP expression. Real-time RT-PCR analysis confirmed that TIMP-1 and -2 (Fig. 7, *I* and *II*) mRNA level increased in radiation enteritis. Detection of TIMP-1 was restricted to the mucosa in control and radiation enteritis samples. In control samples, however, very few TIMP-1 positive cells were found, whereas the mucosa in radiation enteritis samples was heavily stained (Fig. 7*III*). Control samples showed sparse staining for TIMP-2, whereas radiation enteritis samples were highly positive for TIMP-2. Transparietal inflammatory cells and fibrosis myofibroblasts of the submucosa and muscularis propria were strongly immunoreactive for TIMP-2 (Fig. 7*IV*). All immunohistochemical data are summarized in Table 2.

DISCUSSION

To gain further insight into the biological function of various MMPs, their inhibitors, and their involvement in the excessive ECM deposition occurring in chronic radiation fibrosis after radiotherapy in humans, their expression patterns were studied by gene array analysis and immunohistochemistry. Classically, radiation fibrosis has been considered a chronic and progressive process in which normal tissue is replaced by fixed and irreversible fibrotic tissue. This view has however, been challenged, because fibrosis has recently been defined as a dynamic process resembling chronic wound healing. In this study, a marked upregulation of collagen and enzymes involved in ECM remodeling was observed in late radiation enteritis, which concurs with this new definition of radiation-induced fibrosis and is reflective of a continuous repair process.

Collagen is the predominant protein of the intestine's connective tissue. It is secreted by intestinal mesenchymal cells (subepithelial myofibroblasts, smooth muscle cells) located in the lamina propria and in the muscles (14). Fibrillar collagens (type I and III) are found in the lamina propria, the submucosa, and muscles, whereas type IV collagen is found in the basement membrane and the lamina propria (8). Colocalization of collagen deposition and myofibroblasts was demonstrated in one of our previous studies (36). In the present study, we observed a marked increase in type I, III, and IV collagen RNA

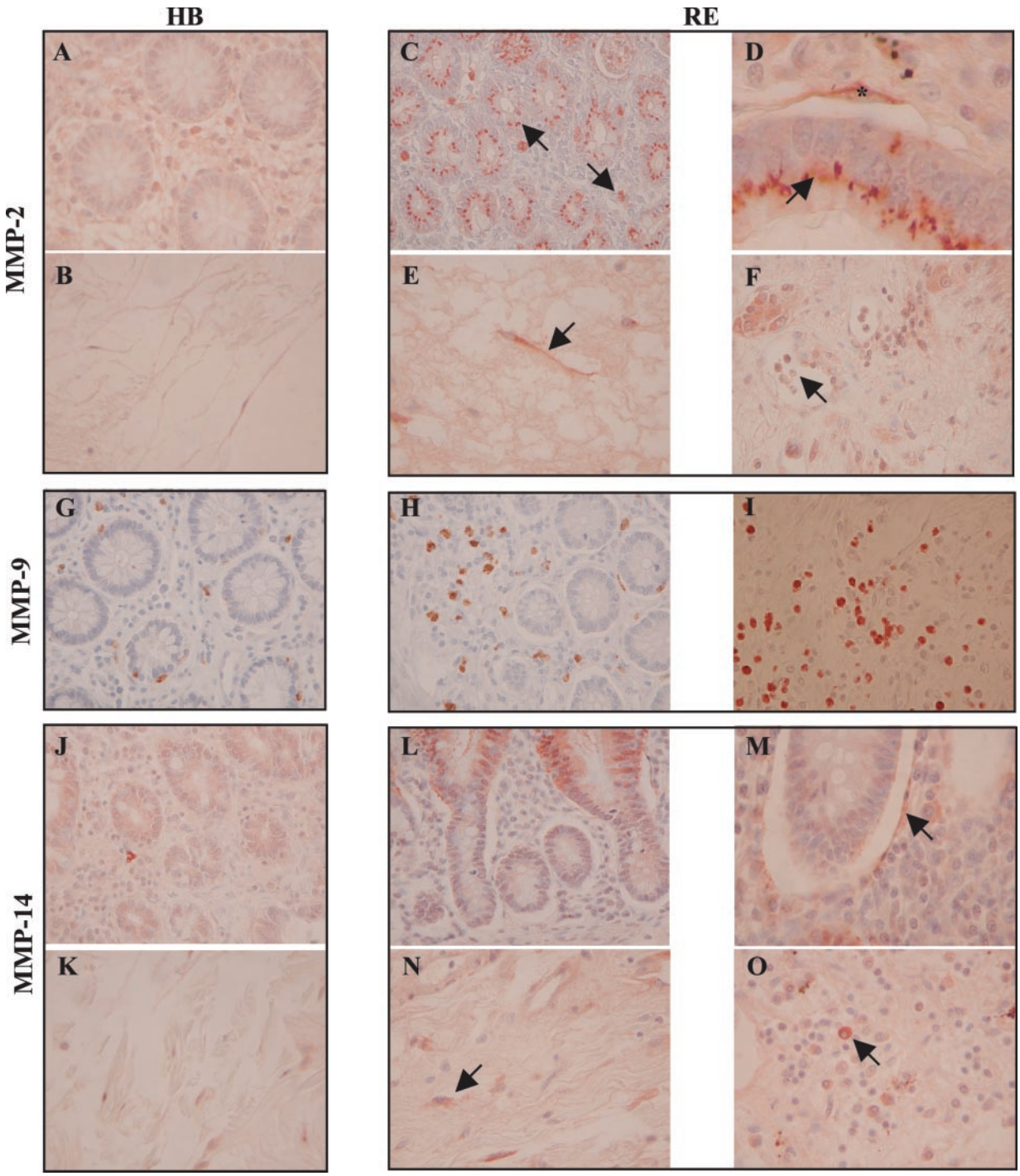


Fig. 5. Gelatinase A (MMP-2), gelatinase B (MMP-9), and membrane-type 1 matrix metalloproteinase (MMP-14) immunostaining in HB and RE specimens. Low MMP-2 immunostaining was seen in the mucosa (*A*; magnification, ×400) and the submucosa (*B*; magnification, ×400) of HB samples. In RE samples, MMP-2 immunostaining increased in mucosal epithelial and inflammatory cells (arrow) (*C*; magnification, ×100). High magnification bright-field image (*D*; magnification, ×400) showed MMP-2 positive staining at the apex of epithelial cells (arrow) and in subepithelial myofibroblasts (*). In the submucosa, increased MMP-2 immunostaining was found in fibrosis myofibroblasts (arrow) (*E*; magnification, ×400) and in infiltrated leucocytes (arrow) (*F*; magnification, ×400). Low MMP-9 immunostaining was seen in the mucosa (*G*; magnification, ×400) and the submucosa (data not shown) of HB samples. In RE samples, MMP-9 immunostaining increased in mucosal (*H*; magnification, ×400) and submucosal inflammatory cells (*I*; magnification, ×400). Low MMP-14 immunostaining was seen in the mucosal inflammatory cells (*J*; magnification, ×400) and the fibroblasts of the submucosa (*K*; magnification, ×400) of HB samples. In RE samples, MMP-14 gradient of expression was observed along the crypt-villus axis in the epithelium (*L*; magnification, ×400) and subepithelial myofibroblasts (arrow) were MMP-14 positive (*M*; magnification, ×400). Submucosal activated fibroblasts (arrow) (*N*; magnification, ×400) and infiltrated leucocytes (arrow) (*O*; magnification, ×400) stained positively for MMP-14.

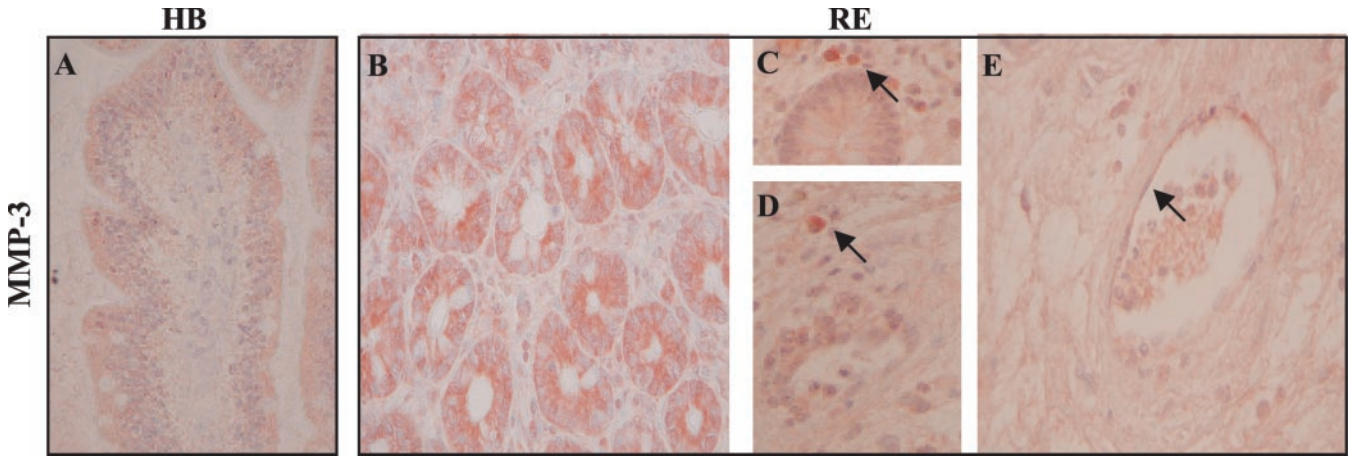


Fig. 6. Stromelysin-1 (MMP-3) immunostaining in HB and RE specimens. Low MMP-3 immunostaining was seen in the mucosa (*A*; magnification, ×400), and no MMP-3 staining was seen in the submucosa of HB samples. In RE samples, MMP-3 immunostaining increased in epithelial cells (*B*; magnification, ×400). High-magnification brightfield image showed MMP-3 positive staining in mucosal (*C*; magnification, ×400) and submucosal (*D*; magnification ×400) leucocytes. Endothelial cells (arrow) stained positively for MMP-3 (*E*; magnification, ×400).

transcripts in strictured ileum resected from patients with radiation enteritis. Moreover, we observed increase in the noncollagenous stromal component tenascin-C, which is produced in immature and newly formed granulation tissue and promotes migration, proliferation, and activation of matrix-producing cells, such as fibroblasts and smooth muscle cells (reviewed in Ref. 12). Induction of type I collagen, normalized for changes in the expression levels of MMPs and TIMPs, is greater than that of type III collagen. This observation is consistent with previous findings showing accumulation of type I collagen in late phases of radiation fibrosis (25). This abnormal deposition of type I/III collagen that consists of large and tensile collagen fibrils, leads to intestinal stenoses and ensuing obstructive symptomatology. Furthermore, abnormal ECM deposition may affect the ability of cells to express and maintain their differentiated phenotype. Besides having a

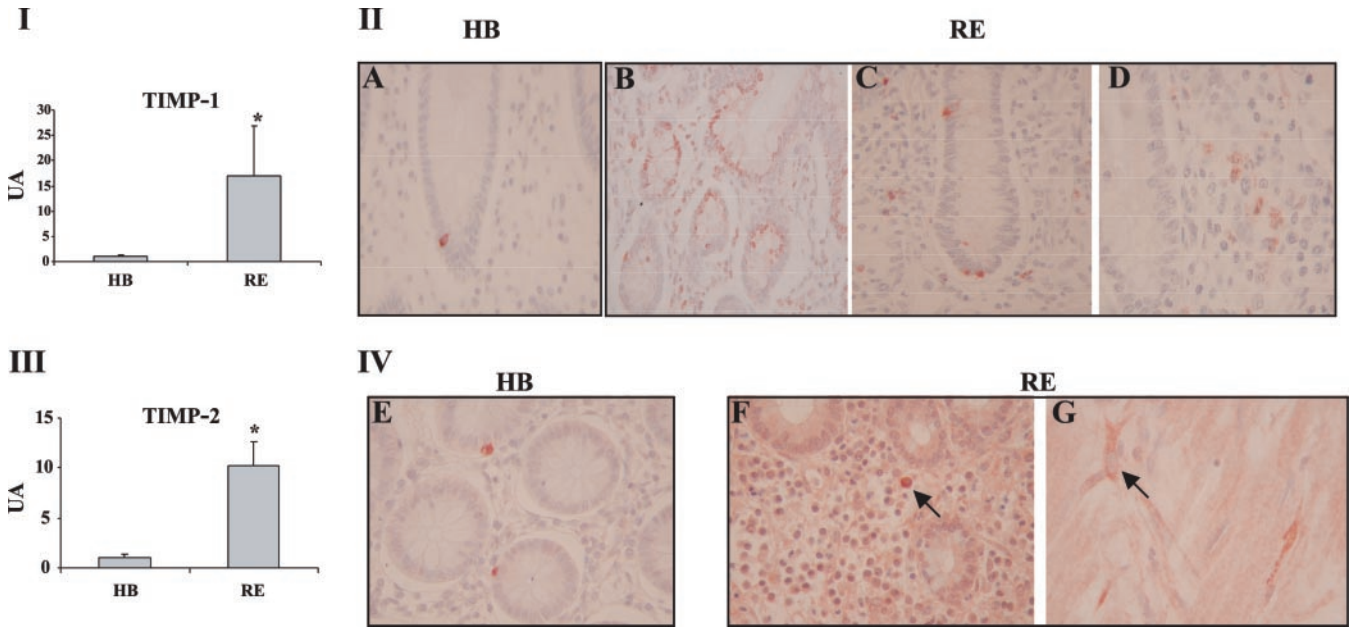


Fig. 7. Tissue inhibitors of metalloproteinase TIMP-1 and -2 mRNA expression and immunolocalization in HB and RE specimens. *I* and *III*: gene expression of TIMP-1 and TIMP-2 determined by real-time RT-PCR, was measured in RE samples ($n = 6$) and compared with the expression in HB samples ($n = 6$). Values were normalized to 18S RNA and are means ± SE (*$P < 0.05$). *II*: immunolocalization of TIMP-1 in RE vs. HB. Both in normal and RE samples, TIMP-1 immunostaining was restricted to the mucosa. Low TIMP-1 immunostaining was seen in the epithelium (*A*; magnification, ×400) of HB samples. In RE samples, TIMP-1 immunostaining increased in epithelial (*B* and *C*; magnification, ×400) and inflammatory cells (*D*; magnification, ×400). *IV*: immunolocalization of TIMP-2 in RE vs. HB. Low TIMP-2 immunostaining was seen in the mucosa (*E*; magnification, ×400) and submucosa of HB samples. In RE samples, TIMP-2 immunostaining increased in leucocytes (arrow) of the mucosa (*F*; magnification, ×400) and of the submucosa. In the submucosa, increased TIMP-2 immunostaining was found in activated fibroblasts (arrow) (*G*; magnification, ×400).

Table 2. *Semiquantitative analysis of MMP-2, -3, -9, -14 and TIMP-1 and -2 in radiation enteritis versus healthy bowel*

Patients	No.	MMP-2	MMP-3	MMP-9	MMP-14	TIMP-1	TIMP-2
Control	6	+/++	−/+	++/+++	+/++	+	+
Radiation enteritis	22	+++	+++	+++	++	+++	++

−, No staining; +, weak staining; ++, mild staining; and +++, strong staining.

structural role, molecules of the ECM are now known to have functional roles, such as storage of growth factors and transmission of differentiation signals to cells (1, 31). Furthermore, MMP activation may lead to the release of growth factors and cytokines from the ECM. For instance, release and activation of TGF-β1 may affect the fibrogenic process (15, 39). Consequently, the nature of the cell microenvironment should not be solely seen as a consequence of tissue fibrosis, but also as a mean to ensure cellular activation responsible for the maintenance of the fibrotic process.

Collagen accumulation in radiation fibrosis has been thought to be associated with a decrease in MMP activity and increased TIMP levels. Our results show an induction of each member of the MMP family, i.e., gelatinases, stromelysin, collagenases, and membrane-type MMPs, in late radiation enteritis. The concomitant induction of MMP inhibitors (TIMP-1, TIMP-2, and PAI-1) counterbalances this induction of MMPs, leading to a net collagen deposition. Because MMPs act locally, studies on whole-tissue lysates provide only partial information about ECM remodeling in late radiation enteritis. We therefore performed immunohistochemical studies on 22 radiation enteritis samples to investigate regulation of ECM metabolism in each bowel layer.

Intense ECM remodeling seems to particularly affect intestinal mucosa in radiation enteritis. We report a coordinated expression of MMP-2, -3, -9, and -14, proportional to the extent of inflammatory cell infiltration. Molecular characterization of inflammatory cell subtypes involved in MMP overexpression in radiation enteritis has not been done so far. However, it seems reasonable to assume that collagen synthesis in mesenchymal cells is stimulated by secretory products of inflammatory cells (14) and that MMP/TIMP secretion may be partly achieved by leucocytes themselves (13, 29). IL-1β can be cited among the inflammatory mediators that control MMP synthesis (21). In a previous study (35), IL-1β mRNA was found to be markedly increased in radiation enteritis samples, suggesting that it may be involved in ECM remodeling in the late phase of radiation fibrosis. Monitoring the T-helper 2 response in radiation enteritis may also be relevant, because T-helper 2 response has been shown to be involved in pneumonitis and subsequent pulmonary fibrosis after thoracic radiotherapy (37) and is known to control ECM remodeling in pathogen-induced pulmonary inflammation (27). Upregulation and colocalization of MMP-2 and -14 in activated mesenchymal cells has already been described in various wound healing models in skin (20), liver (32), and gut (34). Furthermore, in accordance with our findings, increased MMP-2 expression in human subepithelial myofibroblasts within irradiated rectum (9) and derived from IBD biopsies (16) has been demonstrated. Contrary to Hovdenak's findings (9), we report strong MMP-2 and -14 staining in epithelial cells with a crypt-villus gradient of expression for MMP-14. The difference between our findings and Hovdenak's may account for the difference in the anatomical location of the biopsy (ileum vs. colon) and the timing at which resection was performed (several months vs. 2 wk after radiation therapy). Furthermore, colocalization of MMP-3 and -14 has already been described (22) in activated fibroblast-like cells from IBD biopsies, whereas in radiation enteritis samples, MMP-3 and -14 were found to be expressed in intestinal epithelial cells and inflammatory cells in the lamina propria. This increased expression of MMP-2, -3, and -14 provides new insight into the differentiation of intestinal epithelial cells in radiation enteritis. Increased MMP-2 and 3 expression has already been described in migrating keratinocytes during cutaneous wound healing and in monostratified epithelia, increased MMP-2 expression has been reported in lung (41) and mouse colon (23). Furthermore, MMP-2 and -14 are known to process laminin and to regulate migration of lung carcinoma-derived epithelial cells (A549) (24). The present observations suggest that remodeling of the basement membrane occurs in late radiation enteritis and could lead to epithelial anoïkis and chronic ulceration. In addition, production of MMPs by epithelial cells may directly affect differentiation and proliferation of the subepithelial myofibroblasts into fibrosis-activated cells. The significance of MMP-14 gradient of expression along the crypt-villus axis is unknown, but increased MMP-2 activity could be required for accelerated migration of enterocytes toward the top of the villi and to desquamation of differentiated enterocytes in radiation enteritis. In conclusion, we propose that MMP-2, -3, and -14 could be useful markers of epithelium activation and could mediate fibrogenic activation signals from epithelial cells to subepithelial myofibroblasts, as recently described by Xu et al. (41) in pulmonary fibrosis.

Although the most dramatic intestinal collagen deposition occurs in the submucosa and muscles, submucosal and muscular ECM remodeling during intestinal fibrosis has been poorly investigated. In accordance with findings from Matthes and Graham on Crohn's disease (14), in vitro studies performed in our laboratory (17) demonstrated that activated smooth muscle cells derived from the muscularis propria of the bowel of patients with radiation enteritis secretes twofold more type I collagen than their normal counterparts. The present study brings some new insight concerning ECM remodeling in deep intestinal layers, because it shows an increased MMP-2, -3, and -14 staining in fibrosis myofibroblasts within the submucosa and the muscles. ECM turnover depends on the MMPs-to-TIMPs ratio. In this study, mRNA analysis showed that TIMP mRNA levels were higher than the MMPs mRNA level in radiation enteritis, which resulted in inhibition of degradation. Immunohistochemistry brought additional clues to the understanding of the ECM remodeling balance in radiation enteritis, because it enabled us to observe an increased TIMP-1 and -2 staining in the mucosa, whereas only TIMP-2 staining was observed in the deeper layers. Classically, the balance between MMPs and TIMPs has been thought to reg-

ulate proteolytic activities; however, in addition to its inhibitory activity, TIMP-2 can associate with pro-MMP-2 and activate its proteolytic activity (7). Furthermore, TIMPs can play additional functional roles, such as that of anti-apoptotic factors that may be particularly relevant in a fibrotic context. TIMP-2 protects melanoma cells from apoptosis (33), and recently, TIMP-1 has been shown to inhibit apoptosis in activated hepatic stellate cells in established liver fibrosis (18). In this particular model, the anti-apoptotic effect of TIMP-1 was dependent on the prevention of matrix degradation through inhibition of MMPs. In normal conditions, resorption of granulation tissue occurs through apoptosis of myofibroblasts (6), whereas in fibrosis, persistence of myofibroblasts leads to organ failure. The observed TIMP-1 staining in subepithelial myofibroblasts and TIMP-2 staining in submucosal fibrosis myofibroblasts suggest that TIMP-1 and -2 may mediate persistence of myofibroblastic differentiation in late radiation enteritis.

In conclusion, despite the difficulty in assessing the ECM remodeling process in vivo, these observations nonetheless provide the first evidence of active ECM remodeling in late radiation enteritis. These findings further reinforce the concept that fibrotic tissue is dynamic and undergoes constant renewal, thus opening interesting perspectives for the development of antifibrotic interventional therapies.

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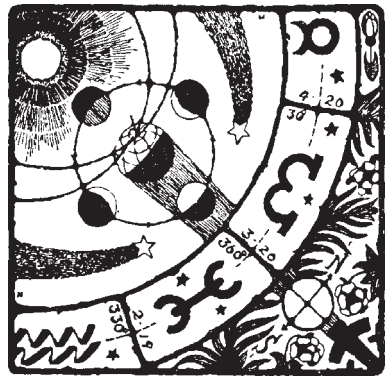

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Gene Expression Profile in Human Late Radiation Enteritis Obtained by High-Density cDNA Array Hybridization

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Late radiation enteritis is a sequela of radiation therapy to the abdomen. The pathogenic process is poorly understood at the molecular level. cDNA array analysis was used to provide new insights into the pathogenesis of this disorder. Gene profiles of six samples of fibrotic bowel tissue from patients with radiation enteritis and six healthy bowel tissue samples from patients without radiation enteritis were compared using membrane-based arrays containing 1314 cDNAs. Results were confirmed with real-time RT-PCR and Western blot analysis. Array analysis identified many differentially expressed genes involved in fibrosis, stress response, inflammation, cell adhesion, intracellular and nuclear signaling, and metabolic pathways. Increased expression of genes coding for proteins involved in the composition and remodeling of the extracellular matrix, along with altered expression of genes involved in cell-to-cell and cell-to-matrix interactions, were observed mainly in radiation enteritis samples. Stress, inflammatory responses, and antioxidant metabolism were altered in radiation enteritis as were genes coding for recruitment of lymphocytes and macrophages. The Rho/HSP27 (HSPB1)/zyxin pathway, involved in tissue contraction and myofibroblast transdifferentiation, was also altered in radiation enteritis, suggesting that this pathway could be related to the fibrogenic process. Our results provide a global and integrated view of the alteration of gene expression associated with radiation enteritis. They suggest that radiation enteritis is a dynamic process involving constant remodeling of each structural component of the intestinal tissue, i.e. the mucosa, the mesenchyme, and blood vessels. Functional studies will be necessary to validate the present results.

INTRODUCTION

The prime goal of radiation therapy is the delivery of lethal doses of radiation to cancer cells with the least toxicity to adjacent normal tissues. However, late normal tissue toxicity may occur several months to several years after radiation therapy, depending on various factors such as the total dose administered, radiation quality, treated volume, fractionation schedule, and individual sensitivity. Development of new techniques such as intensity-modulated radiation therapy (IMRT) aims at delivering higher doses of radiation to tumors through accurate shaping of the radiation beam. IMRT reduces the dose delivered to normal tissues but increases the volume of normal tissue that receives a small dose of radiation. Because the impact of IMRT on late damage to normal tissue in susceptible patients remains unknown, an important issue for the future is to improve the knowledge of the mechanisms involved in the development and persistence of late tissue damage to develop new preventive and curative strategies and to improve the quality of life of cancer survivors.

Because the small bowel is an important dose-limiting organ in radiation therapy of abdominal and pelvic cancers, we were particularly interested in late intestinal toxicity. Stricture formation with subsequent obstruction is one of the most common complications of radiation enteritis. It is characterized at the histopathological level by a severe transmural fibrosis associated with mucosal ulceration and vascular sclerosis. The mainstay of treatment for chronic intestinal obstruction is the inhibition of inflammation with corticosteroids and parenteral nutrition. However, this approach is ineffective in most patients, and surgical resection is often necessary. Different mechanistic models have been proposed to account for late normal tissue damage (*1, 2*). The ''consequential late effect'' model proposes that radi-

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TABLE 1
Characteristics of the Patient Population Studied

Case	Age (years) and sex	Diagnosis	Time after irradiation (month)	Tumor site	Treatment
1	44, male	Ileal stricture (40 cm) with an healthy last ileal loop	3	Rectal adenocarcinoma	Pelvic radiotherapy, 45 Gy + chemotherapy + surgery
2	49, female	Ileal stricture: stricture of the last 3 ileal loops, 40 cm of healthy tissue and stricture of one loop	7	Uterine leimyosarcoma	Surgery + chemotherapy + pelvic radiotherapy, 45 Gy + brachytherapy 10 Gy
3	37, female	Ileal-cecal stricture	4	Cervix carcinoma	Sugery + brachytherapy, 60 Gy
4	51, male	Ileal stricture	75	Hodgkin's disease	Pelvic radiotherapy, 40 Gy + chemotherapy
5	68, female	Ileal stricture	1	Rectal adenocarcinoma	Pelvic radiotherapy, 45 Gy + chemotherapy + surgery
6	42, female	Ileal-cecal stricture (8 cm cecum; 45 cm ileum)	16	Cervix carcinoma	Surgery + pelvic radiotherapy, 45 Gy + chemotherapy
A–F	Male and female	Normal ileum	—	Colon adenocarcinoma	Nonirradiated right hemicolectomy

Note. Control tissues (cases A–F) were nonirradiated, noninflamed and free of malignancy and showed regular histology.

ation-induced fibrosis might result from the initial mucosal injury (*3*). According to the ''target cell'' model, fibrosis occurs in the mesenchyme, where cell turnover and proliferation rates are slow. The ''indirect effect'' model involves the response of cells to vascular damage and paracrine mediators. Late intestinal toxicity generally leads to a chronic wound healing process characterized by a sustained inflammatory response (angiogenesis, granulation tissue formation, re-epithelization) and persistent fibroproliferative response (mesenchymal hyperplasia and extracellular matrix accumulation).

The aim of our study was to get a comprehensive overview of the changes in gene expression associated with late radiation enteritis. Whereas classical approaches allow the examination of individual candidate genes, genome-wide gene expression analysis in tissue samples from affected and normal individuals using cDNA arrays allows the simultaneous examination of hundreds of genes (*4–7*). Characterization of the transcriptional profile could lead to significant advances in the understanding of the molecular pathways involved in chronic radiation enteritis, since the differential gene expression profile may reflect the environmental and/or genetic factors responsible for the pathogenesis of this disorder. This may also help to better define the genetic bases of the risk factors for developing late intestinal complications. The ability to study differential gene expression may provide solid ground for the identification of molecular pathways regulating radiation enteritis. This study showed that despite the heterogeneity of the disease and individual variations, a conserved gene expression pattern can be found in late radiation enteritis.

PATIENTS AND METHODS

Tissue Sampling

Six patients who underwent surgery for delayed radiation-induced complications to the ileum between 1999 and 2002 were included in the study. Diagnosis of radiation-induced enteritis was based on clinical, radiological and histological criteria. Because resection margins could not be considered as normal tissue (*8*), we used samples of healthy, noninflamed and nonirradiated ileum as controls. These control samples were obtained from six patients undergoing surgery for colon cancer; they were free of malignancy and histology was normal. Tissue was obtained after obtaining institutional ethics committee approval and according to French Medical Research Council guidelines. Characteristics of the patients are shown in Table 1. Immediately after surgical resection, 2-cm longitudinal intestinal biopsies composed of mucosa, submucosa and muscularis propria were cut into three equal pieces: one for histology, one for RNA isolation, and one for protein isolation. Serosa was removed to avoid heterogeneity within samples for RNA and protein isolation.

Histopathology

Primary histological examination of the tissue was performed by staining with H&E and Masson's trichrome after fixation in acetic acid-formaldehyde-ethanol.

RNA Extraction

Frozen tissues (500–800 mg) were crushed to powder in liquid nitrogen and total RNA was extracted by homogenization in 4 *M* guanidine isothiocyanate, purified by the method of Chomczynski and Sacchi, and quantified by spectrophotometry (A260/A280 ratio). RNA was treated with RNase-free DNase (0.5 U/μl) to remove contaminating genomic DNA. RNA integrity was assessed by denaturing agarose gel electrophoresis and staining with ethidium bromide.

mRNA Expression Analysis Using cDNA Arrays

Gene expression analysis was performed through hybrid selection of radiolabeled cDNAs on high-density arrays of membrane-bound cDNAs. Two different cDNA arrays were used: the Atlas Human 1.2 (1176 genes + 9 housekeeping genes) and the Atlas Human Cell Interaction (265 genes + 9 housekeeping genes) cDNA expression arrays from Clontech Laboratories (Ozyme, St. Quentin en Yvelines, France) that display 127 genes in common. A list of all the genes included in these two arrays as well as their functions can be found at www.clontech.com/atlas. The Atlas array 1.2 I was used to get a global overview of the genes that are differentially expressed in radiation enteritis. Because excessive deposition of extracellular matrix is observed in radiation enteritis, the Cell

Interaction array was used to get specific information about the remodeling of the extracellular matrix in radiation enteritis.

PolyA RNA was purified from 20 μg of total RNA using the RNA Atlas Pur kit (Clontech) according to the manufacturer's instructions. Radiolabeled cDNAs were prepared according to Clontech's protocol with [α-^{33}P]dATP. Membranes were exposed to PhosphorImager intensifying screens for an optimal period of 10 days, and mRNA expression levels were analyzed by scanning the screens with a PhosphorImager FLA-3000 (Fuji, Kanagawa, Japan). Analysis of differential mRNA expression was performed using the Atlas Image 1.5 software (Clontech).

Radiolabeled cDNAs were prepared from healthy ileum samples and ileum samples from patients with radiation enteritis in parallel and were hybridized to pairs of identical cDNA arrays, so that direct comparison of both sample types could be done after normalization of data with selected housekeeping genes.

Selection of Housekeeping Genes

HPRT, GAPD, TUBA1, RPL13A and the gene encoding the 40S ribosomal protein S9 were selected as housekeeping genes since they did not exhibit significant differential expression according to regression plots. Phospholipase A2 was not selected as a housekeeping gene because it is known to be involved in inflammation and because signals were below background. Ubiquitin, *HLAC* and *ACTB* were differentially expressed between samples.

Sensitivity and Reproducibility of the cDNA Arrays

Although the cDNA array technique using radiolabeled dATP is considered to be reproducible ($0.75 <$ correlation coefficient < 0.85), false positives can be generated from one experiment to another. To minimize these effects and distinguish real signals from noise, we adopted the two-parameter strategy recently proposed by Mills *et al.* (*9*). Duplicate radiolabeled probes were generated from a single preparation of RNA isolated from normal ileum. The two sets of probes were hybridized independently to a pair of membranes. Genes that were found to be up-regulated or down-regulated in one of the two membranes were considered as false positive and were discarded. For each of the two arrays studied, baseline gene expression was established by averaging the six arrays obtained from the six control samples using the Atlas 1.5 software, with 25–35% variation in gene expression in the control group. This allowed us to create a single ''normal composite array'' used to compare the set of normal samples to each radiation enteritis sample. A change in gene expression greater than two times that of the averaged control group in at least three of the six radiation enteritis specimens was considered significant. Finally, data were used only when signal intensities were significantly above background (i.e. 50% or more).

Hybridization intensities were converted into ratios, adjusted for background and housekeeping gene expression. Data are expressed as the ratio of hybridization intensity of radiation enteritis sample (RE*x* with $x =$ patients 1 to 6) to hybridization intensity of normal composite membrane (N composite), according to the following equation: [(gene X intensity on RE*x* array − background RE*x* array)/(average intensity for all six housekeeping genes on RE*x* array − background RE*x* array)]/[(gene X intensity on N composite array − background N composite array)/(average intensities for all six housekeeping genes on N composite array − background N composite array)].

Since the study was based on a small number of patients, statistical analysis was not appropriate. Thus we recorded the lowest and the highest ratios obtained for the six radiation enteritis samples. When the hybridization signal was undetectable or below background signal in normal samples but was detectable in radiation enteritis samples, a value of >10 was assigned to it and the gene was considered as an induced gene. Conversely, when the hybridization signal was undetectable or below background signal in the radiation enteritis samples but was detectable in normal samples, a value of <0.1 was attributed to it and the gene was considered as a repressed gene.

Genes were classified into seven functional groups related to the pathogenesis of radiation enteritis: fibrosis, growth factors, cell adhesion, intracellular signaling, nuclear signaling, metabolic pathways, and stress response, plus one uncategorized group.

Confirmation of Differential Gene Expression

To provide independent confirmation for array data, real time RT-PCR, and Western blot analysis were performed on selected genes.

Quantitative RT-PCR. Real time RT-PCR assays were performed to quantify levels of RNA transcripts. Two micrograms of total RNA was reverse-transcribed with Superscript II reverse transcriptase (Life Technology Invitrogen, Cergy Pontoise, France) and random hexamers according to the manufacturer's protocol. TNFA, IL1B and GAPD primers were purchased from Applied Biosystems (France). RhoB (ARHB) primers were generated with Primer Express software (Applied Biosystems) and purchased from Life Technology Invitrogen. Forward primer: 5′-GTCCCAATGTGCCCATCATC-3′. Reverse primer: 5′-CTGTGCGGACATGCTCGTC-3′. Primer pairs were designed to span intron-exon boundaries. Samples were subjected to 40 cycles of amplification of 15 s at 95°C, followed by 1 min at 60°C using an ABI PRISM 7500 detection system and SYBR green buffer, according to the manufacturer's instructions (Applied Biosystems). The results were normalized by PCR amplification of *GAPD*, a housekeeping gene, for each sample. Both water and genomic DNA controls were included to ensure specificity. Integrity was assessed for each data set through analysis of the amplification plot and dissociation curves. Relative mRNA quantification was performed using the comparative $\Delta\Delta C_T$ method. Relative quantification in radiation enteritis $= 2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is defined as the difference between the mean $\Delta C_{T(\text{radiation enteritis})}$ and the mean $\Delta C_{T(\text{healthy bowel})}$ and ΔC_T as the difference between the mean $C_{T(\text{TNFA, IL1b, RhoB})}$ and $C_{T(\text{GAPD})}$. GAPD is used as an endogenous control.

Protein extraction. Frozen tissues (500–800 mg) were crushed to powder in liquid nitrogen, homogenized in a 50 m*M* Tris-HCl buffer (pH 7.6) containing 150 m*M* NaCl, 10 m*M* $CaCl_2$, 1% Triton X-100, and protease inhibitors (Sigma-Aldrich, St Quentin Fallavier, France), and centrifuged at 10,000 rpm at 4°C for 10 min. Supernatants were collected and the protein concentration was determined according to the Lowry method.

Western blot analysis. Five to 15 μg of total protein was separated by electrophoresis on a 12% SDS-polyacrylamide gel and electrotransferred onto a 0.45 μm nitrocellulose membrane. For HSP27 and JUN determination, membranes were blocked overnight at 4°C in PBST (phosphate-buffered saline with 0.2% Tween 20) containing 5% nonfat dry milk. After 2 h incubation with the primary antibody (1:5000 in PBST with 3% nonfat dry milk), membranes were washed with PBST and incubated with peroxidase-coupled anti-mouse antibody (1:5000). Antibody was detected by enhanced chemiluminescence (ECL) (Amersham, Orsay, France). For a loading control, membranes were then dehybridized as follows: They were incubated in 2% SDS (pH 9.5) at 50°C for 30 min and blocked overnight. Dehybridized membranes were then incubated with the anti-GAPD antibody (1:2000), washed and probed with a peroxidase-coupled anti-mouse antibody (1:5000). Densitometric analyses were performed using a Biocom Analyser (Les Ulis, France) interfaced with the Phoretix software (New Castel, UK).

Primary antibodies against HSP27 (HSPB1) and JUN—SPA-800 and KAP-TF102 (Stressgen Biotechnologies, Victoria, BC, Canada), respectively—and against GAPD—H86504M (Biodesign)—were used.

Statistical Analysis

For Western blots and real-time RT-PCR, statistical differences between the means of the control group and the radiation enteritis group were evaluated using Student's *t* test.

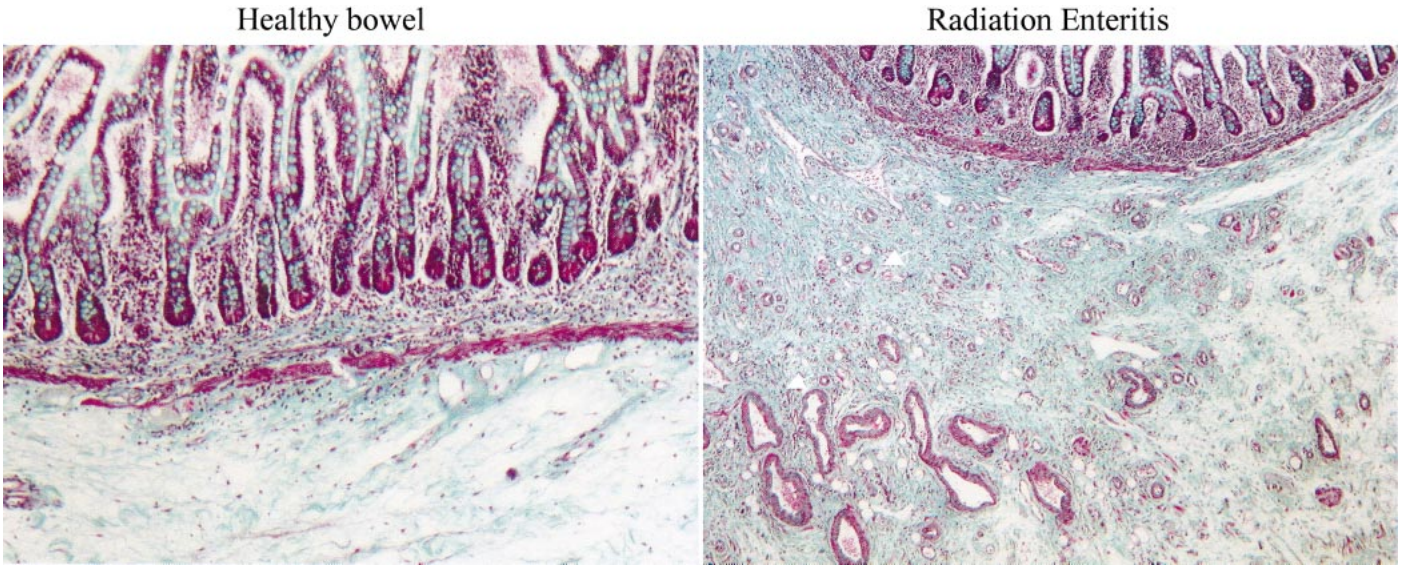

FIG. 1. Masson's trichrome staining of healthy bowel (original magnification 100×) and radiation enteritis (original magnification 100×). In radiation enteritis, the green staining of the collagen fibers showed a severe transmural fibrosis. The submucosa was particularly affected, with zones composed of interlaced fibers of collagen with myofibroblasts and inflammatory cells located around hyalinized vessels (arrows).

RESULTS

Histopathological Examination

Examination of H&E-stained and Masson's trichrome-stained sections (Fig. 1) associated with the scoring system revealed common histological features in all radiation enteritis samples (Table 2). Severe fibrosis affected the whole intestinal wall with a transmural collagen accumulation in the mucosa, submucosa and muscularis propria. Damage to the submucosa and the vessels was always severe whereas the morphology of the mucosa and the muscularis propria was less affected.

Differential Gene Expression in Radiation Enteritis

The intensity of the arrays from the control group was averaged and compared with the intensity of arrays from each radiation enteritis sample. The differentially expressed genes are listed in Table 3 and represent 8% (106/1314) of the genes studied.

Fibrosis

Levels of mRNA coding for the fibrillar type I and III collagens, the microfibrillar type VI collagen, the basement membrane type IV collagen, and the stromal component tenascin C were higher in the radiation enteritis samples. In addition, modulation of several ECM genes not described previously in radiation fibrosis was observed. Type VIII collagen and osteonestin (SPARC) that code for proteins of the perivascular extracellular matrix were increased, whereas the basement membrane coding genes laminin β1 and γ2 and laminin receptor were decreased.

The array technique showed that the level of mRNA coding for the gelatinases MMP2 and the MMP inhibitors TIMP1, TIMP2 and PAI1 were increased in radiation enteritis as already described in various models of radiation fibrosis. In addition, modulation of several MMPs never studied in radiation fibrosis was observed. The interstitial collagenases MMP1 and MMP18/19, membrane-type MMP14, and stromelysin (MMP3) were increased, whereas expression of the macrophage-specific metalloelastase (MMP12) was decreased. Several other factors that regulate ECM remodeling, such as the MMP inducer thymosine β4, were increased in radiation enteritis. Finally, expression levels of genes coding for ephrin B2 and ephrin receptors type B1 and B4, involved in the control of angiogenesis, were decreased in radiation enteritis.

Growth Factors, Cytokines and Chemokines

Expression of the tumor necrosis factor precursor, TNFA and TNF receptor I was decreased, whereas IL1B expression was increased in only three radiation enteritis samples. The gene coding for the profibrogenic cytokine TGFB1 was not detected using the array technique, whereas differential expression of the CTGF did not reach the cut-off value, at 1.8 times the control value.

Expression of genes coding for proteins of the insulin-like growth factor (IGF) axis, IGFBP2, IGFBP4 and IGFBP5, was increased, as well as that of chemokine MIP-2α (CXCL2) and PBP. Conversely, expression of the chemokine SCYA5 (CCL5), BMP4, the NGF receptor, and the receptor of IL2A was decreased in radiation enteritis.

Cell Adhesion

The expression levels of integrins β3, β4, α2B and αL were decreased in radiation enteritis, along with that of genes coding for proteins involved in cell-to-cell interac-

TABLE 2
Histopathological Score

Patient no.	1	2	3	4	5	6
Epithelial damage	2	1	1	1	2	2
Lamina propria inflammation	2	2	2	2	2	2
Thickness of the muscularis mucosa	2	1	2	2	1	2
Fibrosis of the submucosa	2	2	2	2	2	2
Vascular hyalinization	2	2	2	2	2	2
Dystrophy of the muscularis propria	2	1	2	1	2	2

Note. 0 represents normal, 1 mild abnormality, 2 severe abnormality.

tion, such as cadherin 1, desmocollin 1A/1B and 2A/2B, and β-catenin. Expression of integrins β2, α5 (fibronectin/vitronectin receptor), and the integrin-associated protein (IAP), however, was increased in radiation enteritis, and so was that of zyxin, a regulator of actin filament assembly.

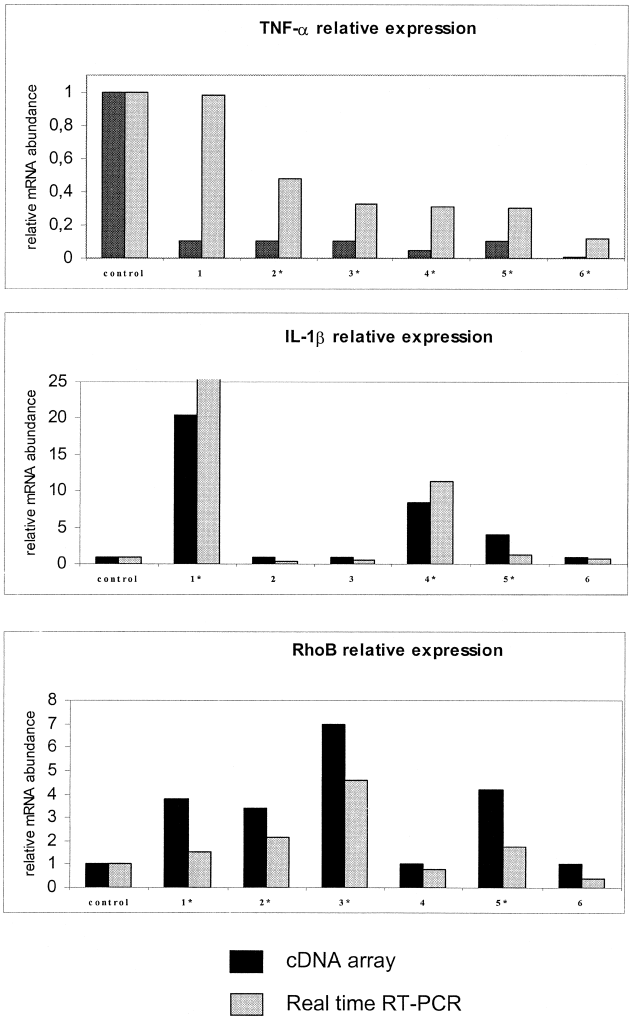

FIG. 2. Expression of IL1B (IL-1β), TNFA (TNF-α) and RhoB was determined by cDNA array and real time RT-PCR. Gene expression was measured individually in each radiation enteritis sample ($n = 6$) and compared to the expression in healthy bowel samples ($n = 6$) (*$P < 0.01$).

Intracellular Signaling

Expression of the focal adhesion kinase and cell adhesion kinase was decreased in radiation enteritis. Expression levels of downstream genes coding for proteins involved in wound healing, contraction signals and stress fibers formation, such as RhoB and the RAS-like protein TC10, were increased, whereas those of RhoHP1 (ARHD) and RhoC (ARHC) were decreased.

Nuclear Signaling

The expression levels of numerous genes coding for transcription factors or transcription-associated proteins were notably increased in radiation enteritis samples. Indeed, we observed increased expression levels of the immediate early gene *JUN* and of the cAMP-related transcription factors ATR1 and CREB2.

Metabolic Pathways

The expression of genes involved in the control of bowel function, including that of enzyme dipeptidyl peptidase IV, was decreased in radiation enteritis samples. Genes coding for neuropeptide receptors, including somatostatin and 5-HT receptors, were mainly down-regulated.

Stress Response

Stress response in radiation enteritis samples was characterized by an increased expression of the heat-shock proteins HSP27 and HSP70 (HSPA1). Expression of the antioxidant enzymes was also altered. mRNA levels of glutathione *S*-transferase theta 1 were decreased, whereas that of *SOD1* and glutathione peroxidase—respectively involved in the dismutation of the superoxide anion into hydrogen peroxide and the breakdown of hydrogen peroxide into water—were strongly increased in radiation enteritis samples.

Expression of apoptosis-related genes was also found to be altered in radiation enteritis samples. Expression of the pro-apoptosis gene caspase 10 was down-regulated, whereas that of anti-apoptosis genes *DAD1*, *GADD153* (*DDIT3*), and *HSP27* was up-regulated, suggesting that the inhibition of apoptosis might play a role in the maintenance of fibrogenic signals.

TABLE 3
Differentially Expressed Genes in Radiation Enteritis

Function	Accession no.	Gene	Symbol	Ratio of radiation enteritis/healthy ileum
Fibrosis				
	L23808	matrix metalloproteinase 12	MMP12	<0.1
	U03056	hyaluronoglucosaminidase	HYAL1	<0.1
	M61916	laminin beta 1 subunit precursor	LAMB1	0.18
	M55172	cartilage-specific proteoglycan core protein (CSPCP)	CSPCP	0.33
	Z15009	laminin gamma 2 subunit precursor	LAMC2	0.44
	U43901	34/67-kDa laminin receptor	LAMR1	0.47
	X04429; M14083	endothelial plasminogen activator inhibitor 1 precursor	PAI1	2–7.5
	X57527	collagen 8 alpha 1 subunit	COL8A1	2–2.11
	D26512; X83535	**matrix metalloproteinase 14 precursor**	**MMP14**	**2.35–6.15**
	J03210; J05471	**matrix metalloproteinase 2**	**MMP2**	**2.47–10**
	J05593	**tissue inhibitor of metalloproteinases 2**	**TIMP2**	**2.50–3.5**
	J04599	biglycan	BGN	2.55
	M17733	thymosin beta 4		2.16–4.16
	U16306; X15998; U26555; D32039	large fibroblast proteoglycan		2.87
	X78565; M55618	tenascin precursor	TN	2.99–10
	X03124	**metalloproteinase inhibitor 1 precursor**	**TIMP1**	**3.1–5.40**
	X52022	collagen 6 alpha 3 subunit	COL6A3	3.3–3.42
	X14420	procollagen 3 alpha 1 subunit precursor	COL3A1	3.87–4.7
	Y08622 + X92521	matrix metalloproteinase 18 (MMP18) + MMP19	MMP18 + MMP19	4.33–13
	X55525; J03464	procollagen 1 alpha 2 subunit precursor	COL1A2	5.16–8.4
	X05231	**matrix metalloproteinase 1**	**MMP1**	**5.3–10**
	J03040	osteonectin/SPARC	ON	5.9
	X05562	*procollagen alpha 2(IV) subunit precursor*	*COL4A2*	*>10*
	X05232	**matrix metalloproteinase 3**	**MMP3**	**>10**
Growth factor, cytokine and chemokine related				
	X01394	**tumor necrosis factor precursor**	**TNFA**	**<0.1**
	M21121	small inducible cytokine A5	SCYA5	0.1–0.45
	M14764	low-affinity nerve growth factor receptor	NGFR	0.1–0,33
	X01057; X01058; X01402	*interleukin-2 receptor alpha subunit precursor*	*IL2Ralpha*	*0.15–0,34*
	M17446	INT-2 proto-oncogene protein precursor	FGF-3	0.17–0.34
	D30751 + M22490	*bone morphogenetic protein 4*	*BMP4*	*0.2–0.4*
	M33294	tumor necrosis factor receptor 1	TNFRI	0.34–0.5
	M54995; M38441	platelet basic protein precursor	PBP	2.0–10
	X53799	macrophage inflammatory protein 2 alpha	MIP2-alpha	2–11.8
	M35410	**insulin-like growth factor binding protein 2**	**IGFBP2**	**2.1–10**
	K02770	*interleukin-l beta precursor*	*IL1B*	*2.5–8.4*
	M65062	insulin-like growth factor binding protein 5 precursor	IGFBP5	3.4–7
	M62403	insulin-like growth factor binding protein 4 precursor	IGFBP4	3.46–12

(Continued on page 305)

TABLE 3
Continued

Function	Accession no.	Gene	Symbol	Ratio of radiation enteritis/healthy ileum
Cell adhesion				
	X56807	desmocollin 2A/2B precursor	DSC2	<0.1
	X53587; X52186; X51841	**integrin beta 4**	**ITGB4**	**0.1–0.4**
	Y00796	integrin alpha L	ITGAL	0.1–0.49
	U53786	envoplakin	EVPL	0.1–0.35
	Z13009	cadherin1	CDH1	0.1–0.40
	M77830; J05211	desmoplakin I and II	DPI and DPII	0.1–0.41
	Y11306	beta-catenin		0.1–0.44
	X72925; Z34522	desmocollin 1A/1B precursor	DSC1	0.1–0.50
	M34480; J02764	integrin alpha 2B	ITGA2B	0.50
	J02703; M25108	integrin beta 3	ITGB3	0.5
	M15395	integrin beta 2	ITGB2	2–15.2
	X94991; X95735	**zyxin + zyxin-2**		**3.48–8**
	X06256	integrin alpha 5	ITGA5	4.1–10
	Y00815 + X69398	**integrin-associated protein**	**IAP**	**4.31**
Intracellular signaling				
	M91815; L26584	cdc 25	CDC25	<0.1
	M25753	G_2/mitotic-specific cyclin B1	CCNB1	0.1–0.5
	D85815	rhoHP1		0.1–0.18
	L07597	*ribosomal protein S6 kinase II alpha 1*	*S6KII-alpha 1*	*0.1–0.36*
	M18391	**ephrin type-A receptor 1 precursor**	**eph**	**0.30**
	L38734	ephrin-B2 precursor	HTK-1	0.33–0.5
	L25081	small GTPase rhoC	rhoC	0.22–0.27
	U43408	tyrosine kinase tnk1		0.22
	X74979	cell adhesion kinase	CAK	0.32
	L49207 + U43522 + U33284	focal adhesion kinase 2	FAK2	0.45
	L40636	ephrin type-B receptor 1 precursor	EPH-2	0.50
	U07695	ephrin type-B receptor 4 precursor	HTK	0.50
	M31470	ras-like protein TC10		2–2.3
	L40027	*glycogen synthase kinase 3 alpha*	*GSK3 alpha*	*2.18*
	X06820	**transforming protein rhoB**	**rhoB**	**3.4–7**
Nuclear signaling				
	X55544	*cyclic-AMP-dependent transcription factor atr-1*	*atr-1*	*<0.1*
	M93255	*fli-1 oncogene; ergB transcription factor*	*ergB; fli-1*	*<0.1*
	X79067	*EGF response factor 1*	*ERF1*	*2–3.5*
	J04111	c-jun proto-oncogene	c-jun	2.1–2.9
	M24069	*DNA-binding protein A*		2.2–7.2
	M62831	transcription factor ETR101	ETR101	2.6–9
	M28372	*cellular nucleic acid binding protein*	*CNBP*	*2.71–9.2*
	U12979	*activated RNA polymerase II transcriptional coactivator p15*		*3.4–8.9*
	D90209	*cAMP-response element binding protein*	*CREB2*	*3.6–6.1*
	X06745	*DNA polymerase alpha catalytic subunit*	*POLA*	*>10*
	M96684	*purine-rich single-stranded DNA-binding protein alpha*	*PURA*	*>10*
Metabolic pathways				
	M74777	**dipeptidyl peptidase IV**	**DPP IV**	**<0.1**
	M81830	somatostatin receptor type 2	SS2R	<0.1
	D49394	*5-hydroxytryptamine 3 receptor precursor*	*5-HT-3*	*<0.1*

(Continued on page 306)

TABLE 3
Continued

Function	Accession no.	Gene	Symbol	Ratio of radiation enteritis/healthy ileum
Metabolic pathways				
	U66661	*gamma-aminobutyric-acid receptor epsilon subunit precursor*	*GABA(A) receptor epsilon*	*<0.1*
	U95367	gamma-aminobutyric-acid receptor pi subunit precursor	GABA(A) receptor pi	<0.1
	M76180	DOPA decarboxylase	DDC	<0.1
	J05252	neuroendocrine convertase 2 precursor	NEC2	<0.1
	D28538	*metabotropic glutamate receptor 5 precursor*	*GRM5*	*<0.1*
	Y07683	*ATP receptor P2X3*		*<0.1*
	A00914	*angiotensin-converting enzyme*	*ACE*	*<0.1*
	U62435	neuronal acetylcholine receptor protein alpha 6 subunit precursor		0.3
	D45248	*proteasome activator HPA28 subunit beta*		*0.21–0.28*
	L25124; D28472	*prostaglandin E2 (PGE) receptor EP4 subtype*	*PTGER4*	*0.23–0.3*
	S56143	*adenosine A1 receptor*	*ADORA1*	*0.26*
	M24795	*platelet glycoprotein IV*	*GPIV*	*0.33*
	M14200	*acyl-CoA-binding protein*	*ACBP*	*2.6–5.6*
	M65212	*membrane-bound and soluble catechol-O-methyltransferase*	*COMT*	*2.8–3.4*
	Y00264	Alzheimer's disease amyloid A4 protein precursor	APPI	>10
	U32944	protein inihibitor of neuronal nitric oxide synthase	PIN	>10
	M12529	apolipoprotein E precursor	APOE	>10
	M34079	proteasome subunit p50		>10
Stress response				
	X79389	glutathione S-transferase theta 1	GSTT1	0.1–0.33
	U60519	*caspase-10 precursor*	*CASP10*	*0.15–0.32*
	M63928	CD27L antigen receptor precursor	CD27	2–8.5
	D15057	*defender against cell death 1*	*DAD1*	*2–3.65*
	S40706; S62138	growth arrest and DNA-damage-inducible protein 153	GADD153	2.4–4.8
	X54079	27-kDa heat-shock protein	HSP27	2.7–8.19
	K00065; X02317	cytosolic superoxide dismutase 1	SOD1	2.85–6.4
	M74816	clusterin precursor	CLU	2.4–13
	M11717	*70-kDa heat-shock protein 1*	*HSP70.1*	*>10*
	Y00483; M21304	*glutathione peroxidase*	*GPX1*	*>10*
Others				
	Y00815 + X69398	***leukocyte antigen-related protein precursor***	***LAR***	***0.1–0.17***
	X90392; L40817; U06846	muscle-specific DNase I-like precursor	DNase1L1	0.1
	M73980	neurogenic locus notch protein homolog 1 precursor	NOTCH1	0.30
	U49089	synapse-associated protein 102	SAP102	0.36
	M73482	*neuromedin B receptor*	*NMBR*	*0.38–0.47*
	U61262	neogenin		0.40
	M74088; M73548	adenomatous polyposis coli protein	APC protein	0.46
	AF003522	delta-like protein 1 precursor	DLL1	0.50
	X75621	*tuberin*	*TSC2*	*2.12–4*
	X69391	*60S ribosomal protein L6*	*RPL6*	*2.16–2.6*

Notes. Bold: genes found to be differentially expressed using the Atlas Human 1.2 and the Atlas Cell Interaction. Italic: genes found differentially expressed in only 3 of 6 or 4 of 6 radiation enteritis samples.

Corroboration of Array Data by Quantitative RT-PCR and Western Blot Analysis

Differential expression of TNFA, IL1B, RhoB, JUN and HSP27 was confirmed for each patient's specimen by quantitative RT-PCR or Western blotting. Array data were in agreement with data obtained by RT-PCR and Western blot analysis. Furthermore, 15 genes (listed in bold in Table 3) had similar expression patterns in both the Atlas Human 1.2 and the Atlas Human Cell Interaction arrays.

Quantitative RT-PCR

The expression of the *TNFA*, *IL1B* and *RhoB* genes was quantified twice by real-time RT-PCR (Fig. 2). Expression of the *TNFA* gene was decreased by two- to eightfold in radiation enteritis samples compared to controls. Expression of IL1B was increased in patients 1, 4 and 5 by 27-, 11.3- and 1.3-fold, respectively. For patients 2, 3 and 6, cDNA array analysis and real-time RT-PCR did not reveal differential expression of IL1B. Expression of the *RhoB* gene was increased by 1.5- to 4.6-fold in radiation enteritis samples compared to controls.

Western Blot Analysis

Western blot analysis of JUN expression was monitored because this protein is involved in the transactivation of profibrogenic cytokines. JUN was detected in total protein extracts from five of six radiation enteritis samples but not from control samples (Fig. 3).

Because HSP27 is an actin chaperone involved in wound contraction, we monitored its expression by Western blotting, which showed an increase in HSP27 expression in total protein extracts from radiation enteritis samples (Fig. 3).

DISCUSSION

We analyzed gene expression profiles in bowel samples from patients with and without radiation enteritis to identify changes in the expression pattern of genes that may be associated with radiation-induced intestinal fibrosis. To obtain a representative overview of all the cellular changes associated with late radiation enteritis, we used total RNA from whole intestinal wall, which consists of a heterogeneous cell population. This approach allowed us to identify potential molecular pathways that may be involved in the pathogenesis of radiation enteritis.

The molecular mechanisms involved in fibrogenesis are not fully understood. However, reactive oxygen species (ROS) have been proposed as potential primary molecular mediators of fibrotic processes that are able to induce secondary fibrogenic molecules (*10*). Our results show that mRNA levels of the antioxidant enzymes SOD1 and GPx increase in radiation enteritis. This finding provides indirect evidence of the involvement of ROS in fibrogenesis and concurs with previous reports (*11, 12*). Moreover, the present results show increased expression of the redox-sensitive transcription factor JUN (*13*), which may be involved in the transcriptional activation of GPx (*14*) and that of inflammatory and fibrogenic cytokines (*15*).

Cytokines and growth factors are important mediators of inflammation, tissue remodeling, and repair. Among the latter, TNFA is considered a key mediator of acute inflammatory processes. In this study, we observed a drop in the TNFA mRNA levels in radiation enteritis samples. This observation was confirmed by real-time RT-PCR and is consistent with recent findings by Quarmby *et al.* (*16*). TNFA plays an important role in homeostasis in limiting the extent of the inflammatory process and tissue injury. Thus the observed decrease in TNFA mRNA levels may be valuable information since it may help us understand the persistence of radiation-induced fibrosis. In addition, we observed an increased expression of genes coding for members of the CC chemokine family (Scya5 and MIP-α). This is consistent with a recent report by Johnston *et al.* (*17*) and supports their hypothesis that activation of lymphocytes and macrophages may be of primary importance in the chronic inflammatory response leading to fibrosis. Finally, we found an up-regulation of the insulin-like growth factor I binding proteins, IGFBP2, 4 and 5. These extracellular associated proteins can either inhibit (IGFBP2) or promote (IGFBP5) interaction of IGF-I with its receptor. They are involved in both smooth muscle cell proliferation and control of vascular and visceral smooth muscle contraction (*18, 19*).

Late radiation enteritis is characterized by extensive transmural fibrosis. Thus abundant expression of interstitial type I, III and VI collagens and tenascin C mRNAs was expected in bowel samples from patients with radiation enteritis. These results confirm biochemical observations reported previously by our group and others in various models of radiation fibrosis (*8, 20–24*), and they tend to validate the array approach. In addition, modulation of the expression of genes coding for vascular ECM components such as type VIII collagen and SPARC, which has never been described in radiation fibrosis, was observed, suggesting that vascular smooth muscle cells are activated in radiation enteritis. Changes in the expression profile of genes coding for basement membrane components—e.g. increase in type IV collagen and decrease in laminins β1 and γ2 and laminin receptor mRNA—associated with a decrease in the expression levels of genes coding for proteins involved in cell-to-cell interaction such as desmocollin 1A/1B and 2A/2B, β-catenin and desmoplakin suggest that the integrity of the intestinal mucosa is altered in radiation enteritis. Since ECM homeostasis is tightly regulated by a complex balance between ECM synthesis and the actions of proteolytic enzymes and protease inhibitors, alteration of the expression pattern of molecules forming the ECM cannot by itself account for the pathological accumulation of ECM observed in radiation enteritis. Thus, to study the molecular balance that is responsible for accumulation of ECM in radiation

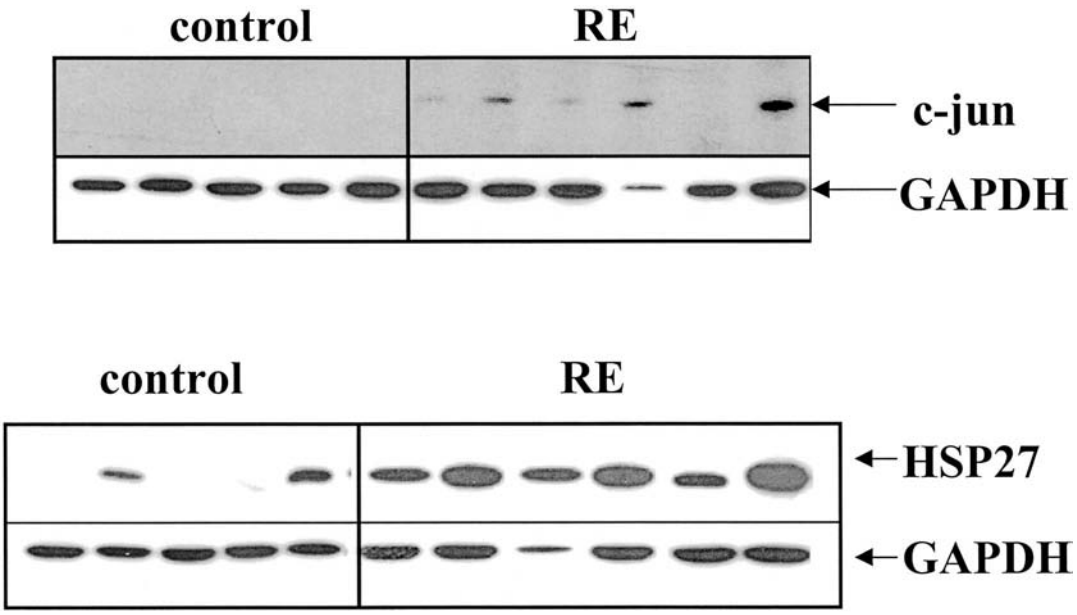

FIG. 3. Expression of JUN (c-jun) and HSP27 protein was determined by Western blot analysis in whole tissue extracts. Protein expression was measured individually in each radiation enteritis sample ($n = 6$) and compared to the expression in healthy bowel samples ($n = 5$) ($P < 0.01$).

enteritis, a global approach was used. cDNA array analysis appears to be a valuable tool since it provides a simultaneous view of collagen/MMP/TIMP expression. The present results show a concomitant mRNA induction of all of the MMP family members, i.e. gelatinases (MMP2, 14), stromelysin (MMP3), and collagenases (MMP1, 18/19), whose expression is regulated mainly at the transcriptional level. This induction may be counterbalanced by the induction of the natural inhibitors of MMPs, TIMP1, TIMP2 and PAI1, which was also observed. In summary, late radiation enteritis ECM consists of a dense fibrillar network of type I and type III collagen associated with immature and newly formed granulation tissue (tenascin C) which promotes migration, proliferation and activation of matrix-producing cells. A concomitant increase in the expression levels of MMPs, TIMPs and PAI1 is also observed. Taken together, these observations suggest that the overall metabolism of the ECM is activated in radiation enteritis, which further supports the hypothesis that fibrotic tissue is dynamic and undergoes constant remodeling (*25*).

The composition of the ECM in radiation enteritis may alter transmission of differentiation signals to cells. Integrin receptors are partly responsible for cell–matrix interactions. They are able to regulate such functions as migration, proliferation and gene expression through direct interaction with the intracellular cytoskeleton or activation of molecular signaling cascades (*26, 27*). However, their role has been poorly investigated in radiation-induced fibrosis. Despite the fact that our findings do not pertain to a specific cell type, they do show a concomitant decreased expression of integrins (ITGB4, ITGAL) and intercellular adhesion molecules (desmocollin 1A/1B and 2A/2B, β-catenin and desmoplakin) that bind to the basement membrane. This suggests that alteration of epithelial cell adhesion to the ECM may be responsible for epithelial damage in radiation enteritis. Up-regulation of the α5, β2 and β3 integrins may be involved in the differentiation of mesenchymal cells into matrix-producing cells, since they are known to regulate the secretion of collagens (*28*) and MMPs (*29*). Expression of ephrin-B2 (HTK1) and ephrin receptors (eph, EPH2, HTK) decreases in radiation enteritis. Ephrins are well-known modulators of endothelial cell adhesion and motility. They are functionally related to integrins and are associated with the activation of intracellular signaling pathways involving the Rho pathway (*30*). This suggests that the ephrin pathway may be involved in the defective vascularization observed in radiation enteritis. This hypothesis is further supported by the fact that expression of small GTPase Rho is altered in radiation enteritis. Our data show increased expression levels of genes coding for RhoB and the RAS-like TC10 protein (ARHQ) and decreased expression levels of genes coding for RhoC and RhoHP1, which may be involved in the aforementioned defective vascularization, in the alteration of smooth muscle contraction, and in the pathological differentiation of fibroblasts into myofibroblasts. Rho-dependent activation of smooth muscle contraction relies on a prior increase in smooth muscle cell sensitivity to Ca^{2+} and phosphorylation of myosin light chain (MLC) by ROCKs. The protein kinases ROCK1 and 2 are considered as specific effectors of Rho. The Rho/ROCK-mediated pathway regulates not only smooth muscle contraction but also the formation of stress fibers and cell motility by controlling actin assembly in fibroblastic cells (*31*). Cytoskeleton reorganization and formation of ac-

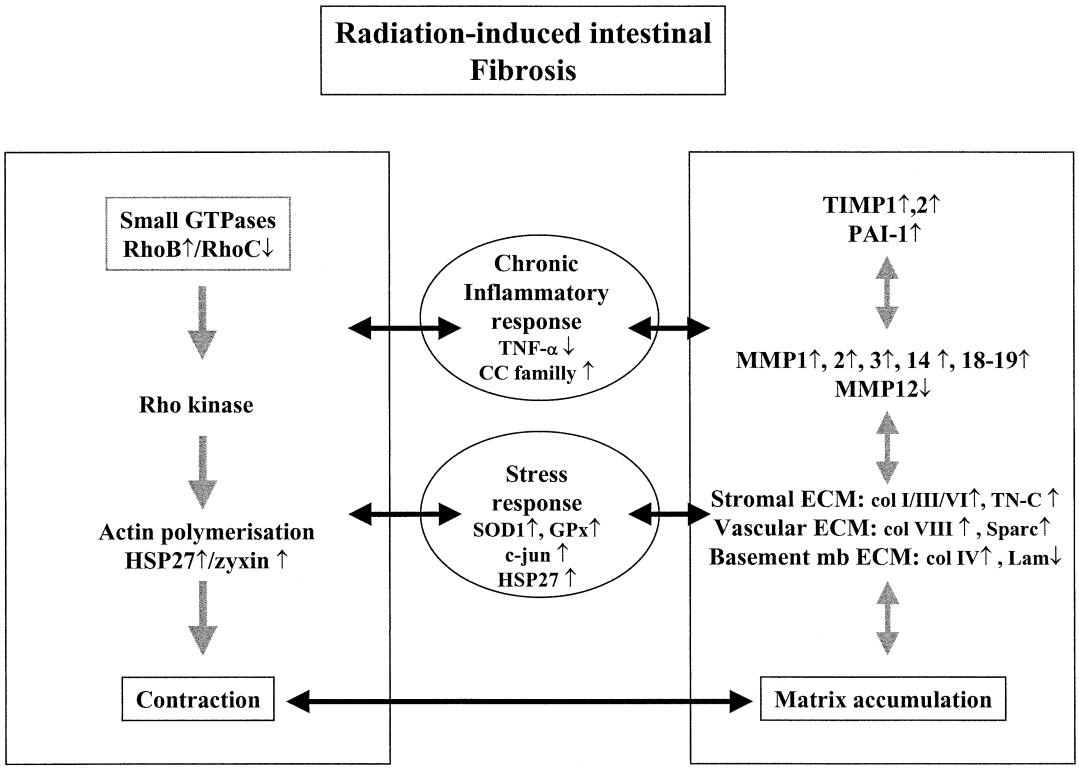

FIG. 4. Hypothesis concerning the pathogenic pathways involved in radiation enteritis.

tin stress fibers are partly controlled by HSP27 (*32*). The actin chaperone HSP27 ensures proper orientation of the contractile apparatus, which is composed of actin, tropomyosin, myosin and caldesmon, and acts in conjunction with zyxin, which is necessary for actin assembly (*33, 34*). In this study, we found an increased expression of the genes coding for HSP27 and zyxin, which suggests that alteration of the Rho/ROCK-mediated pathway may be involved in the pathological contraction and differentiation of smooth muscle cells and fibroblasts into myofibroblasts observed in radiation enteritis.

This highly comprehensive approach, however, has its limitations. For instance, to minimize the effect of potential individual variability and disease heterogeneity, we carefully selected biopsy samples based on histopathological examination. Furthermore, we avoided the commonly used pooling strategy since it may introduce false positives due to interindividual variability, as illustrated by the increase in IL1B mRNA levels found in only three of six patients. Further studies that include a larger number of patients will be necessary to better investigate the expression of IL1B in late radiation enteritis.

Another limitation of this approach is the fact that whole bowel homogenates are used, which makes us unable to determine precisely the type of cell and number of cells which express a given gene. The intestine is a complex tissue composed of epithelial cells, lymphocytes, macrophages, endothelial cells, fibroblasts and smooth muscle cells, all of which may have contributed to the observed results. One illustration of this problem concerns the expression of the profibrogenic cytokine CTGF. In our previous study, an overexpression of CTGF was observed in radiation enteritis, while CTGF was not found to be differentially expressed in radiation enteritis according to the criteria used in this study. A 1.8-fold increase in the level of CTGF mRNA was found in bowel samples from patients with radiation enteritis; however, this value was below the cut-off value. One hypothesis which may account for this discrepancy is that CTGF is expressed mainly by activated mesenchymal cells, which represent only a minor part of the whole bowel cell population. This hypothesis was recently tested and confirmed in primary smooth muscle cells isolated from radiation enteritis samples using the Atlas Human 1.2 array. We found that primary smooth muscle cells isolated from ileum samples from patients with radiation enteritis showed a 2.8-fold increase in the expression levels of CTGF mRNA compared to primary smooth muscle cells isolated from normal ileum (*35*). Finally, another limitation of the cDNA array approach is its relatively poor sensitivity. For instance, genes expressed at low levels, such as TGFB1, cannot be monitored using this technology without prior PCR amplification.

Despite these limitations, the data presented here represent a significant advance in the characterization of the gene expression profile for radiation enteritis. Simultaneous observation of thousands of genes allowed for the identification of both novel and previously described pathogenic pathways, which are summarized in Fig. 4. This study pro-

vides additional support for the hypothesis that radiation-induced fibrotic tissues are active tissues undergoing constant remodeling rather than scarred, quiescent tissues. Functional analyses will be necessary to validate the reported observations.

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Plus de la moitié des patients atteints de cancer sont traités par radiothérapie seule ou en combinaison avec d'autres traitements. La radiothérapie est une méthode de traitement utilisant les rayonnements ionisants dont l'objectif est de délivrer à la tumeur une dose maximale tout en préservant l'intégrité des tissus sains adjacents. Cependant, la toxicité radio-induite aux tissus sains est un facteur limitant dans l'escalade de dose pouvant être délivrée à la tumeur et constitue un problème clinique majeur. Ainsi, la compréhension des mécanismes moléculaires et cellulaires associés aux dommages radio-induits est indispensable dans l'objectif de mettre en place des stratégies thérapeutiques visant à protéger les tissus sains sans compromettre et même améliorer le contrôle tumoral. Les cellules endothéliales contribuent à l'initiation et la progression des dommages tissulaires radio-induits. Ce travail a permis de démontrer que les cellules endothéliales stimulent la prolifération, la migration et la différenciation fibrogénique des cellules musculaires lisses vasculaires après irradiation. D'autre part, le rôle essentiel de l'inhibiteur des activateurs du plasminogène de type I (PAI-1) dans les dommages radio-induits aux tissus sains a été démontré suggérant que PAI-1 constitue une cible thérapeutique prometteuse. De plus, deux nouveaux rôles clefs de la voie TGF-β/Smad dans la pathogénèse des dommages radio-induits ont été mis en évidence. Ainsi, la voie TGF-β/Smad est impliquée à la fois dans le phénotype fibrogénique des cellules musculaires lisses vasculaires induit par les cellules endothéliales et dans la stimulation radio-induite de PAI-1 dans les cellules endothéliales.

Mots Clefs : irradiation, tissus sains, cellules endothéliales, PAI-1, TGF-β, SMAD

More than half of cancers are treated with radiation therapy alone or in combination with surgery and/or chemotherapy . The goal of radiation therapy is to deliver enough ionising radiation to destroy cancer cells without exceeding the level that the surrounding healthy cells can tolerate. Unfortunately, radiation-induced normal tissue injury is still a dose limiting factor in the treatment of cancer with radiotherapy. The knowledge of normal tissue radiobiology is needed to determine molecular mechanisms involved in normal tissue pathogenic pathways in order to identify therapeutic targets and develop strategies to prevent and /or reduce side effects of radiation therapy. The endothelium is known to play a critical role in radiation-induced injury. Our work shows that endothelial cells promote vascular smooth muscle cell proliferation, migration and fibrogenic phenotype after irradiation. Moreover, we demonstrate for the first time the importance of PAI-1 in radiation-induced normal tissue damage suggesting that PAI-1 may represent a molecular target to limit injury following radiotherapy. We describe a new role for the TGF-β/Smad pathway in the pathogenesis of radiation-induced damages. TGF-β/Smad pathway is involved in the fibrogenic phenotype of VSMC induced by irradiated EC as well as in the radiation-induced PAI-1 expression in endothelial cells.

Key words : radiation, normal tissus, endothelial cells, PAI-1, TGF-β, SMAD