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)- β 1 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 interfering RNA targeting Smad3 and a TGF β -RII neutralizing antibody, we demonstrate that a TGF β -1/TGF β -RII/Smad3 pathway is involved in the fibrogenic phenotype of VSMCs induced by irradiated ECs. In con-

clusion, 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-1⁶ after irradiation leads to the increase of leukocyte/EC interactions and leukocyte transmigration. Moreover, irradiation increases the interactions of platelets with the endothelium⁷ 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

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- μ m sections were used to immunolocalize α -smooth muscle actin (α -SMA; Sigma, St. Quentin Fallavier, France), calponin (DAKO, Glostrup, Denmark), proliferating cell nuclear antigen (PCNA; DAKO), collagen I and collagen III (Sigma), transforming growth factor- β TGF- 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- μ m 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 μ g/ml propidium iodide, and analyzed using a FACSsort 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 un-

scratched 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 μ g 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'-CCATTC-CCCAGTGTGTTTCGTGC-3'), COL1A2 (F, 5'-TGAAAACATCCCAGCCAAGAA-3'; R, 5'-AAACTGGCTGCCAGCA-TTG-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- β 1 Enzyme-Linked Immunosorbent Assay (ELISA) Assay

TGF- β 1 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 interfering RNAs (siRNAs) designed to specifically target Smad3 is 5'-ACCUAUCCCGAAUCCGAUdTdT-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 $\pm$ 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 $\pm$ SEM of three experiments realized in triplicate or quadruplicate. * or # $P < 0.05$ 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 $\pm$ 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- β 1 Secretion Is Increased in ECs but not in VSMCs after Irradiation

TGF- β 1 is a well-known growth factor involved in VSMC fibrogenic phenotype. TGF- β 1 secretion was measured in supernatants of ECs and VSMCs by ELISA assay. Interestingly, secretion of total and active forms of TGF- β 1 increased after irradiation of ECs (Figure 5). No difference in the secretion of TGF- β 1 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)₃Lux 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 μ m) 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 $\pm$ 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- β 1 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 irra-

diated 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- β 1/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 $\pm$ SEM of two to four independent experiments realized in duplicate or 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

Figure 5. Irradiation increases TGF- β 1 secretion in ECs. Total and active TGF- β 1 contents were determined by ELISA assay in EC and VSMC supernatants 24 hours after 2 or 10 Gy irradiation. Data are the mean $\pm$ SEM of three experiments realized in triplicate. * $P < 0.05$ versus control.

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 al¹² 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 al¹³ 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 cancer¹⁵ 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 pro-fibrotic 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 sub-mucosal 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 nephrosclerosis²⁶ 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- β 1 mRNA and protein expression in ECs.³⁰ TGF- β signal transduction is initiated by ligand-induced heterodimeric complex formation of TGF β -RII with TGF β -RI, two serine/threonine kinase receptors. Activation of TGF β -RI by phosphorylation with the TGF β -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 knock-down 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 TGF β -RII blunted α -SMA, PAI-1, and collagen up-regulation. All together, these results demonstrate that a TGF β 1/TGF β -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 TGF β -RII neutralizing antibody, increases of CTGF and HSP27 remained unchanged, suggesting that a Smad3-dependent/TGF β 1-independent pathway may be involved. The Smad pathway can also be activated by a TGF β -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 receptors³⁴ and endothelin-1 via the ET_A receptor³⁵ 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.

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