A Hypoxia-Induced Vascular Endothelial-to-Mesenchymal Transition in Development of Radiation-Induced Pulmonary Fibrosis

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Abstract

Purpose: Radiation-induced pulmonary fibrosis (RIPF) is a late side effect of thoracic radiotherapy. The purpose of our study was to gain further insight into the development of RIPF.

Experimental Design/Results: Here, we observed that irradiation of mouse lungs induced collagen deposition, particularly around blood vessels, in the early phase of RIPF. Such deposition subsequently became evident throughout the irradiated tissues. Accompanied by the collagen deposition, vascular EndMT (endothelial-to-mesenchymal transition) began to develop in the early phase of RIPF, before the appearance of EMT (epithelial-to-mesenchymal transition) of alveolar epithelial (AE) II cells in the fibrotic phase. Concomitant with the EndMT, we detected vascular endothelial cell (EC)–specific hypoxic damage.

Introduction

Approximately 60% of patients with non–small cell lung cancer receive radiotherapy. Unfortunately, during conventional radiotherapy or stereotactic body radiotherapy, lung complications such as pneumonitis and fibrosis can cause significant morbidity in cancer survivors. Radiation-induced pulmonary fibrosis (RIPF) triggers physiologic abnormalities (1–3). Despite the pressing medical need, little progress has been made to mitigate the radiation-induced pneumonitis. In the past, several medications that the radiation-induced lung injuries, including in live cell models and human RIPF tissues. In human pulmonary artery endothelial cells (HPAEC), the radiation-induced EndMT via activation of TGFβ-R1/Smad signaling was dependent on HIF1α expression. A novel HIF1α inhibitor, 2-methoxyestradiol (2-ME), inhibited the irradiation-induced EndMT via downregulation of HIF1α-dependent Smad signaling. In vivo, 2-ME inhibited the vascular EndMT, and decreased the collagen deposition associated with RIPF. Furthermore, HIF1α-related EndMT was observed also in human RIPF tissues.

Conclusions: We provide the first evidence that an EndMT occurs in RIPF development and that the EndMT may be effectively inhibited by modulating vascular EC-specific hypoxic damage. Clin Cancer Res. 2015; 21(16):3716–26. ©2015 AACR.
Activated myofibroblasts play central roles in the production of collagen and ECM proteins during pulmonary fibrosis. Myofibroblasts are derived from various different cell types—including resident stromal fibroblasts, bone marrow–derived fibrocytes, and the mesenchymal transition of epithelial cells (the epithelial-to-mesenchymal transition, EMT; ref. 8). Several studies have shown that alveolar type II epithelial cells undergo EMT during development of pulmonary fibrosis, including RIPF (9, 10). Recently, the endothelial-to-mesenchymal transition (EndMT) has been suggested to give rise to cellular phenotypes that closely resemble myofibroblasts.

The EndMT is characterized by loss of cell–cell junctions and the acquisition of invasive and migratory phenotypes. Mesenchymal cell markers such as α-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP-1), and vimentin are upregulated. On the other hand, EC-specific markers, including CD31 and vascular endothelial (VE)-cadherin, are downregulated. There is increasing evidence that TGFβ signaling and the transcriptional activators Snail and Twist are important regulators of the EndMT—hence, the heart and kidney, and in cancer (8, 11, 12). Hashimoto and colleagues (13) demonstrated that EndMT might also serve as a source of fibroblasts in bleomycin-induced pulmonary fibrosis.

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Here, we report that during the development of RIPF in mouse lung (1) endothelial-specific hypoxic damage was evident before formation of fibrotic lesions. In particular, (ii) EndMT occurred principally in large vessels, accompanied by hypoxic damage, and finally, (iii) the EndMT appeared before the development of EMT in alveoli.

Together, our data demonstrated that initial hypoxic vascular damage caused by irradiation leads to chronic pulmonary fibrosis, and that the EndMT may be a novel target for prevention of RIPF.
using Lipofectamine 2000 (Invitrogen). For irradiation, cells were exposed to gamma rays derived from a $^{137}$Cs source (Atomic Energy of Canada, Mississauga, Ontario, Canada) at a dose rate of 3.81 Gy/min. To establish hypoxia, cells were incubated in a Forma 1025/1029 Anaerobic Chamber (Thermo Fisher Scientific) flushed with (all v/v) 1% O$_2$, 5% CO$_2$, and 94% N$_2$.

**In vitro tests**

Immunoblotting was performed as described previously (18) using antibodies against VEGFR1, VEGFR2, CD31, VE-cadherin, collagen I, MMP9, TGFβRI (ALK5), vimentin, E-selectin, VCAM1, ICAM1 (all from Santa Cruz Biotechnology); α-SMA and FSP1 (Abcam); p-Smad2/3, Smad2/3, and Snail (Cell Signaling Technology); HIF1α (BD Biosciences), and β-actin (Sigma-Aldrich).

For immunofluorescence staining, cells were fixed in 4% (v/v) paraformaldehyde, washed with PBS, and next incubated for 15 minutes with 0.01% (v/v) Triton X-100 in PBS. Cells were next incubated with solutions containing 1 μg/mL of antibodies against VE-cadherin, FSP1, CA9, and p-Smad2/3. After washing, fluorescent secondary antibodies (Molecular Probes; Invitrogen) were added at dilutions of 1:500. The cells were again washed with PBS, counterstained with DAPI, and imaged under a confocal laser-scanning microscope (Leica Microsystems). Before counter-staining, cells were stained with Alexa488-conjugated phalloidin (Invitrogen).

TGFβ1 released into culture medium was measured using a human TGFβ1 ELISA kit (Enzo Life Sciences) according to the manufacturer's instructions.

**Statistical analyses**

The Student t test and ANOVA were used to explore the statistical significance of differences between experimental groups. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc.).

**Results**

The pattern of collagen deposition in RIPF

To study RIPF in mouse lungs, we used three different irradiation doses to different tissue volumes, reflecting the various forms of radiotherapy (16). To examine fibrosis development, lung sections prepared at various times after irradiation were stained with Masson's trichrome. When 20 Gy was delivered using a large-beam collimator (7-mm-diameter field) to the left lungs of mice, the collagen deposition was significantly elevated 6 months later, and substantial amounts of fibrotic tissue were noted at 9 months after irradiation (Fig. 1A and Supplementary Fig. S1A). This fibrosis pattern after 20 Gy irradiation was compared with that after ablative focal irradiation with 50 or 90 Gy delivered using a microbeam collimator (3-mm-diameter field; Fig. 1A and Supplementary Fig. S1B). The collagen deposition in lungs receiving 50 Gy using the 3-mm collimator rose slightly 2 months after irradiation, and then rose markedly at 6 months (Fig. 1A). After focal delivery of 90 Gy, significant collagen deposition occurred in the vessels in 2 weeks, which then significantly increased at 4 weeks, indicative of late-stage fibrosis (Fig. 1A).

Interestingly, collagen deposition caused by the three irradiation models commenced principally around blood vessels, especially arteries, but not alveolar capillaries, before the development of substantial fibrosis. Early collagen deposition was rather vascular-specific, particularly at 3 months after 20 Gy irradiation, 2 months after 50 Gy, and 2 weeks after 90 Gy irradiation (Fig. 1A). These results indicated that collagen deposition around vessels may be important in the development of RIPF.

EndMT during development of RIPF

We showed previously that EndMT of human aortic ECs was associated with radiation-induced atherosclerotic tissues (15). To examine the role of EndMT in fibrosis in the development of RIPF, we first determined whether the expression of α-SMA (a fibroblastic marker) and CD31 (an EC-specific marker) are changed in irradiated lung tissue, using immunofluorescence analysis.

Under all three test conditions (20, 50, and 90 Gy), colocalization of α-SMA and CD31 was evident during the development of RIPF (Fig. 1B). A significant increase in the colocalization was evident 2 months after 20 Gy irradiation (Fig. 1B). The extent of overlapping signal (colocalization) steadily increased up to 9 months and decreased at 12 months, at the time of late-stage fibrosis (Fig. 1B, right). It was evident that endothelial and fibroctic markers colocalized as fibrosis progressed and the former marker ultimately disappeared, completing the phenotypic switch (Supplementary Fig. S2A at, 12 month 20 Gy). These time-dependent effects may be attributed to EndMT progression in the lung irradiated with 20 Gy. Similar results were observed after irradiation with 50 or 90 Gy. Upon 50 Gy irradiation, the overlapping signals (the coefficients) of colocalization peaked 4 months after irradiation, thus, before substantial fibrotic changes were evident (at 6 months; Fig. 1B and Supplementary Fig. S2A). Similarly, upon 90-Gy irradiation, the extent of colocalization increased steadily, accompanied by collagen deposition, peaking at 5 days. Thereafter, the colocalization of α-SMA and CD31 continued to decline, as late-stage fibrosis was attained (Fig. 1B and Supplementary Fig. S2A).

Emerging recent evidence indicated that EMT plays an important role in RIPF (9, 10). In general, EMT is characterized by increased expression of α-SMA and vimentin, and decreased expression of the epithelial markers E-cadherin and pro–SP-C in alveolar epithelial (AE) type II cells (19–21). Lung ECs are of both the AE I and II types. The radiation-induced EMT that develops during lung fibrosis occurs principally in AE II cells (9, 10). To elucidate the possible relationship between EndMT and EMT during induction of RIPF, we determined the extent of colocalization of α-SMA and pro–SP-C (pulmonary surfactant Protein C, a marker of AEII cells) in irradiated lung tissue. EndMT occurred principally around vessels, not alveoli.

AE II cells did not express α-SMA in the early stages of RIPF, indicating that EMT was not active when EndMT commenced (Fig. 1B and Supplementary Fig. S2). EMT of AE II cells was prominent at the time of development of substantial fibrosis, particularly at 9 and 12 months after 20 Gy irradiation: 4 and 6 months after 50 Gy, and 3 and 4 weeks after 90 Gy irradiation (Supplementary Fig. S2B). EMT gradually increased in extent until the late stage of RIPF was attained.

Together, the data suggest that the radiation-induced vascular EndMT before EMT may provide an important initial target for prevention of RIPF.

Radiation-induced EndMT via TGFβ receptor/Smad signaling

The mechanism of irradiation-induced EndMT was investigated with human pulmonary artery endothelial cells (HPAEC) in vitro. As shown in Fig. 2A, after irradiation with 10 Gy, the
expression of fibroblast markers, including vimentin, FSP1, and α-SMA, was upregulated and that of EC markers, including VEGFR1, VEGFR2, CD31, and VE-cadherin, was downregulated. The immunofluorescence assay showed that irradiation increased the level of FSP1 and decreased that of CD31. Also, irradiation increased the levels of MMP9 and collagen, indicating that radiation triggered EndMT in HPAECs. In addition, irradiation induced progressive increase in the levels of ICAM-1, VCAM-1, and E-selectin, indicating that the ECs were activated and underwent phenotypic changes (Fig. 2A).

The irradiation-induced EndMT developed in a radiation dose-dependent manner. After 10 Gy irradiation, the expression of α-SMA increased continuously from day 2 to 7. The level of CD31 slightly decreased by 4 hours, and then progressively decreased from day 2 to 7 after irradiation (Fig. 2B). The increase in α-SMA level and decrease in CD31 level after irradiation with 2 Gy or 5 Gy were apparently less than those after 10 Gy irradiation. It has been reported that EndMT-derived cells produce various growth factors, including TGFβ (11). We investigated whether the cells induced to enter EndMT by irradiation release TGFβ by performing ELISA assays of the culture supernatants of various human pulmonary cell lines. Although HPF cells secreted more TGFβ than did ECs before irradiation, ECs (HPAECs and HPMECs, Human pulmonary microvascular ECs) released more TGFβ than did any other cell line tested, 2 days after 10 Gy irradiation (Fig. 2C). Thus, we concluded that ECs are more sensitive to radiation-induced fibroblastic changes than other cell lines.
It has been demonstrated that TGFβ enhances Smad3 transcriptional activity and that Notch and rhBMP-7 are important modulators of the EndMT occurring during tissue fibrosis (8, 12, 22). As it has been reported that TGFβ plays a key role in RIPF, we explored whether the signaling cascade triggered by the TGFβ receptor was associated with the radiation-induced EndMT in HPAECs. As shown in Fig. 2A, the expression of TGFβ receptor 1, and the extent of Smad3 phosphorylation, were increased during the radiation-induced EndMT. Immuno-fluorescence test showed that the addition of TGFβ-R1 inhibitor attenuated the decrease in VE-cadherin level and the increase in FSP-1 level triggered by the radiation-induced EndMT (Fig. 2D, left). Immunoassay revealed that the increases in α-SMA and vimentin levels were inhibited by the TGFβ-R1 inhibitor (Fig. 2D, right).

Next, to examine whether the TGFβ-R1 inhibitor reduces EndMT during RIPF development, we irradiated thoracic regions of C57BL/6 mice with 16 Gy. In line with the in vitro data, trichrome staining showed that the TGFβ-R1 inhibitor reduced the extent of RIPF in vivo, attenuating irradiation-induced collagen deposition in vascular regions (Fig. 2E, top). Consistently, vascular EndMT lesions that appeared during RIPF were significantly decreased by the injection of the TGFβ-R1 inhibitor (Fig. 2E, bottom).
Taken together, the data strongly suggest that irradiation-induced EndMT features in the development of RIPF and may be a potential new target for reducing or eliminating RIPF.

Implication of hypoxic damage to ECs in the development of irradiation-induced fibrosis

As significant fibrosis and EndMT around vessels were evident during the development of RIPF, we sought to define the EC-specific damage pattern triggered by irradiation.

Interestingly, we observed that hypoxic damage specific to vascular ECs occurs before the appearance of substantial fibrosis under all three irradiation conditions by immunostaining for CA9 as a hypoxic marker (Fig. 3A). In the fibrotic stage, hypoxic damage seemed to have propagated to all tissues. Because the time of appearance of EC-specific hypoxic damage was similar to that of development of the EndMT, thus, before the formation of fibrotic tissue, we suggest that radiation-induced hypoxic damage to vascular ECs may be directly associated with the EndMT.
leading to chronic fibrosis. Consistent with this hypothesis, HIF1α-positive ECs were observed in irradiated vessels (Fig. 3B).

**Hypoxia-induced EndMT involves TGFβRI/Smad signaling in RIPF**

HPAECs exhibited high levels of CA9 and actin stress fibers 3 days after 10 Gy irradiation (Fig. 3C, top). Staining with Alexa488-phalloidin revealed the presence of filamentous actin, one of the hallmarks of EndMT (the filaments form actin stress fibers; ref. 23). Western blot assay also revealed an increase in HIF1α expression, and a subsequent increase in the α-SMA level during 7 days after irradiation in HPAECs (Fig. 3C, bottom).

To elucidate the role of EC-specific hypoxic damage in the irradiation-induced EndMT, we exposed HPMECs to hypoxia (1% O2) for 1 to 7 days. The expression of HIF1α increased from 6 to 120 hours of exposure to hypoxia, and the level of CD31 decreased whereas α-SMA expression increased from 24 hours. As the EndMT commenced after an exposure to hypoxia for 24 hours, phosphorylation of Smad3 was significantly increased and expression of TGFβ receptor I and Snail1 were significantly increased (Fig. 3D). Immunofluorescence data showed that the hypoxia-induced morphologic and phenotypic changes were consistent with EndMT phenotype. The exposure to hypoxia caused cells to become elongated, and increased filamentous actin formation and Smad2/3 phosphorylation in the cells treated with control-siRNA (Fig. 3E). On the other hand, the cells treated with Smad3-siRNA did not display EndMT phenotype, and exhibited reduced α-SMA and vimentin expression, compared with control siRNA-treated cells (Fig. 3E). On the other hand, the cells treated with Smad3-siRNA did not display EndMT phenotype, and exhibited reduced α-SMA and vimentin expression, compared with control siRNA-treated cells (Fig. 3E, right). These data indicated that hypoxia, like irradiation, induces EndMT via TGFβ receptor I/Smad signaling. To assess the contribution of HIF1α to the irradiation-induced EndMT, we transfected control-siRNA or HIF1α-specific siRNA into HPAECs. HIF1α siRNA prevented the decrease in VE-cadherin level and increases in CA9 and filamentous actin levels (Fig. 3F, top). Immunoblotting also showed that HIF1α knockdown reduced the irradiation-induced phosphorylation of Smad2/3, and the increases in α-SMA and vimentin expression, compared with control siRNA-treated cells (Fig. 3F, bottom).

Taken together, these findings suggest that in HPAECs, radiation-induced hypoxia triggers EndMT via HIF1α-mediated activation of TGFβ receptor I/Smad signaling.

**2-ME inhibits radiation-induced EndMT during development of RIPF**

Analogues of 2-ME are promising HIF1α-inhibitory agents, and are under active clinical development (24). Several mechanisms have been suggested to account for the action of 2-ME: disruption of microtubules by binding to the colchicine-binding site of tubulin (25) and inhibition of TGFβ-mediated collagen synthesis, α-SMA production, and Smad2/3 phosphorylation (25). These possible mechanisms would all exert inhibitory effects on the HIF1α-dependent EndMT before development of RIPF, and we thus studied the effect of 2-ME on the irradiation-induced EndMT in vitro. Pretreatment of HPAECs with 2-ME inhibited the development of irradiation-induced EndMT phenotype. 2-ME also blocked filamentous actin formation, increased CA9 expression and the loss of VE-cadherin (Fig. 4A, top). In addition, 2-ME inhibited HIF1α expression, phosphorylation of Smad2/3, and increases in β-SMA during irradiation-induced EndMT (Fig. 4A, bottom).

Moreover, in vivo, treatment with 2-ME markedly reduced the vascular deposition of collagen associated with RIPF development and increase in HIF1α expression in vascular ECs after thoracic irradiation (Fig. 4B). Simultaneously, 2-ME inhibited the EndMT, as assessed by the extent of colocalization of α-SMA and CD31 in vascular ECs of irradiated lung tissue. Also, 2-ME inhibited the EndMT, with a concomitant increase in HIF1α levels in vascular ECs (Fig. 4B), followed by decrease of the EMT occurrence and substantial fibrotic phase in the irradiated lung tissue (Supplementary Fig. S3). In addition, treatment with 2-ME significantly reduced RIPF in the lung irradiated with 90 Gy (3-mm collimator; Supplementary Fig. S4).

In conclusion, we suggest that HIF1α expression and the EndMT phenotype may play important roles in the development of RIPF, and that 2-ME may be useful to prevent RIPF.

**HIF1α is upregulated on vascular EndMT in the fibrotic regions of irradiated human lung tissue**

Next, we examined whether HIF1α expression is upregulated on vascular ECs and concomitantly, EndMT occurs in radiation-induced lung fibrotic tissues of human patients with lung adenocarcinoma. Fibrotic normal tissues of patients with lung cancer who underwent surgery following neoadjuvant radiotherapy for lung adenocarcinoma were selected on the basis of H&E staining (Supplementary Fig. S5 and Supplementary Table S1). Most patients underwent the radiotherapy of 45 to 54 Gy in 25 to 30 fractions and surgery about 40 days after radiotherapy. More detailed information with concurrent chemotherapy of human tissues are shown in Supplementary Table S1. Immunofluorescence examination for HIF1α, CD31 and α-SMA was performed on 10 patient tissues samples of RIPF and three samples of normal lung tissues. As shown in Fig. 5B, significant EndMT was observed in the tissues of RIPF. Most EndMT in vascular ECs exhibited upregulated HIF1α, whereas HIF1α-negative vascular ECs did not express α-SMA. For example, see the CD31-positive vessel marked with the open arrow in the tissue of patient #5 (Fig. 5). These clinical results are in accordance with our in vitro data with mouse lung, and support the hypothesis that hypoxia-induced vascular EndMT contributes to RIPF.

**Discussion**

RIPF is a frequently observed side effect of radiotherapy of lung cancer. Pulmonary fibrosis typically develops between 6 and 24 months after radiotherapy, and stabilizes after 2 years (4). Recently, high-dose per fraction hypofractionated radiotherapy such as stereotactic body radiotherapy (e.g., three fractions of 20 Gy) has emerged as a useful modality for various cancers. This new radiotherapy modality has been demonstrated to be highly effective for controlling various cancers, including early stage non–small cell lung cancer. Although this technique is highly confirmative, and thus minimizes normal tissue complications, serious complications have nonetheless appeared (4).

In the present study, we investigated the development of RIPF in mouse caused by three irradiation conditions. Delivery of 90 or 50 Gy with a microbeam collimator (3 mm in diameter) to left lungs induced RIPF in 2 weeks and 6 months later, respectively (Fig. 1A). Delivery of 20 Gy through a 7-mm-diameter beam collimator to left lungs induced RIPF in 9 months (Fig. 1A). EndMT accompanying phenotypic alterations during development of RIPF was common to all irradiation conditions (Fig. 1B).
and Supplementary Fig. S2). Therefore, we suggest that minimizing of the EndMT may be effective to counter RIPF developing after various thoracic radiotherapies.

It is well known that radiation-induced vascular damage plays an important role in the radiation-induced complications of normal tissue (14, 26). The radiosensitivity of various blood vessels has been shown to be dependent on vessel types: radiosensitivity decreases in order of capillaries > small arteries > medium-sized arteries > large arteries > small veins > large veins (26). Vascular fibrosis develops principally in arterioles, arteries, and large veins. The EC response in normal tissues to irradiation is associated with early fibrogenesis (5, 27). Thus, the preventive measure of EC damage is likely to reduce vascular damage during the early phases of tissue injury, and minimizes the late damage in irradiated normal tissues (28).

Previously, Molteni and colleagues (28) reported that pulmonary damage progressed following endothelial detachment and blebs formation several days after 20 Gy irradiation in rats and severe arteritis and interstitial collagen deposition occurred 3 months after the irradiation. Santana and colleagues (29) found that apoptotic death of ECs were evident in mouse lungs 10 hours after whole-body irradiation with 20 Gy. We also observed that whole-body (1 Gy, 5 times) or thoracic (25 Gy) irradiation of mice induced apoptosis and detachment of lung ECs several days later (Supplementary Fig. S6).

In the present study, we focused on the vascular ECs that survived and formed pulmonary vascular structures in irradiated lungs because we hypothesized that the surviving ECs may cause vascular dysfunction or the late pulmonary fibrosis.

Our finding that EndMT occurs during the development of RIPF led us to wonder how the early EC damage triggers the radiation-induced late effects, such as vascular fibrosis (Fig. 1B). We observed that vascular ECs (specifically) were positive for a hypoxic marker, CA9, before substantial fibrogenesis was evident and Supplementary Fig. S2). Therefore, we suggest that minimizing of the EndMT may be effective to counter RIPF developing after various thoracic radiotherapies.

Figure 4. The effects of 2-ME on radiation-induced EndMT and RIPF. A. HPAECs were pretreated for 1 hours with 0 or 10 ng/mL 2-ME and irradiated with 10 Gy. At 72 hours after irradiation, the levels of phalloidin (green), CA9 (white), and VE-cadherin (red) were assessed via immunofluorescence staining (top). Cell proteins were subjected to western blotting using the indicated antibodies (bottom). B. C57BL/6 mice (n = 8/group) were pretreated with 2-ME (60 mg/kg) or vehicle and the thoracic part of the left lung was 16 Gy irradiated. After irradiation, 2-ME treatment was continued on 3 days per week for 2 weeks, and lung samples then obtained 1 month after irradiation. The images show representative HIF1α immunohistochemical data, Masson’s trichrome staining patterns (scale bar, 100 μm) and colocalization (yellow pixels) of CD31 and α-SMA or HIF1α, CD31 and α-SMA. The graphs indicate as the percentage of the vascular area positive for HIF-1α of total vascular area, the relative levels of vascular collagen deposition and colocalization of CD31 and α-SMA or HIF1α, CD31 and α-SMA in vessels, from the five ×100 fields (error bars indicate the SEMs of eight mice/group. **, P < 0.0001; **, P < 0.01 vs. IR alone). Data are representative of three independent experiments.
It thus appeared that hypoxic damage might induce EndMT, thereby forming fibroblasts that cause lung fibrogenesis (Fig. 3C–F and Fig. 4). In our previous reports (16), we showed that when lung tissues of mice were irradiated with 90 Gy (a 3-mm-diameter field), severe hemorrhage with vascular destruction was evident, and the arterial wall thickness increased in 9 days. Increased alveolar wall thickness and destruction were evident after 5 and 7 days, respectively (16). After delivery of 20 Gy of irradiation (a 7-mm-diameter field), fibrosis developed about 6 months later. We therefore hypothesize that direct vascular damage or chronic inflammatory response may trigger vascular dysfunction, leading to tissue hypoxia. Concomitant with the development of vascular dysfunction in irradiated lung tissues, vascular ECs may become hypoxic and specifically positive for CA-9 or HIF1α in the early phase of RIPF development (Fig. 3A and B). In this respect, Fleckenstein and colleagues (7) suggested that radiation-induced hypoxia in lung tissues is caused, in part, by increased oxygen consumption by macrophages, which are activated because of radiation-induced reductions in blood perfusion. Also, it was shown that the initial tissue hypoxia was followed by chronic oxidative stress in irradiated lung tissue, suggesting that hypoxia is one of the driving forces in initiating radiation-induced lung injury (7). Vujaskovic and colleagues (30) also reported that hypoxia was important in triggering continuous production of fibrogenic cytokines and perpetuation of late lung tissue injury.

Relevant to our finding of radiation-induced EndMT during RIPF development, several reports have described EMT-associated RIPF (9, 10). We found that in the development of RIPF, vascular EndMT appeared before an EMT (Fig. 1 and Supplementary Fig. S2). The EMT during development of RIPF was principally in alveolar ECs. The different time course for the occurrence of EndMT and EMT lead us to hypothesize that the initial vascular hypoxic damage after irradiation may propagate to lung tissue, including alveoli, and that EMT in AE cells may be indirectly caused by both fibrogenic cytokines released during the EndMT and hypoxia, apart from the direct damage to AE cells caused by irradiation. In addition, we may not exclude the possibility that EMT is partially independent of EndMT. EndMT was more prominent at 90 Gy than at 20 Gy, although EMT at 20 Gy was more dominant than that at 90 Gy (Supplementary Fig. S2). In addition, the mechanism by which RIPF is caused by 90 Gy irradiation may differ from the mechanism at 20 Gy, as 90 Gy irradiation induced more rapid RIPF in comparison with 20 Gy irradiation. Thus, to determine whether...
EndMT is a direct pathogenesis of RIPF or affects EMT occurrence, we are now studying RIPF in genetically engineered mouse models with modified EC-specific genes that regulate EndMT.

It has been shown that targeting TGFβ receptor I/Smad3 signaling, and downstream targets such as Snail, inhibits EndMT under pathologic conditions (12). In the present work, we also found that the irradiation-induced EndMT was regulated by TGFβ-R1/Smad signaling. Moreover, the EndMT caused by hypoxia was triggered by such signaling, in association with increased expression of HIF-1α. Thus, the irradiation-induced EndMT triggered by TGFβ-R1/Smad signaling could be affected by knockdown of HIF-1α (Fig. 3).

TGFβ receptor (ALK5) kinase activity has recently been reported to require high-level HIF-1α expression in response to TGFβ1 (31). Several reports have suggested that TGFβ and HIF-1α engage in mutual regulation (31–33). In agreement with these reports, we found that the hypoxia-induced EndMT required TGFβ receptor/Smad signaling; we found that Smad3 siRNA decreased HIF-1α expression during the hypoxia-induced EndMT whereas HIF-1α siRNA inhibited radiation-induced EndMT, accompanied by a decrease in TGFβ receptor/Smad signaling. These results suggest that, during irradiation-induced EndMT, the expression of HIF-1α is regulated both by hypoxia-induced TGFβ receptor activity and various. Increases in HIF-1α levels caused by direct hypoxic stress (34) regulated TGFβ receptor/Smad3 signaling (Fig. 3). We are now further elucidating these mutual regulatory mechanisms.

Although much effort has focused on overcoming irradiation-induced complications in normal tissues, the clinical utilities of existing drugs are limited by drug toxicity or the radioprotection of tumors (3). 2-ME, a metabolite of 17beta-estradiol, has been shown to exert marked anticarcinogenic properties in several malignant cell types, and phase I/II clinical trials of 2-ME are currently underway in patients with prostate, breast, and metastatic breast cancer (35). In the present study, we investigate the efficacy of 2-ME to inhibit RIPF because, recently, 2-ME has been suggested to effectively inhibit HIF-1α action even though 2-ME has other effects such as microtubule disruption. In vivo, 2-ME indeed inhibited the radiation-induced increase in HIF-1α expression, showing the decreases of EndMT and concomitant vascular collagen deposits appeared in the development of RIPF (Fig. 4). 2-ME also reduced EMT and the substantial fibrotic phase (Supplementary Fig. S3). Although other investigators also reported that 2-ME enhances tumor radiosensitivity (36–39), further studies are needed for the better understanding of the potential usefulness of 2-ME to radiosensitize tumors. In addition, our studies with human tissues clearly indicated that EndMT mostly was detected on HIF-1α-positive vascular ECs in RIPF tissues and not in normal lung tissues (Fig. 5).

In summary, we provide new insights into the pathogenesis of RIPF. We suggest that irradiation-induced vascular hypoxia trigger vascular EndMT via activation of HIF-1α, thereby leading to chronic tissue fibrosis. Thus, inhibition of EndMT may be an effective strategy to halt RIPF at its early stage. The clinical implication is that targeting irradiation-induced vascular hypoxia may efficiently minimize normal tissue damage.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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3. Sasse AD, Clark LG, Sasse EC, Clark OA. Amifostine reduces side effects and positive vascular ECs in RIPF tissues and needed for the better understanding of the potential usefulness of 2-ME to radiosensitize tumors. In addition, our studies with human tissues clearly indicated that EndMT mostly was detected on HIF-1α-positive vascular ECs in RIPF tissues and not in normal lung tissues (Fig. 5).

In summary, we provide new insights into the pathogenesis of RIPF. We suggest that irradiation-induced vascular hypoxia trigger vascular EndMT via activation of HIF-1α, thereby leading to chronic tissue fibrosis. Thus, inhibition of EndMT may be an effective strategy to halt RIPF at its early stage. The clinical implication is that targeting irradiation-induced vascular hypoxia may efficiently minimize normal tissue damage.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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