A hypoxia-induced vascular endothelial-to-mesenchymal transition in development of radiation-induced pulmonary fibrosis

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Translational relevance

Radiation-induced pulmonary fibrosis (RIPF) is a late side effect of thoracic radiotherapy, causing significant morbidity and mortality. Here, we provide the first evidence that an vascular EndMT caused by initial vascular hypoxic damage leads to RIPF development, prior to EMT in aveoli. Moreover, HIF1-α-related EndMT was prominently observed in the RIPF tissues of lung cancer patients received radiotherapy. In addition, we show that a promising HIF1-α-inhibitory agent (2-ME, currently in phase I/II trials as several cancer therapeutics) prevents RIPF by inhibiting vascular EndMT. In this study, we suggest that a vascular hypoxia-related EndMT may provide an important initial target for RIPF prevention.
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 substantive fibrotic phase.

Concomitant with the EndMT, we detected vascular endothelial-cell-specific hypoxic damage in the irradiated lung tissues. In human pulmonary artery endothelial cells (HPAECs), the radiation-induced EndMT via activation of TGFβ-R1/Smad signaling was dependent on HIF1-α expression. A novel HIF1-α inhibitor, 2-Methoxyestradiol (2-ME), inhibited the irradiation-induced EndMT via downregulation of HIF1-α-dependent Smad signaling. In vivo, 2-ME inhibited the vascular EndMT, and decreased the collagen deposition associated with RIPF. Furthermore, HIF1-α-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 endothelial-cell-specific hypoxic damage.
Introduction

Approximately 60% of non-small-cell lung cancer (NSCLC) patients receive radiation therapy (RT). Unfortunately, during conventional radiotherapy (RT) or stereotactic body radiation therapy (SBRT), lung complications such as pneumonitis and fibrosis can cause significant morbidity in cancer survivors. Radiation-induced pulmonary fibrosis (RIPF) triggers physiological abnormalities (1-3). Despite the pressing medical need, little progress has been made to mitigate the radiation-induced pneumonitis. In the past, several medications have been developed to reduce radiation pneumonitis such as corticosteroids, amifostine, ACE inhibitors or angiotensin II type 1 receptor blockers, pentoxifylline, melatonin, carvedilol, and a manganese superoxide dismutase-encoding plasmid delivered in liposomes (4). Unfortunately, however, their efficacy has been found to be unsatisfactory.

Lung fibrosis develops 6-12 months after lung irradiation, and is characterized by fibroblast proliferation with excessive extracellular matrix (ECM) deposition. It has been demonstrated that the radiation-induced lung fibrosis is preceded by vascular damages within a few weeks of irradiation, such as narrowing and obliteration of capillaries by endothelial cell (EC) swelling, growth of new fibrin plugs, and endothelial hyperplasia, particularly in larger vessels, both arteries and veins (5). Depletion of functional microvasculature would inevitably lead to tissue ischemia and hypoxia.

Although reactive oxygen species (ROS), transforming growth factor-beta (TGF-β), inflammatory cytokines, and hypoxia have been known to participate in the development of chronic fibrosis in the lung, the precise mechanism as to how the initial radiation injuries progress to chronic fibrosis has been elusive (1, 6). Hypoxia has been implicated in radiation-induced lung injuries, including inflammatory and fibrogenic responses. Fleckenstein et al. (7) reported that tissue hypoxia after irradiation was associated with increase in oxygen consumption due to a significant increase in macrophage activation, which in turn caused...
continuous production of ROS. It is likely that the elevation of ROS production stimulates the synthesis of fibrogenic and angiogenic cytokines, thereby triggering chronic radiation-induced lung injury. However, the precise mechanism by which hypoxia affects radiation-induced lung injuries remains unclear.

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, or EMT) (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 fibroblasts during fibrosis of 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.

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, endothelial cell-specific markers including CD31 and vascular endothelial (VE)-cadherin are downregulated. There is increasing evidence that TGF-β signaling and the transcriptional activators Snail and Twist are important regulators of the EndMT in pulmonary fibrosis. However, their precise role in the radiation-induced pulmonary fibrosis has not been clearly understood. Although vascular dysfunctions are well-known to play important roles in the pathogenesis of radiation injury in normal tissues (14, 15), the origins of such dysfunctions remain unclear. We previously reported that radiation-induced atherosclerosis was associated with EndMT. Especially, EC dysfunction was associated with
loss of thromboresistance and increases in cytokine levels. In irradiated human tissues, the prominent fibrosis developing around vascular lesions was characterized by collagen and fibroblast deposition (1, 14).

Here, we report that during the development of RIPF in mouse lung (1) endothelial-specific hypoxic damage was evident prior to formation of fibrotic lesions. In particular, (2) EndMT occurred principally in large vessels, accompanied by hypoxic damage, and finally, (3) the EndMT appeared prior to 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.
**Methods**

**Mice and irradiation**

All procedures were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences, and the Yonsei University Medical School. Radiation was delivered using an X-RAD 320 platform (Precision X-ray, North Branford, CT) as described previously (16). The left lungs of 10-week-old male C57BL/6 mice were irradiated using a 7-mm-diameter field or a 3-mm-diameter field.

ALK5 inhibitor II (Santa Cruz Biotechnology, Dallas, TX) was dissolved in DMSO, further diluted in distilled water, and administrated intraperitoneally (25 mg/kg).

2-ME (Selleckchem, Houston, TX) was dissolved in DMSO and further diluted in 30% (w/v) PEG-400 with 1% (v/v) Tween 80 prior to intraperitoneal injection (60 mg/kg).

**Tissue histology and immunohistochemical staining**

Mice were euthanized and lung tissues harvested and fixed in 10% (v/v) neutral buffered formalin prior to preparation of paraffin sections. Paraffin-embedded sections were deparaffinized and stained with hematoxylin and eosin (H&E; Sigma-Aldrich), or using a Masson’s trichrome stain kit (Sigma-Aldrich) to detect collagen.

Prior to immunohistochemistry, deparaffinized sections were boiled in 0.1 M citrate buffer (pH 6.0) for 30 min and next incubated with 0.3% (v/v) hydrogen peroxide in methanol for 15 min. Sections were blocked in normal horse serum at room temperature for 30 min and immunostained overnight at 4°C with primary antibodies against CD31 (1:100; Santa Cruz), α-SMA (1:100; Abcam, Cambridge, MA), Pro-SPC (1:2,000; Millipore, Schwalbach, Germany), CA9 (1:1,000; Novus Biologicals, Littleton, CO), and HIF1-α (1:100; Santa Cruz). The target proteins were visualized using ABC and DAB kits (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin. For
immunofluorescence staining, sections stained with primary antibodies were incubated with appropriate fluorescently labeled secondary antibodies (1:250; Molecular Probes, Eugene, OR) and counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 3 μM). Images were obtained using a Zeiss microscope. Additional immunohistochemical analyses were performed as described previously (17).

**Human tissues analysis**

The clinical pathological characteristics of the patients analyzed in this study are summarized in Supplementary Table1. The study on specimens of RIPF from patients was approved by Severance Hospital, Yonsei University. Immunostaining, was done for CD31 (1:100; Santa Cruz), α-SMA (1:100; Abcam) and HIF1-α (1:100; Santa Cruz). In addition, tissue microarray including three normal lung tissues was purchased from Biocompare (South San Francisco, CA).

**Cell culture and treatment**

HPAECs (Human Pulmonary Artery ECs), HPMECs (Human Pulmonary Microvascular ECs), HSAEpCs (Human Small Airway Epithelial Cells), HPASMCs (Human Pulmonary Artery Smooth Muscle Cells), and HPFs (Human Pulmonary Fibroblasts), were obtained from PromoCell (Heidelberg, Germany). All cells were used within nine passages.

Smad2/3 siRNA, HIF1α siRNA, and control siRNA were purchased from Santa Cruz Biotechnology and transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For irradiation, cells were exposed to gamma rays derived from a [137Cs] 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, Waltham, MA) flushed with (all v/v) 1% O2, 5% CO2, and 94% N2.
**In vitro tests**

Immunoblotting was performed as described previously (18) using antibodies against VEGFR1, VEGFR2, CD31, VE-cadherin, collagen I, MMP9, TGFβ-RI (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, Beverly, MA); HIF1-α (BD Biosciences, San Jose, CA), and β-actin (Sigma-Aldrich).

For immunofluorescence staining, cells were fixed in 4% (v/v) paraformaldehyde, washed with PBS, and next incubated for 15 min 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). Prior to counterstaining, cells were stained with Alexa488-conjugated phalloidin (Invitrogen).

TGF-β1 released into culture medium was measured using a human TGF-β1 ELISA kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer’s instructions.

**Statistical analyses**

Student’s *t*-test and analysis of variance (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., San Diego, CA).
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 post-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 micro-beam collimator (3-mm-diameter field) (Fig. 1A and Supplementary Fig. S1B). The collagen deposition in lungs receiving 50 Gy employing 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 two 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, prior to 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 endothelial cells was associated with radiation-induced atherosclerotic tissues (15). To examine the role of EndMT in fibrosis in
the development of RIRF, 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), co-localization of α-SMA and CD31 was evident during the development of RIPF (Fig. 1B). A significant increase in the co-localization was evident 2 months after 20 Gy irradiation (Fig. 1B). The extent of overlapping signal (co-localization) 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 fibrotic markers co-localized 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 co-localization 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 co-localization increased steadily, accompanied by collagen deposition, peaking at day 5. Thereafter, the co-localization 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 type (AE) II cells (19). 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 co-localization of α-SMA and pro-SP-c (pro-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 prior to EMT may provide an important initial target for prevention of RIPF.

Radiation-induced EndMT via TGF-β Receptor/Smad Signaling

The mechanism of irradiation-induced EndMT was investigated with human pulmonary artery endothelial cells (HPAECs) 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 endothelial cell 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 h, 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 TGF-β (11). We investigated whether the cells induced to enter EndMT by irradiation release TGF-β by performing ELISA assays of the culture supernatants of various human pulmonary cell lines. Although human pulmonary fibroblast (HPF) cells secreted more TGF-β than did ECs before irradiation, ECs (HPAECs and HPMECs, Human pulmonary microvascular endothelial cells) released more TGF-β 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 are other cell lines.

It has been demonstrated that TGF-β 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 TGF-β plays a key role in RIPF, we explored whether the signaling cascade triggered by the TGF-β receptor was associated with the radiation-induced EndMT in human pulmonary artery endothelial cells (HPAECs). As shown in Fig. 2A, the expression of TGF-β receptor 1, and the extent of Smad3 phosphorylation, increased during the radiation-induced EndMT. Immunofluorescence test showed that the addition of TGF-β-R1 inhibitor attenuated the fall in VE-cadherin level and the rise 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 TGF-β-R1 inhibitor (Fig. 2D, right).

Next, to examine whether the TGF-β-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 TGF-β-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 TGF-β-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 Endothelial Cells 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 prior to 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, HIF1-α positive-endothelial cells were observed in irradiated vessels (Fig. 3B)

**Hypoxia-induced EndMT involves TGFβ-R1/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 GFP-phalloidin revealed the presence of filamentous actin, one of the hallmarks of EndMT (the filaments form actin stress fibers) (23). Western blot assay also revealed an increase in HIF1-α expression, and a subsequent rise 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% [v/v] O₂) for 1-7 days. The expression of HIF1-α increased from 6 to 120 h of exposure to hypoxia, and the level of CD31 decreased while α-SMA expression increased from 24 h. As the EndMT commenced after an exposure to hypoxia for 24 h, phosphorylation of Smad3 was significantly increased and expression of TGF-β receptor I and Snail1 were significantly increased (Fig. 3D). Immunofluorescence data showed that the hypoxia-induced morphological 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 Smad3 phosphorylation under hypoxia. Similar results were found by immunoassay (Fig. 3E, right). These data indicated that hypoxia, like irradiation, induces EndMT via TGF-β receptor I/Smad signaling. To assess the contribution of HIF1-α to the irradiation-induced EndMT, we transfected control-siRNA or HIF1-α-specific siRNA into HPAECs. HIF1-α siRNA prevented the decrease in VE-cadherin level and increases in CA9 and filamentous actin levels (Fig. 3F, top). Immunoblotting also showed that HIF1-α knockdown reduced the irradiation-induced phosphorylation of Smad2/3 and the increases in α-SMA and vimentin expression, compared to control siRNA-treated cells (Fig. 3F, bottom).

Taken together, these findings suggest that in HPAECs, radiation-induced hypoxia triggers EndMT via HIF1-α-mediated activation of TGF-β receptor I/Smad signaling.

2-ME Inhibits Radiation-induced EndMT during Development of RIPF

Analogs of 2-methoxyestradiol (2-ME) are promising HIF1-α-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 TGF-β-mediated collagen synthesis, α-SMA production, and Smad2/3 phosphorylation (25). These possible mechanisms would all exert inhibitory effects on the HIF1-α-dependent EndMT prior to 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 HIF1-α expression, phosphorylation of Smad2/3 and increases in α-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 HIF1-α expression in vascular ECs after thoracic irradiation (Fig. 4B). Simultaneously, 2-ME inhibited the EndMT, as assessed by the extent of co-localization of α-SMA and CD31 in vascular ECs of irradiated lung tissue. Also, 2-ME inhibited the EndMT, with a concomitant increase in HIF1-α 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 HIF1-α expression and the EndMT phenotype may play important roles in the development of RIPF, and that 2-ME may be useful to prevent RIPF.

**HIF1-α is upregulated on vascular EndMT in the fibrotic regions of irradiated human lung tissue**

Next, we examined whether HIF1-α 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 lung cancer patients who received surgery following neoadjuvant radiotherapy for lung adenocarcinoma were selected based on H&E staining (Supplementary Fig. S5 and Supplementary Table S1). Most patients received the radiotherapy of 45-54 Gy in 25-30 fractions and surgery about 40 days after radiotherapy. More detail information with concurrent chemotherapy of human tissues are shown in Supplementary Table 1. Immunofluorescence examination for HIF1-α, 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 HIF1-α, whereas HIF1-α-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 vivo data with mouse lung, and support the hypothesis that hypoxia-induced vascular EndMT contributes to RIPF.
Discussion

Radiation-induced pulmonary fibrosis 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 SBRT (for example, three fractions of 20Gy) 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 micro-beam 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, 33). 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 (33). Vascular fibrosis develops principally in arterioles, arteries, and large veins. The EC response in normal tissues to irradiation is associated with early fibrogenesis (5, 34). 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 (35).
Previously, Molteni et al. (35) 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. Kolesnick et al. found that apoptotic death of endothelial cells were evident in mouse lungs 10 h after whole-body irradiation with 20 Gy (36). We also observed that whole-body (1Gy, 5 times) or thoracic (25 Gy) irradiation of mice induced apoptosis and detachment of lung endothelial cells 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 (Fig. 3A and B). 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 HIF1-α in the early phase of RIPF development (Fig. 3A and B).
In this respect, Fleckenstein et al. suggested that radiation-induced hypoxia in lung tissues is caused, in part, by increased oxygen consumption by macrophages, which are activated due to radiation-induced reductions in blood perfusion (7). 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 irradiation-induced lung injury (7). Vujaskovic et al. also reported that hypoxia was important in triggering continuous production of fibrogenic cytokines and perpetuation of late lung tissue injury (37).

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 prior to 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 alveolar epithelial cells may be indirectly caused by both fibrogenic cytokines released during the EndMT and hypoxia, apart from the direct damage to alveolar epithelial 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 to 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 TGF-β receptor I/Smad3 signaling, and downstream targets such as Snail 1, inhibits EndMT under pathologic conditions (12). In the present work,
we also found that the irradiation-induced EndMT was regulated by TGF-β receptor I/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 TGF-β receptor I/Smad signaling could be affected by knockdown of HIF-1α (Fig. 3).

TGF-β receptor (ALK5) kinase activity has recently been reported to require high-level HIF-1α expression in response to TGF-β1 (38). Several reports have suggested that TGF-β and HIF-1α engage in mutual regulation (38-40). In agreement with these reports, we found that the hypoxia-induced EndMT required TGF-β 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 TGF-β receptor/Smad signaling. These results suggest that, during irradiation-induced EndMT, the expression of HIF-1α is regulated both by hypoxia-induced TGF-β receptor activity and otherwise. Increases in HIF-1α levels caused by direct hypoxic stress (41) regulated TGF-β 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 17-beta-estradiol, has been shown to exert marked anti-carcinogenic 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 (42). 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 depositions 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 (29-32), 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.

Acknowledgements

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References


Figure legends

**Figure 1.** The patterns of collagen deposition and vascular EndMT during development of RIPF. A, Masson’s trichrome staining of lung tissues collected after irradiation. The left lungs of C57BL/6 mice were irradiated with 20Gy through a 7-mm-diameter collimator or the left main bronchi received 50 or 90 Gy through 3-mm-diameter collimators. In mice receiving 20 Gy irradiation, lung samples (n=3 per group) were obtained before (0 month), 2 and 4 weeks, and 3, 6, and 9 months after irradiation. In mice receiving 50 or 90 Gy, lung samples (n=3 per group) were obtained before (0 month); and 1, 2, 3, 4, and 6 months after 50 Gy; or 1 and 9 days; and 2, 3, and 4 weeks after 90 Gy. Collagen stained blue, nuclei purple, and cytoplasm red/pink. The original magnification was ×200. Scale bar = 100 µm. The graphs show the relative levels of collagen deposition in vessels, from five 200× fields. (Error bars indicate the SEMs of three comparable experiments with three mice per group; *P < 0.05; **P < 0.01; ***P < 0.001 vs. no IR. B, The left lungs of C57BL/6 mice were irradiated with 20 Gy or the left main bronchi received 50 or 90Gy. In mice receiving 20 Gy irradiation, lung samples (n=3 per group) were obtained before (0 month), and 1, 3, 6, 9, and 12 months after, irradiation. In mice receiving 50 or 90 Gy, lung samples (n=3 per group) were obtained before (0 month), and 1, 2, 4, and 6 months after 50 Gy; or 1, 3, 5, 9, 11, 14, and 21 days after 90 Gy. Left panels: Representative images of CD31 (green), α-SMA (red), and DAPI (blue). Scale bar = 50 µm. Right panels: The graphs indicate the relative extents of co-localization (yellow pixels) of CD31 and α-SMA, from five 100× fields (Error bars indicate the SEMs of three comparable experiments with three mice per group; *P < 0.05; **P < 0.01; ***P < 0.001 vs. no IR).

**Figure 2.** TGF-β-receptor/SMAD signaling contributes to radiation-induced EndMT. A, HPAECs were irradiated with 10 Gy, and cell lysates were collected before (0 day) and 1, 3,
5, and 7 days after irradiation. The expression levels of endothelial cell marker proteins (VEGFR1, VEGFR2, CD31, and VE-cadherin); mesenchymal cell marker proteins (α-SMA, FSP1, collagen I, Snail, and vimentin); TGF-β signaling proteins (TGF-βRI, p-SMAD2/3, and SMAD2/3); and adhesion proteins (E-selectin, VCAM1, and ICAM1) were analyzed by Western blotting. The band intensity was analyzed using Quantity One software (n=5); *P < 0.05; **P < 0.01 vs. no IR. B, HPAECs were irradiated with 2, 5, or 10 Gy, and cell lysates were collected before (0 h); and 4 and 8 h; and 1, 2, 3, 4, 5, 6, and 7 days after, irradiation. The expression levels of the endothelial cell marker CD31 and the mesenchymal cell marker α-SMA were analyzed by Western blotting. C, ELISA assay of TGF-β1 levels secreted by irradiated HPAECs, HPMECs, HPASMCs, HPFs, and HSAEpCs. Cell culture supernatants were collected 3 days after 10 Gy of irradiation and analyzed using an ELISA kit. Data are representative of four independent experiments.; 'P < 0.005; † † P < 0.05 vs. no IR. D, HPAECs were pre-treated for 1 h with 0 or 10 ng/ml of a TGF-βRI inhibitor, and irradiated with 10Gy. The expression levels of VE-cadherin, FSP1, α-SMA, and vimentin were analyzed via immunofluorescence staining (VE-cadherin: red, FSP1: green) and Western blotting. Scale bar = 20 µm. E, C57BL/6 mice were administered thoracic irradiation (16 Gy), intraperitoneally injected with a TGFβ-R1 inhibitor solution (25mg/kg) six times over 2 weeks, and lung samples (n=3 per group) obtained 4 weeks after radiation. The images show representative HIF1-α immunohistochemical results; Masson’s trichrome staining patterns. Scale bar = 100 µm ; and co-localization (yellow pixels) of CD31 and α-SMA in lung tissue. The graphs show the relative levels of collagen and co-localization of CD31 and α-SMA in vessels, as the average of the five 100× fields (error bars indicate the SEMs of three comparable experiments with three mice per group; † † P < 0.05)
Figure 3. Hypoxic damage patterns during development of RIPF and hypoxia-induced EndMT phenotypic changes in irradiated HPAECs. A, C57BL/6 mice were irradiated in the thoracic region with 20 Gy (7-mm-diameter field) or in the left main bronchus with radiation of 50 or 90 Gy (3-mm-diameter field). In mice receiving 20 Gy irradiation, lung samples (n=3 per group) were obtained before (0 month) and 1, 3, 6, and 9 months after irradiation. In mice receiving 50 Gy irradiation, lung samples (n=3 per group) were obtained before (0 month), and 15 days and 3, 4, and 6 months later. In mice receiving 90-Gy irradiation, lung samples (n=3 per group) were obtained 3 days and 1, 2, and 3 weeks after irradiation. CA9 (carbonic anhydrase IX) stains brown, and nuclei also stain blue. Scale bar = 100 µm. The graphs show the quantification of the areas for CA9. The vascular area positive with CA-9 are shown as % of total vascular area in each sections. Error bars indicate the SEMs of three comparable experiments with three mice per group; *P < 0.05; **P < 0.01; ***P < 0.001 vs. no IR. B, The representative images of HIF1-α immunohistochemical data. Scale bar = 100 µm. The graphs show the quantification of the areas for HIF-1α. The vascular area positive for HIF-1α are shown as % of total vascular area in each sections. Error bars indicate the SEMs of three comparable experiments with three mice per group; **P < 0.01; ***P < 0.001 vs. no IR.. C, HPAECs were irradiated with 10 Gy and, 72 h later, the expression levels of CA9 (red) and phalloidin (green) were analyzed via immunofluorescence staining. At the indicated time points after irradiation, cells were harvested and proteins subjected to western blotting and probing with antibodies against HIF1-α and α-SMA. D, HPAECs were cultured under hypoxic conditions (1% [v/v] O2) for the indicated times and cell lysate proteins subjected to western blotting. E, HPAECs transfected with control or Smad3 siRNA were cultured under normoxic (21% [v/v] O2) and hypoxic (1% [v/v] O2) conditions for 72 hours later, the levels of phospho-Smad2/3 (red) and phalloidin (green) were analyzed via immunofluorescence staining. At the same time, cells were harvested and proteins examined by western blotting
using the indicated antibodies. F, HPAECs transfected with control or HIF1-α siRNA were subjected to 10 Gy of irradiation. Seventy-two hours later, the cells were subjected to immunofluorescence staining detecting phallloidin (green), CA9 (white), and VE-cadherin (red). At the same time, cells were harvested and proteins examined by western blotting using the indicated antibodies. Data are representative of four independent experiments.

**Figure 4.** The effects of 2-ME on radiation-induced EndMT and RIPF. A, HPAECs were pretreated for 1 h with 0 or 10 ng/mL 2-ME and irradiated with 10 Gy. At 72 h after irradiation, the levels of phallloidin (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, C57/Bl6 mice (n=8 per group) were pretreated with 2-ME (60 mg/kg) or vehicle and the thoracic part of the left lung was 16Gy 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 HIF1-α immunohistochemical data, Masson’s trichrome staining patterns (Scale bar = 100 µm) and co-localization (yellow pixels) of CD31 and α-SMA or HIF1-α, CD31 and α-SMA. The graphs indicate as % of the vascular area positive for HIF1-α of total vascular area, the relative levels of vascular collagen deposition and co-localization of CD31 and α-SMA or HIF1-α, CD31 and α-SMA in vessels, from the five 100× fields (error bars indicate the SEMs of eight mice per group . ***P < 0.0001, **P < 0.01 vs. IR alone). Data are representative of three independent experiments.

**Figure 5.** HIF1-α-related EndMT in human RIPF tissues. A, Sections from human normal lung (n = 3) or RIPF tissues (n = 10) were stained for CD31 (green), HIF1-α (white), α-SMA (red), and DAPI (blue). Six representative images out of 10 specimens are shown. Scale
bar = 20 µm. Representative images of HIF1-α-positive EndMT (SMA⁺ and CD31⁺ lesions) are marked with white arrows and an HIF1-α-negative vessel is marked with an open arrow. B, The data from 10 specimens are quantitated and the graph shows the % of SMA⁺ and CD31⁺ lesions in CD31⁺ lesions from at least five 100× fields (error bars indicate the SEM of 3 normal tissues and 10 RIPF tissues, respectively; """"P < 0.0001).
Figure 2

A) HPAEC

B) 0 h  8h  1  2  3  4  6  7 days

C) Released TGFβ1

D) VE-Cadherin (GFP)

E) Trichrome
Figure 3

A

CA9

20Gy

Cont

Cont

50Gy

1m

15d

3m

3m

6m

6m

9m

9m

20Gy

50Gy

90Gy

CA9 vascular lesions per 100 field (%)

Cont

1m

3m

6m

9m

20Gy

50Gy

90Gy

HIF-1α

B

20Gy

50Gy

90Gy

Cont

1w

2w

3w

1w

2w

3w

lesions per 100 field (%)

20Gy

50Gy

90Gy

C

Phalloidin / CA9 / DAPI

Control

IR 10Gy

α-SMA

Vimentin

TGFβ-RI

(ALK5)

D

Hypoxia: 0 6 24 72 120 (hr)

HIF-1α

CD31

α-SMA

Vimentin

TGFβ-RI

(ALK5)

p-Smad2/3

Smad2/3

Snail1

β-Actin

E

Phalloidin / Phospho-smad2/3 / DAPI

Normoxia

Hypoxia

siControl

siSmad3

siHIF-1α

F

Phalloidin (Green) / CA9 (White) / VE-cadherin (red) / DAPI

No IR

IR

siControl

siHIF-1α
Figure 4

A

No IR  IR

DMSO  2-ME

Phalloidin (Green) / CA9 (Red) / VE-Cadherin (white) / DAPI

B

IR  IR+2-ME

HIF-1α

Trichrome

CD31 (Green) / α-SMA (Red) / DAPI

2-ME

DMSO  0.5 μM  1 μM

10Gy

α-SMA

HIF-1α

P-Smad2/3

Smad 2/3

β-actin

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Figure 5

A

CD31(Green) / α-SMA(Red) / HIF-1α(White) / DAPI

Patient #1

Patient #2

Patient #3

Normal lung tissue

Patient #4

Patient #5

Patient #6

B

α-SMA+ and CD31+ lesions per x 100 field (%)

- Negative lesions
- Positive lesions

HIF-1α

(P<0.0001)***

Normal lung tissues

Fibrotic lung tissues

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# Clinical Cancer Research

## A hypoxia-induced vascular endothelial-to-mesenchymal transition in development of radiation-induced pulmonary fibrosis

Seo-Hyun Choi, Zhen-Yu Hong, Jae-Kyung Nam, et al.

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