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Ionizing Radiation Promotes Epithelial–to–Mesenchymal Transition in Lung Epithelial Cells by TGF-β-producing M2 Macrophages

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Abstract. *Background/Aim: Ionizing radiation induces pulmonary fibrosis, which is a common dose-limiting complication in patients receiving radiotherapy. Fibrosis occurs through the accumulation of large amounts of ECM components, synthesized by myofibroblasts in damaged lung tissue. Epithelial cells serve as one of the cellular sources of myofibroblasts via the epithelial–to–mesenchymal transition (EMT) process. In this study, we investigated the role of TGF-β-secreting M2 macrophages in association with ionizing radiation-induced EMT. Materials and Methods: The lung epithelial cell line MLE12, was irradiated and the expression of EMT markers and chemokines was examined. Moreover, the mouse lung macrophage MH-S cell line was cultured with conditioned media from irradiated MLE12 cells, to examine the effects of the secreted factors on the migration ability of macrophages. For the murine pulmonary fibrosis model, mice were locally irradiated and the levels of M1 or M2 macrophage-related markers and cytokines were measured in bronchoalvelolar lavage (BAL) fluid and lung tissue. Results: In MLE12 cells, irradiation directly induced expression of EMT-related markers and secretion of various chemokines, which lead to macrophage migration. Interestingly, the sub-population of macrophages recruited in the lung of mice after thoracic irradiation was M2 macrophages that expressed Arg-1 and CD206. M2 macrophages induced the MLE12 to undergo phenotypic conversion to form fibroblast-like cells, which leads to a down-regulation of epithelial markers and an up-regulation of new EMT-related markers. In thoracic irradiated mice, pro-inflammatory cytokines such as IL-1β, IL-4 and IL-10 were increased at 2 weeks, but returned to normal levels from 16 weeks or 24 weeks after irradiation. However, thoracic irradiation led to a rapid increase of TGF-β and IGF-1 levels, which lasted up to 24 weeks. It was confirmed that M2 macrophages secreted the high levels of TGF-β. Moreover, the elimination of TGF-β from M2 macrophages attenuated mesenchymal transition of MLE12. Conclusion: TGF-β-secreting M2 macrophages play an important regulatory role in mesenchymal transition of epithelial cells in the lung of irradiated mice, thus contributing to radiation-induced pulmonary fibrosis.*

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Radiation-induced pneumonitis and fibrosis are the major adverse responses of the lung during radiation therapy for cancer patients. Myofibroblasts have been suggested to play a central role in the pathogenesis of fibrosis through producing large amounts of ECM components, including collagens (especially types I and III), glycoproteins and proteoglycans (fibronectin, laminin, and tenascin) in and around inflamed or damaged tissue (1, 2). Myofibroblasts can arise from resident stromal cells such as fibroblast and pericytes, as well as from circulating fibrocytes (3). However, several studies suggest that injured/damaged epithelial cells may directly serve as a source of myofibroblasts in a highly regulated process termed epithelial-to-mesenchymal transition (EMT) (4-7). During EMT process, epithelial cells undergo a partial morphological alternation from round cobblestone-like to fibroblast-like cells and thus transdifferentiate into myofibroblast cells expressing the ECM markers (α-smooth muscle actin (SMA) and type I collagen). This transition is characterized by down-regulation of cell-surface epithelial markers, such as E-cadherin and cytokeratins, and up-regulation of new mesenchymal markers, including the cell-surface protein N-cadherin, cytoskeletal markers (fibronectin,

α-SMA, and Vimentin), and transcription factors regulating EMT (Snail and Slug) (8, 9). In the lung, abnormal repair and transdifferentiation have been frequently observed in damaged epithelial cells, for example after irradiation; this phenomenon has been linked with the development of lung fibrosis (10, 11).

Injured/damaged epithelial cells secret various chemokines that allow recruitment of inflammatory cells (*e.g.*, neutrophils, eosinophils, macrophages, and lymphocytes) to the site of damage. Actually, infiltrating macrophages correlate with the degree and severity of tissue damage and fibrosis (12-15). At the earliest stages of wound healing after tissue damage, neutrophils are the most abundant inflammatory cell, but are quickly replaced by macrophages. Macrophages are responsible for removing dead and dying cells (such as neutrophils and lymphocytes), and controlling the inflammatory process via the secretion of anti-inflammatory cytokines. However, if the irritant persists, control of inflammation by macrophages is disturbed, leading to an excess production of inflammatory mediators and subsequently to tissue injury, accompanied by fibrosis. In a fibrotic process, macrophages play an important role in fibroblast migration and differentiation/proliferation into myofibroblasts, which are the key mediators of fibrotic tissue remodeling (2, 16, 17). For this reason, macrophages are crucial in the orchestration of the wound healing process. Several interesting studies showed that macrophage depletion at an early stage of inflammation improves kidney or liver injury and reduce the development of fibrosis (18, 19).

Recent studies showed that resident and infiltrating macrophages are activated and then may be polarized according to the cytokine micro-environmental signals (20-22). Classically activated macrophages (M1) are mainly activated by Toll-like receptor ligands and Th1 cytokines such as interferon (IFN)-γ, while alternatively activated macrophages (M2) are mainly stimulated by Th2 cytokines such as interleukin (IL)-4 and IL-13. M1 macrophages are involved in the generation of proinflammatory cytokines, whereas M2 macrophages are responsible for tissue remodeling and often associated with fibrotic conditions, including pulmonary fibrosis, when the damage lesion is persistent (20-26). During the wound healing process, a tightly regulated balance between M1 and M2 macrophages is crucial for tissue integrity. Moreover, IL-4 and IL-13, which are necessary for the polarization of M2 macrophages, are known as the powerful profibrotic cytokines because of the direct stimulation of collagen synthesis in fibroblasts (23, 27-29). Interestingly, M2 macrophages are known to secrete large amounts of pro-fibrotic factors such as TGF-β, IGF-1 and Galactin-3 (30). Actually, TGF-β, one of the key drivers of fibrosis through inducing ECM-producing myofibroblasts, is secreted by several inflammatory, epithelial, mesenchymal cells M1 macrophages as well as M2 macrophages at injured/damaged sites.

Although the exact mechanism leading to radiation-induced pulmonary fibrosis is unclear, we observed that ionizing radiation directly induced EMT in lung epithelial cells *in vitro*, and then irradiated epithelial cells induced the migration of macrophages, especially of M2. We also showed that TGF-β blockage from M2 macrophage attenuated mesenchymal transition of the epithelial cell line. Our present data strongly suggests that the exacerbation of radiation-induced pulmonary fibrosis might depend on the mesenchymal transition of epithelial cells, promoted by the TGF-β-secreting M2 macrophages.

Materials and Methods

Cell lines and cell culture. The mouse lung macrophage MH-S cell line and lung epithelial cell line, MLE12, were obtained from American Type Culture Collection (Manassas, VA, USA). MH-S cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol and penicillin/streptomycin. MLE12 cells were cultured in Dulbecco's modified Eagles's medium (DMEM)/F12 medium supplemented with 2% heat-inactivated FBS, 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, 10 mM HEPES, 1 mM L-glutamine, penicillin/streptomycin. Cells were cultured in a standard humidified incubator at 37˚C in a 5% CO_2 atmosphere. All culture reagents were purchased from GIBCO BRL (Paisley, UK).

Animals and ethics statement. Six-week-old female C57BL/6N (H-2b) mice were purchased from Orient Inc. (Charles River Technology, Seoul, Republic of Korea). The mice were housed at a controlled temperature of 22±2˚C and at 50±5% relative humidity, under a specific pathogen-free condition. Mice were fed with a standard animal diet and water ad libitum. The animal experiments were conducted according to the guidelines for the use and care of laboratory animals of Ministry of Health and Welfare, Republic of Korea. The protocols were approved by the Institutional Animal Care and Use Committee of the Korea Atomic Energy Research Institute (KAERI-IACUC) and included criteria for euthanasia to minimize suffering. The number of mice used for each analysis is shown in the figure legends.

Irradiation of epithelial cell line and thoracic irradiation of mice. Once cells reached >60% confluence, MLE12 cells were irradiated with a single dose of 5 Gy or 10 Gy γ-rays using a ^{137}Cs γ-ray source (Gammacell 40 Exactor, Nordin International Inc., Ottawa, Canada) at a dose rate of 0.95 Gy/min. Irradiations were performed at room temperature, while non-irradiated control cells were studied in parallel, at the same conditions.

For thoracic irradiation, the mice were irradiated once with 14 Gy of using a Gammacell 40 lead collimator with a ^{137}Cs γ-ray source at a 0.6 Gy/min dose rate. The mice were anesthetized with Zoletil (20 mg/kg body weight, Virbac S.A, France) just before irradiation and then were confined in specifically designed plastic jigs and placed such that the thoraces were in the 3 cm diameter field defined by lead collimators (Nordin International Inc.). Age-matched normal controls (4, 8, 16, and 24 weeks aged control mice) were maintained under identical conditions for the course of the experiment.

RNA extraction, reverse transcriptase (RT)-polymerase chain reaction (PCR), quantitative real-time PCR. Total RNA was isolated from cells using TRIzol Reagent (Invitrogen, CA, USA) according manufacturer's protocol. Reverse transcription from 3μg of total RNA was implemented using random primer and MMLV reverse transcriptase (Promega, Madison, WI, USA). One microliter of the reverse transcription product was used as a template for PCR amplification. PCR was performed using the Taq DNA polymerase (Promega) and 100 nmole/l of primers. The primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the sequences were as follows: β-actin, 5'-AGCCATGTACGTAGCCATCC-3' and 5'-TCTCAGCGTG GTGGTGA AG-3'; CCL-2, 5'-CCCAATGAGTAGGCT GGAGA-3' and 5'-AGACCTTAGGGCAGATGCAG-3'; CCL-4, 5'-TGTC TGCCCT CTCTCTCCTC-3' and 5'-TCAGTTCAACTCCAAG TCACTCA-3'; CCL-7, 5'-GATCTCTGCCACGCTTCTGT-3' and 5'-CTTTGGAG TTGGGGTTTTCA-3'; CXCL-10, 5'-AAG TGCTGCCGTCATT TTCT-3' and 5'-TTCATCGTGGCAA TGATCTC-3'; CXCL-17, 5'-TCATGTCCATGGTCTTCAGC-3' and 5'-AAAGCTTGCCAGGTG ACATC-3'; CD32, 5'-TGTCGCAGCCATTGTTATTATC-3' and 5'-TGTGGTTCTGGTAA TCATGCTC-3'; Arg-1, 5'-CAGAAGAA TGGAAGAGTCAG-3' and 5'-CAGATATGCAGGGAGTCACC-3'; CD206, 5'-ACGACA ATCCTGTCTCCTTTGT-3' and 5'-TCAGC TTTGGTTG TAATGGATG-3'. The PCR amplifications were carried out in 20-μl final reaction volume containing PCR buffer, dNTP, and Taq polymerase (Promega), in a thermal cycler (Applied Biosystems, CA, USA). The PCR protocol for the amplification of β-actin, CCL-2, CCL-4, CCL-7, CXCL10, and CXCL17 included 35 cycles of 20 s at 95˚C, 30s at 60~64˚C, and 40 s at 72˚C. PCR products were resolved on 1.5% agarose gel stained with ethidium bromide by electrophoresis. Quantitative real-time PCR was performed by a StepOne Real-Time PCR (Applied Biosystems) with SYBR Green reagent (Applied Biosystems). The amplification conditions of CD32, Arg-1, and CD206 genes were as follows: 95˚C for 15 min, followed by 40 cycles at 95˚C for 15 s, 60˚C for 10 s, and 72˚C for 15 s in a thermal cycler (Applied Biosystems). The comparative Ct method was used and relative mRNA expression level was calculated based on normalization to β-actin. All experiments were repeated three times independently.

Preparation of bronchoalvelolar lavage (BAL). Mice were sacrificed by cervical dislocation at 2, 4, 8, 16, and 24 weeks after thoracic irradiation. The trachea was cannulated and BAL was obtained by flushing three times with 0.5 ml of PBS including with 1mM EDTA and 10% FBS through a tracheal cannula. BAL was centrifuged and supernatant was stored at –30˚C for determination of cytokine levels.

Measurement of cytokines in cell culture supernatant and BAL fluid. The levels of CCL-2 (MCP-1), IL-1β, IL-4, IL-10, and TGF-β in culture supernatant and BAL were measured by BD OptEIA™ ELISA set according to the manufacturer's instructions (http://www.bdbiosciences.com). All OptEIA™ sets were purchased from BD PharMingen (SanDiego, CA, USA).

Macrophages migration assay. MH-S macrophages were seeded into the upper chamber of a trans-well (Corning Incorporated, NY, USA) at a density of 6×10^4 cells/well in 200 μl of serum-free medium and placed on the 24-well plate containing the conditioned medium obtained from non-irradiated or irradiated MLE12. After a 16-h incubation, the cell suspension in the upper chamber was aspirated, and the upper surface of the filter was carefully cleaned with cotton plugs. Cells that migrated through the polycarbonate membrane were fixed with 3.7% formaldehyde in PBS 100% and were permeabilized with 100 % methanol and then were stained with 0.5% crystal violet for 15 min. The membrane was cut away from each chamber and migrated cells on the lower surface of the filter were counted in six representative fields of microscope at a 200× magnification (Leica, Heidelberg, Germany). Experiments were performed in triplicate and data are reported as mean±SD of cell numbers.

Induction and evaluation of M1 or M2-plarized macrophages. For MH-S polarization, the cells were cultured in RPMI 1640 media supplemented with IFN-γ 20 ng/ml or IL-4 and IL-13 4 ng/ml, for polarization towards the M1 or the M2 type, respectively. After 48 h, the media were collected and prepared as M1- or M2-polarized macrophage conditioned media (CM). For confirmation of macrophage polarization, the expression levels of iNOS, CD32, Arg-1, and CD206 were measured through RT-PCR and western blot.

Western blotting. Total cell lysates were prepared using RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) containing 10 mM phenylmethanesulphonylfluoride (PMSF), 10 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4) and a complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) for 30 min on ice. The protein concentrations of the cell extracts were measured using the BCA (bicinchoninic acid) method (Pierce, Rockford, USA). Protein extract (20 μg) was boiled in a Laemmli sample buffer containing 2-mercaptoethanol (Bio-Rad Laboratories, Inc.) and then separated on SDS-polyacrylamide gel. Proteins were transferred onto a Hybond™-P membrane (Amersham, Seoul, Korea). After blocking in a blocking buffer (50 g/l skimmed milk or bovine serum albumin (BSA; Sigma-Aldrich) in tris-buffered saline containing 1 ml/l tween-20), the membranes were incubated overnight at 4˚C with primary antibodies. Antibodies against fibronectin, α-SMA, Snail, Slug, and β-actin were purchased from Cell Signaling (Banvers, MA, USA). Anti-E-cadherin, anti-N-cadherin, and anti-vimentin antibodies were purchased from Thermo Scientific (Rockford, IL, USA) and anti-iNOS and anti-Arg-1 antibodies were purchased from Abcam (Gyeonggi-do, Republic of Korea). After washing, the membranes were incubated with anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG secondary antibodies conjugated with horseradish peroxidase (HRP) (Cell Signaling) for 1 h. Chemiluminescence was used for the detection, according to the manufacturer's protocol (Immobilon™ Western, Millipore, Billerica, MA, USA).

Statistical analysis. Data were expressed as mean±standard deviation (SD) from at least three independent experiments. The statistical significance of differences between two groups (control mice vs thoracic irradiated mice) was analyzed by using a two-tailed Student's *t*-test. Statistical probability of $p<0.05$ or $p<0.01$ was considered significant.

Results

Ionizing radiation directly induced the expression of EMT markers in lung epithelial cells, in vitro. To examine the effects of ionizing radiation on the MLE12 lung epithelial cells, cells were irradiated at a dose of 5 Gy or 10 Gy. Cell

morphology was observed at 3 days following irradiation; MLE12 lost their round cobblestone-like appearance and showed an elongated mesenchymal-like morphology, which is typical of EMT morphological phenotypes (Figure 1A). Moreover, radiation was shown to directly modulate EMT-associated proteins in MLE12 cells (Figure 1B). The expression levels of E-cadherin showed a marked reduction in cells irradiated at the single dose of 10 Gy when compared to control levels, but no difference in cells irradiated with 5 Gy. Loss of E-cadherin expression is thought to be one of the most important events in EMT (8, 9). Meanwhile, the protein levels of N-cadherin, vimentin, fibronectin, α-SMA, Snail, and Slug, which are used as markers of mesenchymal cells, were enhanced by irradiation. These data suggest that ionizing radiation directly induced MLE12 trans-differentiation into a mesenchymal-like phenotype.

Damaged epithelial cells following irradiation induce macrophages to migrate. It is known that various immune cells, especially macrophages, are implicated in the induction of pulmonary fibrosis by ionizing radiation (13, 31, 32). Herein, we examined whether epithelial cells that are damaged and induced to undergo EMT by irradiation affect the mobility of macrophage by analyzing the expression of chemokines related to macrophage attraction. MLE12 epithelial cells were irradiated with 10 Gy, and mRNA levels of several chemokines were evaluated at specific time-points 3-48 h post-irradiation. Ionizing radiation increased the mRNA expression levels of CCL2, CCL4, CCL7, CXCL10, and CXCL17 in a time-dependent manner, compared to non-irradiated cells (Figure 2A). Moreover, ELISA analysis also showed that radiation exposure significantly elevated the secretion of CCL2 chemokine ($p<0.001$) in culture supernatant from MLE12 cells at 2 and 3 days after irradiation, compared to the control cells (Figure 2B). Similarly, thoracic irradiation in mice induced a significant increase in CCL2 production in the BAL fluid at 2-24 weeks post-radiation ($p<0.05$), compared to that of age-matched normal control mice (Figure 2C). Interestingly, the migration of MH-S macrophages was significantly enhanced ($p<0.01$) in conditioned media obtained from irradiated epithelial cells (Figure 2D), suggesting that irradiated epithelial cells secreted various chemokines to attract MH-S macrophages.

The activated macrophages recruited into the lung after thoracic irradiation were M2 type. Generally, activated macrophages are classified into two types with distinct functions: the M1 and the M2 (20, 22, 26). We examined how thoracic irradiation affected the polarization of activated macrophages that were recruited into the lung of mice. CD32 mRNA expression was decreased at 4 months after thoracic irradiation ($p<0.01$) and was sustained at a low level until 6 months ($p<0.05$); whereas the mRNA expression levels of Arg-1 and CD206 were significantly enhanced in the lungs of thoracic irradiated mice at 4 months ($p<0.0001$ for both markers), and then maintained at a high level until 6 months ($p<0.05$ for both markers) (Figure 3). These results demonstrated that M2 macrophages were recruited from middle stage to late stage following thoracic irradiation, suggesting that damaged epithelial cells by irradiation induced to attract M2 macrophages.

M2 macrophages induced the expression of EMT markers in lung epithelial cells. Despite many reports that the classical and alternative activated macrophages have been proposed to play a role in radiation-induced fibrosis (31-34), it is not clear how they affect the differentiation of fibroblasts and lung epithelial cells. To determine that, we examined the mesenchymal transition of lung epithelial cells in conditioned media of M1 macrophages or M2 macrophage. Culture of MH-S lung macrophage cell in the presence of IFN-γ or IL-4 and IL-13 successfully induced their polarization towards M1 or M2 macrophage, respectively. MLE12 cells were co-cultured for 14 days in conditioned media obtained from non-treated (NT-CM), M1 (M1-CM), or M2 macrophages (M2-CM). The changes in cell morphology and EMT-associated marker expressions were investigated. As shown in Figure 4A, MLE12 cultured in M2-CM appeared a spindle-shaped and elongated morphology and dissociated from their neighbors, compared to the morphology of MLE12 in NT-CM or M1-CM. Regarding the protein expression of EMT-related markers, M2-CM decreased expression of the EMT-negative marker E-cadherin in MLE12 cells, and increased the EMT-positive markers, fibronectin, α-SMA, Vimentin, N-cadherin, Snail, and Slug (Figure 4B). However, no differences in the levels of the EMT-related markers were observed between MLE12 cells cultured with NT-CM and M1-CM. These results suggested that proteins secreted from M2 macrophages induced mesenchymal differentiation of lung epithelial cells.

Thoracic irradiation induced pro-inflammatory and pro-fibrotic cytokine secretion. Several pro-inflammatory and pro-fibrotic cytokines regulate pneumonitis and pulmonary fibrosis (17, 35). Therefore, we examined the levels of pro-inflammatory and pro-fibrotic cytokines, such as IL-1β, IL-4, IL-10, and TGF-β in BAL fluid and IGF-1 in the lung tissue of thoracic irradiated mice. Thoracic irradiation rapidly increased the levels of IL-1β ($p<0.05$), IL-4 ($p<0.05$), IL-10 ($p<0.05$) in BAL fluid 2 weeks after thoracic irradiation, compared to those of age-matched normal mice (Figure 5). However, the high levels of their cytokines turn down to normal levels from 4 to 16 weeks or 24 weeks following thoracic irradiation. Moreover, TGF-β in BAL fluid and IGF-1 in the lung tissue of thoracic irradiated mice showed markedly high levels (TGF-β: 2

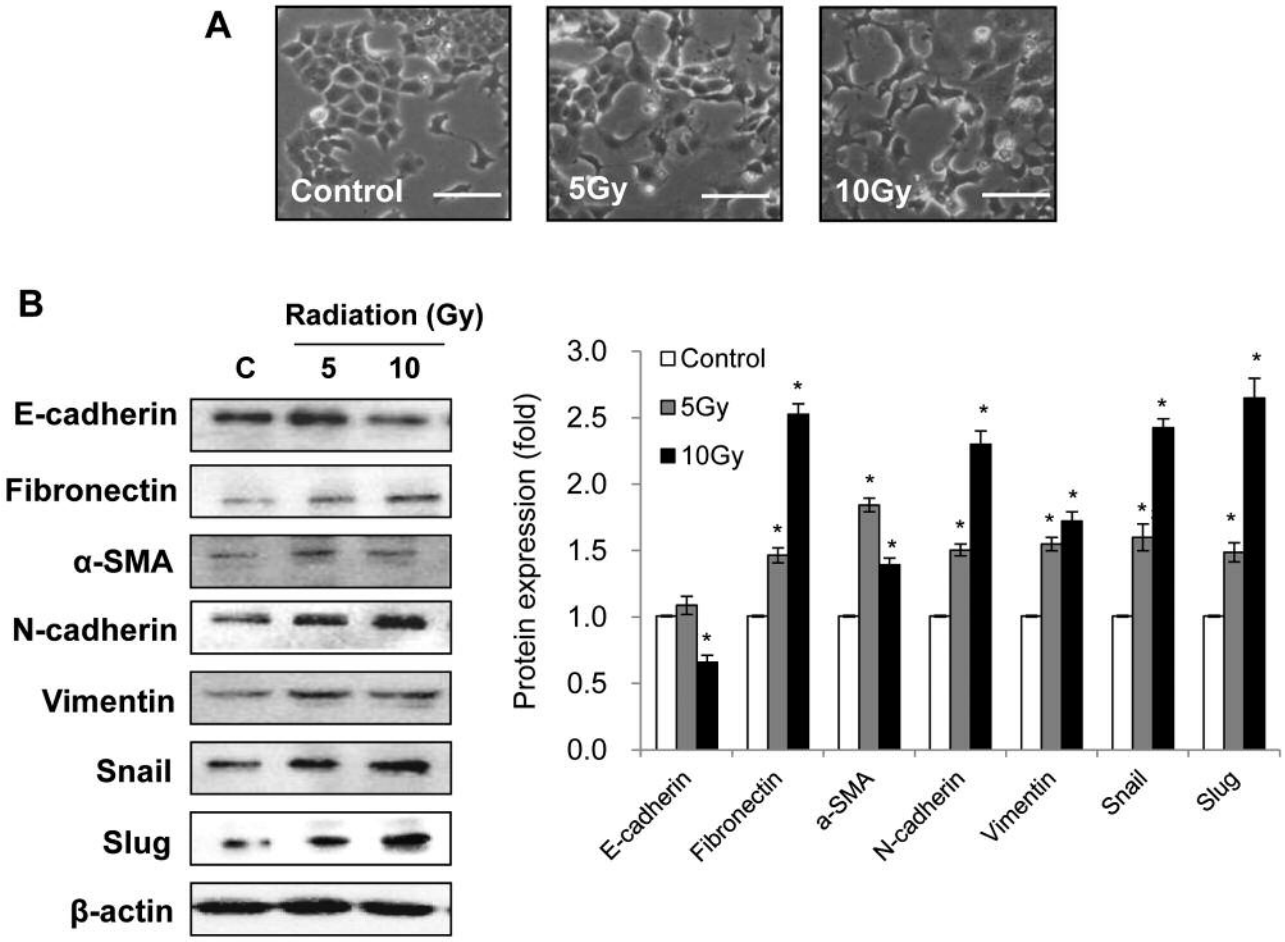

Figure 1. *Ionizing radiation induced a mesenchymal phenotype and increased epithelial–to–mesenchymal transition (EMT) markers in MLE12 in vitro. MLE12 cells were irradiated at a dose of 5Gy or 10Gy and then analysis was conducted at 7 days after irradiation. Representative cell morphology images of MLE12 after single-dose irradiation are shown (A). Scale bar, 50 μm. Expression levels of EMT-related markers in MLE12 after single-dose irradiation were examined by western blot (B). The experiment was repeated three times independently and the bars indicate the means±SD.*

weeks after radiation, $p<0.0001$; IGF-1: 8 weeks after radiation, $p<0.0001$) compared to those of age-matched normal mice (Figure 5). Interestingly, the high levels of TGF-β and IGF-1 lasted up to 24 weeks after thoracic irradiation, suggesting that TGF-β and IGF-1 plays a vital role in the overall stage of radiation-induced fibrosis development.

Inhibition of TGF-β derived from M2 macrophages attenuated mesenchymal transition of epithelial cells. In the damage-healing response, macrophages are major producers of TGF-β and IL-10, which is, indisputably, showing wound-healing and pro-fibrotic activity (25, 36). Based on this information, we measured the levels of IL-10 and TGF-β secreted from M1 macrophages or M2 macrophages. As shown in Figure 6A, M2 macrophages secreted high levels of IL-10 ($p<0.005$) and TGF-β ($p<0.01$), whereas in M1 macrophages only a slight increase in TGF-β secretion was observed, compared to the control macrophages that were cultured without stimulants (no statistical significance). Next, we investigated the change in the expression of EMT-related markers by IFN-γ, IL-4/IL-13, or TGF- β in the MLE12 cell line (Figure 6B). As we expected, MLE12 cells that were cultured in the presence of TGF-β showed decreased expression of E-cadherin (only at 2 ng/ml TGF-β, $p<0.01$), but significantly increased the cytoskeletal markers α-SMA ($p<0.01$) and Vimentin ($p<0.01$), the cell-surface protein N-cadherin ($p<0.01$), as well as the EMT-related transcription factors Snail ($p<0.01$) and Slug ($p<0.01$), compared to the control cells. However, the expression levels of EMT-related markers did not differ among the MLE12 cells cultured with IL-4 and IL-13, except elevated expression of Vimentin

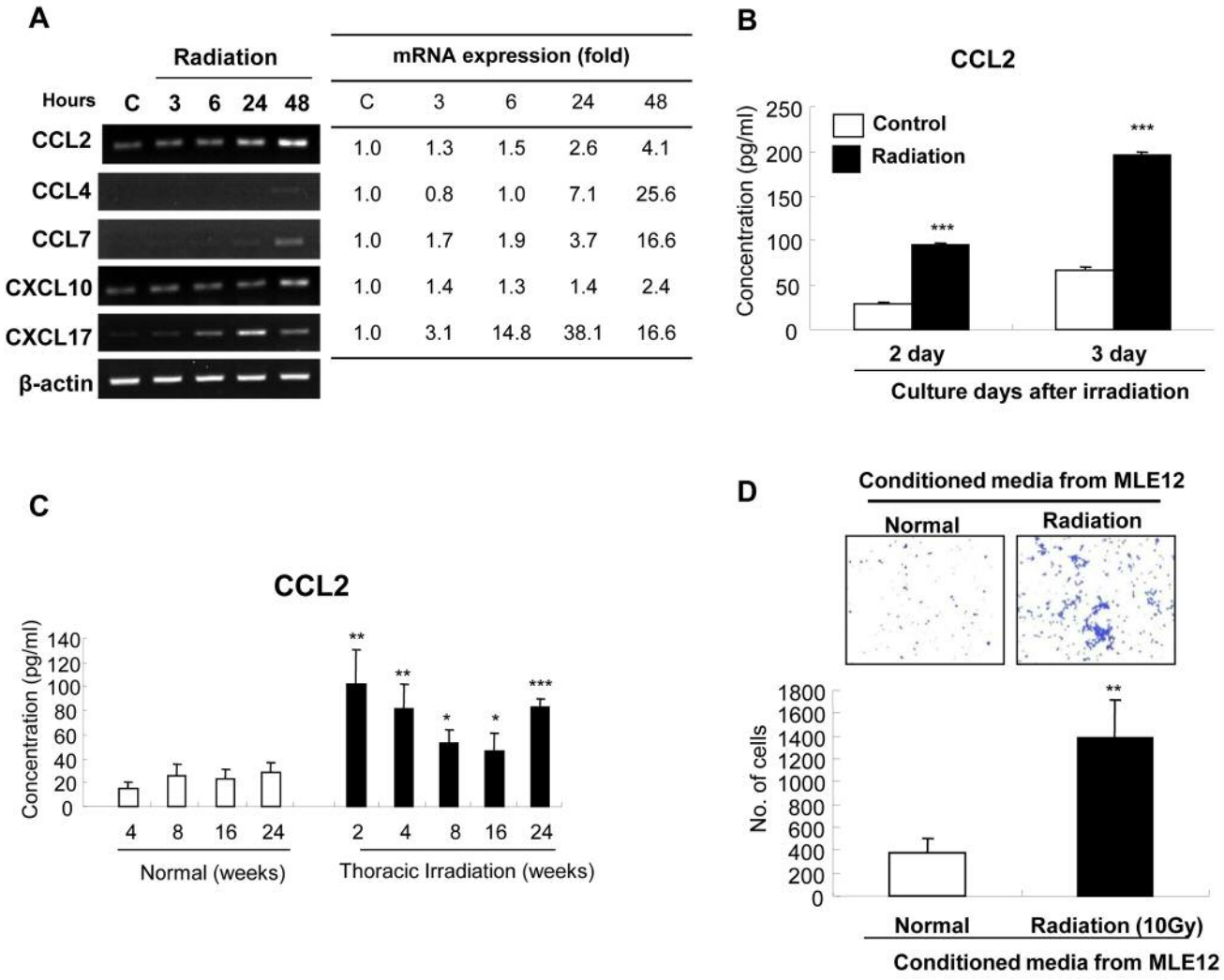

Figure 2. *Irradiation induced the expression of various chemokines in epithelial cells and promoted macrophage migration. A representative of the mRNA levels of various chemokines in MLE12 cells at indicated time points after irradiation (10 Gy) is shown (A). After irradiation at a dose of 10 Gy, CCL2 levels in culture supernatants of MLE12 cells were analyzed by ELISA (B). CCL2 levels in the BAL fluid at 2, 4, 8, 16, and 24 weeks after thoracic irradiation were tested using ELISA. Each group consisted of five mice and the bars indicate the means±SD (C). Representative images (upper) and statistical column plots (lower) demonstrated increased potential of migration of MH-S cells cultured in conditioned media from irradiated MLE12 cells, compared to MH-S cells cultured in control media (from non-irradiated MLE12 cells) (D). C, Control; *p<0.05, **p<0.01, ***p<0.001.*

($p<0.05$) and Slug ($p<0.01$). In the MLE12 cells cultured with IFN-γ, only α-SMA ($p<0.001$), N-cadherin ($p<0.001$), Snail ($p<0.01$) and Slug ($p<0.01$) showed decreased expression levels, compared to the control group.

Based on the above findings, we speculated that the main cause of the radiation-induced pulmonary fibrosis was TGF-β secreted by M2 macrophages which were recruited into the lung after radiation exposure. To test this hypothesis, the lung epithelial cells MLE12, were cultured in conditioned media (NT-CM, M1-CM, or M2-CM) with or without anti-TGF-β or anti-IL-10 antibody in M2-CM, and then the expression of EMT-associated markers was investigated. All EMT-related markers induced by M2-CM were dramatically reversed when TGF-β was neutralized in M2-CM, compare to MLE12 cells in M2-CM without anti-TGF-β mAb, while anti-IL-10 mAb did not alter the expression of EMT-markers when compared to MLE12 cells in M2-CM without anti-IL-10 mAb (Figure 6C). Moreover, both anti-TGF-β mAb and anti-IL-10 mAb treatment markedly inhibited the increase of N-cadherin, Slug, α-SMA induced by M2-CM. This result suggested that TGF-β secreted from M2 macrophages plays an important role on EMT.

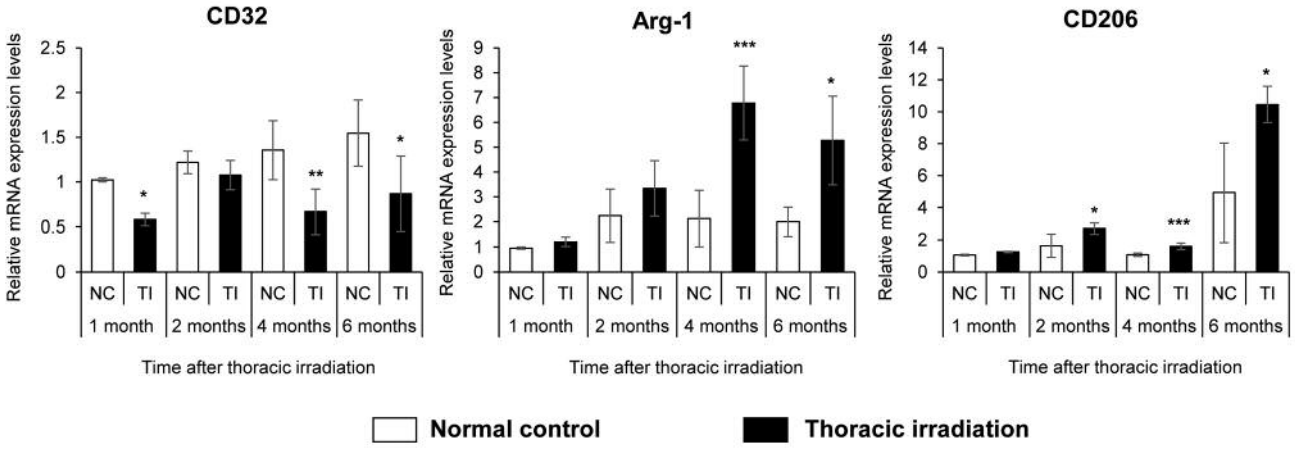

Figure 3. *Thoracic irradiation increased the expression of Arg-1 and CD206 in the lung. At 1, 2, 4, and 6 months after irradiation, the RNA expression levels of CD32, Arg-1, and CD206 in the lung tissue were measured through quantitative real-time PCR. There were five or six mice in each group. The bars indicate the means±SD. *p<0.05, **p<0.005, ***p<0.001 compared to the age-matched control mice. NC, Normal control; TI, thoracic irradiation.*

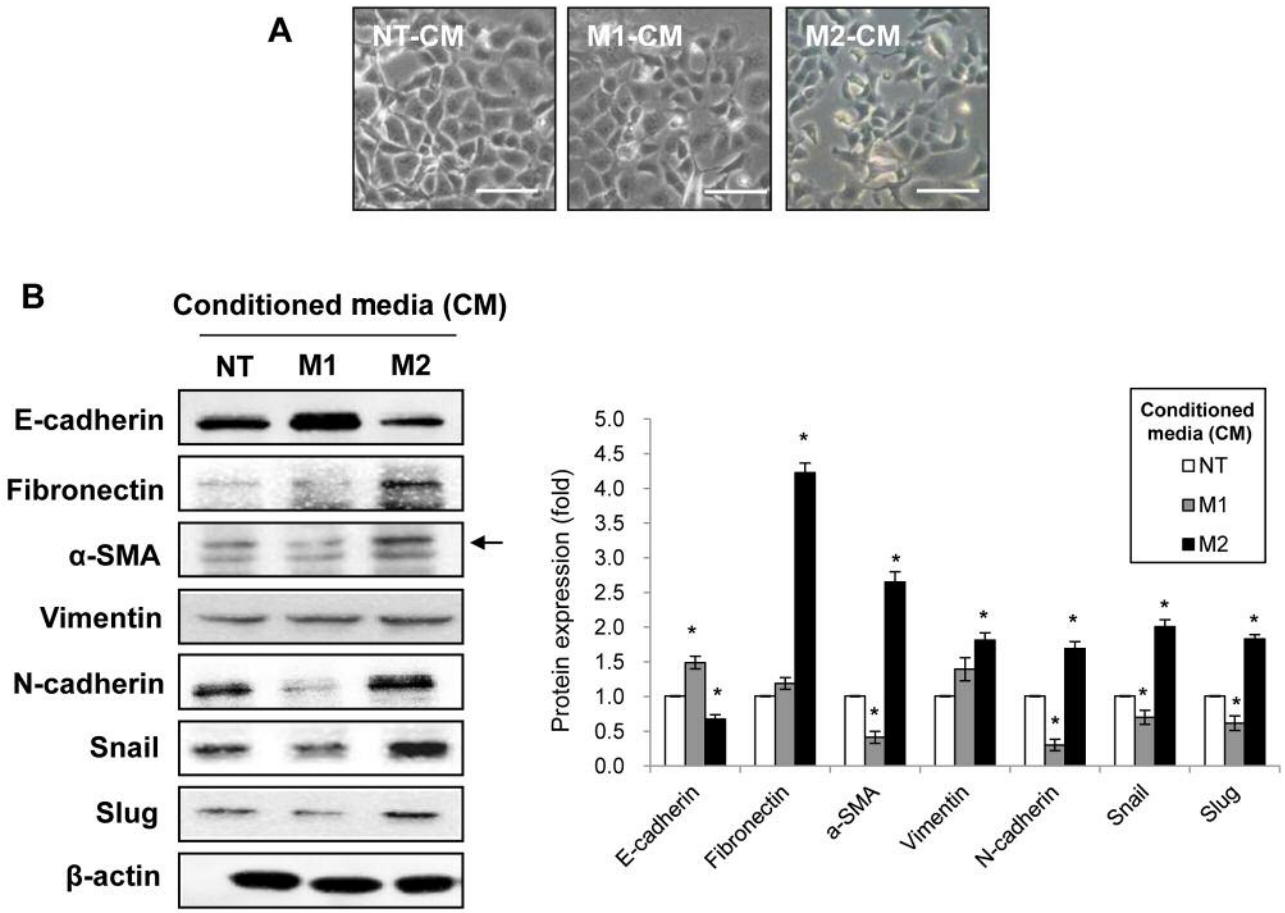

Figure 4. *Soluble protein of M2 macrophages induced a mesenchymal phenotype and increased epithelial–to–mesenchymal transition (EMT) markers in MLE12 cells, in vitro. MLE12 cells were cultured with conditioned media of M1 polarized, M2 polarized, or non-stimulated macrophages, for 14 days. Representative cell morphology images of MLE12 cells cultured in conditioned media of M1-polarized or M2-polarized macrophages are shown (A). Scale bar, 50 μm. Expression of EMT-related markers in MLE12 cells cultured in conditioned media obtained from non-treated (NT-CM), M1 (M1-CM), or M2 macrophages (M2-CM) (B). Experiment was repeated three times independently and the bars indicate the means±SD.*

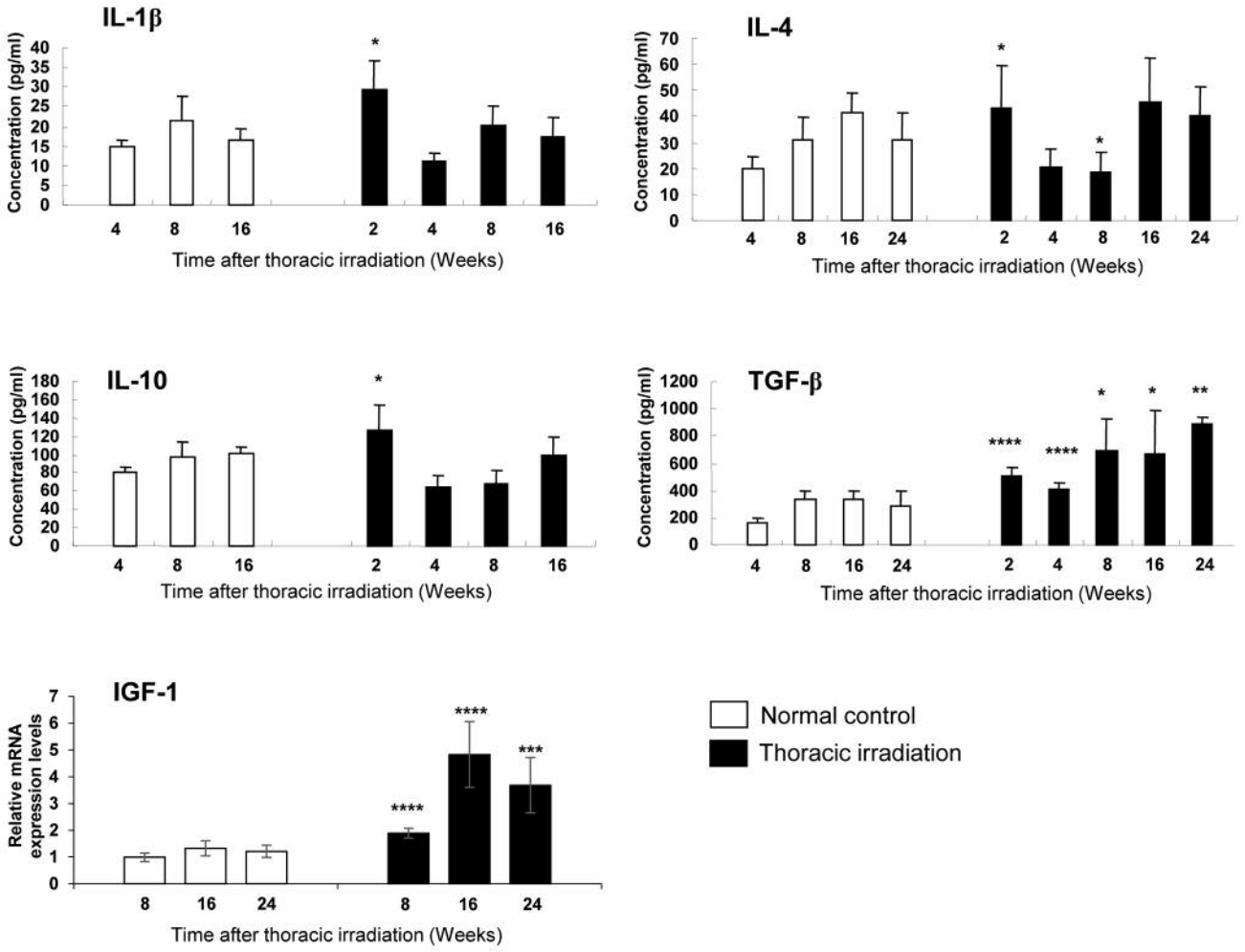

Figure 5. *Thoracic irradiation increased pro-inflammatory and pro-fibrotic cytokines in the lung of mice. At 2, 4, 8, 16, and 24 weeks after irradiation, interleukin (IL)-1β, IL-4, IL-10, and transforming growth factor (TGF)-β in bronchoalveolar lavage (BAL) fluid of mice were measured through ELISA, and relative mRNA expression levels of insulin-like growth factor (IGF)-1 in the lung tissue were examined through quantitative real-time PCR. There were four or five mice in each group. The bars indicate the means±SD. *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001 compared to the age-matched control mice.*

Discussion

The aim of this study was to document the effects of ionizing radiation on EMT of lung epithelial cells *in vitro* and *in vivo*, as well as the underlying mechanisms, focusing on the role of TGF-β-secreting M2 macrophages. Although many factors have been demonstrated as causes of radiation-induced pneumonitis and pulmonary fibrosis until recently, the exact mechanisms have not yet been identified. However, the obvious cause of radiation-induced pneumonitis and fibrosis is the excessive accumulation of ECM due to the imbalance between ECM synthesis and breakdown (33). Large amounts of ECM are synthesized from myofibroblasts, which are derived from various sources, such as resident fibroblasts, circulating fibrocytes, and damaged epithelial or endothelial cells (17). We focused on epithelial cells as the source of myofibroblasts since literature evidence supports that about 30% (in the kidney) and up to 45% (in the liver) of myofibroblasts can arise via local EMT under inflammatory stress, whereas approximately 12% come from the bone marrow (37, 38).

The current study indicated that ionizing radiation directly induced the morphological transition of lung epithelial cell from round cobblestone-like into mesenchymal-like phenotype, and significantly increased the expression of EMT-associated markers, suggesting that MLE12 cells after exposure to ionizing radiation were differentiated into myofibroblasts. This is in consistence with previous studies,

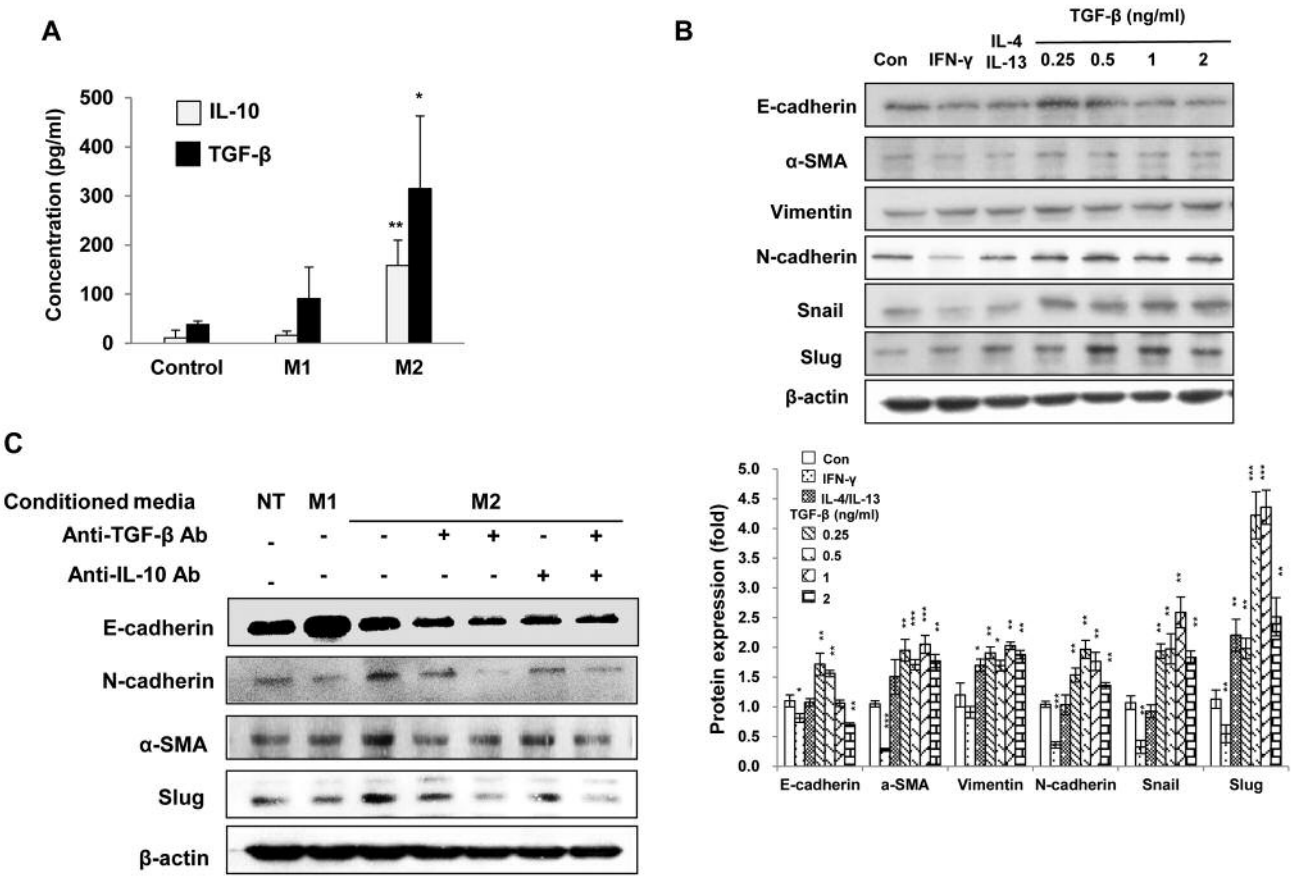

Figure 6. *Inhibition of transforming growth factor (TGF-β) derived from M2 macrophages attenuated epithelial–to–mesenchymal transition (EMT) in MLE12 cells. The levels of interleukin (IL)-10 and TGF-β from M1-polarized or M2-polarized MH-S macrophages are presented (A). The bars indicate the means ± SD. *p<0.01 and **p<0.005 compared the control macrophages that were cultured without stimulants. Protein levels of EMT-related markers in MLE12 cells after treatment with interferon (IFN)-γ, IL-4/IL-13, TGF-β, on untreated (Con) were evaluated by western blot (B). Protein levels of E-cadherin, N-cadherin, α-SMA, and Slug in MLE12 cells treated with anti-TGF-β antibody or anti-IL-10 antibody under M1 or M2-conditioned culture media were evaluated by western blot (C). The bars indicate the means±SD. *p<0.05, **p<0.005 and ***p<0.0005 (compared the control MLE12 cells). NT, Non-treatment (cultured in RPMI 1640 media supplemented with 10% FBS).*

which reported that radiation directly induced EMT in alveolar type II epithelial cells through ERK/GSK3β/Snail signaling pathway (39).

Fibrosis is the final result of chronic inflammatory reactions. All wound healing processes depend on whether the irritant persists or not, i.e. if the tissue-damaging irritant persists after the exposure of ionizing radiation, the sequential inflammatory reactions eventually lead to fibrosis (10, 12, 13). We have previously shown that the sequential influx of neutrophils and macrophages was observed in the lung of thoracic irradiated mice, leading finally to pulmonary fibrosis at six months after irradiation (34). This result indicated that cells damaged by the exposure to ionizing radiation attracted neutrophils and macrophages. We hypothesized that ionizing radiation directly affected epithelial cells and then, the damaged epithelial cells attracted inflammatory cells, such as neutrophils and macrophages, around injured sites. In this study, irradiated lung epithelial cells secreted various chemokines that can recruit macrophages. In fact, infiltration of macrophage into the damaged tissue is necessary during wound healing process, because macrophages are responsible for surveillance and tissue homeostasis through removing toxic materials, as well as dead and dying cells (1, 2, 17). However, the degree and severity of damage correlates with infiltrating macrophages. For this reason, there are many studies that showed depletion of macrophages to reduce the pro-fibrotic process (19, 40, 41). Interestingly, a study on renal fibrosis demonstrated that the infiltrating angiotensin II type 1 receptor (AGTR1)-positive macrophages have an anti-fibrotic role (42). This result suggests that there are diverse populations of macrophages with pro- and anti-

fibrotic capacities; such sub-populations are crucial in the orchestration of the wound healing process.

Gordon and Taylor first suggested that IL-4 (or IL-13)-stimulated macrophages develop an alternative activation state that is distinct from the IFN-γ-induced classical activation state (43). Many *in vitro* and *in vivo* studies have shown that macrophages activated with IL-4 (or IL-13) are characterized by elevated expression of the mannose receptor (CD206), the chitinase-3-like protein-3 (Ym1), the resistin-like molecule-α (FIZZ-1), the major histocompatibility complex class II antigens and the enzyme arginase-1 (Arg1) and are designated as M2 macrophages. Actually, M2 macrophages are intended to create an anti-inflammatory environment and promote healing and regeneration of wounds. However, chronic activation of M2 macrophages results in the release of large amounts of pro-fibrotic factors such as TGF-β, PDGF, VEGF, IGF-1, and Galactin-3, leading to an opposite effect through the activation of quiescent resident fibroblasts (25, 44). In addition, expression of Arg1 by M2 macrophages is very important; this enzyme controls L-proline production, which is required for collagen synthesis by activated myofibroblasts (45). Groves and colleagues showed the role of M2 macrophages in the development of pneumonitis and pulmonary fibrosis after thoracic irradiation in IL-4-/- mice (31). Following thoracic irradiation, the major source of increased production of IL-4 in the lung is of macrophage origin (32). In our present study, macrophages in the lungs of thoracic irradiated mice highly expressed the M2 macrophage markers, Arg-1 and CD206. Interestingly, we have previously shown that at the same time point after thoracic irradiation, there is a dramatic increase of infiltrating macrophages in the lungs and animals start to die (34). Therefore, we hypothesized that infiltrated and alternatively activated macrophages after thoracic irradiation play an important role in inducing the mesenchymal differentiation of lung epithelial cells. In support of this hypothesis, the results here showed that M2 macrophages secrete various powerful pro-fibrotic cytokines as well as Arg-1. Our results showed that soluble proteins secreted from M2 macrophages induced lung epithelial cells to express mesenchymal markers, but not those of M1 macrophages.

M2 macrophages are commonly observed during the peak of the profibrotic immune response, and they tightly control between wound healing and fibrosis through secreted proteins (23-27). After lung damage, an irritant maintenance or an insufficient repair is associated with the persistence of M2 macrophages that continue to produce growth factors. M2 macrophages contribute to the control of the inflammatory process through the release of IL-10, TGF-β and HO-1, and also these cells can activate resident fibroblasts through the release of TGF-β, IGF-1, and galactin-3 (26). Interestingly, our present results showed that from 2 to 6 months after irradiation, TGF-β and IGF-1 concentrations were high in the lung of thoracic irradiated mice, whereas other cytokines related to inflammation, such as IL-1β, IL-10, and IL-4, were down-secreted. Actually, inhibition of signaling pathway related with TGF-β, one of the key drivers of fibrosis, has been shown to completely protect from the development of fibrosis in various experimental models (46-50). Although TGF-β is produced by numerous inflammatory, mesenchymal, and epithelial cells, we hypothesized that TGF-β in radiation-induced lung fibrosis was secreted by M2 macrophages because concentration of TGF-β and the expression level of M2 macrophage-related protein were increased at the same time-point. We confirmed that macrophages cultured with IL-4 and IL-13 secreted high levels of TGF-β and TGF-β elimination in culture supernatant of M2 macrophages attenuated mesenchymal transition of lung epithelial cells, suggesting that the elimination of TGF-β secreted from M2 macrophages is likely to prevent pulmonary fibrosis.

Our results showed that mesenchymal transition of epithelial cells was directly induced by ionizing radiation or indirectly by the M2 macrophages that were attracted into the irradiated area. Importantly, TGF-β secreted from M2 macrophages was a key role on mesenchymal transition of epithelial cells, although the detailed mechanisms by which they promote EMT are still unclear. Ongoing studies aiming to determine the mechanism, by which M2 macrophages control pulmonary fibrosis after ionizing radiation, may foster the new immunological approaches for understanding radiation-induced pulmonary fibrosis.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

HRP carried out all *in vitro* and *in vivo* experiments and wrote the manuscript. UHJ and SKJ participated in the design of study. All authors have read and approved the manuscript.

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