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Inhibitory effect of CXC chemokine receptor 4 antagonist AMD3100 on bleomycin induced murine pulmonary fibrosis

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Abbreviations: BAL, bronchoalveolar lavage; BM, bone marrow; BMDHSC, bone marrow derived hematopoietic stem cells; BMDMSC, bone marrow derived mesenchymal stem cells; CXCR4, CXC chemokine receptor 4; IPF, idiopathic pulmonary fibrosis; KC, cytokine-induced neutrophil chemoattractant; SDF-1, stromal cell derived factor-1

Abstract

CXC chemokine receptor 4 (CXCR4), which binds the stromal cell-derived factor-1 (SDF-1), has been shown to play a critical role in mobilizing the bone marrow (BM)-derived stem cells and inflammatory cells. We studied the effects of AMD3100, CXCR4 antagonist, on a murine bleomycin-induced pulmonary fibrosis model. Treatment of mice with AMD3100 in bleomycin-treated mice resulted in the decrease of SDF-1 in bronchoalveolar lavage (BAL) fluids at an early stage and was followed by the decrease of fibrocytes in the lung. AMD3100 treatment decreased the SDF-1 mRNA expression, fibrocyte numbers in the lung at an early stage (day 3) and CXCR4 expression at the later stage (day 7 and 21) after bleomycin injury. The collagen content and pulmonary fibrosis were significantly attenuated by AMD3100 treatment in later stage of bleomycin injury. AMD3100 treatment also decreased the murine mesenchymal and hematopoietic stem cell chemotaxis when either in the stimulation with bleomycin treated lung lysates or SDF-1 *in vitro*. In BM stem cell experiments, the phosphorylation of p38 MAPK which was induced by SDF-1 was significantly blocked by addition of AMD3100. Our data suggest that AMD3100 might be effective in preventing the pulmonary fibrosis by inhibiting the fibrocyte mobilization to the injured lung via blocking the SDF-1/CXCR4 axis.

Keywords: bleomycin; chemokine CXCL12; chemotaxis; JM 3100; pulmonary fibrosis; receptors, CXCR4

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and irreversible fibrosing lung disease characterized by the remodeling of the lung parenchyma and collagen deposition (Gross and Hunninghake, 2001). The hallmark lesions are the fibroblastic foci representing focal areas of active fibrogenesis featuring fibroblast replication and extracellular matrix deposition. Activated fibroblasts, or myofibroblasts, are known to be heterogeneous with respects to a number of phenotypic features. Although it is assumed that the myofibroblasts arise from intrapulmonary fibroblasts, there is recent evidence of circulating fibrocytes and BM-derived progenitor cells may be recruited in lung injury and play an important role in the pathogenesis of pulmonary fibrosis (Epperly *et al*., 2003; Dunsmore and Shapiro, 2004; Garantziotis *et al*., 2004; Phillips *et al*., 2004; Mizuno *et al*., 2005; Lama and Phan, 2006). Fibrocytes are BM-derived cells with monocyte morphology, that express surface markers of leukocytes and hematopoietic stem cells but also collagen I; and are capable of differentiating into diverse cell types

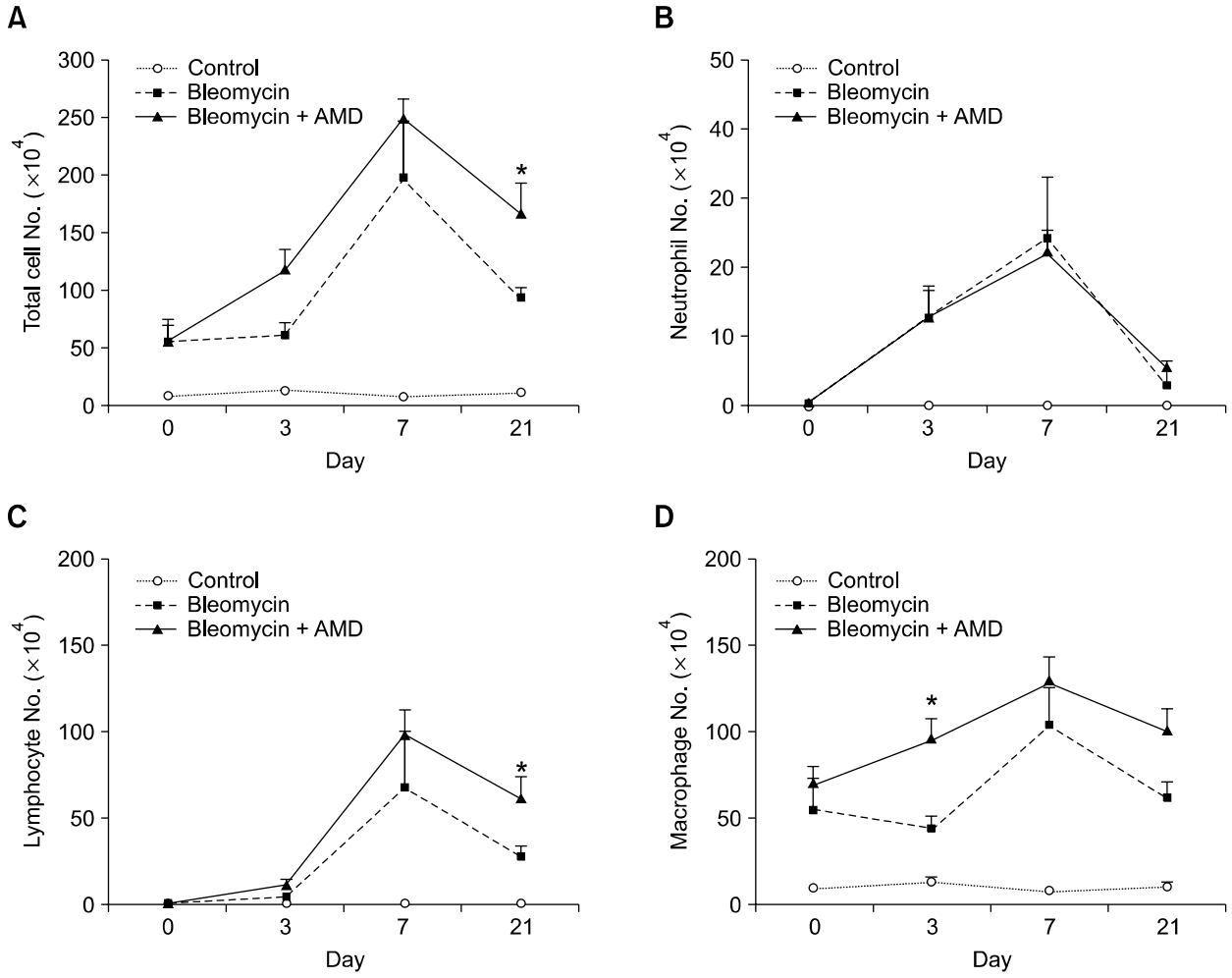

Figure 1. The total inflammatory cell and differential cell numbers in the BAL fluids were measured from the mice. Total inflammatory cells were increased by AMD3100 treatment at day 3 and 21 than bleomycin injury alone (*: $P < 0.05$). No difference of neutrophil counts ware noted between bleomycin group and bleomycin plus AMD3100 group.

(Metz, 2003; Quan *et al*., 2004; Hong *et al*., 2005).

SDF-1, also called CXCL-12 is a CXC chemokine, acting via its receptor, CXCR4, is a chemoattractant for a number of leukocyte populations (Bleul *et al*., 1996; Aiuti *et al*., 1997; Nagase *et al*., 2000; Hashimoto *et al*., 2004; Mizuno *et al*., 2005) including BM derived stem cells. It has become evident that the SDF-1-CXCR4 signaling axis also plays an important role in the homing and engraftment of hematopoietic stem/progenitor cells (Peled *et al*., 1999; Kahn *et al*., 2004). Mobilization of stem cell from BM to peripheral blood, and thence to injured tissues, may be down an SDF-1 concentration gradient (Guo *et al*., 2005; Wright *et al*., 2005).

AMD3100 is a bicyclam derivative and selectively antagonizes the CXCR4 (Schols *et al*., 1997). AMD3100 inhibits the intracellular calcium influx responses to SDF-1 (Schols *et al*., 1997) and also attenuates allergic lung inflammation (Lukacs *et al*., 2002). In this study, we hypothesized that AMD3100 might be effective in controlling the bleomycin-induced pulmonary fibrosis in mice by blocking the SDF-1/CXCR4 axis.

Results

BAL findings in the bleomycin-treated mice and effect of AMD3100

As shown in Figure 1, bleomycin administration to the lung increased the neutrophil and total cell number in the BAL fluids. Treatment of AMD3100 more increased the total cell numbers without change of neutrophil numbers at 3 days after bleomycin administration although the content of

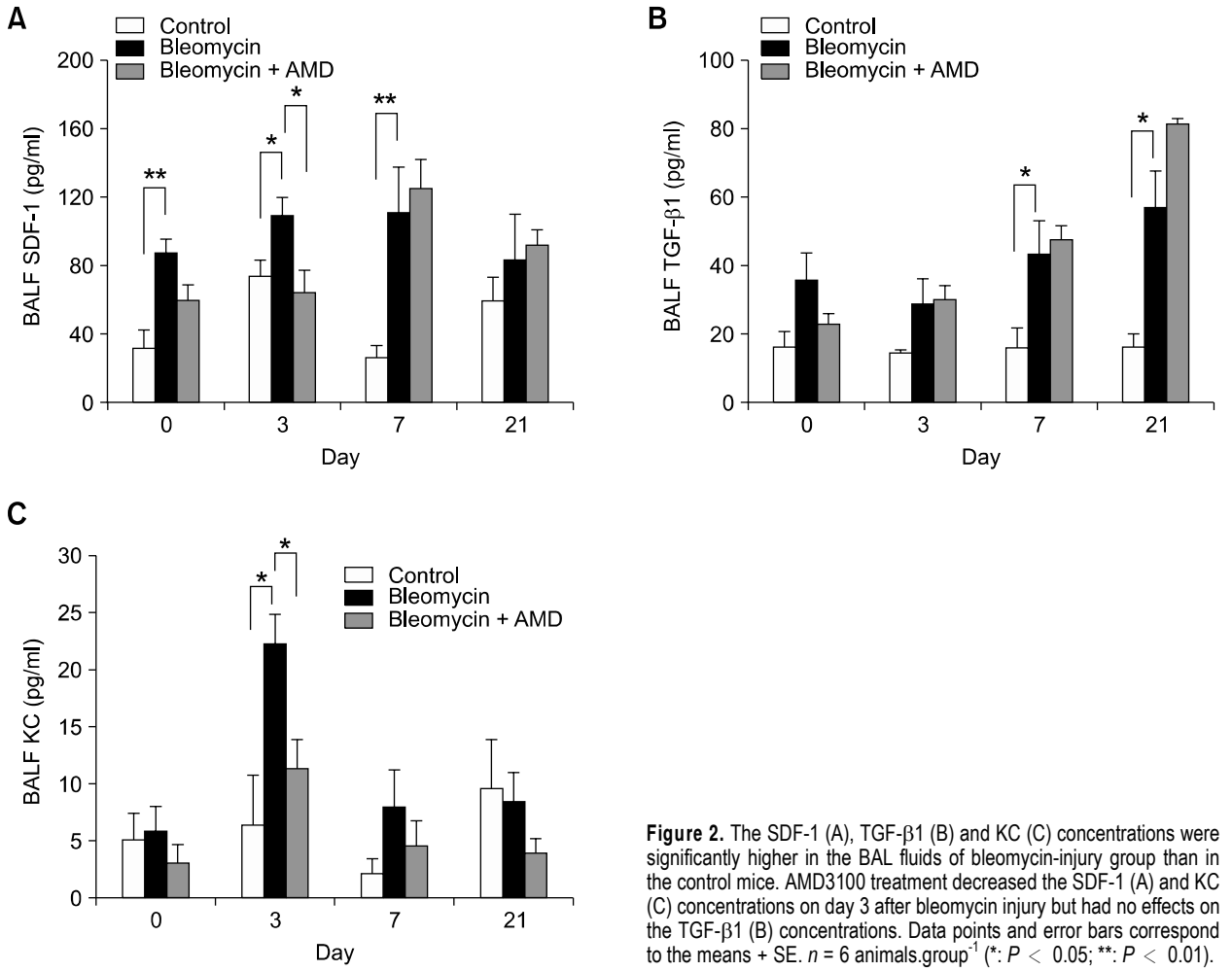

Figure 2. The SDF-1 (A), TGF-β1 (B) and KC (C) concentrations were significantly higher in the BAL fluids of bleomycin-injury group than in the control mice. AMD3100 treatment decreased the SDF-1 (A) and KC (C) concentrations on day 3 after bleomycin injury but had no effects on the TGF-β1 (B) concentrations. Data points and error bars correspond to the means + SE. n = 6 animals.group^{-1} (*: $P < 0.05$; **: $P < 0.01$).

cytokine-induced neutrophil chemoattractant (KC) was decreased in AMD3100 treated mice on day 3 (Figure 2C). Bleomycin administration increased the SDF-1, and transforming growth factor (TGF)-β in the BAL fluids and AMD3100 treatment decreased the SDF-1 concentrations on day 3 (Figures 2A and 2B).

Flow cytometry analysis

Fibrocytes in the lung were identified as triple staining with Col I, CD45, CXCR-4 and analyzed with flow cytometry. Murine fibrocytes in the lung were maximally increased at 3 days after bleomycin administration. AMD3100 treatment significantly blocked the accumulation of the fibrocytes to the lung at 3 days after bleomycin administration (Figures 3A and 3B). Despite the SDF-1 in BAL fluid (Figure 1A) and SDF-1 mRNA (Figure 5) is already increased since day 0 after bleomycin injury, fibrocytes in the lung only increased on day 3 after injury.

Effect of AMD3100 on the collagen content in the lung and pulmonary fibrosis of bleomycin-treated mice

The collagen contents in the lung were increased from the 7 days after bleomycin administration until day 21. Treatment of AMD3100 significantly decreased the total collagen contents in the lung on day 21 without change on day 7 after bleomycin administration (Figure 4A). Pulmonary fibrosis score which was measured by Ashcroft method showed that AMD3100 treatment decreased the fibrosis significantly in bleomycin injury model (Figure 4B). Histological examinations also revealed that AMD3100 apparently modified the bleomycin-induced lung inflammation and fibrosis (Figure 4C).

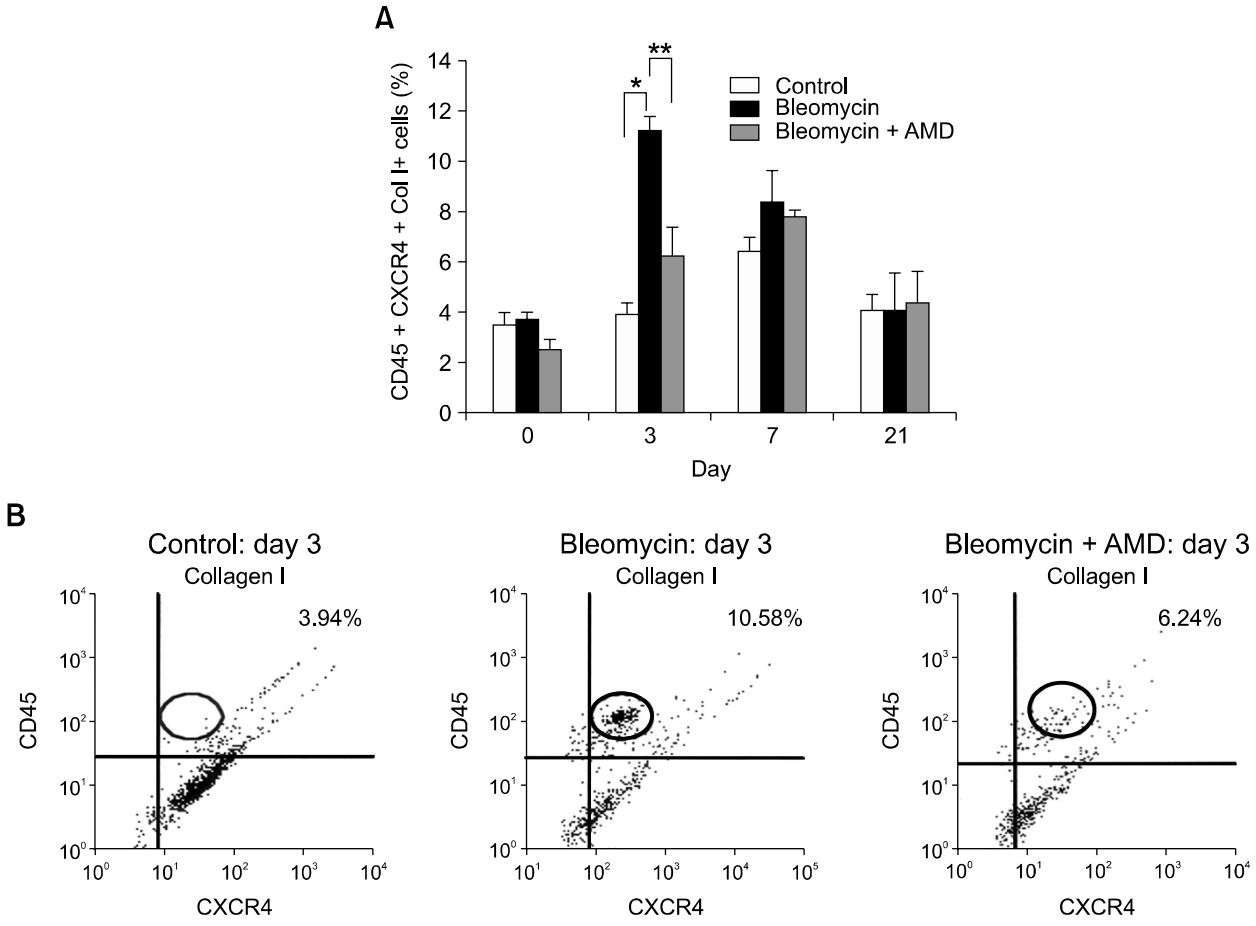

Figure 3. Intrapulmonary CD45+CXCR4+Col I+ fibrocytes recruitment after bleomycin injury. CD45+CXCR4+Col I+ fibrocytes were significantly increased on day 3 after bleomycin injury. AMD3100 treatment significantly decreased the fibrocytes recruitment on day 3 after bleomycin injury (A, B). Single cell suspensions from the lung in the bleomycin injury group, bleomycin plus AMD3100 group and control group were made and triple stained for CD45, Col I, and CXCR4, then examined by FACS analysis. n = 5 samples.group^{-1} (*: $P < 0.05$; **: $P < 0.01$).

SDF-1 mRNA expression in the lung

It has been described that SDF-1 concentrations in the serum and BAL fluids were increased in the bleomycin-treated mice (Xu *et al*., 2007). As shown in Figure 5, SDF-1 mRNA expression in the lung was increased on day 0, 3 and 7 after bleomycin injury and AMD3100 treatment significantly decreased the SDF-1 mRNA expression on day 3.

CXCR-4 levels in the lung

The CXCR-4 protein expression in the lung was increased from day 3 to day 21 after bleomycin administration and treatment of AMD3100 decreased the CXCR-4 expression on day 7 and 21 (Figure 6). The time course of the increase in CXCR4 expression in the lungs was delayed relative to the time course of BAL SDF-1 concentrations (Figure 2). This finding suggested that AMD3100 treatment inhibited the CXCR-4 positive cell accumulation which was induced by increased SDF-1 in the lung after bleomycin injury.

Chemotaxis of the BM stem cells

To elucidate the role of SDF-1/CXCR-4 axis in homing of BM derived mesenchymal stem cell (BMDMSC) or hematopoietic stem cells (BMDHSC) to the lung in bleomycin-treated mice, we performed a chemotaxis assay using the cultured murine BMDMSC (Figure 7A) or BMDHSC (Figure 7B) with different lung extracts obtained from mice on day 3 after bleomycin treatment. SDF-1 (50 ng/ml) significantly induced chemotactic migration of MSC and HSC as much as from the bleomycin treated lung lysates in migration experiments. Treatment of AMD3100 markedly blocked the migration of MSC and HSC which was induced by

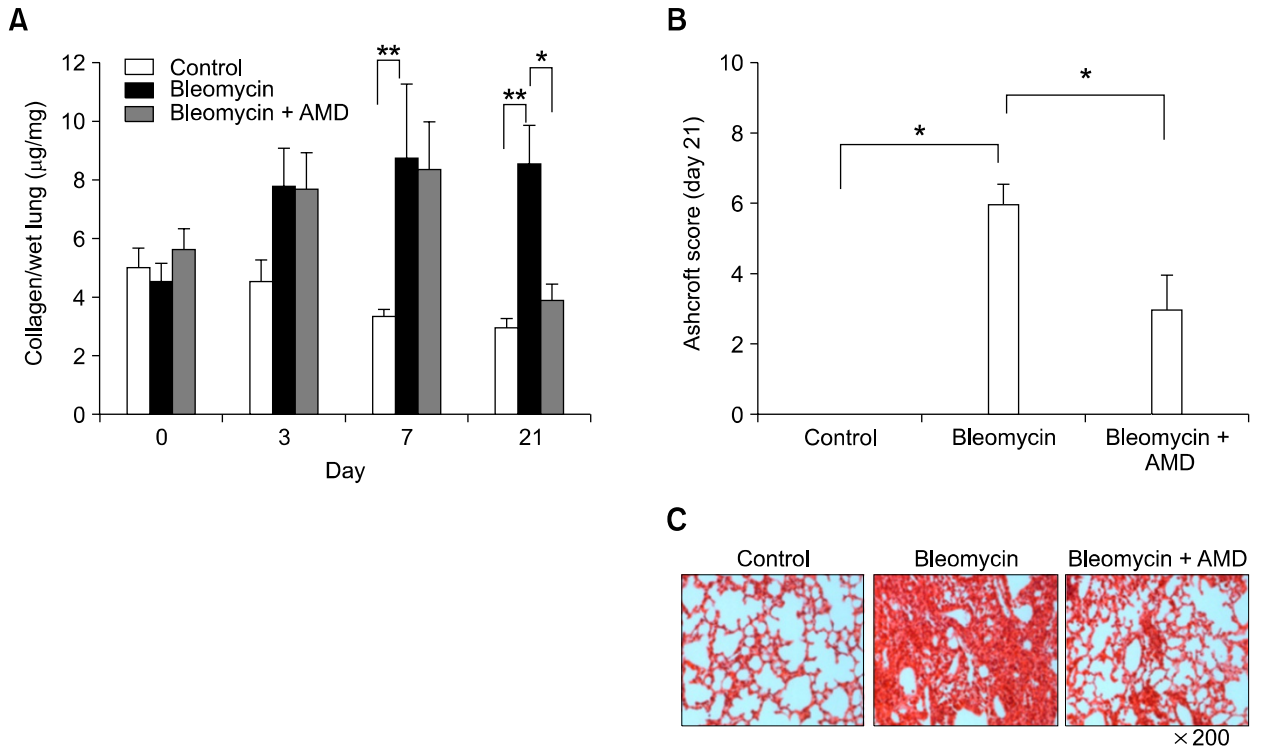

Figure 4. Collagen content and representative histopathology. The collagen content in the lung was increased from 3 days after bleomycin injury until day 21. The collagen content was decreased by AMD3100 treatment on day 21 after bleomycin injury (A). Total collagen content was determined by the Sircol assay. Severe pulmonary inflammation and fibrosis occurred after bleomycin injury on day 21 and this histological change was markedly reduced by AMD3100 treatment (B). Pulmonary fibrosis was scored by Ashcroft method. Representative photomicrographs of lung tissue stained with a hematoxyline-eosin (C). Original magnification X 200. n = 4 lungs.group^{-1} (*: $P < 0.05$; **: $P < 0.01$).

SDF-1 or bleomycin treated lung lysates. Furthermore, lysates of the AMD3100-treated lung after bleomycin injury decreased the migration of MSC and HSC which was induced by bleomycin-injured lung lysates. These results again suggest that SDF-1 is produced in the injured lung from the bleomycin treated mice which recruits BMDMSC or HSC to the lung through the CXCR4 receptors and AMD3100 treatment effectively blocked this migration of MSC and HSC *in vitro* experiments by blocking the CXCR4 receptors.

Phosphorylation of MAPK analysis in BMDMSC and HSC

Activation of p38 MAPK occurs in the bleomycin-treated mouse model (Nick *et al*., 2000). As shown in Figure 7, SDF-1 (50 ng/ml) phosphorylated p38 MAPK from the MSC (Figure 8A) and HSC (Figure 8B) but not the p44/42 MAPK of the mouse BM (data not shown). The phosphorylation level of p38 MAPK was apparently decreased by addition of AMD3100 (100 μg/ml) as much as SB203580, a specific p38 MAPK inhibitor, in mice MSCs (Figure 7A) and HSCs (Figure 7B).

Discussion

MSCs have generated a great deal of interest as a potential source of cells for cell-based therapeutic strategies (Tocci and Forte, 2003; Hashimoto *et al*., 2004; Le Blanc and Pittenger, 2005). However, recent evidence suggests that a population of BM derived cells called "fibrocytes" may be recruited in lung injury and plays an important role in the pathogenesis of pulmonary fibrosis (Dunsmore and Shapiro, 2004; Garantziotis *et al*., 2004; Hashimoto *et al*., 2004; Phillips *et al*., 2004; Ishii *et al*., 2005; Lama and Phan, 2006). Circulating fibrocytes might act as a significant sources of lung fibroblasts in response to lung injury (Epperly *et al*., 2003; Dunsmore and Shapiro, 2004; Phillips *et al*., 2004; Ishii *et al*., 2005) and express a characteristic markers, including collagen I (Col I), CD45, CD34, CXCR4 and CCR7 (Bucala *et al*., 1994; Abe *et al*., 2001).

In this study, we demonstrated that treatment of AMD3100 (a specific CXCR4 antagonist) starting one day before the bleomycin administration for 21 days, the bleomycin induced pulmonary fibrosis was decreased and the fibrocyte recruitment to the

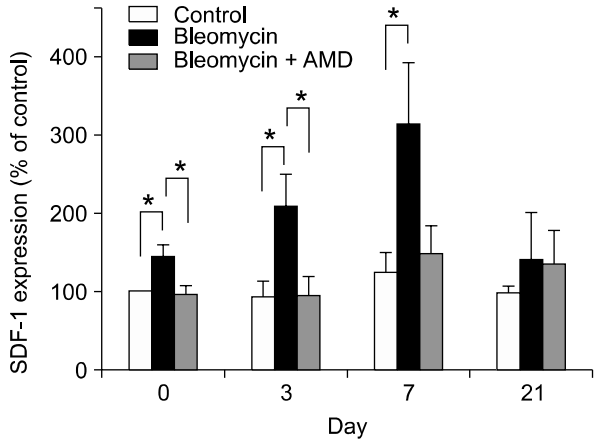

Figure 5. Expression of SDF-1 mRNA in mouse lung. SDF-1 mRNA expression in the lung was increased at 0, 3 and 7 days after bleomycin injury than in control mice. AMD3100 treatment decreased the SDF-1 mRNA expression in the lung at 0 and 3 days after bleomycin injury. In each group, mRNA levels were analyzed using real-time RT-PCR and β-actin as housekeeping gene. Nonparametric Kruskal-Wallis H test. n = 3 lungs in each group (*: $P < 0.05$).

lung was also decreased in the early stage. Treatment of AMD3100 to the bleomycin-injured mice, the total inflammatory cell numbers were increased without change of neutrophil numbers in BAL fluids, although the content of neutrophil chemoattractant KC was decreased on day 3. This phenomenon is interesting and somewhat similar to other paper that the mobilization of neutrophils from the bone marrow by the KC was enhanced by blocking the SDF-1 by using a CXCR4 antagonist (Martin *et al*., 2003). If this is true, we suggest that the suppression of KC by AMD3100 treatment might be due to negative feedback phenomenon because KC-induced neutrophil mobilization from the bone marrow was enhanced by AMD3100 treatment. In addition, despite KC content was decreased by AMD3100 treatment, other neutrophil chemoaattractant such as MIP-2 may affect the neutropohil mobilization to the lung after bleomycin injury.

Increased expression of SDF-1 in the lungs of patients with idiopathic interstitial pneumonia (IIP) was demonstrated which suggest the importance of the axis SDF-1/CXCR4 in the development of pulmonary fibrosis (Yang *et al*., 2007). In view of the 64% and 77% increase in lung SDF-1 in lung tissue from patients with usual interstitial pneumonia (UIP) and fibrotic nonspecific interstitial pneumonia (NSIP) than from normal lungs, enhanced expression of SDF-1 was associated with higher number of circulating fibrocytes in pulmonary fibrosis (Mehrad *et al*., 2007). Stromal cell-derived factor-1 (SDF-1/CXCL12), a CXC chemokine, was originally cloned from BM stromal cells (Tashiro *et al*., 1993) binds only to one receptor, CXCR4. The SDF-1/CXCL12 plays an important role in hematopoiesis, as well as in vascular and cerebellar development and cardiogenesis (Nagasawa *et al*., 1996; Tachibana *et al*., 1998; Zou *et al*., 1998).

In this investigation, SDF-1 levels in BAL fluid were increased early after bleomycin-injury and was accompanied by an increase in CXCR4 expression in lungs until three weeks after inducing lung injury. This temporal relationship is consistent with the concept that SDF-1 recruits CXCR4 expressing cells from BM to the injured lung. Inhibition of interaction between CXCR4 and SDF-1 by AMD3100 pre-treatment in our bleomycin-administred murine model resulted in the decrease of fibrocytes in the lung tissue. Because the fibrocytes are BM-derived cells that express surface markers of leukocytes and hematopoitic stem cells but also collagen I, we identified lung fibrocytes by FACS analysis through the triple staining with CD45, CXCR4 and collagen I. Since circulating fibrocytes are capable of differentiating into myofibroblasts both in culture and *in vivo* (Abe *et al*., 2001; Schmidt *et al*., 2003), we suggest that this increased fibrocytes were invol-

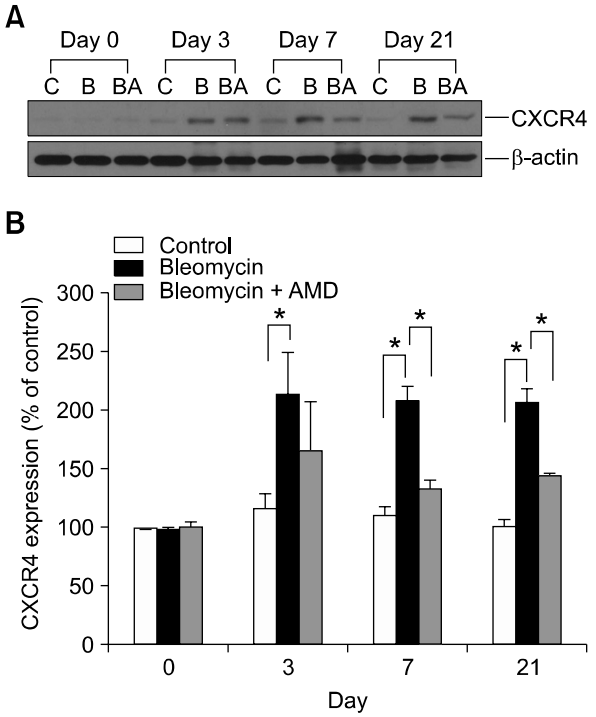

Figure 6. Western immunoblotting showing CXCR4 protein in the lung was increased from 3 days to 21 days after bleomycin injury. AMD3100 treatment decreased the CXCR4 protein at 7 and 21 days after bleomycin injury. C: control, B: bleomycin, BA: bleomycin plus AMD3100. Nonparametric Kruskal-Wallis H test. n = 3 lungs.group^{-1} (*: $P < 0.05$).

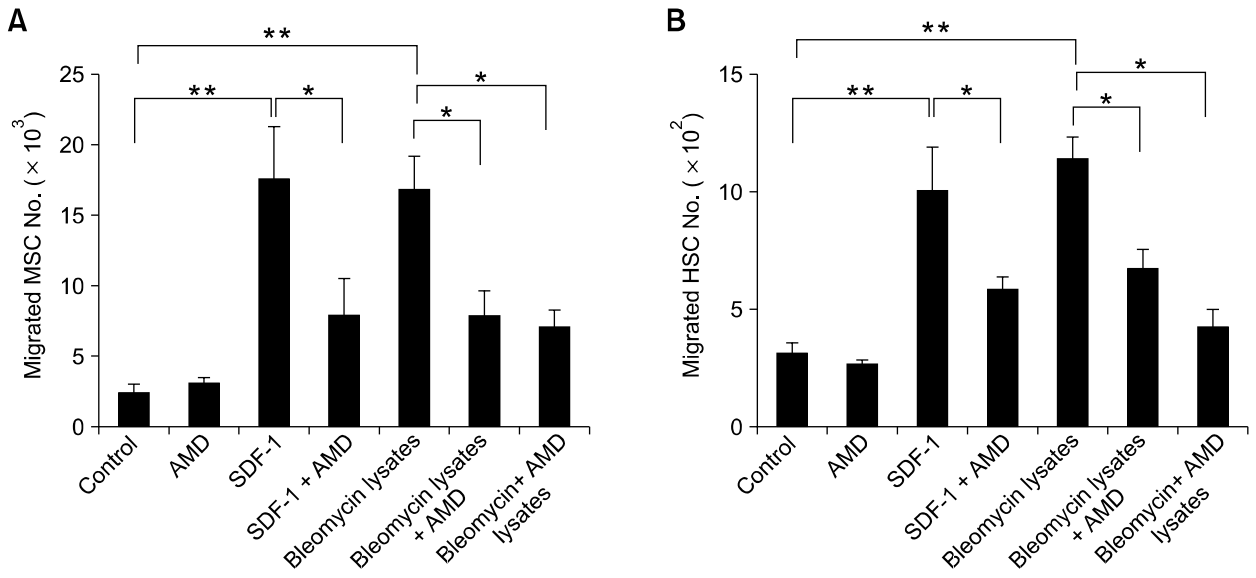

Figure 7. Chemotaxis of MSCs and HSCs across the filter of HTS Transwell®-96 Permeable Support Systems. The MSC and HSC were collected from the mouse femur and allowed to migrate to the lower chamber toward several stimulants in Transwell Systems. Cells that migrated to the lower chamber were counted microscopically. SDF-1 (50 ng/ml) and bleomycin-injured lung lysate markedly increased the migration of MSCs (A) and HSCs (B). Adding the AMD3100 (100 μg/ml) to the bleomycin-injured lung lysates or SDF-1 treated samples inhibited the stem cell migration. Lung lysates from the AMD3100 treated groups in bleomycin-injured mice also showed inhibition of stem cell migration compared with bleomycin-injured lung lysates. Nonparametric Kruskal-Wallis H test. Results are mean of at least three independent experiments (*: $P < 0.05$; **: $P < 0.01$).

ved in murine pulmonary fibrosis after bleomycin injury.

We found that despite the SDF-1 in BAL fluids increased immediately after bleomycin injury, fibrocytes in the lung increased at 3 days after injury. This point is already discussed in the recent paper that the time course of the increase in CXCR4+ bone marrow derived mesenchymal cells in the lungs was delayed relative to the time course of BAL SDF-1 concentrations (Xu *et al*., 2007). The source of lung fibroblasts and myofibroblasts is a critical question in the pathogenesis of human fibrotic lung diseases. While these cells were classically thought to be derived exclusively from resident lung fibroblasts, recent studies indicate that they can differentiate from pulmonary epithelial cells (Kim *et al*., 2006) and from a circulating precursor cell, the fibrocyte (Phillips *et al*., 2004). On the basis of the expression of CD34 by fibrocytes (Bucala *et al*., 1994; Abe *et al*., 2001), the presence of CD34+ cells that also express COL I and α-smooth muscle actin in the bronchial mucosa of patients with allergic asthma (Schmidt *et al*., 2003) suggests that circulating fibrocytes are potential precursors for the fibroblast/myofibroblast in the remodeling airway wall. The mechanism by which fibrocytes are induced to undergo phenotypic transformation into fibroblasts and myofibroblasts and contribute of fibrogenesis in lung are poorly understood, although recently both haptoglobin and cysteinyl leukotrienes have been implicated (Larsen *et al*., 2006; Vannella *et al*., 2007). CXCR4 is a G protein-linked seven transmemtrane spanning receptor that was first identified as a co-factor for T cell-tropic HIV-1 and -2 viral entry into cells (Feng *et al*., 1996). A variety of stem cells express CXCR4 (Peled *et al*., 1999; Rosu-Myles *et al*., 2000; Lapidot and Kollet, 2002), including hematopietic stem cells (Aiuti *et al*., 1997) as well as progenitor cells committed to neural (Zou *et al*., 1998), myocardial (Damas *et al*., 2000), and endothelial (Dar *et al*., 2005) differentiation pathways. Thus, CXCR4+ stem cells recruited to the lungs after injury may be precursors of inflammatory cells as well as of mesenchymal-derived cells.

We also measured total collagen content in lung using the Sircol assay, where significant collagen deposition was detectable within 3 days after bleomycin injury. This finding was consistent with the concept that collagen is detectable very early in the inflammatory process during acute lung injury in patients (Clark *et al*., 1995; Pugin *et al*., 1999). Lung fibrosis was prominent at 21 days after bleomycin injury and was quantified by Ashcroft score. We confirmed that daily AMD3100 treatment reduced the total collagen content and pulmonary fibrosis in murine bleomycin injury model. Despite the TGF-β level in BAL fluid was increased from 7 days to 21 days after bleomycin injury and AMD3100 treatment did not decrease the TGF-β,

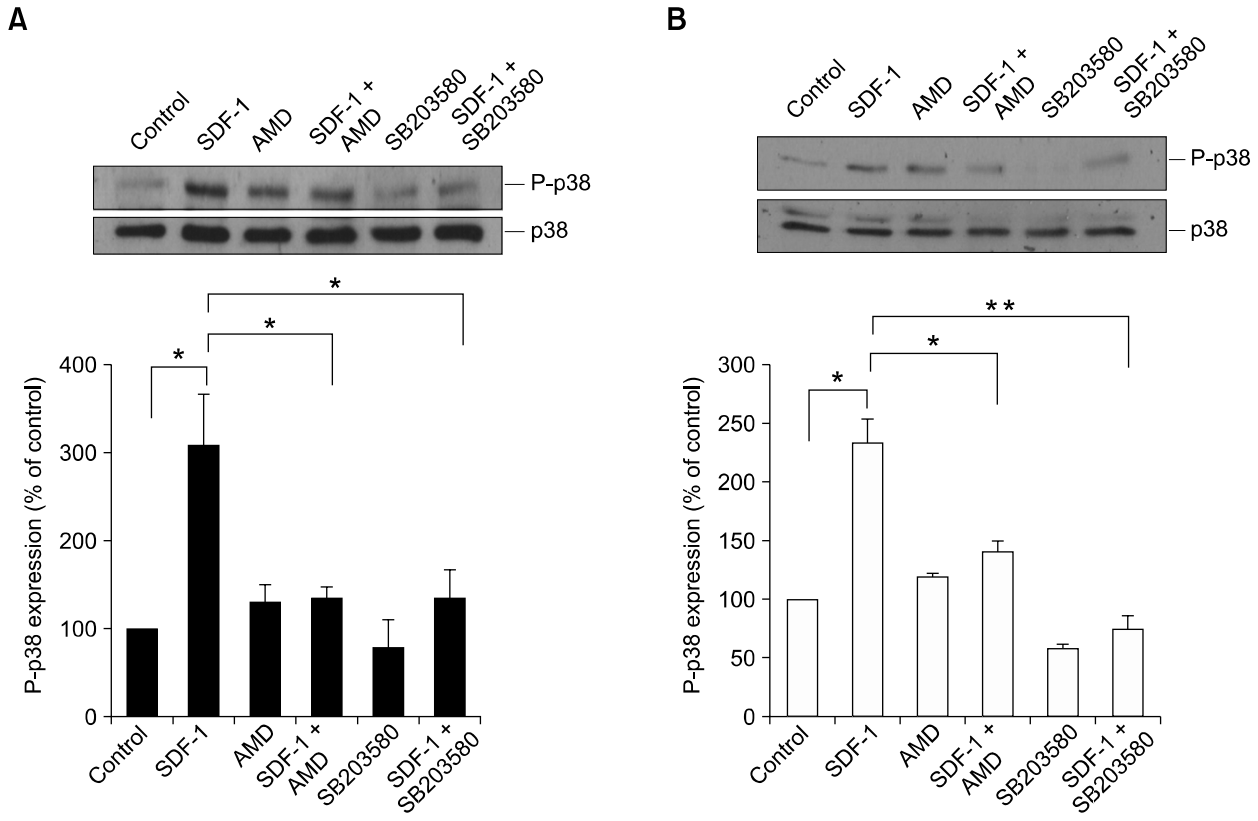

Figure 8. MAPK phosphorylation analysis of mesenchymal stem cells. The SDF-1 phosphorylated the p38 MAPK in MSCs (A) and HSCs (B). The phosphorylation level of p38 MAPK was apparently decreased by addition of AMD3100 as much as SB203580, a specific p38 MAPK inhibitor, in mice MSCs and HSCs. The mesenchymal or hematopoitic stem cells were purified from the mouse femur. Results are representative of at least three independent experiments (*: $P < 0.05$; **: $P < 0.01$).

the increased collagen content after bleomycin injury was decreased by AMD3100 treatment at 21 days after injury. We think that this discrepancy between TGF-β level and collagen content is developed because the origin of collagen at day 21 is mainly from the migrated fibrocytes to the lung at day 3. The increased collagen content might be originated from the epithelial to mesenchymal transition or resident fibroblast rather than from fibrocytes on day 7 but the newly formed collagen on day 21 might be formed mainly from the recruited fibrocytes. Isolated fibrocytes have the capacity to differentiate to collagen secreting myofibroblasts *in vitro* when stimulated by TGF-β (Abe *et al*., 2001).

To confirm whether the injured lung release the substances which made an influx of BM-derived cells into the lung, we did a chemotaxis experiment using the HSC and MSC. Lung lysates from the bleomycin injured lung markedly migrated the isolated HSC and MSC similar to that degree of SDF-1 addition and this chemotaxis of stem cells were markedly suppressed by addition of AMD3100. These findings taken together would be consistent with the possibility that increasing expression of SDF-1 like substances in bleomycin induced lung fibrosis can lead to migratory recruitment of BM derived precursor cells into the lung and contribute to the active fibrotic lesions. This concept was supported from the findings that neutralization of SDF-1 results in reduced fibrocyte recruitment to the bleomycin-injured lung, which is accompanied by reduced lung fibrosis (Phillips *et al*., 2004). Others have reported similar results. For example, treatment of mice with a CXCR4 antagonist, TN14003, with bleomycin-induced lung injury significantly attenuated lung fibrosis (Xu *et al*., 2007).

The possible cellular sources for these chemokines are lung fibroblasts, endothelial cells or epithelial cells (Ponomaryov *et al*., 2000). In our experiment, SDF-1 phosphorylated p38 MAPK but not the p44/p42 MAPK in HSC and MSC where AMD3100 inhibited the p38 MAPK activation like p38 specific inhibitor, SB203580. This finding suggested that increased SDF-1 in bleomycin-in-

jured lung could recruit the HSC and MSC by activating the p38 MAPK after binding to CXCR4 on their cell surface. SDF-1 is a chemokine implicated in the recruitment of HSC principally; only recently has it been described as an important element in the recruitment of MSC (Aiuti *et al*., 1997; Peled *et al*., 2000; Hattori *et al*., 2003). Because p44/42 ERK MAPK is a downstream signaling molecule of SDF-1/CXCR4 signaling pathway in prostatic cancer line (Wang *et al*., 2005), we suspect that signal pathway is different in BM stem cells.

Taken together, our observations provide evidence that AMD3100 or specific inhibitors of SDF-1/CXCR4 axis may be useful in the prevention of pulmonary fibrosis by inhibiting the recruitment of CD45+, CXCR4+, collagen I+ fibrocytes that was originated from the BM to the injured lungs.

Methods

Animals

Eight week old C57BL/6 female mice were used. They were randomized into three groups before initiating experimental protocols. Animals were maintained in specific pathogen-free conditions and were approved by the Catholic University Ethics Committee for Animal Experiments.

Treatment of animals

The mice were anesthetized by intra-peritoneal (i.p) administration of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg) and then trachea was exposed via a cervical incision. Bleomycin (Sigma, St. Louis, MO; 2 U/kg in mice) in 50 μl of PBS or PBS vehicle were injected into the tracheal lumen with a 24 gauge needle on day 0. AMD3100 (Sigma-Aldrich; 200 μg in 250 μl of PBS) were injected i.p. for 21 days from day -1 to day 20 and then sacrificed on day 0, 3, 7, and 21 (Supplemental Data Figure S1). Animal experimental procedures were performed according to guidelines of the Animal Subjects Committee of The Catholic University of Korea.

BAL

The trachea was exposed by incising the skin and cannulated with a 24-gauge intravenous catheter. Lavage was performed with a 500 μl aliquot of phosphate-buffered saline (PBS) and was repeated for 2 times. The collected fluids were centrifuged at 400 × *g* for 5 min. The supernatants were collected and stored at -70°C until analyses of cytokines. The BAL cell pellet was resuspended in 500 μl of PBS and the cell numbers were counted using a hemocytometer. Cells were dispersed on a slide glass using cytospin 2 (Shandon, Pittsburgh, PA) and stained with Wright-Giemsa. 300 cells were differentially counted to determine populations of each cell fraction.

ELISA for assessment of SDF-1, TGF-β1, and KC levels in BAL fluids

The levels of SDF-1, TGF-β1, and KC were quantified in BAL fluids using an ELISA kit (DuoSet® ELISA development system, R&D systems, Minneapolis, MN) according to the manufacturer's protocol. All samples were centrifuged at 8,000 rpm for 15 min before assay, and each level was measured in duplicate. The absorbance at 450 nm wavelength was measured by microplate reader (Molecular device Corp., Sunnyvale, CA).

Fluorescence-activated cell sorting analysis (FACS)

Lung cells were isolated from the whole lungs of mice. Whole lung tissues were mechanically macerated, constantly agitated in 0.2% (w/v) type IV collagenase in RPMI 1640 with 10% FBS for 2 h at 37°C. After total cells were washed in PBS, RBCs of cell suspensions were lysed by distilled water and then the remnant cells were counted with a hemocytometer. More than 10^6 cells were fixed with 4% paraformaldehyde for 15 min on ice and permeabilized using 100% methanol. The cells were washed in PBS, blocked with PBA (PBS, 5% FBS, 0.01% NaN_3), and then incubated with PerCP-conjugated anti-mouse CD45 (BD Pharmigen, San Jose, CA), PE-conjugated anti mouse CXCR4 (BD Pharmigen) and rabbit anti-collagen I (Abcam, Cambridge, UK) for over night at 4°C. For the observation of collagen I, FITC-conjugated rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The cells were analyzed on a FACS (30000 cells count, BD Biosciences, Franklin Lakes, NJ, CA) and the data were analyzed by using the CellQuest™ 3.3 software (BD Biosciences).

Morphological analysis

The left lung was fixed by 4% paraformaldehyde at 4°C for 48 h. The fixed lung was embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin-eosin. For the quantitative analysis of fibrotic changes induced by bleomycin, the Ashcroft score was used. 5 fields within each lung section were observed at a magnification of ×100, and the degree of fibrosis was graded on a scale from 0 (normal) to 8 (total fibrosis), using the average of microscopic field scores. Fibrosis was defined as areas that had an Ashcroft grade of 7 or 8 (Ashcroft *et al*., 1988).

Sircol collagen assay

Total soluble collagen was measured in right middle lobe of lung tissue using the Sircol assay kit (Biocolor Ltd., Carrickfergus, Northern Ireland) according to the manufacturer's protocol. 1 ml of Sircol dye reagent was added to 100 μl test sample and mixed for 30 min at room temperature. The collagen-dye complex was precipitated by centrifugation at 10,000 × *g* for 10 min; and then washed twice with 500 μl of ethanol. The pellet was

dissolved in 500 μl of alkali reagent. The absorbance was measured at 540 nm by microplate reader. The calibration curve was set up on the basis of collagen standard provided by the manufacturer.

Real time RT-PCR for SDF-1 gene expression analysis

Total RNA from each sample (right lower lobe) was isolated and reverse transcribed into cDNA using TRI Reagent (guanidium isothiocyanate-phenol mixture) and SuperScript III (200 U/μl, Invitrogen, Carlsbad, CA). 50 ng of cDNA was amplified in iQ5 cycler (Bio-Rad, Hercules, CA) with SYBR Green Real-Time Premix (RBC Bioscience, Chung Ho City, Taiwan) using the following specific primer pairs. SDF-1: 5'-GAAAGGAAGGAGGGTGGCAG-3' (forward)/5'-TCCCC-GTCTTTCTCGAGTGT-3' (reverse), GAPDH: 5'-TGCCAA-AGCTGCTGCTAAGGCT-3' (forward)/5'-AGTCCAAAGCC-AGGTCTTGCTG-3' (reverse). The cycling conditions were 45 cycles at 60°C and amplified DNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot for CXCR4 and MAPK protein

Right upper lobe of lung was homogenized with RIPA buffer containing protease inhibitors (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mg/ml aprotinin, 1 mM PMSF, 0.1 mM sodium vanadate and 10 mM sodium fluoride) and the supernatant was isolated by centrifugation. For clarification of the signal pathway via CXCR4 in the stem cells, hematopoietic and mesenchymal stem cells were isolated from the mice femur and then both cells were incubated with SDF-1 (50 ng/ml, R&D systems), AMD3100 (100 μg/ml, Sigma) or lung lysates from the mice 3 day after treatment with bleomycin or bleomycin plus AMD3100. Each samples were lysed by chaps cell extract buffer (0.1% chaps, 50 mM pipes-HCl (pH 6.5), 2 mM EDTA, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 5 mM DTT). The concentration of total protein in the lung and cell lysates was quantitated by Bradford (Bio-Rad, Hercules, CA) analysis, as per the manufacturer's instructions. 50 μg protein samples were loaded onto a 10% PAGE gel and separated by electrophoresis at 10 mA. Proteins were transferred to nitrocellulose membrane at 70 V for 2 h. The membrane was blocked for 1 h in Tris-buffered solution (TBS; 10 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 5% nonfat dry milk and was incubated overnight with CXCR4 antibody (1:200; Santa Cruz Biotechnology) or phosphorylated p38 MAPK antibody (1:1,000, Cell Signaling, danvers, MA) in blocking solution at 4°C. The membrane was washed 3 times with washing buffer (TBS with 0.1% NP-40) and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) at room temperature for 2 h. The target protein was detected using the ECL$^+$ kit (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK) and x-ray film.

Migration assay

Hematopoietic and mesenchymal stem cells were obtained from the mouse BM. BM cells were isolated by flushing with DMEM/F-12 media through the end of both femurs. After 10^6 cells were plated in Ø100 mm culture dish, cells were incubated with DMEM/F-12 media containing 20% FBS, 200 IU/ml penicillin and 250 μg/ml streptomycin for 48 h. The HSCs were purified from nonadherent cells after removing the RBCs by using the distilled water and adherent cells were collected as MSCs. The purified BMDMSC was identified by CD 44 staining (Tian *et al*., 2008) and BMDHSC was identified by CD45 staining. The fourth to sixth passages were used for individual chemotactic experiments. Cells of inhibition group were incubated with AMD3100 (100 μg/ml), 30 min before SDF-1 or lung lysates treatment. The HSCs or MSCs (5×10^4 cells each) were loaded into the upper chamber of 8-μm pore transwell insert (HTS Transwell®-96 Permeable Support Systems, Corning Incorporated, Corning, NY) for migration assay. PBS, SDF-1 (50 ng/ml) or lung lysates (425 μg/ml) from the mice 3 day after treatment with bleomycin or bleomycin plus AMD3100, were added to the lower chamber. After incubation for 24 h, cells of lower chamber were collected and counted by a hemocytometer.

Statistical analysis

All data were expressed as mean ± SE. Differences between two groups were compared using the Mann-Whitney U test. Comparisons among multiple groups were analyzed using the nonparametric Kruskal-Wallis H test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software, version 10.0.7 (SPSS Inc., Chicago, IL). Differences were considered statistically significant if *P* values were less than 0.05.

Supplemental data

Supplemental Data include a figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-6-07.pdf.

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