Bone marrow cells repair cigarette smoke-induced emphysema in rats

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Huh JW, Kim S-Y, Lee JH, Lee J-S, Van Ta Q, Kim M, Oh Y-M, Lee Y-S, Lee S-D. Bone marrow cells repair cigarette smoke-induced emphysema in rats. Am J Physiol Lung Cell Mol Physiol 301: L255–L266, 2011. First published May 27, 2011; doi:10.1152/ajplung.00253.2010.—The therapeutic potential of stem cells in chronic obstructive pulmonary disease is not well known although stem cell therapy is effective in models of other pulmonary diseases. We tested the capacities of bone marrow cells (BMCs), mesenchymal stem cells (MSCs), and conditioned media of MSCs (MSC-CM) to repair cigarette smoke-induced emphysema. Inbred female Lewis rats were exposed to cigarette smoke for 6 mo and then received BMCs, MSCs, or MSC-CM from male Lewis rats. For 2 mo after injection, the BMC treatment gradually alleviated the cigarette smoke-induced emphysema and restored the increased mean linear intercept. The BMC treatment significantly increased cell proliferation and the number of small pulmonary vessels, reduced apoptotic cell death, attenuated the mean pulmonary arterial pressure, and inhibited muscularization in small pulmonary vessels. However, only a few male donor cells were detected from 1 day to 1 mo after BMC administration. The MSCs and cell-free MSC-CM also induced the repair of emphysema and increased the number of small pulmonary vessels. Our data show that BMC, MSCs, and MSC-CM treatment repaired cigarette smoke-induced emphysema. The repair activity of these treatments is consistent with a paracrine effect rather than stem cell engraftment because most of the donor cells disappeared and because cell-free MSC-CM also induced the repair.

chronic obstructive pulmonary disease; paracrine; stem cell; smoking oxidative stress, persistent inflammation, extracellular matrix degradation by overwhelming protease activity, and an altered alveolar maintenance program leading to apoptosis and abnormal cell repair (28, 56). In addition to alveolar destruction, chronic smoking induces endothelial dysfunction and pulmonary vascular remodeling with abnormal intimal proliferation of poorly differentiated smooth muscle cells (7, 13). These changes lead to pulmonary hypertension.

Although present COPD therapies reduce airway obstruction, limit COPD exacerbation, and improve quality of life (1, 9), no curative therapy for COPD is available. However, recent advances in stem cell therapy encourage its clinical use in the treatment of several kinds of disease, possibly because of the capability of these cells to differentiate along multiple lineages (2) or to repair damaged tissue by paracrine action (18).

Several studies showed that bone marrow cells (BMCs), which comprise multiple heterogeneous populations including mesenchymal stem cells (MSCs), hematopoietic stem cells, and endothelial progenitor cells, have beneficial effects in the treatment of myocardial infarction (14, 45, 48), limb ischemia (33), and cerebral ischemia (5).

The therapeutic potential of bone marrow-derived MSCs has also been demonstrated in myocardial infarction (12, 31) and limb ischemia (25). Bone marrow-derived MSCs also have beneficial effects in several pulmonary disease models. Bone marrow-derived MSCs ameliorate bleomycin-induced pulmonary fibrosis (35, 40) and reduce monocrotaline-induced pulmonary hypertension (4) and endotoxin-induced acute lung injury (19). In addition, bone marrow-derived MSCs induce regeneration of alveolar structures in an experimental broncho-pulmonary dysplasia (BPD) model (3, 51). Interestingly, some of these beneficial effects are thought to be mediated by the paracrine action of MSCs (3, 4, 19, 51).

However, no study has focused on the therapeutic potential of BMCs, bone marrow-derived MSCs, and conditioned media of MSCs (MSC-CM) in COPD treatment despite the wide prevalence of COPD. The aim of this study was to evaluate whether BMCs, MSCs, and MSC-CM have beneficial effects in a rat model of smoking.
METHODS

Exposure of rats to cigarette smoke. The Institutional Animal Care and Use Committee of Asan Medical Center approved this study. Eight-week-old inbred female Lewis rats (Orient Bio, Seongnam, Korea) were exposed to the mainstream smoke of 20 filtered commercial cigarettes per day, each containing 8.5 mg tar and 0.9 mg nicotine (Eighty Eight Lights, KT&G, Daejeon, Korea). The rats were exposed to cigarette smoke 5 days per week for 6 mo according to the protocol in Cavara et al. (10) with modifications. Ten to twelve rats were settled in an inhalation box (50 × 40 × 30 cm) connected to a pump and were exposed for 10 min to mainstream cigarette smoke generated simultaneously from 10 cigarettes. The rats remained in the box for an additional 10 min after the cigarettes had burned. The box was then ventilated to remove the cigarette smoke, and the rats breathed normal room air for 5 min. A second exposure was performed in the same manner. After exposure to cigarette smoke, the rats were returned to their cages. Control animals inhaled clean room air only in the cages.

BMC isolation. BMCs were isolated from four to six 7-wk-old male Lewis rats for each set of experiments as described previously (6) with slight modification. After the rats were euthanized with halothane, the femurs and tibias were excised, and the connective tissue attached to the bones was removed carefully under sterile conditions. Bone marrow plugs were extracted from the bones by flushing the bone marrow cavity with serum-free DMEM (GIBCO, Carlsbad, CA). The marrow plugs were dispersed by pipetting, and the suspension was sieved through a 40-μm mesh (BD Biosciences, San Jose, CA) and then centrifuged at 1,000 g for 5 min. Red blood cells were lysed with 150 mM ammonium chloride and 10 mM potassium bicarbonate, pH 7.4. After being washed with DMEM, BMCs isolated from the total suspension were suspended in DMEM at 2 × 10^6 cells/ml.

MSC culture and preparation of MSC-CM. Bone marrow-derived MSCs were cultured as described previously (6) with slight modifications. BMCs were plated in plastic culture dishes (SPL Life Science, Pocheon, Korea) in complete medium (DMEM containing 10% FBS, penicillin, and streptomycin) and incubated at 37°C in a humidified atmosphere with 5% CO_2_. Three days later, unattached cells were removed by a wash with complete medium, which was replaced and changed once or twice per week. At 90% confluence, the plastic adherent cells were trypsinized with 0.25% trypsin-EDTA (Invitrogen) and passaged at a 1:3 ratio. Fourth-passage MSCs from four male rats were pooled and administered to female rats.

To verify the multipotent differentiating capability of MSCs, fourth-passage MSCs were cultured in adipogenic medium containing 10^-6 M dexamethasone, 10 μg/ml insulin, and 100 μg/ml 3-isobutyl-1-methylxanthine, or in osteogenic medium containing 10^-7 M dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. After 3 wk, adipocytes and osteoblasts were stained with 3.75% Oil Red O and 2% Alizarin Red S, respectively (19).

Surface markers of MSCs were identified using a fluorescence-activated cell sorter (FACS). MSCs were detached with trypsin-EDTA, incubated in FACS buffer (2% BSA, and 0.01% sodium azide in PBS) for 30 min on ice for blocking and then incubated with anti-CD34, -CD45, -CD73, or -CD90 antibodies (1:100) for 30 min on ice. Prelabeled anti-CD34 (FITC), -CD45 (phycoerythrin; PE), and -CD90 (FITC) antibodies were purchased from BD Biosciences, and anti-CD73 antibody (BD Biosciences) was labeled with PE using a kit (Dojindo, Kumamoto, Japan). Nonimmune mouse IgG was used for negative staining. After incubation with the antibodies, MSCs were washed with FACS buffer and analyzed using a FACSCalibur flow cytometer, and the data were analyzed using the CellQuest analysis program (BD Biosciences).

MSC-CM was collected from third- to fifth-passage MSCs obtained from five male Lewis rats under aseptic conditions. At 90% confluence, MSCs in 15-cm culture dishes were washed three times with PBS and incubated in 50 ml of fresh serum-free DMEM. After 24 h, MSC-CM was collected, filtered through a 0.22-μm filter, frozen in liquid nitrogen, and stored at −70°C. Just before use, the frozen MSC-CM was thawed and concentrated aseptically 20-fold in a 70% alcohol-washed Amicon Ultra centrifugal filter device with a molecular weight cutoff of 3 kDa (Amicon, Beverly, MA).

Administration of BMCs, MSCs, and MSC-CM. After exposure to cigarette smoke for 6 mo, rats were given BMCs, MSCs, or MSC-CM (Supplemental Fig. S1; supplemental material for this article is available online at the American Journal of Physiology Lung Cellular and Molecular Physiology website). In the first set of experiments (Set 1, n = 6–10 per group), lung repair was observed 2 mo after administration of BMCs. To assess the time course of lung repair by BMCs, rats were killed 1 day, 1 wk, 2 wk, 1 mo, and 2 mo after BMC administration (Set 2, n = 5–7 per group). In addition, to compare the reparative effects of MSCs and MSC-CM with those of BMCs, rats were killed 2 mo after administration (Set 3, n = 5–9 per group). At the end of the cigarette smoke exposure, a single injection of BMCs (6 × 10^6 cells in 0.3 ml) or MSCs (6 × 10^6 cells in 0.3 ml) was given using a retrobulbar approach. Concentrated MSC-CM (0.3 ml) or an equal volume of serum-free DMEM was injected into the tail vein twice per week for the first 5 wk after cessation of cigarette smoke exposure.

Hemodynamic monitoring and tissue preparation. Pulmonary arterial pressure was measured as in our previous report (26). Each rat was anesthetized with ketamine/xylazine, and the right jugular vein was cannulated with a Silastic catheter (Saint-Gobain Plastics, Lincoln, IL) connected to a pressure monitor (Hewlett-Packard, Palo Alto, CA). The catheter was advanced into the right ventricle and pulmonary trunk, and the mean pulmonary arterial pressure (MPAP) was measured after stabilization. The heart and the lungs were then removed en bloc, and the left lung was inflated by intratracheal infusion of 0.3% low-melting agar at 25 cmH₂O. The inflated lung was fixed with 4% paraformaldehyde in PBS and embedded in paraffin. In the third set of experiments, the right ventricular systolic pressure (RVSP) was measured by a direct puncture method with modification (52). Rats were anesthetized by intraperitoneal injection of Zoletil (Virbac, Carros, France) at 35 mg/kg. The trachea was intubated, and the lungs were ventilated with room air at 60 strokes/min and a 2.0-ml tidal volume. After a midline abdominal incision and a midline incision of the diaphragm, a 22-gauge needle connected to a disposable pressure-monitoring kit (Biosensors International, Kampong, Singapore) was placed into the right ventricular cavity by direct puncture of the right ventricle.

Histological analysis. Lung sections of 4 μm thickness were used for histological analysis. The mean linear intercept (MLI) and the degree of pulmonary vascular muscularization were determined in paraffin-embedded lung sections by staining with hematoxylin and eosin (H&E) (for MLI) and with the elastin-van Gieson method (for pulmonary vascular muscularization) (17), as described previously. All histological examinations were made by two people in a blinded manner.

Identification of donor cells in recipient lungs by fluorescent in situ hybridization. The presence of male donor cells in recipient lungs was confirmed by fluorescent in situ hybridization (FISH) using a FITC-labeled probe for the rat Y chromosome (Cambio, Cambrige, UK) according to the manufacturer’s instructions with slight modification. Paraffin sections (4 μm) were dewaxed in xylene, hydrated with decreasing concentrations of ethanol (100%, 95%, 70%, and 50%), and equilibrated with 2× SSC. The sections were boiled in 10 mM sodium citrate (pH 6.0), washed with 2× SSC, and dehydrated with increasing concentrations of ethanol (70%, 95%, and 100%). The Y chromosome probe (8 μl per slide) was added to the lung sections, and the sections were sealed under cover glasses with rubber cement. After being heated at 70°C for 10 min on a heating block and incubated overnight at 37°C, the cover glasses were carefully peeled off, and the sections were washed sequentially with 50% formamide in 2× SSC, 2× SSC, and 4× SSC buffer at 37°C. Nuclei were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and normal saline.
stained with 1 μg/ml Hoechst 33258 (Calbiochem, San Diego, CA) and washed with 4× SSC. Lung sections from male rats were stained in the same way as a positive control. Stained cells were observed and analyzed using a confocal laser microscope (LSM 510, Carl Zeiss, Jena, Germany).

Western blot analysis. Lung tissue was homogenized with a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 2 mM P-nitrophenyl phosphate, and a protease inhibitor cocktail) on ice with a Polytron tissue homogenizer (Brinkman, Riverview, FL). After centrifugation at 14,000 g for 20 min at 4°C, the proteins were separated in a 10% or 12% SDS-PAGE gel and then transferred to a nitrocellulose membrane. The membranes were blocked with 5% skim milk for 1 h at room temperature and then incubated with primary antibody (1:1,000) overnight at 4°C. After being washed with 0.5% Tween-20 in Tris-buffered saline (TBS): 50 mM Tris, 150 mM NaCl, pH 8.0, TBS-T), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000). Proteins were visualized using ECL reagents (Amersham, Piscataway, NJ) and detected with a LAS-3000 imaging system (Fuji, Tokyo, Japan). The following primary antibodies were used: cleaved caspase-3, Akt, phosphorylated Akt (Cell Signaling Technology, Beverly, MA), hepatocyte growth factor (HGF) (Santa Cruz Biotechnology, Santa Cruz, CA), VEGF (Abcam, Cambridge, MA), angioptin-2 (Novus Biological, Littleton, CO), keratinocyte growth factor (KGF; Abbivetec, San Diego, CA), and GAPDH (AbFrontier, Seoul, Korea).

TUNEL assay. Apoptosis was assessed using a terminal dUTP nick-end labeling (TUNEL) assay kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, paraffin sections were dewaxed, hydrated, heated in 0.1 M citrate buffer, pH 6.0, and permeabilized with PBS containing 0.1% Triton X-100. The sections were incubated with 50 μl of the TUNEL mixture in the dark at 37°C for 60 min, then rinsed, and incubated overnight at 4°C with anti-von Willebrand factor (vWF, Abcam) or anti-surfactant protein-C (SP-C) (Abcam, Cambridge, MA) antibody (1:100). The sections were rinsed with TBS-T and then incubated with Alexa Fluor 488-conjugated anti-rabbit antibody (1:100, Invitrogen) at room temperature for 1 h. Nuclei were stained with Hoechst 33258, and images were observed with a fluorescence microscope (LSM 510, Zeiss).

Assessment of vascularity and muscularization in pulmonary vessels. Vascularity was assessed by immunohistochemical staining with anti-vWF antibody (1:100, Abcam) using a procedure similar to that described above. After incubation with the primary antibody (anti-vWF, 1:100) and secondary antibody (Cy3-labeled goat anti-rabbit, 1:1,000, Invitrogen), images were observed at ×10 magnification. Pulmonary blood vessels showing a tubular structure were classified by size as small (<50 μm in external diameter), medium (50–100 μm), or large (>100 μm), as measured with imaging software (Carl Zeiss). The total numbers of vessels in each class were counted in five randomly chosen fields. To observe vascular remodeling, α-smooth muscle actin (α-SMA) and vWF were stained simultaneously with anti-α-SMA (1:100, Abcam) and anti-vWF (1:100). Measurement of cellular proliferation. Proliferating cells in the lungs were detected by immunostaining with anti-PCNA antibody (1:1,000, Cell Signaling Technology) as described above. The total numbers of PCNA-positive cells in five randomly chosen fields were counted at ×100 magnification. Proliferating type 2 alveolar epithelial (AT2) cells, or vascular endothelial cells were identified with double staining with anti-SP-C, or anti-vWF antibody together with anti-PCNA antibody. The total numbers of the double-positive cells in five randomly chosen fields were counted at ×100 magnification with a fluorescence microscope (Carl Zeiss). The localization of PCNA, SP-C, and vWF were confirmed in Z-sections with a laser confocal microscope (LSM 510, Zeiss).

CSE preparation. Cigarette smoke extract (CSE) was prepared as previously described (34). Briefly, 40 ml of cigarette smoke (Eighty Eight Light containing 8.5 mg tar and 0.9 mg nicotine/cigarette) drawn into a 50-ml plastic syringe through a three-way cock was mixed with 10 ml of DMEM by vigorous shaking. One cigarette was used per 10 ml CSE, and CSE was prepared no more than 30 min before use in experiments. CSE solution filtered through an aseptic 0.22-μm filter was considered to be 100%.

Preparation of conditioned media. Normal human lung fibroblasts (NHLFs; Lonza, Rockland, ME) and human pulmonary artery smooth muscle cells (HPASMCs, Lonza) of passage 6-8 were cultured in DMEM containing 10% FBS and penicillin-streptomycin. At 90% confluence in 10-cm-diameter culture dishes, the cells were washed three times with PBS and cultured in 10 ml fresh serum-free DMEM. After 24 h, the conditioned medium was filtered through an aseptic 0.22-μm filter and concentrated using a 3-kDa cutoff ultrafiltration membrane (YM-3, Amicon).

Treatment of CSE-damaged HPAECs with conditioned media. Human pulmonary artery endothelial cells (HPAECs) (Lonza) were cultured in supplemented endothelial growth medium-2 (EGM-2, Lonza) containing 5% FBS. To assess the reparative effect of the conditioned media of MSCs, NHLFs, and HPASMCs, HPAECs of passage 5 were used as target cells. HPAECs were seeded at a density of 3,000 cells per well in a 96-well plate. The next day, HPAECs were washed with PBS and exposed to 0% and 4% CSE for 24 h in supplemented EGM-2 containing 5% FBS. Thereafter, the cells were washed with PBS, and the media were replaced with the conditioned media of each type of cell for 24 h. The 20-fold concentrated conditioned media was half diluted with supplemented EGM-2 containing 5% FBS. A mixture of serum-free DMEM and supplemented EGM-2 containing 5% FBS (1:1) was used as a control medium. Cell proliferation was checked with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid (MTT) assay.

Detection of SP-C/PCNA/p-Akt-positive cells. Lung sections of 4-μm thickness were used for triple immunostaining with anti-SP-C, anti-PCNA, and anti-p-Akt antibodies. For triple staining, anti-p-Akt antibody (Rockland Immunochemicals, Gilbertsville, PA) was labeled with PE by using a kit (R-Phycoerythrin Labeling Kit-NH2, Dojindo). As described above, lung sections were deparaffinized and hydrated, and antigen retrieval was performed. The sections were incubated with a blocking solution (5% normal goat serum, Abcam) in TBS-T at room temperature for 1 h, and then incubated overnight with anti-SP-C (1:100) and anti-PCNA (1:100) antibodies in the blocking solution at 4°C. The next day, the sections were washed with TBS-T and incubated with the secondary antibodies (1:100) in the blocking solution at room temperature for 2 h. After being washed, the sections were incubated with PE-labeled anti-p-Akt antibody (1:10) for 1 day at 4°C. After being washed, images were observed with a confocal microscope (Zeiss). The total numbers of the triple-positive cells in five randomly chosen fields were counted.

Statistical analysis. The data are presented as the means ± SE. One-way ANOVA and the Mann-Whitney test were used to compare data between groups. A P value of <0.05 was considered significant.

RESULTS

Repair of cigarette smoke-induced emphysema by BMCs. Female Lewis rats exposed to cigarette smoke for 6 mo were treated with BMCs. Two months later, we evaluated the lung repair activity of BMCs by histological examination. Untreated female Lewis rats exposed to cigarette smoke for 6 mo showed severe alveolar destruction even after cessation of cigarette smoke exposure (Fig. 1A). The MLI was significantly higher in the smoking group (SM, 95.6 ± 3.2 μm) than in the control group (CON, 68.6 ± 3.1 μm) (Fig. 1B). In rats exposed to cigarette smoke and treated with BMCs (SM+BMC group), the alveolar structures showed dramatic regeneration (Fig. 1A) and the MLI returned to a near-normal level (70.9 ± 2.3 μm).
In rats treated with BMCs but not exposed to cigarette smoke (BMC group), MLI was normal and no significant microscopic changes were observed compared with the CON group.

We next assessed the time course of lung repair by observing the morphological changes 1 day, 1 wk, 2 wk, 1 mo, and 2 mo after BMC administration. In the SM group, histological evidence of lung repair did not appear until 2 mo after cessation of cigarette smoke exposure (Fig. 2A). The MLI did not change significantly, despite a tendency to increase early and to decrease later (Fig. 2B). By contrast, smoke-exposed rats treated with BMCs showed morphological evidence of progressive time-dependent repair of the emphysema after treatment and
reduction in the MLI. Even after 1 wk, the average MLI was slightly, although nonsignificantly, lower in the SM+BMC group than in the SM group. Two weeks after BMC administration, the MLI was significantly lower in the SM+BMC group (83.2 ± 3.9 µm) than in the SM group (100.3 ± 5.3 µm) (P < 0.05), and it remained significantly lower up to 2 mo after the treatment. At 2 mo, the MLI was significantly lower in the SM+BMC group than in this group on day 1.

BMC-induced cell proliferation and BMC-reduced apoptosis in lungs with emphysema. Using immunostaining for PCNA and the TUNEL assay, we measured the extent of cell proliferation and apoptosis, respectively, in the repaired tissue. The number of PCNA-positive cells in the SM group increased slowly after cessation of cigarette smoke exposure (Fig. 3, A and B). By comparison, the number of PCNA-positive cells in the SM+BMC group increased markedly from day 1 and remained significantly higher than in the SM group through the second week after BMC administration. After 2 wk, the number of PCNA-positive cells decreased but remained higher than in the SM group. Two months after BMC administration, the number of PCNA-positive cells did not differ significantly between the SM and SM+BMC groups.

In double staining, some of PCNA-positive cells were identified as AT2 cells (SP-C-positive cells) and others as vascular endothelial cells (vWF-positive cells) (Supplementary Figs. S2 and S3). The number of proliferating AT2 cells was significantly greater 1 day and 1 wk after administration in the SM+BMC group than in the SM group. At 2 mo, the number of PCNA-positive cells did not differ significantly between the SM and SM+BMC groups.

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Fig. 3. Induction of cell proliferation and reduction in apoptosis after BMC administration. A and B: induction of cell proliferation by BMC administration. A: numbers of PCNA-positive cells in 5 randomly chosen fields, counted at ×100 magnification. *Statistically significant difference (P < 0.05) between the SM and SM+BMC groups. B: representative images of PCNA-positive cells in the SM and SM+BMC groups at 1 day and 2 mo. Arrowheads indicate PCNA (green) in the nuclei (blue). Scale bar = 100 µm. C and D: reduction in apoptosis after BMC administration. C: changes in numbers of terminal dUTP nick-end labeling (TUNEL)-positive cells. D: representative images showing TUNEL-positive (red) and von Willebrand factor (vWF)-positive (green) cells and nuclei (blue). E: confocal images of TUNEL-positive type 2 alveolar (AT2) cells. Arrows indicate surfactant protein-C (SP-C)/TUNEL-positive cells; nuclei (blue), TUNEL (red), and SP-C (green). In the cell indicated by the yellow arrow, Z-section shows relative localization of the nucleus, TUNEL, and SP-C. F: numbers of SP-C/TUNEL-positive and vWF/TUNEL-positive cells in the SM and SM+BMC groups on day 1. The number of double-positive cells was counted at ×200 magnification in 5 randomly chosen fields. *Statistically significant difference (P < 0.05) between the SM and SM+BMC groups. G: Akt phosphorylation and caspase-3 cleavage on day 1. Phosphorylated Akt and cleaved caspase-3 were quantified after normalization to total Akt and GAPDH, respectively. H: levels of keratinocyte growth factor (KGF) on day 1. KGF was quantified after normalization to GAPDH. In G and H, * and ** denote significant differences (P < 0.05) between the CON and SM groups, and between the SM and SM+BMC groups, respectively. I: confocal images of p-Akt in proliferating AT2 cells on day 1. p-Akt was detected in proliferating AT2 cells by triple immunostaining with anti-SP-C, anti-PCNA, and anti-p-Akt antibodies. Arrows indicate SP-C/PCNA/p-Akt-positive cells: SP-C (blue), PCNA (green), and p-Akt (red). In the cells indicated by the yellow arrow, Z-section shows relative localization of the SP-C, PCNA, and p-Akt. The number of double-positive cells was counted at ×200 magnification in 5 randomly chosen fields. *Statistically significant difference (P < 0.05) between the SM and SM+BMC groups.
Fig. S4). Similarly, more proliferating endothelial cells were detected 1 and 2 wk after BMC administration in the SM+BMC group than in the SM group. Other proliferating cells were not identified as either AT2 cells or vascular endothelial cells.

After cessation of cigarette smoke exposure, the number of TUNEL-positive cells in the SM group decreased from 285.5 ± 25.4 cells per five fields on day 1 to 164.5 ± 12.1 cells per five fields at 1 wk and remained within this range for 2 mo (Fig. 3, C and D). The number of TUNEL-positive cells decreased more rapidly in the first day after treatment in the SM+BMC group (to 139 ± 5.9 cells per 5 fields) than in the SM group and remained significantly lower than in the SM group at 1 wk, 1 mo, and 2 mo. On day 1, the number of TUNEL-positive AT2 cells was significantly reduced in the SM+BMC group compared with the SM group; however, the number of TUNEL-positive endothelial cells showed nonsignificant reduction (Fig. 3, E and F).

In Western blot analysis, the BMC treatment similarly suppressed the cleavage of caspase-3, corresponding to the decrease in the number of TUNEL-positive cells (Fig. 3G). Consistent with the marked cell proliferation on day 1, Akt phosphorylation also increased significantly in the SM+BMC group. To investigate an underlying mechanism for reduced apoptosis and enhanced proliferation with increased Akt phosphorylation in alveolar epithelial cells, levels of KGF were assessed by Western blotting because KGF induces proliferation (50) and inhibits apoptosis in AT2 cells in an Akt-dependent manner (39) and prevents elastase-induced pulmonary emphysema (37). On day 1, KGF levels were significantly elevated in the SM+BMC group compared with the SM group (Fig. 3H). However, there was no statistically significant difference between the two groups at later observation times (data not shown). In immunohistochemistry, the numbers of SP-C/PCNA/p-Akt-positive cells were significantly higher in the SM+BMC group than in the SM group, and stronger Akt phosphorylation in PCNA-positive AT2 cells was seen in the SM+BMC group than in the SM group (Fig. 3I). However, Western blot analysis and immunochemistry showed no further significant changes in Akt phosphorylation or caspase-3 cleavage at later observation times (data not shown).

**Fate of donor BMCs.** In the lungs of the SM+BMC group, only a few donor BMCs (2–3 cells per entire lung section) were observed from 1 day to 1 mo after BMC administration (Fig. 4), and this number did not change throughout the month (data not shown). These scarce donor BMCs did not seem to proliferate in clusters. Moreover, donor cells were not detected at all in lung sections from the BMC and SM+BMC groups 2 mo after BMC administration (data not shown).

**Increase in small pulmonary vessels after BMC administration in cigarette smoke-induced emphysema.** Although the numbers of large and medium pulmonary vessels in the SM and SM+BMC groups did not differ significantly (data not shown), the number of small pulmonary vessels in the SM+BMC group increased significantly beginning 1 wk after BMC administration (Fig. 5). The number of these small vessels reached a maximum at 2 wk, and this increased vascularity was maintained for up to 2 mo. By contrast, the number of small vessels in the SM group increased to a lesser degree for 1 mo after smoking cessation and then declined to reach the day 1 level at 2 mo.

**Alleviation of pulmonary hypertension and pulmonary vascular remodeling by BMCs.** In addition to increasing the number of small pulmonary vessels, BMCs alleviated cigarette smoke-induced pulmonary hypertension and vascular remodeling. After 2 mo, the MPAP was significantly lower in the SM+BMC group (15.6 ± 0.8 mmHg) than in the SM group (21.1 ± 1.7 mmHg) (Fig. 6A). In addition, the BMC treatment reversed the decrease in the percentage of nonmuscularized vessels, which returned nearly to the control level (Fig. 6B). The increased formation of double elastic lamina (Fig. 6C) and α-SMA expression in pulmonary vessels (Fig. 6D) were both attenuated in the SM+BMC group.

**Repair of cigarette smoke-induced emphysema by MSCs and MSC-CM.** As in the experiments with BMCs, we treated female Lewis rats exposed to cigarette smoke for 6 mo with MSCs isolated from male Lewis rat BMCs by plastic adherence. The isolated plastic adherent MSCs were differentiated into adipogenic and osteogenic lineages in the corresponding differentiation culture media (Supplemental Fig. S5). Flow cytometry showed that the cells were negative for CD34 and CD45 and that most of the cells were positive for CD73 and CD90 (Supplemental Fig. S6). Considering the scarcity of donor MSCs in the recipient lungs for the first month and the absence of donor MSCs after 2 mo, we hypothesized that MSCs repair emphysema by a paracrine mechanism, and we tested this.
hypothesis using cell-free MSC-CM in an in vitro cell experiment and the same animal model of cigarette smoking. In the in vitro experiment, HPAECs were treated with a 10-fold concentrated conditioned media of MSCs, NHLFs, or HPASMCs after exposure to CSE. MSC-CM significantly induced proliferation of HPAECs after CSE exposure, whereas the conditioned media of the other kinds of cells did not (Fig. 7A). In the animal experiments, 2 mo after MSC administration, histological examination showed that the MSCs, like the BMCs, restored alveolar architecture in the SM+/H11001 group (Fig. 7B) and reduced the MLI nearly to the control level (70.0 ± 2.6 μm), which was significantly lower than in the SM group (89.8 ± 1.8 μm) (Fig. 7C). As in the previous experiments with BMCs, however, donor male MSCs were not detected by FISH in the recipient lungs 2 mo after administration (data not shown). The MSC-CM-induced histological improvements were similar to those induced by BMCs or MSCs (Fig. 7B), and MSC-CM treatment normalized the MLI (71.6 ± 2.1 μm) (Fig. 7C). In addition, both MSCs and MSC-CM increased the number of small vessels (Fig. 8, A and B), similar to the effect of BMCs. Although MSCs significantly suppressed the cigarette smoke-induced elevation in RVSP, MSC-CM caused a small nonsignificant reduction in RVSP (Fig. 8C).

DISCUSSION

In this study, we showed for the first time that BMC therapy alleviates the emphysema, pulmonary hypertension, and pulmonary vascular remodeling caused by chronic cigarette smoke exposure. Previous studies have shown that autologous bone marrow mononuclear cells and bone marrow-derived MSCs repair the emphysema induced by elastase and papain, respectively (57, 58). However, models of cigarette smoke-induced COPD may be more relevant to human disease in terms of the slow progression of cumulative damage, changes in tissue repair activity, and pathological findings (54).

In the earlier part of the experiment, we did not select specific BMC subpopulations to maximize the therapeutic effects. Others have reported that BMCs comprising multiple heterogeneous populations, including hematopoietic stem cells, endothelial progenitor cells, and MSCs, have therapeutic potential in several experimental models, including myocardial infarction (14, 45, 48), limb ischemia (33), and cerebral ischemia (5).

In this study, BMC administration induced the proliferations of AT2 cells, pulmonary vascular endothelial cells, and unidentified cells in the lung parenchyma most markedly from 1 day to 2 wk after BMC administration. Using FISH, however, we detected few donor BMCs from 1 day to 1 mo after BMC administration and no donor BMCs at 2 mo although the MLI had returned almost completely to the control levels after 2 mo. Consistent with our results, in other studies, the engraftment of bone marrow-derived MSCs was too low to explain their therapeutic effect in hyperoxia-induced BPD in neonatal rats (3, 51). Schweitzer et al. (43) reported a dramatic decrease in the number of administered adipose stem cells in the lungs 7 days or 21 days after systemic administration compared with 1 day. They also mentioned that exposure to cigarette smoke before the systemic administration of the cells led to decreased lung trapping at 21 days.

One possible explanation for the lung repair with scarce engraftment of donor BMCs is the fusion of donor BMCs with recipient AT2 cells. Herzog et al. (21) reported that the Y
Chromosomes of donor BMCs are lost from fused cells after 6 mo, possibly by nuclear reprogramming. However, although we did not directly address cell fusion in this study, fusion and subsequent loss of Y chromosomes does not explain the observed scarcity of donor cells. The male donor BMCs remained at a relatively constant low frequency (2–3 cells per lung section) from 1 day to 1 mo after BMC administration, whereas the MLI decreased and the numbers of small pulmonary vessels increased in the emphysematous lungs during this period.

As an alternative to cellular fusion, paracrine action may explain the effects of BMCs or MSCs on lung repair. Treatment with cell-free MSC-CM restored the MLI to a similar degree to that induced by BMCs and MSCs in our study. Consistent with our results, another study found that MSC-CM induced lung regeneration in hyperoxia-induced BPD (3). MSCs release soluble factors that promote cell proliferation, inhibit cell death, and attenuate inflammation (49). For example, HGF released from bone marrow stromal cells (53) stimulates AT2 cell proliferation in vivo (42) and in vitro (29). MSCs also secrete adrenomedullin (32), a paracrine factor that promotes alveolar and vascular regeneration when administered in elastase-induced emphysema (30). Alternatively, KGF may play a role in the inhibition of apoptosis and proliferation of AT2 cells with Akt phosphorylation in the SM+BMC group. KGF is known to induce proliferation (50) and inhibit apoptosis of AT2 cells in an Akt-dependent manner (39). Furthermore, KGF released from bone marrow-derived MSCs is suggested to alleviate endotoxin-induced acute lung injury by the finding that transfection of KGF siRNA into MSCs reduced the therapeutic effect of MSC-CM, and recombinant KGF restored the therapeutic effect of MSC-CM, which were transfected with KGF siRNA (27).

Another possible lung repair mechanism involves the capacity of the donor cells to recruit BMCs. In elastase-induced emphysema, adrenomedullin promotes lung regeneration through the recruitment and incorporation of BMCs into the lung, and some of the incorporated BMCs bear markers of alveolar epithelial cells or vascular endothelial cells (30). Intranasal administration of HGF ameliorates elastase-induced emphysema, leading to the appearance of AT2 cells, vascular endothelial cells, and fibroblasts of bone marrow origin in the lung (20).

We found that BMCs, MSCs, and MSC-CM increased pulmonary vascularity, whereas BMCs and MSCs, but not MSC-CM, suppressed the elevation in RVSP. It is possible that the amount of MSC-CM administered was not sufficient to inhibit pulmonary hypertension. Other studies have shown that MSCs attenuate pulmonary hypertension (51) but that MSC-CM resolves pulmonary vascular remodeling and right ventricular hypertrophy (3). The discrepancy between our results and those from a BPD model (3, 51) may stem from differences between disease models, ages of recipient animals, or administration protocols. Interestingly, all of the treatments with BMCs, MSCs, or MSC-CM increased the numbers of small pulmonary vessels and restored MLI to a similar degree.

Fig. 6. Attenuation of pulmonary hypertension and pulmonary vascular remodeling by BMCs. A: mean pulmonary arterial pressure (MPAP). B: proportion of small muscularized pulmonary vessels. Peripheral pulmonary vessels with a 50–200 μm external diameter were categorized as fully muscular, partially muscular, or non-muscular, and the percentage of each relative to the total number of vessels counted was calculated. C: morphology of small pulmonary vessels stained by the elasin-van Gieson method. Insets show magnified images of vascular walls. D: expression of α-smooth muscle actin (α-SMA) in small pulmonary vessels. Nuclei appear blue; vWF, red; and α-SMA, green. * and **, Significant difference (P < 0.05) compared with the CON and the SM groups, respectively.
Therefore, increased vascularity does not seem to be necessary to relieve cigarette smoke-induced pulmonary hypertension. Wright et al. (55) suggested that structural and functional changes in the pulmonary vasculature, rather than loss of the vascular bed, lead to pulmonary hypertension in COPD.

On the other hand, the increased pulmonary vasculature does seem to support the repair of emphysema. Although it is not clear whether lung regeneration requires angiogenesis, wound healing generally accompanies angiogenesis. Hence, an adequate blood supply through the pulmonary vascular bed probably contributes to normal lung architecture and repair of emphysema. The importance of the pulmonary vascular bed is supported by the findings that blockade of the VEGF receptor leads to loss of the vascular bed followed by emphysema (23) and that adenoviral VEGF expression in a BPD model induces both angiogenesis and alveolarization (46).

In our study, the numbers of proliferating AT2 and endothelial cells were unexpectedly low compared with total numbers of PCNA-positive cells at earlier times. If the PCNA-positive cells originated from donor cells, the Y chromosomes would have been detected in FISH assay. Even though these unidentified cells are assumed to originate from donor hematopoietic cells, we did not observe augmented infiltration of inflammatory cells in alveolar structures, or in peribronchial...
and perivascular areas in the SM+BMC group. Another possibility is that cells other than AT2 and endothelial cells, such as fibroblasts, may have proliferated. Togo et al. (47) reported that fibroblasts from patients with COPD showed reduced chemotaxis and collagen gel contraction, which were mediated through activation of the prostaglandin E pathway, suggesting that alterations in fibroblasts contribute to the development of emphysema. In this regard, BMC administration might stimulate proliferation and/or normalize the dysfunction of interstitial fibroblasts. However, we did not notice prominent thickening of the alveolar septal wall in H&E-stained sections following administration of BMSCs, MSCs, or MSC-CM (Figs. 1, 2, and 7) or exaggerated staining of collagen and vimentin in the alveolar septa (data not shown). The identity and significance of cells other than AT2 and endothelial cells in alveolar regeneration remain to be clarified.

Even though the absolute numbers of proliferating AT2 and endothelial cells were low, the numbers of those cells were significantly higher in the BMC-treated group than in the SM group at the earlier times, and the numbers of TUNEL-positive cells decreased markedly at the earlier times. Similar to these changes, Akt phosphorylation was elevated by BMC administration at 1 day. Akt is a prosurvival factor that inhibits proapoptotic proteins including BCL2-antagonist of death protein, caspase-9, and X-linked inhibitor of apoptotic proteins (15, 16). Additionally, Akt regulates the cell cycle by inhibiting proteins related to cell cycle arrest including p21\(^{Cip1/WAF1}\), p27\(^{kip1}\), and p53. For example, the phosphorylation of p21\(^{Cip1/WAF1}\) by Akt inhibits its interaction with PCNA and promotes the assembly of the cyclin D1/cyclin-dependent kinase 4 complex (11, 59). However, despite many investigations, the pathophysiologic significance of Akt remains unknown in COPD because of multiple roles of Akt in various biological phenomena including inflammation and cell survival (8). Even assuming that Akt phosphorylation 1 day after BMC administration was related to the aggravation of inflammation, we did not notice differences between the SM and SM+BMC groups in inflammatory infiltration in H&E-stained sections or in the total number of macrophages stained with anti-CD68 antibody (data not shown).

Many questions remain to be answered about the clinical application of stem cells. First, the therapeutic effect of stem cell therapy on small airway remodeling must be determined. Second, although we found in this study that unfractionated BMCs resolved multiple features of COPD, the component populations in the BMCs may have different potencies for treating the various features of COPD, such as emphysema, pulmonary hypertension and vascular remodeling, small airway remodeling, and inflammation. Individual patients may need treatment with different types of cells on the basis of the features of their COPD. The potential adverse effects of cell therapy must also be evaluated. For example, although MSCs may have a therapeutic advantage in their capacity to evade an immune response, they might also undergo malignant transformation during long-term culture (41) or transition into tumor-associated fibroblasts (44). Use of cell-free conditioned media or a purified effective component in place of MSCs may avert these potentially grave outcomes. Administration of autologous BMCs in COPD would also avoid an immune response; however, we have yet to determine whether self-BMCs participate in lung repair, whether the reparative capacity of self-BMCs is impaired, or whether self-BMC mobilization to the lungs is deranged in COPD patients. In this regard, a recent study is notable in showing that chronic exposure to cigarette smoke suppressed the number and proliferation of progenitor cells in bone marrow (43). Moreover, previous data imply suppression of bone marrow by the indirect finding that circulating bone marrow-derived progenitor cells were reduced in patients with COPD (22, 36). Interestingly, angiogenic factors including VEGF, HGF (22, 36), and angiopoietin-2 (22) are elevated in patients with COPD. The elevation of these angiogenic factors may not be sufficient to induce alveolar repair, or other factors may play a more important role in the regeneration of alveolar structures. However, we did not observe an increase in HGF, VEGF, or angiopoietin-2 expression in the Western blots or immunohistochemistry (data not shown).
Finally, if allogeneic stem cells must be used, the donor age may be important. In a model of myocardial infarction in aged mice, MSCs from younger mice showed a greater capacity for tissue repair (24).

In conclusion, we report here for the first time that BMCs, MSCs, and MSC-CM may alleviate emphysema and that BMCs or MSCs may attenuate pulmonary hypertension in a rat model of cigarette smoking. The effectiveness of MSC-CM was similar to that of BMCs and MSCs, supporting a paracrine mechanism underlying the therapeutic effect. The clinical relevance of our model, in terms of chronic exposure to an etiological factor and corresponding complicated pathology, supports the further development of stem cell therapy for the treatment of human COPD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.


