The authors investigated whether autologous bone marrow mononuclear cell (BMC) transplantation via the left and right main bronchi would mitigate elastase-induced pulmonary emphysema in rabbits. Four weeks after elastase administration, rabbits also receiving BMCs showed significantly better pulmonary function (FVC, FEV100, FEVPEF) and smaller alveolar airspaces, as indicated by a smaller mean linear intercept, than those receiving porcine pancreatic elastase (PPE) (200 U/kg) alone via the left and right main bronchi. BMCs also significantly reduced cell counts in bronchoalveolar lavage fluid, the incidence of apoptotic (TUNEL-positive) cells and matrix metalloproteinase (MMP)-2 expression, while increasing numbers of proliferative (Ki-67-positive) cells. Thus, BMCs may inhibit the progression to emphysema by attenuating inflammation, MMP-2 expression, and apoptosis, while enhancing alveolar cell proliferation.

Keywords: emphysema, immunohistochemistry, matrix metalloproteinase, pulmonary function tests

Pulmonary emphysema is a chronic, progressive obstructive pulmonary disease pathologically defined as “dilation of the alveolar space without desmoplasia by breakdown of an alveolar wall and the degradation of gas exchange” [1–6]. Among other factors, smoking, defects in α1-antitrypsin, and disequilibrium between elastase (the enzyme that breaks down elastin fibers) and anti-elastase, as well as between matrix metalloprotease (MMP)
and tissue MMP inhibitor (TIMP), reportedly play important roles in the pathogenesis of emphysema [7–9].

It is controversial whether bone marrow stem cells can differentiate into alveolar epithelial cells in the lung [10–14]. In addition, recent reports have shown that, in the heart, the beneficial effects of bone marrow cell transplantation are dependent upon paracrine effects of the cells rather than regeneration of cardiomyocytes [15]. To our knowledge, the use of cell transplantation to treat pulmonary emphysema is very rare. The purpose of the present study, therefore, was to determine whether transplantation of autologous bone marrow mononuclear cells (BMCs) via the left and right main bronchi is an effective treatment for pulmonary emphysema using a rabbit elastase-induced emphysema model [16].

MATERIALS AND METHODS

Experimental Animals

Japanese white male rabbits (2.5 to 3.0 kg; Chubu Kagaku, Nagoya, Japan) were used for this experiment. All of the rabbits received humane care in accordance with the Guide for Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication 8523, revised 1985). The study protocol was approved by the Ethics Committee of Gifu University School of Medicine, Gifu, Japan.

Experimental Design

Rabbits were divided into 3 groups (n = 10 in each). (1) The sham group was treated with saline. (2) The PPE group received porcine pancreatic elastase (PPE) (200 U/kg) alone suspended in 2 mL of 1 M phosphate-buffered saline (PBS) via the left and right main bronchi by a new method modified from the previous one [16], in order to make the injected PPE distribute uniformly within the entire lung. In the new method a catheter was initially wedged in the left main bronchus and used to instill 1 mL into 2 mL of 1 M PBS in which PPE was suspended into the lung. Thereafter, the catheter was wedged in the right main bronchus and the instillation was repeated. (3) The BMC group was transplanted with BMCs in the left and right main bronchi 24 hours after PPE administration.

In the BMC group, rabbits were anesthetized by intravenous injection with 30 mg/kg of sodium pentobarbital, after which approximately 10 mL of iliac bone marrow were aspirated and suspended in 20 mL of RPMI-1640 medium (Shigma-Aldrich, St. Louis, MO, USA) containing 2000 U of heparin sodium. The BMCs were then isolated by centrifugation on a ficoll gradient (JIMRO, Takasaki, Japan), and approximately $1.0 \times 10^8$ BMCs were suspended in 2 mL of 1 M PBS and used for transplantation. In order
to make the injected BMCs also distribute uniformly within the entire lung, the instillation was repeated by the same method as PPE was injected.

**Measurement of Pulmonary Function**

Before and 4 weeks after treatment, each rabbit was connected to a respiratory function machine via a soft rubber tracheal tube (Toray Medical, Tokyo, Japan) inserted under anesthesia. The rabbit was then slowly inspired to a tracheal pressure of 20 cm H$_2$O and then forced to expire as quickly as possible. We then measured the forced vital capacity (volume expired during the fast expiration; FVC), the volume expired in first 100 ms of the fast expiration (FEV100), and the volume expired at the peak expiratory flow (FEVPEF) from the expiration waveform. These pulmonary function parameters were assessed using an EMNS (Bordon, UK) forced maneuver system, as previously described [17].

**Bronchoalveolar Lavage**

After measuring pulmonary function 4 weeks after treatment, the rabbits were killed and exsanguinated by puncture of the carotid artery under pentobarbital anesthesia. Thereafter, the trachea, lungs, and heart were removed en masse, a soft rubber tracheal tube was inserted into the trachea of the resected organs, saline was infused and immediately aspirated, and the numbers of cells in the lavage fluid were counted.

**Histological Evaluation**

After collecting the bronchoalveolar lavage fluid, the lungs were fixed by infusing 10% buffered formalin into the trachea for 24 hours at a pressure of 25 cm H$_2$O. The lung tissues obtained from the left and right posterior lobes were embedded in paraffin, cut into 4-μm-thick sections, and stained with hematoxylin and eosin (H&E) or Masson trichrome. Using the H&E-stained sections, the average alveolar diameter was calculated using the method of Thurlbeck [18]. In addition, a pathological grade was assigned according to the following criteria: 0 = no lung abnormality; 1 = presence of inflammation and fibrosis involving <25% of the lung parenchyma; 2 = lesions involving 25% to 50% of the lung; and 3 = lesions involving >50% of the lung [19]. Comparisons were made by two persons (Y.M. and X.C.) blinded to the conditions.

**Immunohistochemical Staining Using Paraffin-Embedded Sections**

Using the indirect immunoperoxidase method, immunohistochemical analysis of anti–Ki-67 (a monoclonal mouse anti-rat Ki-67 antibody; Daka,
Glostrup, Denmark) staining was used to assess cell proliferation, whereas terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL: TACS 2 TdT DAB kit Trevigien; Gathersburg, MD, USA) staining was used to detect apoptotic cells. Moreover, MMP-2 and MMP-9 staining were used to investigate the degree of degradation of the extracellular matrix. We used mouse monoclonal antibodies (mAbs) against human MMP-2 and MMP-9 (mouse anti-human MMP-2, clone 42-5D11 IgG1 isotype, purified antibody, and mouse anti-human MMP-9, clone 56-2A4 IgG1 isotype, purified antibody, from Fuji Chemical Industries, Takaoka, Japan), each of which cross-reacts with rabbit tissues [20]. The sections were incubated for 18 hours at 4°C with mouse mAbs raised against human MMP-2 or MMP-9 diluted 1:100 in 0.01 M PBS. They were then incubated for 30 min at 37°C with biotinylated anti-mouse immunoglobulin G (IgG) (1:500 dilution; Dako) Morphometric analyses carried out by two persons (Y.M. and G.T.) blinded to the conditions.

Western Blotting and Immunofluorescent Staining

Western blotting and immunofluorescent staining were carried out using other animals from the sham, PPE, and BMC groups (n = 5 in each). In the BMC group, BMCs were labeled using PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Shigma-Aldrich, St. Louis, MO, USA) [21] and then were injected into the left and right lungs via a catheter wedged in the main bronchi. Four weeks later, the rabbits were sacrificed under anesthesia, and the lungs were immediately excised, placed in iced PBS (< 4°C), and cut into two parts for Western analysis and laser-scanning confocal microscopy.

For Western analysis, tissue samples of the posterior lobe in the lung (∼200 mg) were snap-frozen in liquid nitrogen immediately after sacrifice. For the measurement of MMP-2 and MMP-9, 50 mg of frozen lung tissue were homogenized in lysis buffer and centrifuged for 10 min at 10,000 × g and 4°C. Western analysis was carried using previously described anti-MMP-2 and anti-MMP-9 antibodies (mouse anti-human MMP-2, clone 42-5D11, purified antibody, and mouse anti-human MMP-9, clone 56-2A4, purified antibody, from Daiichi Fine Chemical Industries, Takaoka, Japan) and standard procedures [22–25]. The resultant signals were quantified by densitometry.

Other lung tissue specimens (two specimens from each lobe, approximately 3 × 3 × 2 mm) from each lobe in whole lung were embedded in OCT compound (Sakura Finetechanical, Tokyo, Japan), snap-frozen in liquid nitrogen, and cut into 5-μm-thick sections using a cryostat for immunohistochemical analysis. The sections were stained with a 1:100 dilution of anti-cytokeratin antibody (Chemicon, Temecula, CA), a specific epithelial cell marker, and a 1:500 dilution of Alexa Flour 488 goat anti-mouse IgG.
The tissues were then observed using a laser-scanning confocal microscope (LSM510 NLO; Zeiss, Tokyo, Japan). Morphometric analyses were performed by two persons (Y.M. and M.A.) blinded to the conditions.

**Statistical Analysis**

All values are presented as means ± SD. Within each group, we evaluated the time-dependent changes in pulmonary function parameters using paired t-tests. Multiple comparisons among groups were made using 1-way analysis of variance (ANOVA) with post hoc Tukey’s tests. Values of \( P < .05 \) were considered significant.

**RESULTS**

**Pulmonary Function**

In the sham group, FVC, FEV100, and FEVPEF were all significantly increased 4 weeks after saline administration (Figure 1), which is consistent with the normal growth of the rabbits over the course of the 4-week period of the experiment. In the PPE group, by contrast, none of the 3 pulmonary function parameters were increased 4 weeks after administration. Notably, rabbits receiving BMC transplantation via the left and right bronchi 24 hours after PPE administration showed significant increases in FVC, FEV100, and FEVPEF similar to those seen in the sham group (Figure 1).

**Bronchoalveolar Lavage Fluid**

As shown in Figure 2, the total cell and macrophage counts were significantly higher in bronchoalveolar lavage fluid from the PPE group (1027 ± 302/mL and 991 ± 290/mL, respectively) than from the sham (456 ± 218/mL and 441 ± 215/mL, respectively) or BMC (629 ± 114/mL and 604 ± 110/mL, respectively) group. Moreover, there was no significant difference in cell counts between the sham and BMC groups.

**HISTOLOGICAL EVALUATION**

Examination of lung tissue sections from the PPE group reveals clear expansion of the alveolar spaces and destruction of the alveolar walls that is not seen in sections from the sham group (Figure 3). These lesions appeared to be significantly attenuated in the BMC group, a finding that was confirmed by the mean linear intercepts, which were 86.1 ± 25.2 μm in the sham group, 127.4 ± 52.3 μm in the PPE group and 94.8 ± 32.3 μm in the BMC group, respectively (Figure 4A) [18]. The mean linear intercepts
were similar for the left and right posterior lobes (left and right posterior lobes: 81.9 ± 27.9 and 91.3 ± 20.8 μm in the sham group, 124.6 ± 54.0 and 131.0 ± 51.9 μm in the PPE group, and 92.9 ± 25.7 and 97.3 ± 39.6 μm in the BMC group, respectively). In addition, the PPE group showed significant increase in the degree of inflammation and fibrosis of lung parenchyma compared with the sham group, and the increase significantly attenuated in the BMC group (Figure 4B) [19].

**Immunohistochemical Staining**

The incidence of proliferative (Ki-67–positive) cells in the alveolar wall was significantly diminished in the PPE group (7.8 ± 2.1/200 alveoli), as compared to the incidence in the sham group (15.0 ± 4.8/200 alveoli),
whereas the incidence of Ki-67–positive cells in the alveolar walls in the BMC group (29.5 ± 7.2/200 alveoli) was markedly higher than in either the sham or PPE group (Figure 5A). Conversely, the incidence of TUNEL-positive apoptotic cells in the alveolar walls was significantly higher in the PPE group (3.8 ± 1.8/200 alveoli) than in either the sham (0.6 ± 0.9/200 alveoli) or BMC (1.1 ± 1.0/200 alveoli) group (Figure 5B). Likewise, the incidences of MMP-2– and MMP-9–positive cells were significantly higher in the alveolar walls of the PPE group than the other 2 groups. And although the frequency was less than in the PPE group, the incidence of MMP-positive cells was still higher in the BMC group than in the sham group (Figure 5C and D).

**Western Blotting**

Western analysis showed significantly greater expression of MMP-2 in the PPE group than in either the sham or BMC groups (Figure 6A). Likewise, we observed significantly greater expression of MMP-9 in the PPE group than in the sham group (Figure 6B). MMP-9 expression in the PPE group also tended to higher than in the BMC group, but the difference did not reach the level of significance.

**Immunofluorescent Staining**

Confocal microscopic examination revealed no cytokeratin-positive alveolar epithelial cells that were also positive for PKH26, a marker of

![Graph showing total cell and macrophage counts in bronchoalveolar lavage fluid 4 weeks after the indicated treatment. Note the increases in total cell and macrophage counts in the PPE group, as compared to the sham group, and the improvement in the BMC group. See Figure for definitions of sham, PPE, and BMC.](image)
transplanted BMCs, in the sham or PPE groups. These cells were seen only in the BMC group, the frequency was small, and moreover their distribution among lobes in whole lung was reasonably uniform (the number of PKH26-positive cells per 200 alveoli: 5.1 ± 3.3 in left anterior, 6.4 ± 3.9 in left posterior, 4.9 ± 3.2 in right anterior, 4.9 ± 3.7 in right middle, and 6 ± 4.1 in right posterior lobe, respectively). A part of them clustered as shown in Figure 7.

**DISCUSSION**

**Effect of BMC Transplantation on Pulmonary Function and Pathology**

The present study has shown that BMC transplantation via the left and right bronchi significantly improves pulmonary function (FVC, FEV100, and FEVPEF) in a PPE-induced rabbit model of pulmonary emphysema.

![Figure 3](image-url)  
**Figure 3** Histology and immunohistochemical analysis of the lungs 4 weeks after the indicated treatment. A (upper panel, low magnification): Photomicrographs showing enlargement of the airspace, emphysematous changes, in a wedge-shaped area in a representative lung from a rabbit in the PPE group (A2-1). Such changes are clearly absent in the sham (A1-1) and BMC groups (A3-1) (H&E stain). Scale bars, 200 μm; PL, pleural surface. (Lower panel, high magnification): Note the severe inflammatory and fibrotic change of lung parenchyma in the PPE group (A2-2) compared with those of the sham (A1-2) and BMC group (A3-2) (H&E stain). Scale bars, 50 μm. B, Significantly fewer in Ki-67–positive (brown) alveolar cells (arrows) were seen in the PPE group (B2) than the sham group (B1), whereas there was a marked increase in the BMC group (B3). C, The incidence of TUNEL-positive (brown; apoptotic) alveolar cells was significantly higher in PPE-treated lungs (C2) than in sham- (C1) or BMC-treated (C3) lungs. D and E, Greater incidences of both MMP-2–positive (D1-3) and MMP-9–positive (E1-3) cells were seen in PPE-treated lungs (D2, E2) than in sham- (D1, E1) or BMC-treated (D3, E3) lungs. Scale bars, 50 μm (B–E). See Figure 1 for definitions of sham, PPE, and BMC.

![Figure 4](image-url)  
**Figure 4** Mean linear intercept of the alveoli and grade of alveolar fibrosis 4 weeks after the indicated treatment. There was a significant increase in the mean linear intercept only in the PPE group (A). There was a significant increase in the inflammation and fibrosis grade in the PPE group (B). Data are means ± SD, *P < .05. See Figure 1 for definitions of sham, PPE, and BMC.
FIGURE 5 Incidences of Ki-67–, TUNEL–, MMP-2–, and MMP-9–positive cells in lung tissues 4 weeks after the indicated treatment. Note that there are significantly fewer Ki-67-positive cells in the PPE group and significantly more in the BMC group (A), and that there are significantly higher incidences of TUNEL–(B), MMP-2–(C), and MMP-9–(D) positive cells in the PPE group than in either the sham or BMC group. See Figure 1 for definitions of sham, PPE, and BMC.

FIGURE 6 Western analysis. Note the significantly higher MMP-2 (A) and MMP-9 (B) levels in the PPE group. The increase in MMP-2 expression was significantly inhibited in the BMC group (A). Data are means ± SD, *P < .05. See Figure 1 for definitions of sham, PPE, and BMC.
In addition, it also reduces the mean linear intercept of the alveolar wall, a specific pathological finding affected by emphysema. Thus, BMC transplantation appears to inhibit the progression of PPE-induced pulmonary emphysema.

**Possible Mechanisms of the Protective Effect**

There are several mechanisms by which BMC transplantation could slow the progression of pulmonary emphysema. First, it may exert an anti-inflammatory effect, as evidenced by the diminished total cell and macrophage counts in the bronchoalveolar lavage fluid of rabbits receiving BMC transplantation after PPE. The molecular mechanism of this anti-inflammatory effect remains unclear, however. Second, it is well-known that
MMP is secreted from fibroblasts, alveolar and airway epithelial cells, and inflammatory cells, such as alveolar macrophages [22–28], and we found that expression of both MMP-2 and MMP-9 is up-regulated in rabbits administered PPE. These two enzymes dismantle type IV collagen (the major component of basal membrane), denatured collagen (gelatin), type V collagen, and insoluble elastin, and contribute to the progression of pulmonary emphysema via elongation of the alveolar wall [24, 26]. We found that BMC transplantation inhibited the expression of MMP-2 and MMP-9 otherwise enhanced by PPE, suggesting inhibition of MMP expression may contribute to the protective effect exerted by BMCs against pulmonary emphysema.

Third, it is well-known that apoptosis among alveolar epithelial cells plays a key role in PPE-induced pulmonary emphysema [29]. Our TUNEL assays showed that BMC transplantation inhibited PPE-induced apoptosis among cells within the alveolar wall and, at the same time, there was a significant increase in the numbers of proliferative (Ki-67–positive) cells. Thus, inhibition of apoptosis and increased cell proliferation within the alveolar wall may be related to the beneficial effects of BMC transplantation.

Finally, although it is presently controversial whether BMCs can transdifferentiate into alveolar cells in vivo [30, 31], we did observe some cells that were positive for both cytokeratin, a marker of alveolar epithelial cells, and PKH26, a marker of injected BMCs. These cytokeratin\(^+\)/PKH\(^+\) cells clustered partly as shown in Figure 7. This may be indicative of the proliferation of BMC-derived alveolar epithelial cells. However, it is difficult to differentiate precisely between transdifferentiation of injected BMCs into alveolar epithelial cells and fusion between the injected BMCs and resident alveolar epithelial cells. Recent studies have detailed the difficulties of identifying stem cells based on immunohistochemical or immunofluorescence analyses [30, 31]. As a result, the extent of transdifferentiation from BMCs into alveolar epithelial cells remains unclear. In any case, our findings suggest that regeneration is likely not the primary mechanism underlying the protective effects of BMC transplantation against pulmonary emphysema, because the numbers of the cells positive for both cytokeratin and PKH26 were too small.

**Methodological Problem of Injection via Left and Right Main Bronchi**

In the present study, the mean linear intercepts were similar for the left and right posterior lobes, which indicates similar elastase-induced emphysematous changes in each lobe. The frequency of cells positive for both cytokeratin and PKH26 also was similar among lobes in whole lung. This
suggests the uniform distribution of the instilled cells. Most likely, the injection of elastase or BMCs via the wedged catheter into the left and right main bronchi in the present study is largely responsible for the uniformity of the cytokeratin+/PKH+ cells distributions. But migration of the injected BMCs also may have contributed to the uniformity.

**CONCLUSION**

BMC transplantation via the left and right main bronchi may inhibit the progression to emphysema through down-regulation of inflammatory responses, MMP-2 expression, and apoptosis and the up-regulation of cell proliferation.

**REFERENCES**


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