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ORIGINAL ARTICLE

Therapeutic effects of adipose-derived stem cells pretreated with pioglitazone in an emphysema mouse model

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There is no therapy currently available that influences the natural history of disease progression in patients with chronic obstructive pulmonary disease (COPD). Although stem cell therapy is considered a potential therapeutic option in COPD, there are no clinical trials proving definitive therapeutic effects in patients with COPD. Recently, it was reported that pioglitazone might potentiate the therapeutic effects of stem cells in patients with heart or liver disease. To test the capacity of pioglitazone pretreatment of stem cells for emphysema repair, we evaluated the therapeutic effects of pioglitazone-pretreated human adipose-derived mesenchymal stem cells (ASCs) on elastase-induced or cigarette smoke-induced emphysema in mice. We also investigated the mechanisms of action of pioglitazone-pretreated ASCs. Pioglitazone-pretreated ASCs had a more potent therapeutic effect than non-pretreated ASCs in the repair of both elastase-induced and smoke-induced emphysema models (mean linear intercept, 78.1 ± 2.5 μm vs 83.2 ± 2.6 μm in elastase models and 75.6 ± 1.4 μm vs 80.5 ± 3.2 μm in smoke models, $P<0.05$). Furthermore, we showed that pioglitazone-pretreated ASCs increased vascular endothelial growth factor (VEGF) production both *in vitro* and in mouse lungs in the smoke-induced emphysema model. Pioglitazone-pretreated ASCs may have more potent therapeutic effects than non-pretreated ASCs in emphysema mouse models.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible.[1] COPD is a major cause of morbidity and mortality worldwide, and its incidence is estimated to rise to the third leading cause of death worldwide by 2030.[2] Although the pathogenesis of COPD is incompletely understood, emphysema and small-airway disease are known to be the main pathological processes for developing airflow limitations.[3] The mechanisms involved in emphysema development are persistent inflammation, extracellular matrix proteolysis, apoptosis and ineffective repair.[4,5] Although COPD is an important public health problem, there is no curative therapy for patients with COPD, and current therapeutic approaches are mostly focused on improving symptoms and decreasing the frequency of exacerbations.[1]

Recent advances in stem cell therapy may lead to a promising therapeutic option for more broad and definitive treatments of COPD considering that stem cells can differentiate into multiple lineages[6] and repair injured tissue via paracrine actions.[7] The therapeutic effects of stem-cell-based cell therapy have been shown in various organ diseases, such as diseases of the heart, bone, liver and lung.[8–12] Mesenchymal stem cells (MSCs) from bone marrow and adipose tissue have also demonstrated a therapeutic effect for COPD in an experimental cigarette smoke-induced model and elastase-induced emphysema model.[13–17] Our previous research also demonstrated that bone-marrow-derived MSCs repaired cigarette smoke-induced emphysema.[18] To date, clinical trials for stem cells in the area of COPD have not demonstrated definitive therapeutic effects, such as pulmonary function improvement or mortality reduction, although systemic administration of MSCs was found to be safe in patients with COPD.[19] However, clinical trials are ongoing.

It is suggested that the therapeutic impact of MSCs is mediated by their ability to differentiate into tissues and,

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potentially, by their trophic, paracrine and anti-apoptotic functions.[20] More advanced methods of manufacturing MSCs, including increasing differentiation efficiency, more persistent engraftment and/or augmentation of the paracrine effect, may be required to increase the therapeutic effects of MSCs for application in clinical practice. We have previously reported that paracrine effects might be more important than stem cell engraftment,[18] the distribution of intravenously injected MSCs,[21] and the optimal dose of MSCs in elastase-induced emphysema models;[22] these data may be helpful for further research on MSCs for patients with COPD. However, much remains to be determined for the clinical application of MSCs in patients with COPD.

Because the preferred culture methods to develop the maximal paracrine effects of human MSCs are still undetermined,[20] clinical trials may show a substantial paracrine effect of MSCs if an adequate method to potentiate stem cells is identified. Recently, it has been suggested that peroxisome proliferator-activated receptor-γ (PPAR-γ) activation changed the phenotype of mesenchymal cells during cell differentiation. Furthermore, it was reported that transplantation of pioglitazone-pretreated MSCs significantly improved cardiac function and may be a promising cardiac stem cell source to promote cardiomyogenesis or augmentation of paracrine functions.[23]

Therefore, we aimed to identify the capacities of pioglitazone pretreatment of stem cells in COPD. In this study, we intravenously injected with adipose-derived stem cells (ASCs) pretreated with pioglitazone into mice with elastase-induced or smoke-induced emphysema, identified the therapeutic effects and evaluated the mechanisms of action of pioglitazone-pretreated ASCs (pioASCs).

MATERIALS AND METHODS

Cell culture and pretreatment with pioglitazone

ASCs were purchased from Invitrogen (Carlsbad, CA, USA) and cultured using MesenPRO RS Medium with supplied supplements (Invitrogen). ASCs were fed every 3–4 days with fresh media and were subcultured with 0.05% trypsin-EDTA (Gibco Life Technologies, Grand Island, NY, USA). For pioASCs, the ASC culture medium was treated with 3 $\mu mol\,l^{-1}$ pioglitazone for 1 week. MLE-12 airway-epithelial cells were purchased from ATCC (Manassas, VA, USA), cultured with the recommended complete growth medium and subcultured with 0.05% Trypsin-EDTA (Gibco Life Technologies).

Preparation of conditioned media

At 70% confluence, ASCs were washed three times with phosphate-buffered saline and incubated with MesenPRO RS Medium. After 24 h, ASC-conditioned media (ASC-CM) were collected, filtered through a 0.22-μm filter, and concentrated aseptically using an Amicon Ultracentrifugal filter device with a molecular weight cutoff of 10 kDa (Amicon, Beverly, MA, USA).

Proliferation assay

We performed a CCK-8 assay to measure proliferation rates. The MLE-12 cell line was incubated for 24 h with ASC-CM or pioASC-CM; then, CCK-8 was mixed with medium (1:10, CCK-8:medium) and incubated for 2 h. Absorbance at 450 nm was measured using a microplate reader (PerkinElmer, Waltham, MA, USA).

Mouse model

The Institutional Animal Care and Use Committee of Asan Medical Center approved all mouse experiments in this study. C57BL/6 mice were purchased from Orient Bio (Seongnam, Korea). Mice were housed in a specific pathogen-free facility. For the elastase-induced mouse model, as described previously,[21] 6-week-old female C57BL/6 J mice were intratracheally injected with 0.4 U of porcine pancreatic elastase (Sigma-Aldrich, St Louis, MO, USA) at day 0, followed by an intravenous injection of 1×10^5 ASCs at day 7, and then killed at day 14 for lung harvest. For the smoke-induced emphysema model, as described previously,[18] the mice were exposed to cigarette smoke 5 days per week for 6 months from commercially purchased cigarettes that contained 8.5 mg of tar and 0.9 mg of nicotine (Eighty Eight Lights, KT&G, Daejeon, Korea). After exposure to cigarette smoke for 6 months, the mice were intravenously injected with 1×10^5 ASCs and then killed on day 7.

Histology and quantification of emphysema

Lung samples for histology were obtained using a previously described method.[21] In brief, the perfused lungs were inflated with 0.5% low-melting agarose, fixed with 4% formalin and embedded in paraffin. Lung sections of 6-μm thickness were stained with hematoxylin and eosin. Mean linear intercepts (MLIs) were determined using microscopy images.

HGF, FGF2 and VEGF measurement

Ten micrograms of protein from lung tissue were examined using enzyme-linked immunosorbent assay (ELISA). The hepatocyte growth factor (HGF), fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) levels in the lung protein lysates were measured using ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Caspase-3/7 activity measurement

Caspase-3/7 measurement has been previously described.[21] Ten micrograms of protein, prepared with a cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and quantified based on the Bradford assay, were incubated with caspase-3/7 substrate diluted with caspase-3/7 buffer in black multiwell plates (Promega, Madison, WI, USA). After 5 h, the fluorescence of each sample was measured using a fluorometer.

Data analysis

Statistical analyses were performed using GraphPad Prism ver. 5 software (GraphPad Software Inc., La Jolla, CA, USA). The data are presented as the mean ± standard error. The Mann–Whitney U test was used to compare both groups, and statistical significance was set at $P<0.05$.

RESULTS

Lung epithelial cell proliferation *in vitro*

To identify the effects of pioglitazone pretreatment on ASCs *in vitro*, the murine lung epithelial MLE-12 cell line was cultured in ASC-CM and pioASC-CM. Proliferation of lung epithelial cells was higher in the group treated with pioASC-CM than in the group treated with ASC-CM ($P=0.028$; Figure 1a and b).

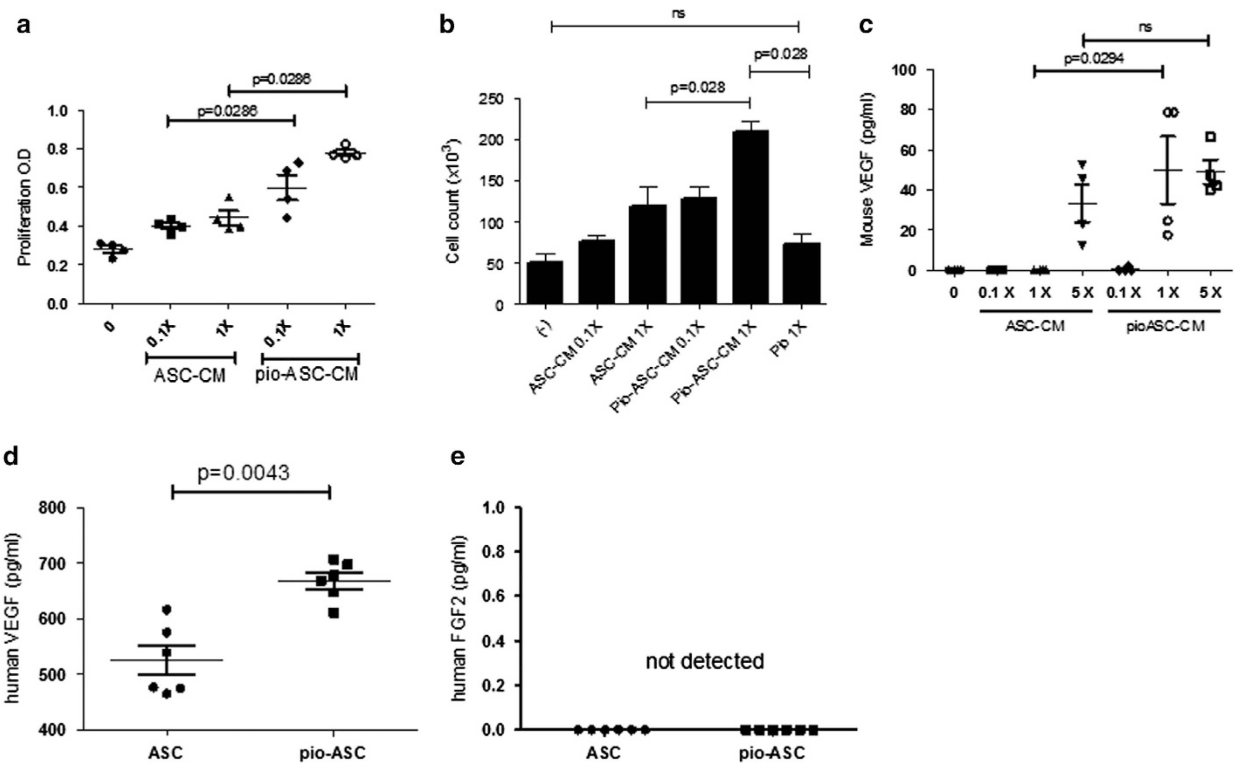

Figure 1 The effects of pioglitazone pretreatment on human adipose-derived stem cell (ASCs) *in vitro*. The murine lung epithelial MLE-12 cell line was cultured in conditioned media with ASCs (ASC-CM) or pioglitazone-pretreated CM with ASCs (pioASC-CM). Proliferation of lung epithelial cells was increased in the pioASC-CM compared with the ASC-CM. ($P=0.028$) (**a** and **b**). Mouse vascular endothelial growth factor (VEGF) production in pioASC-CM (×1) was significantly higher than in ASC-CM, ($P=0.029$) (**c**), and the human VEGF level in lung epithelial cells was higher in pioASC-CM than in ASC-CM. ($P=0.004$) (**d**) And, human fibroblast growth factor 2 (FGF2) production was not detected in either group (**e**).

Mouse VEGF levels from lung epithelial cells (×1) were higher in mice treated with pioASC-CM than in mice treated with ASC-CM ($P=0.029$) (Figure 1c). Production of human VEGF was significantly higher in mice treated with pioASCs than ASCs ($P=0.004$) (Figure 1d). However, human FGF2 production was not detected in either group (Figure 1e).

Repair of elastase-induced emphysema by pioASCs

In the elastase-induced mouse emphysema model, mice treated with ASCs and pioASCs showed improved alveolar regeneration compared with elastase-only mice (Figure 2a). Alveolar destruction was measured quantitatively by MLI. The elastase-only mice showed markedly increased MLI (90.8 ± 3.3) compared with the MLI (58.4 ± 0.7 μm) of control mice, and the MLI levels were decreased (83.2 ± 2.6 μm) in the mice treated with ASCs and further decreased (78.1 ± 2.5 μm) in the mice treated with pioASCs ($P<0.05$) (Figure 2b).

The mechanisms of pioASCs in the elastase-induced emphysema model

To identify the mechanisms of action of pioASCs in the mice with elastase-induced emphysema, proteins and messenger RNA were obtained from lung tissue of the four groups (control group, elastase-only group, ASC-treated elastase group and pioASC-treated elastase group). The mechanisms of action on regeneration were evaluated by assessing growth factors and apoptosis. First, production of the growth factors HGF, FGF2 and VEGF were measured in lung homogenate using ELISA. HGF and FGF2 levels were not significantly different among the four groups (Figure 3b and c). The VEGF level in lung tissue was significantly decreased in the elastase-only group compared with the control group, and it was increased in the pioASC-treated and ASC-treated mouse groups ($P<0.05$; Figure 3a). There was no significant difference between the pioASC-treated and ASC-treated mouse groups. Second, caspase-3/7-dependent apoptosis was evaluated. Caspase-3/7 activity was significantly increased in the elastase-only group compared with the control group, and it was significantly decreased in the pioASC-treated and ASC-treated groups compared with the elastase-only group ($P<0.05$). No significant difference was seen between the elastase-only group and the pioASC-treated group (Figure 3d).

Effect of pioASCs on smoke-induced emphysema

For the smoke-induced emphysema mouse model, similar to the elastase model, pioASC-treated mice showed improved alveolar regeneration compared with ASC-treated mice (Figure 4a). In addition, pioASC-treated mice had significantly

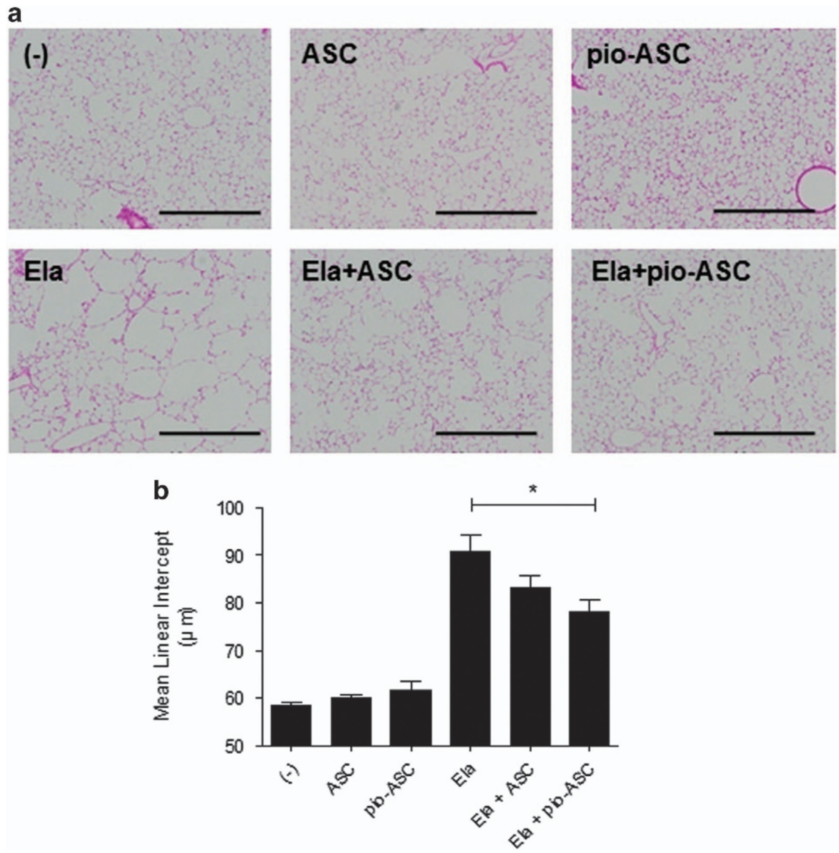

Figure 2 The therapeutic effects of pioglitazone pretreatment on human adipose-derived stem cells (ASCs) in mice with elastase-induced emphysema. C57BL/6 J mice were intratracheally injected with 0.4 U of elastase on day 0 and then intravenously injected with ASCs or pioglitazone-pretreated ASCs (pioASCs) on day 7. Data are measures of the histological staining with hematoxylin and eosin in lung sections on day 14. (**a**) Control (−, $n=4$), Ela (elastase only, $n=9$), ASC (ASC only, $n=7$), pioASC (pioASC only, $n=10$), Ela+ASC (elastase+ASC, $n=8$) and Ela+pioASC (elastase+pioASC, $n=9$). Scale bars, 0.5 mm. (**b**) Morphometic analysis of the mean linear intercept (MLIs). Values are presented as the mean ± s.e.m. $*P<0.05$ was statistically significant in the comparison between groups.

lower MLI (75.6 ± 1.4) than the MLI (80.5 ± 3.2) of ASC-treated mice ($P<0.05$; Figure 4b). In the smoke-induced emphysema model, HGF, FGF2 and VEGF production related to lung regeneration were also measured in lung homogenate using ELISA. HGF, FGF2 and VEGF levels in lung tissue were significantly increased in the pioASC-treated mice compared with the elastase-only mice and the ASC-treated mice (Figure 4c–e).

DISCUSSION

In this study, we showed that pioASCs have more potent therapeutic effects than ASCs for repair of alveolar destruction in emphysema mouse models. In addition, we showed that these effects may be mediated via restored imbalance of protease/antiprotease, decreased apoptotic activity and increased production of growth factors, suggesting augmentation of paracrine effects by pioASCs. Furthermore, we suggest that VEGF may play an important role in potentiating pioASCs because increased VEGF production was shown in pioASC-CM compared with ASC-CM *in vitro*, and mice treated with pioASCs had increased VEGF production compared with the mice treated with ASCs in the smoke-induced emphysema model. We showed, for the first time, that pioASCs were more effective in alleviating emphysema compared with ASCs. These data may be of use in expanding the efficacy of ASCs in clinical studies of COPD.

In COPD, stem cell therapy is an interesting alternative therapy because there is no curative therapy in current treatments of COPD, and stem cell therapy may also be a candidate therapy because of its capacity for repairing injured tissue. The therapeutic effects of MSCs derived from bone marrow and adipose tissue have been shown in mouse models of emphysema.[13–18] However, because there has been no demonstrated evidence of clinical benefit in patients with COPD, we attempted to elucidate the mechanisms of action and/or find more effective methods of manufacturing stem cells. The reported mechanisms of action of stem cells in COPD studies are inconsistent. However, many studies have focused on the paracrine effects of MSCs versus effects by differentiation or engraftments when explaining stem cell mechanisms of action in COPD models; these studies reported increased levels of growth factors,[16] similar therapeutic effects of cell-free MSC-CM[18] and identification of limited engraftment rates of MSCs.[21] Because MSCs produce many

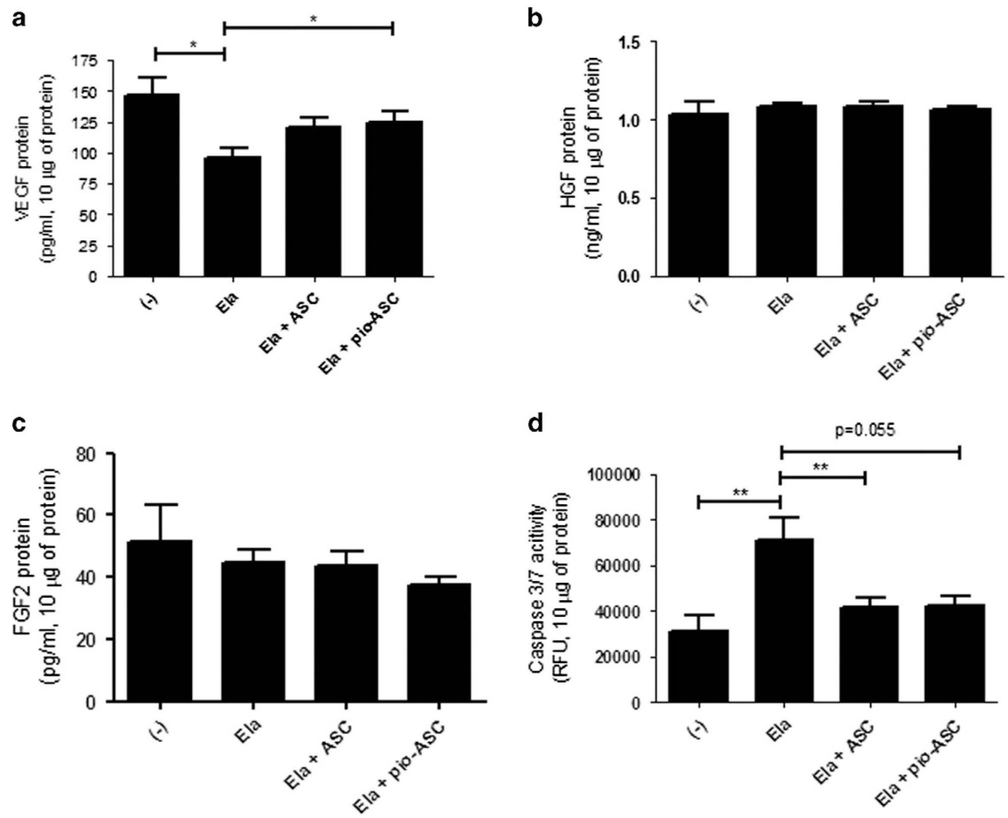

Figure 3 The production of growth factors in elastase-induced emphysema with adipose-derived stem cells (ASCs) or pioglitazone-pretreated ASCs (pioASCs). C57BL/6 J mice were intratracheally injected with 0.4 U of elastase on day 0 and then intravenously injected with ASC or pioASCs on day 7. Lung tissue was collected on day 14 ($n=5$–9 per group). The growth factors were measured with enzyme-linked immunosorbent assay (ELISA) for vascular endothelial growth factor (VEGF) (**a**), hepatocyte growth factor (HGF) (**b**) and fibroblast growth factor 2 (FGF2) (**c**). Caspase-3/7 activity was measured with fluorimetric enzymatic assay and normalized by protein concentration in lung homogenates. (**d**) Values are presented as the mean ± s.e.m. * and ** denote statistical significance ($P<0.05$) for the comparison between groups. (−): control; Ela, elastase only; Ela+ASC, elastase+ASC; Ela+pioASC, elastase+pioASC.

inflammatory cytokines and growth factors,[20,24] it is understood that MSCs assist and modulate the regenerative environment via paracrine mechanisms.[20] Among growth factors HGF, FGF2 and VEGF are known to be critical growth factors to repair lung destruction in emphysema models.[25–27] In particular, VEGF is known to be an important factor in airway and pulmonary vasculature development.[25] Furthermore, according to previous reports that inhibition of VEGF receptors induced emphysema features,[28] and ASC delivery was protective of damage in the VEGF inhibition model of emphysema,[17] VEGF induced by stem cells is crucial for protection of lung damage. Our previous research also showed that the therapeutic effects of MSCs were related to an increase in VEGF production. In addition, our current study emphasizes the role of VEGF in therapeutic effects of ASCs and in potentiating the effects of pioASCs, showing greater levels of VEGF production in pioASC-CM *in vitro* and in the smoke-induced mouse model. Our current study, however, did not show differences in the levels of VEGF production between ASC- and pioASC-treated groups in the elastase-induced model, although the level of VEGF production was increased in the ASC- and pioASC-treated groups. VEGF signaling is mediated by its receptors, such as VEGF receptor-1, -2 and -3. Therefore, there may be differences in the effects of VEGF following expression of each VEGF receptor. However, we did not confirm VEGF receptor expression in lungs from elastase- and smoke-induced models. It remains to be studied further.

Pioglitazone acts as an agonist of PPAR-γ and is known to enhance adipocyte differentiation.[29] Because PPAR-γ is also known to be expressed in MSCs,[30] it has been suggested that PPAR-γ activation, using pioglitazone, changes the phenotype of mesenchymal cells during cell differentiation. Several studies have reported that pioglitazone enhances transdifferentiation of stem cells and improves therapeutic functions. Shinmura *et al.*[23] reported that transplantation of pioglitazone-pretreated bone-marrow MSCs repaired heart tissue in an myocardial infarction model *in vivo*. Werner *et al.*[31] reported that pioglitazone increased the number and function of bone-marrow-derived endothelial progenitor cells in patients with coronary artery disease and that this effect represents a potential regenerative mechanism in atherosclerosis. Recently, Vosough *et al.*[32] also reported that transplantation of MSCs with pioglitazone in cirrhotic patients was safe and feasible. These clinical studies showed that stem cell therapy combined

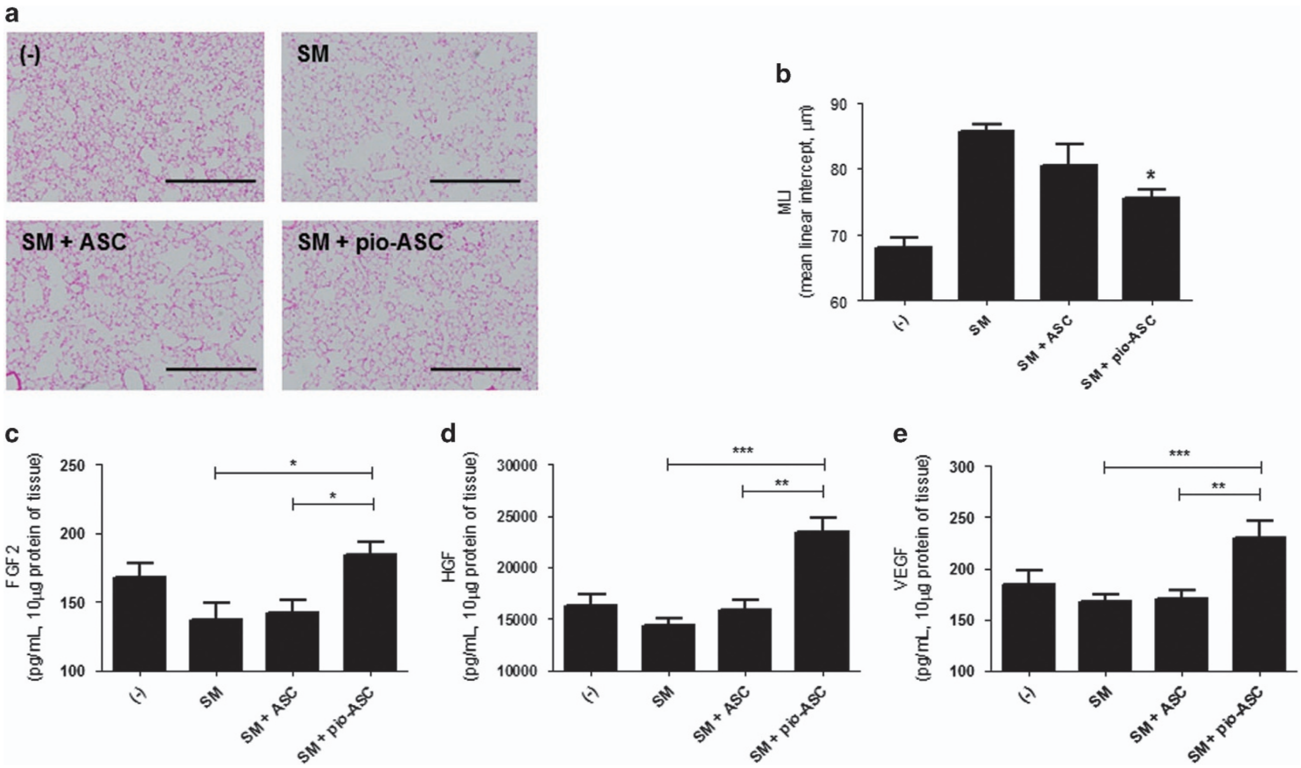

Figure 4 The therapeutic effects and mechanisms of action of human adipose-derived stem cells (ASCs) or pioglitazone-pretreated ASCs (pioASCs) in mice with smoke-induced emphysema. C57BL/6 J mice were exposed to cigarette smoke for 6 months and were then treated with ASCs or pioASCs. Shown here is the histological assessment at 2 months of lung sections stained with hematoxylin and eosin. (**a**) Control (−, $n=5$), SM (smoking only, $n=4$), SM+ASC (smoking+ASC, $n=4$) and SM+pioASC (smoking+pioASC, $n=5$). Scale bars, 0.5 mm. (**b**) Morphometic analysis of the mean linear intercept (MLIs). Values are presented as the mean ± s.e.m. *$P<0.05$ was statistically significant in the comparison between groups. The growth factors were measured with enzyme-linked immunosorbent assay (ELISA) for vascular endothelial growth factor (VEGF) (**c**), hepatocyte growth factor (HGF) (**d**) and fibroblast growth factor 2 (FGF2) (**e**). Values are presented as the mean ± s.e.m. *, ** and *** denote statistical significance ($P<0.05$) for the comparison between groups. (−): control; SM, smoke only; SM+ASC, smoke+ASC; SM+pioASC, smoke+pioASC.

with pioglitazone might potentiate the therapeutic effects of stem cells in heart or liver disease. Moreover, Shinmura *et al.*[23] reported that pioglitazone increased the cardiomyogenic transdifferentiation of human bone-marrow MSCs via PPAR-γ activation *in vitro*, and Wang *et al.*[33] also reported that pioglitazone stimulated the transdifferentiation of bone-marrow MSCs into adipocytes in a rat model. These studies suggest that pioglitazone facilitates MSCs to core cells of injured tissue and improves organ function through differentiation. However, the precise mechanisms of action of pioglitazone on stem cells remain to be elucidated. Recently, Hou *et al.*[34] reported that activation of PPAR-γ enhanced survival and therapeutic efficacy of MSCs via upregulation of Cx43 expression. Another possible explanation is that that the beneficial effects of pioglitazone on stem cell therapy outcomes is that pioglitazone augments the paracrine effect of MSCs. In our current research, we showed that pretreatment with pioglitazone in stem cell culture media increased therapeutic effects in damaged lung tissue and increased production of growth factors. These findings suggest that the beneficial effects of pioglitazone on lung tissue may be because of augmentation of paracrine effects of ASCs. ASC potentiation by means of pioglitazone may lead us to expand the efficacy of ASCs in clinical studies.

In conclusion, we report that pioASCs have more potent therapeutic effects than ASCs in emphysema mouse models. Furthermore, we suggest that the paracrine effects of ASCs, particularly on the production of growth factors such as VEGF, may have an important role in the mechanism that potentiates ASCs by pioglitazone. These data may be helpful in expanding the efficacy of ASCs in a clinical study of COPD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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