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Intravenous administration of adipose-derived stromal cells does not ameliorate bleomycin-induced lung injury in rats*

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ABSTRACT

Background: Mesenchymal stromal cells (MSCs) have been studied intensively in regenerative medicine. Among MSCs, adipose tissue-derived stromal cells (ASCs) are relatively easy to obtain from a patient. Since ASCs are ideal candidates for use in the treatment of disease states including pulmonary fibrosis, we investigated whether intravenous injection of ASCs could exert a therapeutic effect against bleomycin-induced lung injury in rats. Methods: Rats were intratracheally administered bleomycin, and one week later ASCs were isolated and cultured. Two weeks after bleomycin treatment ASCs or PBS (phosphate-buffered saline) were injected to the rats. Three or six weeks after bleomycin instillation, the total cell counts and their profile in bronchoalveolar lavage fluid (BALF) were measured, and a histological evaluation was semi-quantitatively assessed for the injured lungs, followed by cell tracing. Results: The BALF cell counts and its profiles were not significantly different in the ASCs and PBS groups. Furthermore, ASC treatment led to no significant histological effect compared with the PBS treatment. Using a fluorescent cell tracer, it was noted that the ASCs homed to the injured lung areas, but some ASCs accumulated around scars, and scarcely migrated into the fibrotic areas. Conclusions: In the present study, the intravenous administration of ASCs could not reduce the severity of bleomycin-induced lung injury in a rat model. Although the ASC counts and passage numbers were suitable, the older age and fibrotic disease stage of the rats were likely responsible for the treatment failure.

Keywords: Adipose Tissue; Aged; Mesenchymal Stromal Cells; Migration; Pulmonary Fibrosis

1. INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a refractory and lethal form of pulmonary fibrosis characterized by fibroblast proliferation, extracellular matrix deposition, and progressive lung scarring, and exhibits a histopathological pattern consistent with usual interstitial pneumonia (UIP) [1,2]. Despite extensive research and rapid expansion of scientific knowledge, the pathogenesis of IPF remains unclear. Recent data strongly suggest that the mechanisms driving IPF reflect abnormal, deregulated wound healing in response to multiple sites of ongoing alveolar epithelial injury, involving increased activity, and possibly exaggerated responses by a spectrum of proinflammatory and profibrogenic factors [3,4]. Most treatments, such as corticosteroids, and immunosuppressive, immunomodulatory, or antifibrotic agents, seek to suppress inflammation, but none has been proven to alter this process [5,6].

Mesenchymal stromal cells (MSCs), of different cellular origins (umbilical cord, bone marrow, adipose tissue), represent one of the most challenging and promising areas of research in terms of novel therapeutic strategies. MSCs have recently been applied for the treatment

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of several chronic, incurable diseases [7,8]. Under certain circumstances, MSCs can differentiate into chondrocytes, myoblasts, endothelial cells, epithelial cells, and neurocytes [9]. Furthermore, MSCs have been observed to display immunomodulatory properties [10-13].

Adipose-derived stromal cells (ASCs) have been identified as an alternative source of bone marrow-derived MSCs (BMMSCs) because of their easy extraction, high content of MSCs and *ex-vivo* expandability. ASCs, similar to BMMSCs, not only possess the ability to self-renew and differentiate into various mesenchymal cell types, but also secrete significant levels of many potent growth factors and cytokines [14-16]. However, little information exists about the effects of ASCs treatment on bleomycin-induced lung injury. The purpose of this study was to examine whether ASC treatment could attenuate bleomycin-induced lung injury in rats.

2. MATHELIALS AND METHODS

2.1. Bleomycin-Induced Lung Injury Model

A total of 10 mg bleomycin chloride (Nippon Kayaku, Japan) was dissolved in 1.0 ml of sterile 0.9% saline (BLM). Male Wistar rats, aged more than 24 weeks and weighing from 370 to 540 g (Shimizu Laboratory Supply, Kyoto, Japan), were intratracheally administerd 1.0 ml BLM/kg or 1.0 ml saline/kg under anesthesia induced by an intraperitoneal injection of sodium pentobarbital (3 mg/100g body weight). The institutional animal care and use committee approved this study, and all experiments abided by the Principles of Laboratory Animal Care advocated by the Animal Experiment Committee of Kyoto University (2007).

2.2. Isolation and Culture of Rat ASCs

White adipose tissue (WAT) was collected under anesthesia induced by the intraperitoneal injection of sodium pentobarbital (3 mg/100g body weight). The anterior abdominal wall was opened 2 cm lateral to the spine, and WAT weighing from 3 to 7 g was collected from the abdominal cavity. The WAT was washed with Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic/antimycotic (Gibco, Los Angeles, Calif., USA) two times to remove blood cells. After chopped into pieces, the WAT was digested with 10 ml collagenase solution in a water bath at 37˚C for 60 min under continuous shaking. To make the collagenase solution, 100 ml of Hanks' balanced salt solution with calcium and magnesium (Sigma, St. Louis, Mo., USA), pH 7.4, containing 4 g of bovine albumin (fraction V), 300 mg of collagenase type VIII (40% ammonium sulfate fraction from *Clostridium histolyticum*, Sigma) and 1.3 mg/ml of glucose were sterilized using a 0.22-μm filter. After collagenase treatment, the enzyme activity was neutralized with an equal volume of DMEM. The suspension was filtered through 250-μm nylon mesh and centrifuged at 300 g for 5 min at room temperature to separate the pellet. The pellet was treated with red blood cell lysis buffer (Sigma) for 5 min at 37˚C, then filtered through a 100-μm nylon mesh, followed by centrifugation (300 g for 5 min at room temperature) to obtain the pellet of ASCs (also referred to as the stromal-vascular fraction). The cells were cultivated in 75 cm^2 culture flasks, and the culture medium was changed twice a week. Upon reaching 60% - 90% confluence, the ASCs were detached using 0.25% trypsin-EDTA (Gibco), and divided 1:2. After 7 days in culture, the ASCs were isolated using trypsin, followed by centrifugation (300 g for 5 min at room temperature) to obtain a pellet of ASCs, which was then suspended in PBS to be autologously injected into the same recipient rats. The ASC suspension (1.0×10^7/0.5 ml) was gently infused within a few minuites via the external carotid vein of rats under local anesthesia. Control animals received the same volume of PBS.

2.3. Experimental Groups

The rats were randomly assigned to 6 weight-matched experimental groups. Group A included 7 animals that received intratracheal administration (IT) of bleomycin chloride solution (BLM) on day 0, intravenous injection (IV) of 0.5 ml phosphate buffer saline (PBS) via an external vein on day 14, and were sacrificed on day 21. Group B included 8 animals that received IT BLM on day 0, had ASCs harvested on day 7, underwent IV of 1.0×10^7 ASC/0.5 ml PBS on day 14, and were sacrificed on day 21. Group C included 7 animals that received IT saline on day 0, IVPBS on day 14, and were sacrificed day 21. Group D included 7 animals that received IT BLM on day 0, IVPBS on day 14, and were sacrificed on day 42. Group E included 6 animals that received IT BLM on day 0, had ASCs harvested on day 7, received IV of 1.0×10^7 ASC/0.5 ml PBS on day 14, and were sacrificed on day 42. Group F included 7 animals that received IT saline on day 0, IVPBS on day 14, and were sacrificed on day 42.

2.4. DiI and DAPI Staining

For cell tracing, ASCs were labeled with CM-DiI (Invitrogen, Eugene, Oreg., USA) according to the manufacturer's protocol. Briefly, CM-DiI (in DMSO solution) was diluted in PBS to obtain a concentration of 10 μg/ml. The ASCs were incubated with the dye solution for 3 min at 37˚C followed by 15 min at 4˚C. The labeled ASCs were washed once with PBS, and then were cultured in DMEM as mentioned above at 37˚C under 5%

CO_2 in humidified air. The labeling efficiency was confirmed to be greater than 90% by fluorescence microscopy. Nuclei were stained with DAPI (Sigma) on the glass slides.

2.5. Bronchoalveolar Lavage (BAL)

At 3 or 6 weeks after intratracheal administration of BLM, the rats were sacrificed by an intraperitoneal overdose injection of sodium pentobarbital. Immediately after the animals were killed, a 16-gauge cannula was inserted into the incised trachea. BAL was performed using 3 ml × 5 infusions of 0.9% normal saline with withdrawal via the cannula. The cells in the BAL fluid were counted using a hemocytometer. Differential cell counts were performed using slides prepared by cytocentrifugation and May-Grünwald-Giemsa staining.

2.6. Preparation of the Lung Tissues and Histological Evaluation by the SMI

Following BAL, each left lung was inflated at a constant pressure of 20 cm H_2O of 10% paraformaldehyde for five minuites, removed from each animal, and fixed in 10% paraformaldehyde for several days. For the histological examination, each left rat lung was transversely sectioned into 7 pieces. The specimens were then dehydrated and embedded in paraffin. Four-μm thick sections were cut using a rotary microtome, placed on glass slides, deparaffinized, and sequentially stained with hematoxylin & eosin and Masson's trichrome stains. Thereafter, the sections were examined using a standard light microscope (BX-40, Olympus, Tokyo, Japan). Among 6 independent cut planes, the three most severely injured were selected for a histological assessement by a semiquantitative morphological index (SMI) scaling, without knowledge of the treatment groups, using a grading scheme reported by Lossos *et al*. [17]. We used a fluorescence microscope (BZ-9000, Keyence, Tokyo, Japan) to examine the fluorescences, DiI and DAPI signals. The histological assessement by an SMI scaling was as follows: 0, normal lung; 1, minimal areas of inflammation, epithelial hyperplasia and fibrosis, usually limited to subpleural foci in just 1 or 2 sections; 2, more frequent lesions; 3, all three sections exhibit lung lesions which are not limited to subpleural foci; 4, extensive lesions in at least 2 of 3 sections; 5, the majority of each of the three lung sections are affected by inflammation and fibrosis.

2.7. Statistical Analysis

The data are expressed as the means ± standard deviation. Upon detection of significant differences by the Kruskal-Wallis test, post-hoc pairwise comparisons were conducted using the Tukey-Kramer test, with the level of statistical significance set at $P < 0.05$.

3. RESULTS

3.1. Total Cell Counts and the Profile in BALF (Figure 1)

The total cell counts and cell profile in the BALF of groups A, B and C were not significantly different. In addition, no significant differences in the total cell counts and cell profile in the BALF were observed among groups D, E and F. Moreover, there were no significant differences in the total cell counts between groups A and D or between groups B and E. The number of neutrophils, lymphocytes, and macrophages in the BAL fluid of the ASC-treated rats in groups B and E were not significantly different from the PBS-treated rats in groups A and D, respectively.

3.2. Semiquantitative Morphological Evaluation of the Lung Injury in Rats Treated with ASC (Figure 2)

The ASC-treated rats in groups B and E did not have significantly different scores compared to the PBS-treated rats in groups A and D, respectively. A comparison of the histological changes between the rats in groups

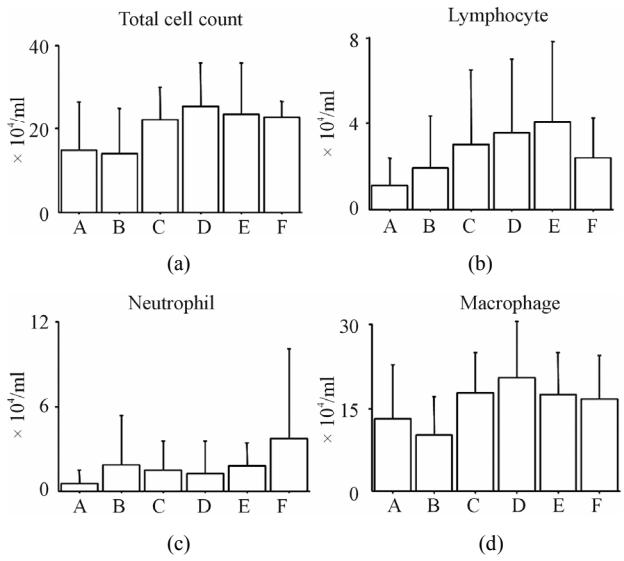

Figure 1. The time-dependent changes in the inflammatory cell profile in the BAL fluid the total cell count (a), lymphocyte counts (b), neutrophil counts (c), and macrophage counts (d) in the BAL fluid are shown. ASC treatment could not affect inflammatory cell counts following BLM-induced lung injury at 3 and 6 weeks after injury. No significant differences were found between any of the groups. Group A: BLM IT (day 0) + PBS IV (day 14) + sacrificed (day 21); Group B: BLM IT (day 0) + ASC harvest (day 7) + ASC IV (day 14) + sacrificed (day 21); Group C: Saline IT (day 0) + PBS IV (day 14) + sacrificed (day 21); Group D: BLM IT (day 0) + PBS IV (day 14) + sacrificed (day 42); Group E: BLM IT (day 0) + ASC harvest (day 7) + ASC ASC IV (day 14) + sacrificed (day 42); Group F: Saline IT (day 0) + PBS IV (day 14) + sacrificed (day 42).

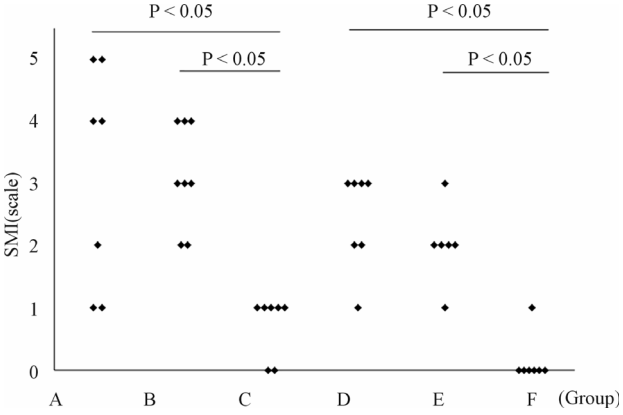

Figure 2. The BLM-induced histological changes in the lungs assessed by the SMI after ASC transfer. The ASC-treated rats in groups B and E did not have significantly different scores compared with PBS-treated rats in groups A and D, respectively. A comparison of the histological changes between groups B and E, as well as between groups A and D revealed that there was temporal improvement in the natural course of BLM-induced lung injury. Group A: BLM IT (day 0) + PBS IV (day 14) + sacrificed (day 21); Group B: BLM IT (day 0) + ASC harvest (day 7) + ASC IV (day 14) + sacrificed (day 21); Group C: Saline IT (day 0) + PBS IV (day 14) + sacrificed (day 21); Group D: BLM IT (day 0) +PBS IV (day 14) + sacrificed (day 42); Group E: BLM IT (day 0) + ASC harvest (day 7) + ASC IV (day 14) + sacrificed (day 42); Group F: Saline IT (day 0) + PBS IV (day 14) + sacrificed (day 42).

B and E, as well as between those in groups A and D revealed that there may be a temporal improvement in the bleomycin-induced lung injury.

3.3. Distribution of DiI-Labeled ASCs in the Lung Fields (Figure 3)

The ASCs were found in the BLM-injured lung field. However, some ASCs were present in the surrounding fibrotic areas and did not migrate into the scar (a representative photo is shown in **Figure 3**). DiI-labeled ASCs, fluoresced red, are emphasized by arrows. Nuclei labeled with 4',6-diamidino-2-phenylindole (DAPI) fluoresce blue.

4. DISCCUSION

In the present study, ASCs harvested from abdominal white adipose tissues could not reduce the severity of the bleomycin-induced lung injury in male rats. Many authors have reported benefits from using BMMSC for bleomycin-induced lung injury (**Table 1**) [10-13]. There are several possible explanations for this failure.

First, the ASCs in the present study were isolated and cultured using methods that have been proven to be effective [18-21]. Therefore, the number of cells used for intravenous transplantation is a very important quantity. The efficacy of using different total stromal cell counts

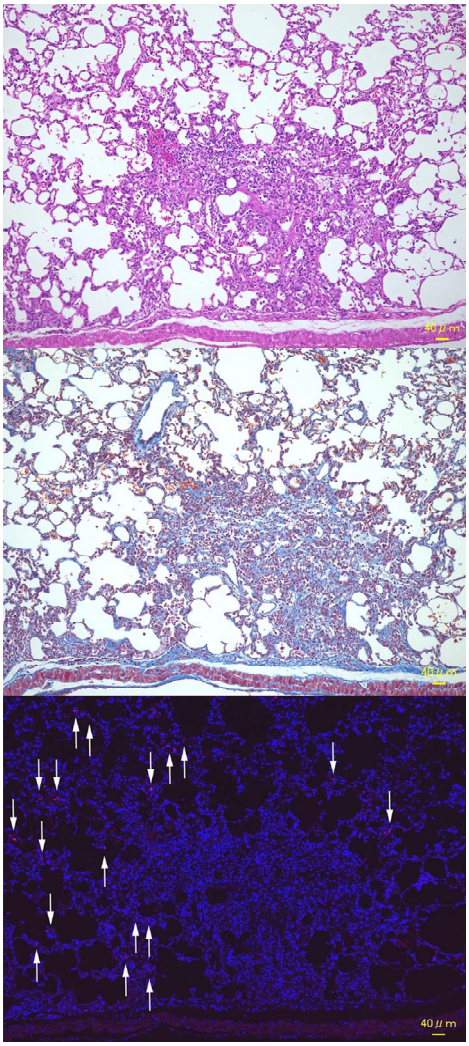

Figure 3. The ASCs surrounded the fibrotic tissue and did not migrate into the scar. The upper panel shows the results of hematoxylin & eosin staining, middle panel shows the Masson's trichrome staining, and the lower panel shows the fluorescence. Nuclei were labeled with DAPI, and fluoresced blue. DiI-labeled ASCs (red fluoresence, indicated by arrows) surrounded the fibrotic tissue, but barely migrated into the scar, in which interstitium was thickened by fibrosis, and the alveolar architecture was distorted (a representative photograph is shown).

has been previously documented in various reports (**Table 1**). Since the stromal cell count per body weight in our study was similar to that used in many of the previous reports, the cell count was still suitable for stromal cell therapy. Therefore, we do not believe that the cell count was the major cause of the lack of efficacy.

Patients with IPF are often middle aged, usually between 40 and 70 years of age [1]. The incidence of the disease increases with older age. The rats used in our

Table 1. The effects of intravenous MSC administration on bleomycin-induced lung injury.

Author (Ref)	Animal model	Period	Cell type	Passage	Num	Animal weight	Num/weight (kg)	Outcome
Lee (10)	6-wk female SD rats	4 days	BMMSC	ND	1×10^7	ND *250 gram	4×10^7	effective
Zhao (11)	4-wk male SD rats	12 hours	BMMSC	3 to 10	5×10^6	200 - 250 gram	$2 - 2.5 \times 10^7$	effective
Ortiz (12)	6 - 10 wk female C57BL/6 mice	0 or 7 days	BMMSC	ND	5×10^5	ND *20 gram	2.5×10^7	effective
Rojas (13)	6 - 8 wk C57BL/6 mice	6 hours	BMMSC	ND	5×10^5	ND *20 gram	2.5×10^7	effective
Present	over 24-wk male Wister rats	14 days	ASC	0 to 1	1×10^7	370 - 540 gram	$1.9 - 2.7 \times 10^7$	ineffective

Ref: reference; Period: the duration from bleomycin instillation to cell therapy; Num: Number of cells; wk: week old; SD: Sprague-Dawley; BMMSC: bone marrow-derived mesenchymal stromal cell; ASC: adipose-derived stromal cell; ND: no data included in the report; *: likely body weight based on animal age.

study were over the age of 24 weeks, which was relatively older than the rodents used in previous reports (**Table 1**). Many authors have shown the BMMSCs activities to decrease in older humans or animals [22-25]. Alt *et al*. isolated human ASCs from young, middle aged, and aged healthy volunteers, and demonstrated that the number of growing adherent cells/gram of adipose tissue was reduced with age, and their ability to form colonies was decreased, despite the identical expression of surface markers characteristic of ASCs [26]. Moreover, aging decreases the multi-lineage differentiation potential, and proliferative capacity in association with increases in cellular senescence, and significantly increased the quiescence of G2 and S phase in the cell cycle. The older animals used in the present study may therefore be one of the reasons for the failure of stromal cell therapy.

More males have been reported with IPF than females [1]. Recent studies have addressed gender differences in the pro- and anti-inflammatory properties of MSCs [27, 28]. For example, Crisostomo *et al*. have demonstrated gender differences in apoptosis and vascular endothelial growth factor, tumor necrosis factor, and interleukin-6 expression under lipopolysaccharide- and hypoxia-induced stress in murine BMMSC [27]. Since only male rats were used in the present study, we could not conclude whether the gender of the rats affected the outcome of therapy, but this is a possibility that should be examined in future studies.

With regard to cell transplantation into the lungs, some authors have investigated the survival rate of intravenously administerd MSCs, and found high percentages [29-31]. Because the duration from MSC injection to estimation of the cell counts in these studies was much shorter than the 3 and 6 weeks used in our study, our cell survival rate cannot be directly compared with the previous reports. A longer observation for stromal cell survival *in vivo* is needed to confirm the viability of cells following administraion.

It has been suggested that with increasing time in cell culture, and with increasing passages, cultured MSCs lose their proliferation and differentiation potential, and senesce [32,33]. As the cell passage number used in the present study was low, this was not one of the causes of treatment failure.

The intratracheal administration of bleomycin is a documented method for inducing pulmonary fibrosis in rodents. Thrall *et al*. reported time-dependent changes in the lung histopathology following intratracheal instillation of bleomycin in rats [34]. Within the first 3 to 5 days, focal areas of intraalveolar hemorrhage were present. Over the course of the first week, these changes gave way to the appearance of atypical alveolar lining cells and confluent interstitial inflammatory cell invasion. By the second week, the interstitial infiltrates were obviously associated with an increase in fibroblasts and the start of deposition of interstitial extracellular collagen. From the third week after instillation, the amount of collagen in the interstitial areas became increasingly prominent, with some evident condensation. In the previous studies that documented successful BMMSC treatment [10-13], therapy was initiated within hours or within one week after bleomycin instillation. However, in the present study, the stromal cell treatment was initiated two weeks after bleomycin administration when the fibroblasts had already started to appear and interstitial extracellular collagen had started to be deposited.

Okada *et al*. examined the effects of neural stem/progenitor cells (NSPCs) transplantation for spinal cord injury during both the acute and delayed phases in mice [35]. In the acute group, the NSPCs were mainly distributed within the scar area, while in the delayed group, the NSPCs were found around the scar. The timing of cell transplantation may therefore be a key determinant of the fates and function of integrated cells, since cell survival and migration depended on the time of transplantation relative to injury. Although the ASCs were noted to home to the lesion sites in our study, they accumulated around the scar and only scarcely migrated into fibrotic areas (**Figure 3**). Taken together, these findings indicate that it would not be likely that the ASCs surrounding the fibrotic tissue would secrete factors in a paracrine fashion to affect the inflammation and fibrosis. Instead, it ap-

pears that the ASCs could not sufficiently migrate into the fibrotic areas in order to inhibit increasing interstitial fibrosis when they were administered from two to three weeks after bleomycin instillation. The precise mechanism responsible for the lack of migration into fibrotic areas is not clear. Baek *et al*. indicated that prior *in vitro* modulation by several chemokines or growth factors affected the homing capacity of human ASCs by changing the expression of receptors, and that this could stimulate the movement of the ASCs into injured areas *in vivo* when they were administered intravenously, thereby improving their therapeutic potential [36].

5. CONCLUSION

In our present study, the intravenous administration of ASCs could not reduce the severity of bleomycin-induced lung injury in rats. Although the stromal cell counts and passage number were suitable, the older age and fibrotic disease stage of the rats used in this study were likely responsible for the treatment failure.

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