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ORIGINAL ARTICLE

Mesenchymal stem cells attenuate ischemia–reperfusion injury after prolonged cold ischemia in a mouse model of lung transplantation: a preliminary study

Tatsuaki Watanabe[1] · Yasushi Hoshikawa[1] · Naoya Ishibashi[1] · Hirotoshi Suzuki[1] · Hirotsugu Notsuda[1] · Yui Watanabe[1] · Masafumi Noda[1] · Masahiko Kanehira[1] · Shinya Ohkouchi[2] · Takashi Kondo[1] · Yoshinori Okada[1]

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Abstract
Purpose Mesenchymal stem cells (MSCs) suppress inflammation and immune responses. We conducted this study to find out if MSCs attenuate ischemia–reperfusion injury in a mouse model of lung transplantation.
Methods C57BL/6J mouse lungs perfused with low-potassium dextran glucose solution were preserved at 4 °C for 18 h. Human MSCs were slowly injected into the left pulmonary artery of the lung grafts, and orthotopic left lung transplantation was then performed. The lung isografts were reperfused for 6 h, and bronchoalveolar lavage fluid (BALF) from the left lung graft was collected. We measured the protein concentration, cell count, and proinflammatory cytokine concentrations in the BALF.
Results The protein concentration and cell count in the BALF were significantly lower in the MSC-administered grafts than in the PBS-administered controls. Concentrations of proinflammatory cytokines, including IL-1β, IL-17A, and TNF-α, in BALF tended to be lower in the MSC-administered grafts than in the controls, but the difference was not significant.
Conclusion The pre-transplant administration of MSCs via the pulmonary artery of the lung graft attenuated ischemia–reperfusion injury after prolonged cold ischemia in this mouse model of lung transplantation.

✉ Tatsuaki Watanabe
tatsuaki_watanabe@yahoo.co.jp

[1] Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

[2] Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan

Keywords Mesenchymal stem cells · Ischemia–reperfusion injury · Lung transplantation

Introduction

Ischemia–reperfusion injury (IRI) is a leading cause of morbidity and mortality in the early phase of lung transplantation and has been identified as an important risk factor in the development of bronchiolitis obliterans syndrome [1, 2]. Following ischemic and reoxygenation damage, lung grafts are exposed to inflammatory insults and innate and adaptive immunity, triggering the expression of inflammatory cytokines and the infiltration of leukocytes, all of which lead to structural damage of the graft with the development of interstitial and alveolar edema [3–6].

Mesenchymal stem cells (MSCs) are fibroblast-like cells, isolated from the bone marrow and connective tissue of almost all organs [7]. MSCs are characterized by their ability to propagate in vitro and differentiate into several cellular phenotypes, including bone, cartilage, and adipose tissue. MSCs have also been found to suppress the inflammation and immune responses that are mediated by paracrine secretions of anti-inflammatory and immunoregulatory factors [8–10]. Recent studies suggest that MSCs ameliorate various forms of experimental acute lung injury [11, 12].

MSCs are known to ameliorate acute graft failure induced by IRI in various organs, including the kidney, heart, and liver [13–15]. MSCs have also been applied therapeutically to treat many diseases of the lung, cardiovascular system, bone and cartilage, and the nervous system [16]. Despite their promising results, the effect of MSCs in reducing IRI after lung transplantation has been poorly investigated. Therefore, we conducted an experimental

study to investigate if pre-transplant MSC administration can prevent IRI in a mouse model of lung transplantation.

Materials and methods

Animals

Pathogen-free 9–13-week-old male C57BL/6J mice were obtained commercially from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and used in compliance with the rules of the Institutional Animal Care and Use Committee [17]. The mice served as donors and recipients of left syngeneic single lung transplantation.

Mesenchymal stem cells

Human MSCs (hMSCs) isolated from healthy men were kindly provided by Professor Darwin Prockop of Texas A&M Health Science Center. Frozen hMSCs were thawed and expanded according to a previous report [18]. Briefly, cells were cultured at 37 °C for 24 h with 5 % CO_2 using a complete culture medium consisting of minimum essential medium alpha (Invitrogen, Carlsbad, CA) supplemented with 17 % of fetal bovine serum (Nichirei, Tokyo, Japan). Viable cells were recovered with trypsin/EDTA, replated at a density of 60 cells/cm^2, and cultured again with media that was replaced every 3 days. After 9 days of culture, cells were harvested as passage 2 and frozen. We used hMSCs at passage 3 in the present experiment.

Left lung transplantation

Orthotopic mouse left lung transplantation was performed as described previously [19]. Briefly, donor mice were anesthetized with an intraperitoneal injection of ketamine (0.1 mg/g) and xylazine (0.01 mg/g) and intubated through tracheostomy and mechanically ventilated. A median laparosternotomy was performed after the administration of 100 U heparin via the penile vein of the donor. The lungs were flushed through the pulmonary trunk with 2 mL of cold (4 °C), low-potassium dextran glucose solution. Subsequently, the heart–lung block was harvested and stored in a petri dish and immersed in low-potassium dextran glucose solution at 4 °C for 18 h. The recipient mice were anesthetized with an intraperitoneal injection of ketamine (0.1 mg/g) and xylazine (0.01 mg/g) and were intubated orally and mechanically ventilated. Orthotopic and syngeneic left lung transplantation was performed using a cuffed technique. The animals were woken after the thoracotomy had been closed. Animals were killed for analyses 6 h after reperfusion.

MSC administration

Before left lung transplantation, hMSCs (5×10^5 cells in 100 μL of PBS) or PBS alone ($n = 7$ in each group) were infused slowly into the left pulmonary artery through the cuff. Immediately after infusion, left lung transplantation was performed.

Measurement of protein concentration and cell count in the bronchoalveolar lavage fluid (BALF)

Following 6 h of reperfusion, recipient mice were killed. A median laparosternotomy was performed, the right hilum was clamped, and BALF was collected via an intratracheal injection of 400 μL of PBS into the left lung graft. This procedure was repeated once. The collected BALF was centrifuged at 400*g* for 5 min at 4 °C. Furthermore, the Cell-free BAL supernatant was processed to measure the protein concentration using a BCA Protein Assay Kit (Thermo Scientific Japan, Yokohama, Japan). The pelleted cells were suspended again in 100 μL of PBS. Cells were counted using Countess (Invitrogen, Carlsbad, CA). The Cell-free BAL supernatant and lung graft were stored at −80 °C until further analysis.

Measurement of murine cytokines

The proinflammatory cytokines, IL-1β, TNF-α, IL-17A, IL-6, and IFN-γ, in Cell-free BAL supernatant were quantified with a multiplex cytokine panel assay using the Bioplex Bead Array technique (Bio-Rad Laboratories, Hercules, CA). The samples were analyzed as instructed by the Bioplex array reader, a fluorescent-based flow cytometer employing a bead-based multiplex technology, each of which was conjugated with a reactant specific for a different target molecule. The lower limits for the quantitation of the cytokines were 10.36 pg/mL for IL-1β, 5.8 pg/mL for TNF-α, 2.65 pg/mL for IL-17A, 0.74 pg/mL for IL-6, and 1.84 pg/mL for IFN-γ.

Histology and immunohistochemistry

For histological and immunohistochemical analyses, experiments other than the BALF study were performed for each group ($n = 3$ in the PBS group, $n = 3$ in the MSC group). The lung grafts harvested 6 h after reperfusion were immediately fixed in 4 % paraformaldehyde. After 24 h, they were embedded in paraffin, and then sectioned and stained with hematoxylin and eosin. To evaluate the extent of lung injury, several pathological categories (perivascular edema, intra-alveolar hemorrhage, capillary congestion, and neutrophil in small vessels) were scored on a scale

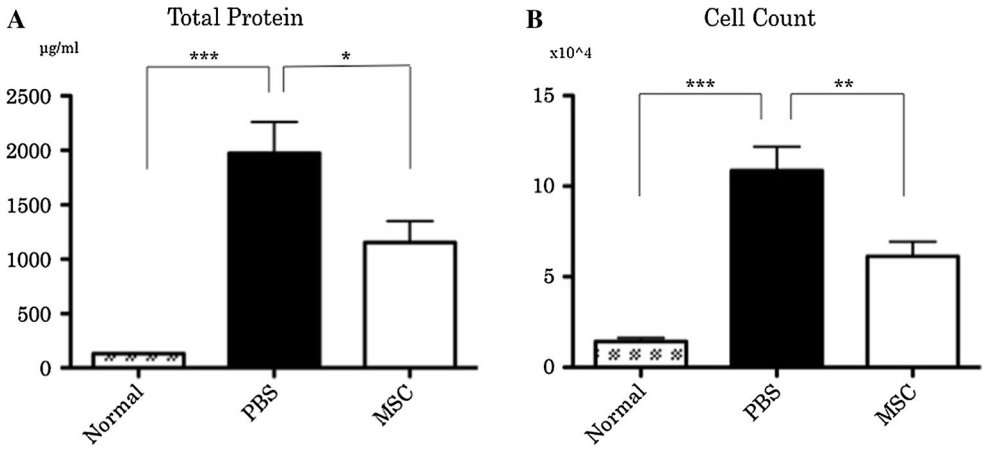

Fig. 1 The total protein concentration (**a**) and total cell count (**b**) in bronchoalveolar lavage fluid (BALF) were measured to evaluate pulmonary vascular permeability. The total protein concentration and cell count in the BALF were higher in left lung grafts preserved for 18 h and reperfused for 6 h than in normal left lungs. The total protein concentration and cell count in the BALF were lower in the human mesenchymal stem cell-administered lung grafts via the pulmonary artery before transplantation than in the PBS-administered controls. ***$P < 0.005$, *$P < 0.05$. *Normal* normal left lung ($n = 6$), *PBS* lung grafts administered with PBS before transplantation ($n = 7$), *MSC* lung grafts administered with 5×10^5 hMSCs before transplantation ($n = 7$)

of 0–4, with 0 = 0 % involvement, 1 = 1–25 % involvement, 2 = 26–50 % involvement, 3 = 51–75 % involvement, and 4 = 76–100 % involvement [20]. To examine the localization of hMSCs, immunohistochemical staining for the human MHC class I antigen that is expressed on hMSCs was performed [21]. Sections were deparaffinized and incubated for 5 min at 120 °C. Primary antibody reactions were performed using an anti-human MHC class I antibody (ab52922; Abcam, Santa Cruz, CA) overnight in dilution of 1:600 at 4 °C. Antibody depositions were visualized using diaminobenzidine. Nuclei were counterstained with hematoxylin.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis for the detection of human tumor necrosis factor-inducible gene 6 protein (TSG-6)

Total RNA was purified individually from lung grafts ($n = 3$ in each group) using Isogen (Nippon Gene, Tokyo, Japan). RT-PCR was performed using ReverTra Ace (Toyobo, Tokyo, Japan) and GoTaq (Promega, Madison, WI), according to the manufacturers' instructions. The RT reaction was conducted in one cycle at 42 °C for 20 min and at 99 °C for 5 min. The PCR reaction was conducted in three steps as follows: at 96 °C for 2 min (one cycle); at 96 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s (30 cycles for GAPDH, 36 cycles for TSG-6); and at 72 °C for 5 min and 4 °C for 5 min (one cycle). The primer pairs used in this study were as follows: GAPDH, forward: 5′-GTC TTC ACC ACC ATG GAG A-3′ and reverse: 5′-AAG CAG TTG GTG GTG CAG-3′, size of products: 170 base pairs; TSG-6, forward: 5′-CCA GGC TTC CCA AAT GAG TA-3′ and reverse: 5′-TTG ATT TGG AAA CCT CCA GC-3′, size of products: 284 base pairs.

Statistical analyses

Data were expressed as mean ± SE. Comparisons between groups were performed using the *t* test. All statistical analyses were performed using GraphPad PRISM (GraphPad Software Inc., San Diego, CA). Values of $P < 0.05$ were considered significant.

Results

Total protein concentration and cell count in BALF

Figure 1 shows the total protein concentration and cell counts in the BALF of normal lungs and lung grafts administered hMSC or PBS via the pulmonary artery, then transplanted, and reperfused for 6 h. The BALF total protein concentration and cell count of the lung grafts were significantly higher than those of the normal lungs. The total protein concentration and cell counts in the BALF of the hMSC-administered grafts were significantly lower than those of PBS-administered controls (total protein: 1.095 vs. 1.965 mg/ml; cell count: 0.61×10^5 vs. 1.09×10^5 cells).

Concentrations of murine cytokines in BALF

Figure 2 shows the BALF concentrations of murine cytokines measured by BioPlex. The concentrations of IL-1β, TNFα, IL-6, and IFN-γ in the normal left lungs were

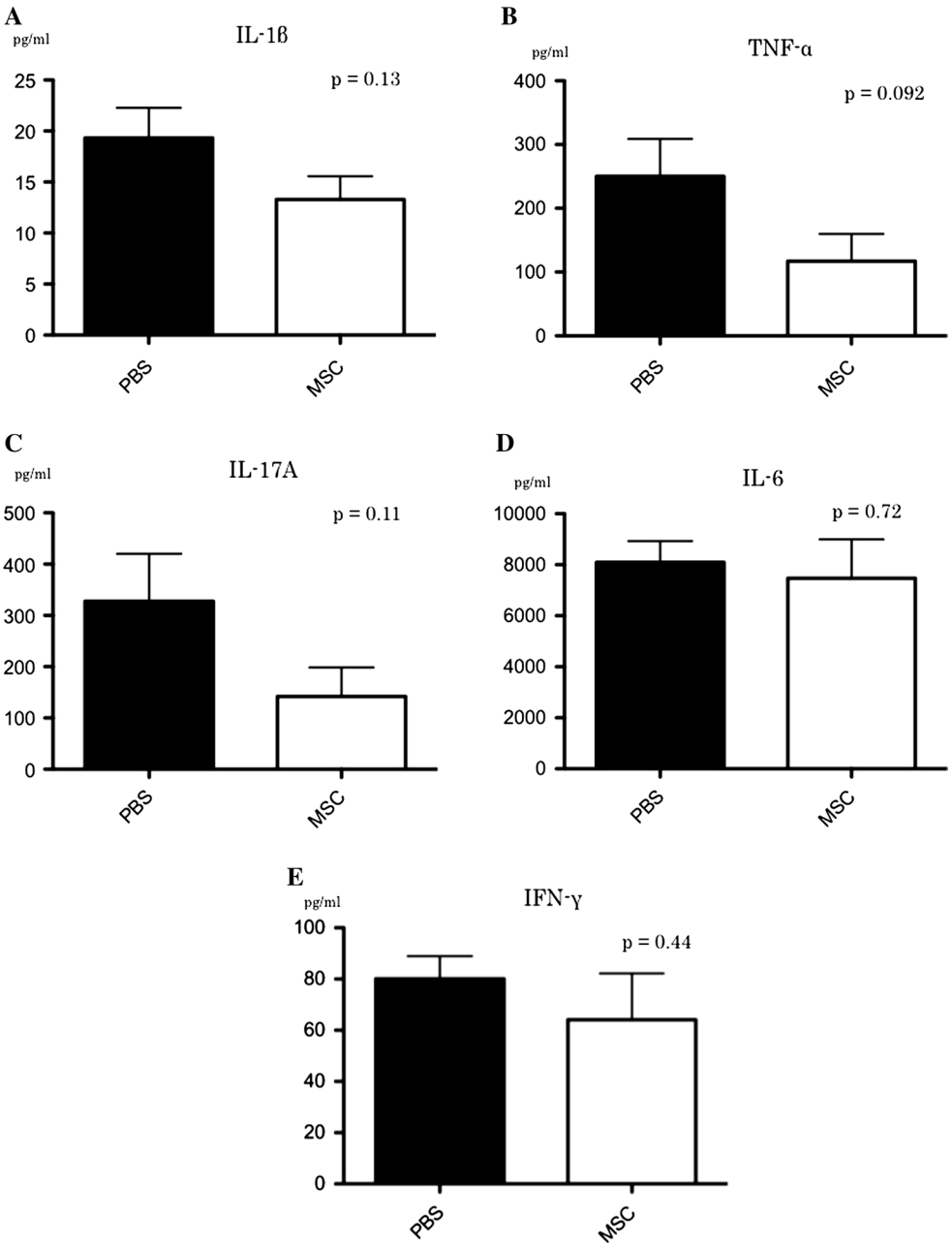

Fig. 2 Comparison of bronchoalveolar lavage fluid proinflammatory cytokine concentrations between the groups. The concentrations of proinflammatory cytokines in bronchoalveolar lavage fluid, as measured by multiplex immunoassay, tended to be lower in the human mesenchymal stem cell-administered lung grafts than in the PBS-administered controls; however, the difference in the concentrations of any cytokines did not reach significance. **a** IL-1β, **b** TNF-α, **c** IL-17A, **d** IL-6, **e** IFN-γ. *PBS* lung grafts administered with PBS before transplantation ($n = 7$), *MSC* lung grafts administered with 5×10^5 hMSCs before transplantation ($n = 7$)

under detectable limits (data not shown), whereas the concentration of IL-17A was 3.4 pg/ml. The concentrations of proinflammatory cytokines in the BALF of the hMSC-administered grafts showed a decreasing trend compared with those of PBS-administered controls; however, the difference was not significant.

Histology and immunohistochemistry

PBS-administered grafts that were harvested 6 h after reperfusion showed severe perivascular edema with capillary congestion and moderate intra-alveolar hemorrhage (Fig. 3a; Table 1). In contrast, these findings were minimal in the hMSC-administered lung grafts. Neutrophils in small vessels were also seen less frequently in the hMSC-administered lung grafts than in the PBS-administered grafts.

Immunohistochemically, cells positive for human MHC class I were found in the pulmonary arterioles and alveolar septae of the hMSC-administered lung grafts harvested 6 h after reperfusion but not of the PBS-administered controls (Fig. 3b).

Human TSG-6 gene expression in the lung grafts

Human TSG-6 gene expression was readily detectable by RT-PCR in the hMSC-administered lung grafts, but was not seen in the PBS-administered controls (Fig. 4).

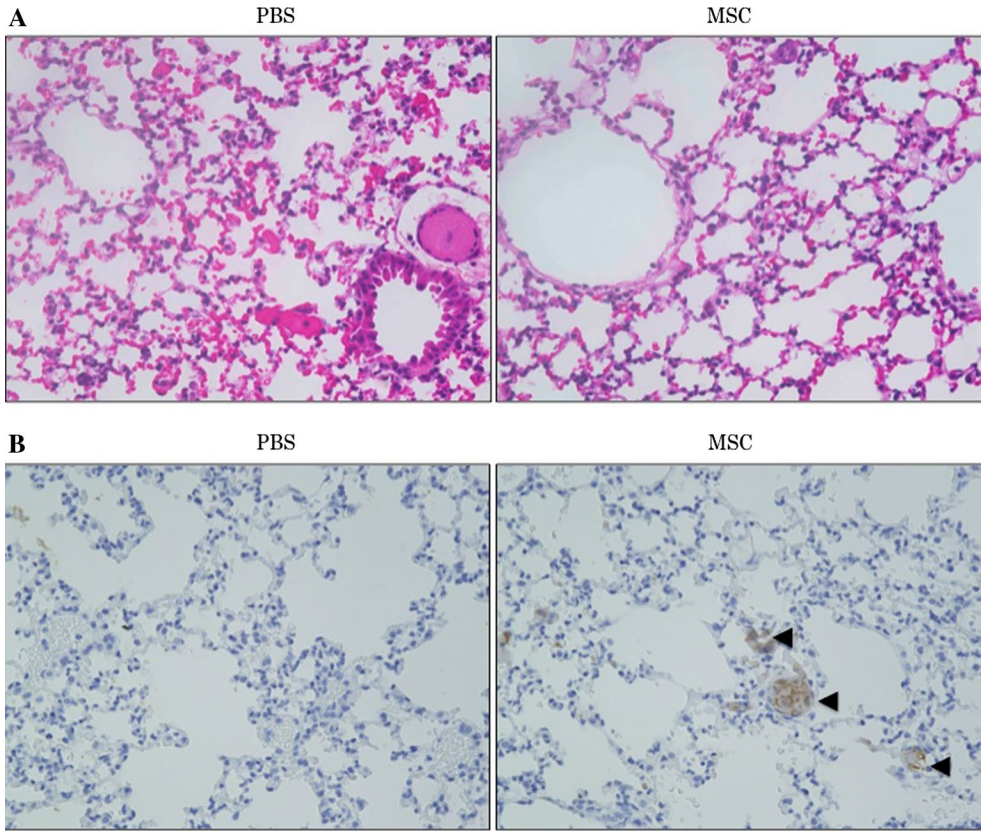

Fig. 3 Photomicrograph of the pathologic findings of the left lung grafts in both groups. Histology revealed significantly less intra-alveolar hemorrhage and capillary congestion in the human mesenchymal stem cell-administered lung grafts than in the PBS-administered control lung graft (hematoxylin and eosin; ×40) (**a**). Immunohistochemistry revealed cells positive for MHC class I in the pulmonary arterioles and alveolar septae of the hMSC-administered lung grafts (*arrow*) but not of the PBS-administered controls (**b**). Photomicrographs were obtained with a BZ9000 microscope (Keyence, Tokyo) with a ×40 objective. *PBS* lung grafts administered with PBS before transplantation, *MSC* lung grafts administered with 5×10^5 hMSCs before transplantation

Table 1 Histological lung injury scores

Group	Perivascular edema	Intra-alveolar hemorrhage	Capillary congestion	Neutrophil in small vessels
PBS	3, 2, 3	2, 2, 2	3, 3, 3	4, 2, 2
MSC	2, 1, 1	1, 1, 1	1, 1, 2	1, 1, 1

Numbers represent individual lung grafts

Discussion

The present study found that the pre-transplant administration of MSCs via the pulmonary artery of a lung graft significantly attenuated IRI in a mouse model of lung transplantation after prolonged cold ischemia. This was demonstrated by significantly lower protein concentrations and cell counts in BALF collected from hMSC-administered grafts than from PBS-administered controls. BALF analysis has been used extensively to evaluate lung injury because plasma proteins and inflammatory cells are

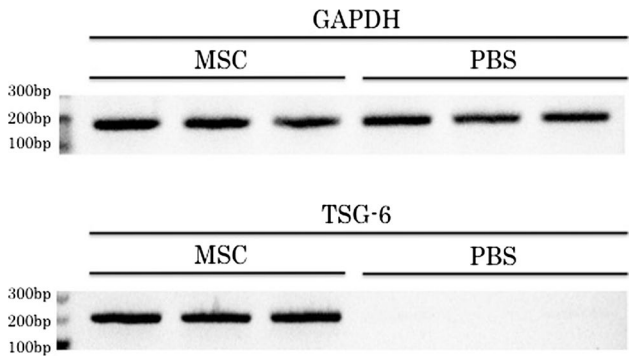

Fig. 4 RT-PCR analysis of the lung grafts for TSG-6. The human TSG-6 gene is readily identified in the grafts administered with human mesenchymal stem cells before transplantation. *PBS* lung grafts administered with PBS before transplantation, *MSC* lung grafts administered with 5×10^5 hMSCs before transplantation

present in very low quantities in the alveolar spaces of normal lungs. Increased protein concentrations indicate a loss of endothelial and alveolar barrier function, whereas

increased cell counts indicate the activation of inflammatory responses in the lungs [22, 23]. Pathologic examination in the present study also revealed that the administration of hMSCs ameliorated intra-alveolar hemorrhage and capillary congestion of the grafts, which were harvested 6 h after reperfusion.

IRI is caused by an imbalance in metabolic supply and demand in ischemic organs. It can activate innate and adaptive immune responses and trigger cell death processes after reperfusion [24, 25]. Recent research showed that MSCs had anti-inflammatory and anti-apoptotic effects in experimental models of IRI [13–15, 26]. MSCs activated by inflammatory cytokines are known to introduce two negative feedback loops. One negative feedback loop is upregulation of cyclooxygenase, increasing the secretion of prostaglandin E2 (PGE2), which drives resident macrophages to an anti-inflammatory phenotype [27]. The other negative feedback loop is secretion of TSG-6, which inhibits macrophage production of proinflammatory cytokines. The activated MSCs secrete TSG-6, which interacts with CD44 on resident macrophages to decrease TLR2/NFκ-B signaling, and thereby decrease the secretion of proinflammatory mediators [28]. In our model, the expression of human TSG-6 was readily detectable in the grafts treated with hMSCs that were harvested 6 h after reperfusion. Secretion of TSG-6 from MSCs might lead to attenuated lung injury in this model. Mitochondrial transfer from MSCs to the alveolar epithelium was reported to protect against lung injury [11]. Finally, microvesicles released by MSCs contain mRNA and mitochondria and can reduce inflammatory injury, including acute lung injury [29]. This mechanism may contribute to the ameliorative effects of MSCs seen in our model; however, this will require further investigation.

MSCs have been demonstrated to attenuate acute lung injury when delivered by intravenous or intratracheal routes [30]. In the present study, we infused the lung graft with MSCs through the pulmonary artery before transplantation, which may be another potential route when administrating MSCs in lung transplantation. In a rodent single left lung transplant model, the blood flow ratio to the left lung graft was significantly decreased in the early phase of transplantation, particularly when the grafts were preserved for a long time [20]. The direct infusion of MSCs into the pulmonary artery of the graft will encompass larger numbers of cells than the intravenous administration. For this reason, the pulmonary artery would be a practical delivery route in the clinical setting.

In this study, we used hMSCs; not mouse MSCs for the following reasons: first, although hMSCs express MHC class I, they are considered to possess low immunogenicity [31]. Second, hMSCs would not trigger a strong host inflammatory response in a rat brain [32]. Third, hMSCs are known to ameliorate acute lung injury in immunocompetent mice [33, 34]. In considering hMSCs for clinical application, we investigated their potential in the acute phase of IRI after lung transplantation. The longer effect of MSCs in IRI after lung transplantation, as well as their immune response against MSCs in the lung milieu, should be investigated using both human and rodent MSCs in the future.

In summary, the findings of the present study demonstrated the effect of the pre-transplant administration of MSCs via the pulmonary artery on ameliorating IRI of lung transplants exposed to prolonged cold ischemia. Before the clinical application of MSCs in lung transplantation, other potential routes, including intrabronchial injection and incorporation with ex vivo lung perfusion system [35]; the optimal timing of administration; and the appropriate number of cells to be administered should be investigated in large animal models so we can obtain more detailed physiological parameters.

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Compliance with ethical standards

Conflict of interest Tatsuaki Watanabe and his co-authors have no conflicts of interest.

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