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Microvesicles Derived From Human Mesenchymal Stem Cells Restore Alveolar Fluid Clearance in Human Lungs Rejected for Transplantation

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The need to increase the donor pool for lung transplantation is a major public health issue. We previously found that administration of mesenchymal stem cells ''rehabilitated'' marginal donor lungs rejected for transplantation using *ex vivo* lung perfusion. However, the use of stem cells has some inherent limitation such as the potential for tumor formation. In the current study, we hypothesized that microvesicles, small anuclear membrane fragments constitutively released from mesenchymal stem cells, may be a good alternative to using stem cells. Using our well established *ex vivo* lung perfusion model, microvesicles derived from human mesenchymal stem cells increased alveolar fluid clearance (i.e. ability to absorb pulmonary edema fluid) in a dose-dependent manner, decreased lung weight gain following perfusion and ventilation, and improved airway and hemodynamic parameters compared to perfusion alone. Microvesicles derived from normal human lung fibroblasts as a control had no effect. Co-administration of microvesicles with anti-CD44 antibody attenuated these effects, suggesting a key role of the CD44 receptor in the internalization of the microvesicles into the injured host cell and its effect. In summary, microvesicles derived from human mesenchymal stem cells were as effective as the parent mesenchymal stem cells in rehabilitating marginal donor human lungs.

Abbreviations: AFC, alveolar fluid clearance; Ang1, angiopoietin-1; CD44, extracellular matrix receptor type III for hyaluronic acid; EVLP, *ex vivo* lung perfusion; IFNγ, interferon gamma; IL-1β, interleukin 1 beta; KGF, keratinocyte growth factor; MSC, mesenchymal stem cells; MV, microvesicles; NHLF, normal human lung fibroblasts; NO, nitric oxide; TNFα, tumor necrosis factor alpha

Introduction

Lung transplantation remains the standard of care for an increasing number of patients with irreversible lung diseases. Since 1990, the number of lung transplantations in the world has multiplied by four, reaching 3700 cases in 2011, with a median age of recipients increasing from 45 to 55 years (1). Unfortunately, in the United States, the number of new patients on the waiting list for lung transplantation has grown from 1500 in 2006 to 2200 in 2012, with a mortality rate of 300 patients on the waiting list per year over the same period (2). Nevertheless, due to improvements in medical and surgical care, recipient survival rate at 5 years increased from 45% to 55% in the past 20 years (1).

Recently, a new technique of *ex vivo* lung perfusion (EVLP) has been developed to extend the donor pool size (3,4). EVLP allows ''rehabilitation'' of marginal donor lungs initially rejected for transplantation by allowing a short duration of perfusion and oxygenation with ventilation prior to transplantation, which in preliminary studies has reduced the incidence of primary graft dysfunction (3,4). In addition, EVLP has become an ideal method to test the effects of pharmacologic and/or gene- or cell-based therapy prior to surgery to improve the success of lung transplantation (5,6). Using a preclinical model of EVLP, we reported that intravenous administration of human bone marrow-derived mesenchymal stem (stromal) cells (MSC) restored alveolar fluid clearance (AFC) in lungs rejected for transplantation (7); in patients with acute respiratory distress syndrome, impaired AFC rate is associated with higher mortality (8). Based on our previous studies in a human EVLP model of acute lung injury induced by *Escherichia coli* bacteria or endotoxin (5,9), we postulated that the primary mechanisms underlying the therapeutic effect of MSC were through the secretion of soluble factors with reparative

properties. However, the use of MSC or cell-based therapy in clinical practice has some potential limitations such as the risk of tumor formation, immunogenicity, and need of a bone marrow transplant facility to store and process the stem cells (10,11).

Recently, MSC have been found to release microvesicles (MV) that were as biologically active as the cells themselves. MVs are anuclear plasma membrane bound circular fragments, 50–200 nm in size, constitutively released from multiple cell types from the endosomal compartment as exosomes or shed from the plasma membrane (12). Microvesicles derived from human mesenchymal stem cells (MSC MV) express very low levels of MHC I or II antigens allowing them to become ''immunoprivileged'' and carry mRNA, miRNA and proteins for soluble factors with reparative properties. Bruno et al found that MSC MV accelerated the morphologic and functional recovery of glycerol-induced acute kidney injury in mice by inducing proliferation of renal tubular cells (13). MSC MV homed and incorporated into the injured tubular cells in part via the surface receptor CD44 (extracellular matrix receptor type III for hyaluronic acid), allowing the transfer of MSC MV mRNA. We also found that human MSC MV reduced pulmonary edema and lung protein permeability in an *E. coli* endotoxin-induced acute lung injury in mice in part through the expression of keratinocyte growth factor (KGF) mRNA in the injured alveolus (14). In the current study, we hypothesize that MSC MV would be effective in restoring AFC in human lungs rejected for transplantation using EVLP.

Materials and Methods

Selection criteria for human lungs
We used human lungs rejected for transplantation by the Northern California Transplant Donor Network and approved for research. Lungs were resected *en bloc* without preservative flush but heparinized, gently inflated, and stored on ice at 4°C. Lungs were used if they met these criteria: (1) a cold ischemia time <48 h, (2) no obvious parenchyma lesions, and (3) AFC >0% but <10%/h.

Ex vivo *perfused human lung and measurement of alveolar fluid clearance*
The pulmonary artery and bronchus was cannulated from either the right or left lung and placed in a bioreactor (Harvard Apparatus, Holliston, MA) as previously described (7). The rate of perfusion, with a solution containing DMEM without Phenol Red + 5% bovine serum albumin and kept at 37°C, was slowly increased to 250 mL/min over 30 min, allowing progressive rewarming of the lung. When the perfusate temperature reached 30°C, ventilation with a large animal respirator (Harvard Apparatus) was started ($V_T = 300$ mL, respiratory rate = 10/min, $FiO_2 = 21\%$, and positive end-expiratory pressure = 5 cmH$_2$O, Figure 1A). Perfusion flow, pulmonary arterial pressure (PAP), pulmonary vascular resistance (PVR), tidal volume (V_T), tracheal pressure, and compliance were recorded in real time using Pulmodyn Data Acquisition software (Harvard Apparatus).

After steady state was reached, a small catheter (Intramedic, BD Bioscience, Franklin Lakes, NJ) was introduced into the bronchus and advanced into the lung lobe of interest until gentle resistance was encountered. A volume of 125 mL of warmed normal saline solution containing 5% albumin was instilled, and the bronchoalveolar lavage (BAL) fluid was sampled after 5 and 35 min. The AFC was measured by the change in protein concentration of the BAL fluid over 30 min using the following equation: AFC (%/h) = 2(1-Ci/Cf) × 100 (Ci and Cf = protein concentration 5 and 35 min after BAL instillation respectively). If 0 <AFC <10%/h, either 100 μL of MSC MV, 200 μL of MSC MV, 200 μL of MSC MV + anti-CD44 antibody (Ab), 200 μL of MSC MV + polyclonal goat IgG, or 200 μL of microvesicles derived from normal human lung fibroblasts (NHLF MV, Lonza, Basel, Switzerland) was administered into the perfusate. The control group received perfusion only. At 6 h (T6), a second BAL was performed into the lower lobe to measure AFC (Figure 1A).

Measurement of blood gas and metabolites in the perfusate
Perfusate pH, PO$_2$, and PCO$_2$ were measured every hour from T0 to T6 using a blood gas machine (OptiMedical, Roswell, GA). Lactate, nitric oxide (NO), and Syndecan-1 levels were measured in the perfusate at T0 and T6 using colorimetric kits (Fisher Scientific, Hampton, NH, and Abcam, Cambridge, UK).

Measurement of angiopoietin-1 and tumor necrosis factor-α in the BAL fluid
Angiopoietin-1 (Ang1) and TNFα concentrations were measured in the BAL fluid at T0 and T6 using ELISA kits (R&D Systems, Minneapolis, MN).

Isolation of microvesicles derived from human mesenchymal stem cells or normal human lung fibroblasts
Human bone marrow derived MSC were obtained from a NIH repository at Texas A&M Institute for Regenerative Medicine; these cells fulfilled the criteria of MSC as defined by the International Society of Cellular Therapy (15).

MSC and NHLF MV were obtained from the supernatant of human MSC and NHLF respectively using ultracentrifugation (16). MSC or NHLF were first serum starved for 48 h in conditioned medium composed of α-MEM without nucleosides and 0.5% bovine serum albumin (MP Biomedicals, Santa Ana, CA). The conditioned medium was collected and centrifuged at 300 g for 20 min to remove cellular debris and then at 100 000 g for 1 h at 4°C twice (Beckman Coulter [Brea, CA] Optima L-100XP) to sediment the MV. MSC or NHLF MV were resuspended in phosphate buffered saline according to the final cell count (10 μL per 1×10^6 cells) and stored at −80°C until further use.

Characterization of microvesicles derived from human mesenchymal stem cells
MV released by monolayers of MSC were photographed with a JEOL 1200 EX transmission electron microscope operating at 80 kV as previously described (9). The protein content of 100 μL of MSC MV was measured by BCA protein assay. Western Blot analysis was performed to confirm the presence of CD44 on the MSC MV (13) using the primary anti-CD44 Ab (1 μg/mL anti-CD44, Abcam). Total RNA was isolated from MSC MV using RNeasy Mini Kit (QIAGEN Sciences, Venlo, the Netherlands), and PCR primer for human Ang1 was purchased from SABiosciences.

Uptake of fluorescent labeled MSC MV: Primary cultures of human alveolar type II cells were isolated from human lungs as previously described (17). The cells were plated on collagen I coated 24-well plates in DMEM high glucose 50%/F-12 50% mix medium containing 10% FBS and antibiotics at a concentration of 1×10^6 cells/well. Following 72 h from isolation, type 2 cells were cultured without FBS for 24 h and then exposed to 50 ng/mL of cytomix, a mixture of IL-1β, TNF-α, and IFN-γ (R&D Systems) often used as a surrogate for inflammatory injury.
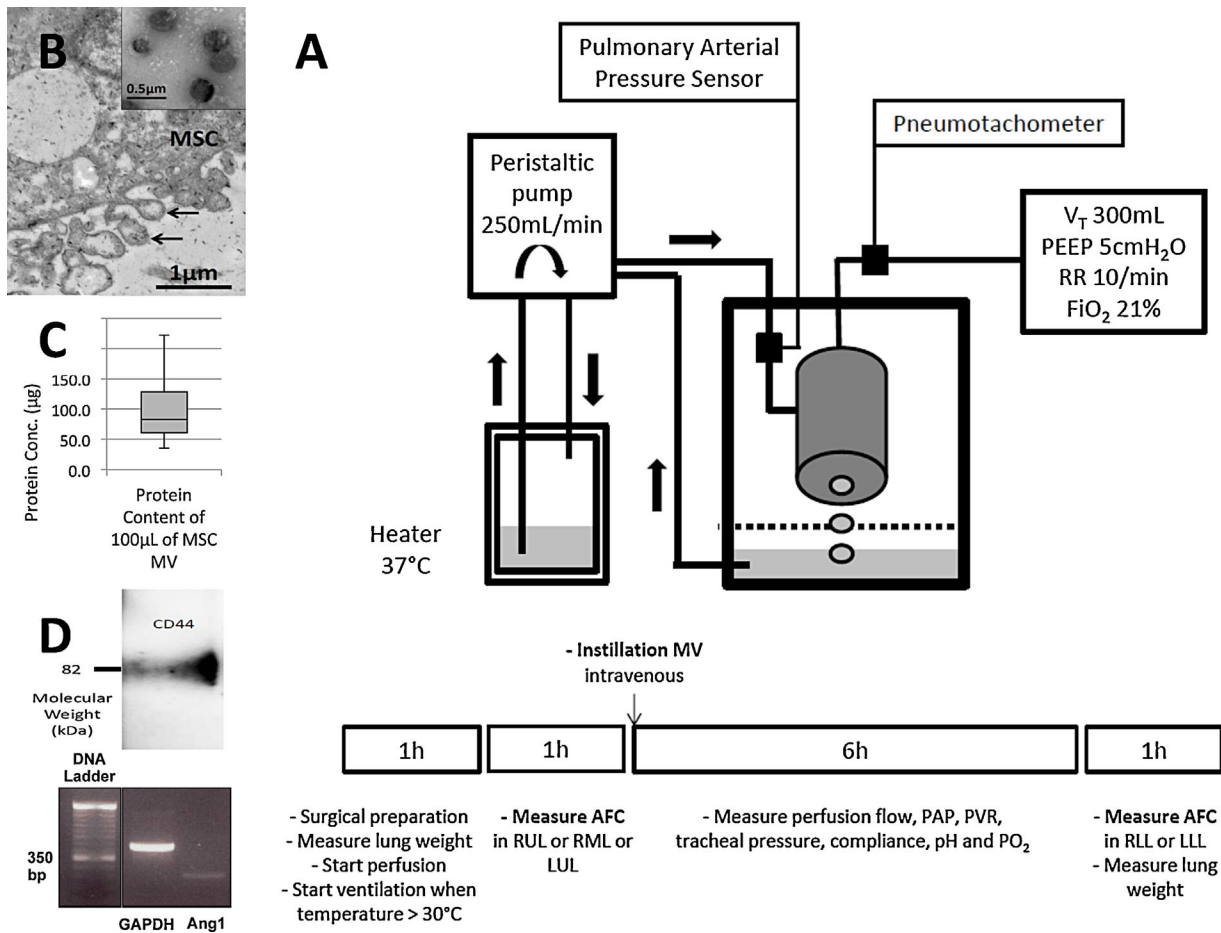
Figure 1: Characterization of mesenchymal stem cell microvesicles and the *ex vivo* lung perfusion model. (A) Schematic view of the *ex vivo* human lung preparation as well as lung perfusion and ventilation protocol used for the experiments. (B) Electron microscopy image of human mesenchymal stem cells (MSC) constitutively releasing microvesicles (MV) after 48 h of serum starvation. Black arrows designate MV, which appear as spheroids budding off the plasma membrane. Insert shows a homogenous collection of MSC MV, which is 50 to 200 nm in size. (C) Protein concentration of 100 μL of MSC MV. Data represent the median of 24 samples of MSC MV. (D) Western Blot showing the expression of the membrane receptor CD44 by MSC MV. CD44 plays an important role in MSC MV internalization into the host cell. PCR showing the expression of Ang1 mRNA by RT-PCR.

For the uptake experiments, human type 2 cells were exposed with prestained MSC MV (cellmask-fluorescent labeled MV: 30 μL/well) the same day as cell injury. To investigate the role of CD44 in MV uptake, MSC MV were pre-incubated with anti-CD44 blocking Ab (BD Biosciences) or with negative control IgG (R&D Systems). After 24 h, cells were washed twice with PBS, cytospined on glass slides, fixed 10 min with 4% paraformaldehyde and mounted with fluorescent medium (Vectaschield fluorescent mounting medium, VWR, Visalia, CA). The cells were examined by fluorescence microscopy (Leica DM 1000 microscope). Fluorescence intensities from images of 20 randomly selected microscopic fields of cells from each condition were analyzed by densitometry (ImageJ software, NIH Image, Bethesda, MD).

CD44 neutralizing antibody and polyclonal goat IgG control

In CD44 neutralizing Ab experiments, 1 μg/mL of CD44 neutralizing Ab (BD Biosciences) was mixed with 200 μL of MSC MV and incubated at 4°C for 30 min prior to administration. For control, 1 μg/mL of polyclonal goat IgG (R&D Systems) was used.

Statistical analyses

Results are expressed as mean ± SD if normally distributed and as median [Q1;Q3] if not. Comparisons between several groups were made using the analysis of variance (ANOVA). Comparisons between two groups were made using either ANOVA or the unpaired Student t-test. Comparisons with a sample over time were made by repeated measures of ANOVA using the Bonferroni correction. We used the software GraphPad Prism.

Results

Baseline data for donor lungs

The demographic, clinical data, and ischemia time for 30 donor lungs are listed in Table 1. There was no significant differences between groups for PaO_2/FiO_2 ratio, compliance, chest radiograph infiltrates, or lung injury score prior to organ harvest (18).

Table 1: Donor clinical data and ischemia time

Variable	Perfusion only (n = 6)	MSC MV 100 μL (n = 6)	MSC MV 200 μL (n = 6)	MSC MV 200 μL + anti-CD44 Ab (n = 4)	MSC MV 200 μL + IgG (n = 4)	NHLF MV 200 μL (n = 4)
Mean age (year)	57 ± 8	49 ± 11	43 ± 16	56 ± 9	57 ± 9	46 ± 18
Male (%)	33	67	67	75	50	25
Chest radiograph infiltrates	2 ± 0	2 ± 1	2 ± 1	2 ± 1	2 ± 1	2 ± 2
Donor PaO_2/FiO_2 ratio (mmHg)	259 ± 107	275 ± 155	294 ± 137	309 ± 102	270 ± 142	414 ± 73
PEEP (cmH_2O)	8 ± 3	6 ± 2	8 ± 4	8 ± 2	7 ± 2	6 ± 2
Donor lung compliance (ml/cmH_2O)	35.6 ± 4.9	40.5 ± 10.1	34.0 ± 11.2	36.8 ± 7.6	41.6 ± 13.3	35.5 ± 14.8
Lung injury score	1.75 ± 0.40	1.71 ± 0.90	1.75 ± 0.97	1.69 ± 0.52	1.63 ± 0.48	1.44 ± 0.31
Ischemic time (hours:minutes)	21:26 ± 10:29	20:35 ± 16:40	28:01 ± 11:03	33:26 ± 4:16	27:25 ± 16:35	34:55 ± 8:36

Ab, antibody; MSC MV, microvesicles derived from mesenchymal stem cells; NHLF MV, microvesicles derived from normal human lung fibroblasts; PEEP, positive end-expiratory airway pressure.

Data are expressed as mean ± SD.

Lung Injury Score based on maximum of 4 points for the PaO_2/FiO_2 ratio, the level of positive end-expiratory airway pressure, the quasi-static respiratory compliance and the extent of chest radiograph infiltrates.

Characterization of MSC microvesicles

Using electron microscopy, we found that MV was released constitutively from MSC after 48 h of serum starvation. MSC MV appeared as homogeneous spheroids between 50 and 200 nm in size (Figure 1B). The median protein concentration of 100 μL of MSC MV was 82.8 [61.0;128.4] μg; the dose used was equivalent to the dose used in other studies using MSC MV as a therapeutic (13,16) (Figure 1C). We confirmed that MSC MV expressed the membrane receptor, CD44, by Western Blot analyses and the mRNA for Ang1 by RT-PCR (Figure 1D).

MSC MV improve AFC and decrease lung weight gain following perfusion

With lung perfusion and ventilation, AFC significantly decreased in the Control group from time T0 to T6. However, administration of MSC MV 100 μL or 200 μL significantly increased AFC rate in a dose-dependent manner compared to T0 (Figure 2). Lung weight increased during EVLP in all groups. However, the lung weight gain was significantly lower in the Treatment groups (MSC MV 100 μL ± 200 μL) compared to the Control at T6 (Figure 3). Administration of NHLF MV had no therapeutic effects. Perfusion alone increased Syndecan-1 levels, a marker of endothelial injury, from 38 ± 37 ng/mL (mean ± SD) at time 0 h to 139 ± 67 ng/mL at 6 h. Although not statistically significant, administration of either 100 or 200 μL MSC MV reduced Syndecan-1 levels numerically by approximately 25% at 6 h.

MSC MV improve airway and hemodynamic parameters

Over 6 h, the tracheal pressure in the Control group significantly increased by 37%, which was associated with a nonsignificant decrease in lung compliance at T6. However, treatment with MSC MV restored the tracheal pressure to baseline, significantly increasing the lung compliance at T6 as compared to T0 (p = 0.029 by t-test for MSC MV 100 μL and p = 0.046 by t-test for MSC MV

100 μL and 200 μL combined as a group at T6 vs. T0) (Figure 4). There was no significant effect on pulmonary artery pressure or pulmonary artery resistance with perfusion over 6 h. However, treatment with MSC MV reduced both hemodynamic parameters in a dose-dependent manner. Treatment with 200 μL MSC MV significantly reduced both PAP and PVR by 50% at T6 compared to T0 (Tables 2 and 3). Perfusate NO levels were unchanged at T6 compared
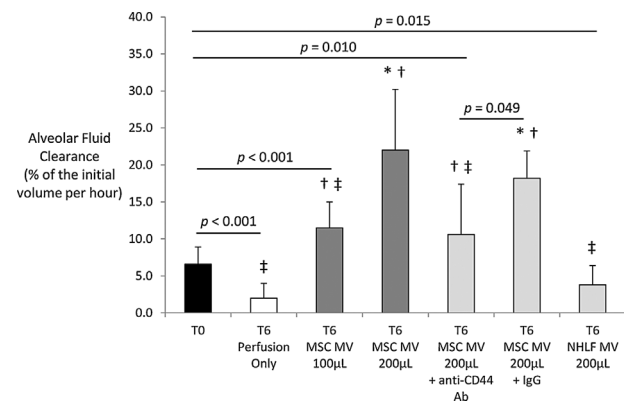

Figure 2: Effect of microvesicles derived from human mesenchymal stem cells on alveolar fluid clearance in donor human lungs rejected for transplantation. Administration of MSC MV significantly improved AFC in a dose-dependent manner compared to the Control (Perfusion only) at T6. Administration of NHLF MV as a cellular control had no effect on AFC compared to the Control at T6. Inhibition of CD44 with a neutralizing Ab significantly reduced the therapeutic effect of MSC MV 200 μL, whereas administration of IgG control with MSC MV 200 μL had no significant effect. Data are presented as mean ± SD, N = 4-6 per group. *p significant by ANOVA vs. T0, †p significant by ANOVA versus T6 Perfusion Only, ‡p significant by ANOVA versus T6 MSC MV 200 μL. Abbreviations: Ab, antibody; AFC, alveolar fluid clearance; MSC, mesenchymal stem cells; MV, microvesicles; NHLF, normal human lung fibroblast.
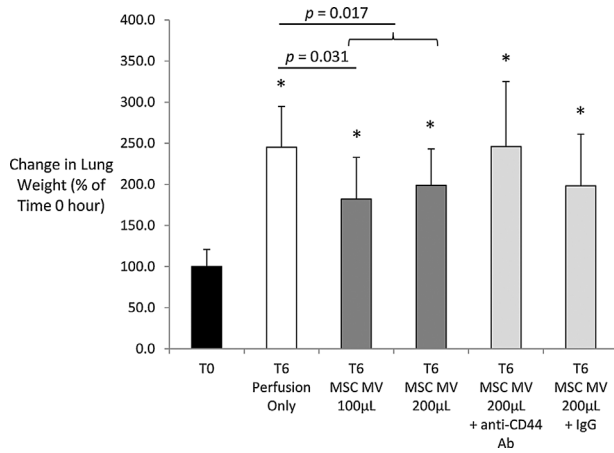
Figure 3: Effect of microvesicles derived from human mesenchymal stem cells on lung weight gain. Perfusion with ventilation significantly increased the total lung weight by 150%. Administration of MSC MV (100 μL or 100 μL + 200 μL groups) significantly reduced lung weight by 20–25% as compared to the Control (Perfusion only) at T6. Data was normalized to the initial weight of the lung to account for any differences in baseline lung injury or weight. Data (normalized to T0) are presented as mean ± SD, N = 4–6 per group. *p significant by ANOVA versus T0.

to T0 in the Control group (118 ± 7% at T6 as % of Control at T0), whereas it significantly increased in both treatment groups (137 ± 13% for MSC MV 100 μL and 148 ± 9% for MSC MV 200 μL, p < 0.001 by ANOVA).

MSC MV effects on PO_2, PCO_2, pH, and lactate in perfusate

Perfusate PO_2 levels decreased during EVLP with ventilation in all groups from T0 to T6 (Figure 5A). There were no significant differences between Control and Treatment groups for PO_2 or PCO_2 levels at T6 (Figure 5B). Perfusate pH decreased between T0 and T6 for both Treatment groups, while it remained unchanged in the Control group. At T6, pH levels were significantly lower for both Treatment groups compared to Control (p = 0.004 by ANOVA for MSC MV 100 μL and p = 0.010 by ANOVA for MSC MV 200 μL) (Figure 6A). The increase in AFC was correlated with the decrease in perfusate pH between T0 and T6 (Spearman's rank correlation coefficient, $r_s = -0.7657$) (Figure 6B). Perfusate lactate levels increased at T6 compared to T0 in the Control group (149 ± 36% at T6 as % of Control at T0, p = 0.010 by ANOVA), whereas it was significantly lower in both Treatment groups at T6 compared to Control group at T6 (107 ± 25% for MSC MV 100 μL, p = 0.010 by t-test, and 80 ± 24% for MSC MV 200 μL, p = 0.004 by ANOVA).

MSC MV uptake is CD44-dependent

The uptake of fluorescent-labeled MSC MV was increased by 80% in human alveolar epithelial type 2 cells with injury (cytomix). Exposure to the anti-CD44 neutralizing Ab
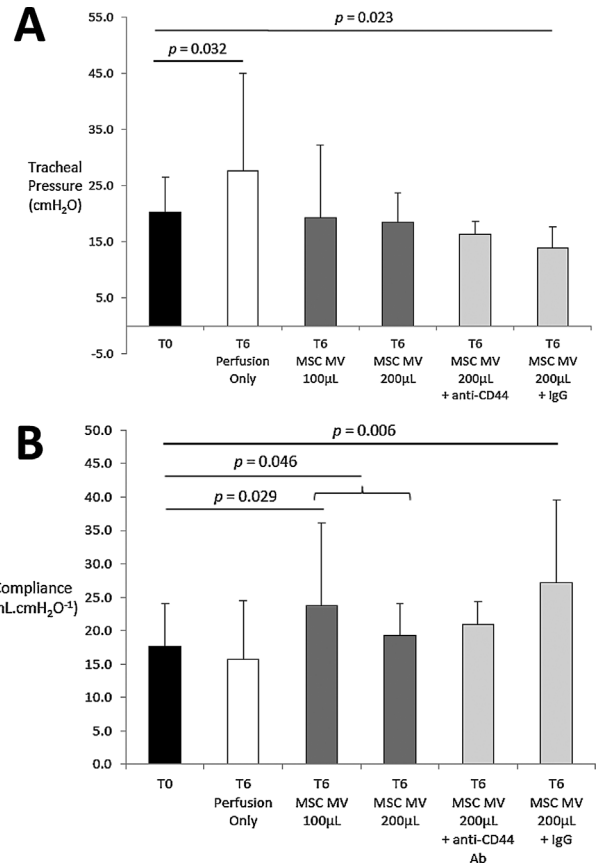

Figure 4: Effect of microvesicles derived from human mesenchymal stem cells on lung (A) tracheal pressure and (B) compliance. Perfusion with ventilation significantly increased tracheal pressure at T6. Administration of either doses of MSC MV reduced the tracheal pressure to baseline, increasing the compliance of the lung compared to T0. Data are presented as mean ± SD, N = 4–6 per group. Abbreviations: Ab, antibody; MSC, mesenchymal stem cells; MV, microvesicles.

suppressed this inflammatory uptake of MSC MV (Figure 7).

MSC MV improve AFC in part by a CD44-dependent mechanism

The increase in AFC in EVLP using MSC MV was attenuated by administration of an anti-CD44 neutralizing Ab (p < 0.001 by ANOVA MSC MV 200 μL vs. MSC MV 200 μL + anti-CD44 Ab) but not by a control IgG (Figure 2). Co-administration of the anti-CD44 Ab with MSC MV 200 μL also eliminated any benefit with change in lung weight (Figure 3), compliance (Figure 4), or with pulmonary artery pressure and pulmonary artery resistance (Tables 2 and 3).

MSC MV effect on Ang1 and TNFα levels in the alveolus

TNFα levels in the BAL fluid significantly increased with perfusion and ventilation. However, there was no

Table 2: Time dependent values of mean pulmonary arterial pressure

Time (hours)	Perfusion only (n = 6)	MSC MV μL (n = 6)	MSC MV 200 μL (n = 6)	MSC MV 200 μL + anti-CD44 Ab (n = 4)	MSC MV 200 μL + IgG (n = 4)
0			11.0 ± 6.3		
1	12.2 [4.6;20.9]	9.0 ± 5.1	6.4 ± 2.3*	9.6 ± 4.4	5.6 ± 2.0
2	5.4 [4.0;22.5]	7.9 ± 5.9	5.6 ± 2.2*	9.1 ± 6.4	5.0 ± 1.8*
3	8.4 [2.8;26.6]	11.2 ± 7.7	5.2 ± 2.2*	9.4 ± 8.0	4.7 ± 1.4*
4	7.2 [3.4;23.8]	8.5 ± 7.0	5.3 ± 2.7*	9.7 ± 8.6	4.7 ± 1.3*
5	8.2 [2.2;26.7]	9.3 ± 6.9	5.4 ± 3.0*	10.0 ± 9.0	4.8 ± 1.2*
6	7.0 [2.5;25.2]	9.3 ± 5.1	5.6 ± 3.0*	10.7 ± 8.8	5.0 ± 1.3*

Ab, antibody; MSC MV, microvesicles derived from mesenchymal stem cells.
Data are expressed as mean ± SD when normally distributed and as median [Q1;Q3] if not (in mmHg).
Pulmonary arterial pressure at T0 corresponds to the mean value of each group pooled together.
*p significant by Student t-test vs T0.

significant effect of MSC MV administration on inflammation at T6 (778 [253;3493] pg/mL for Control vs. 951 [533;4453] pg/mL for MSC MV 100 μL at T6 for TNFα level, p = 0.368 by t-test), suggesting that immunomodulation was not critical for the restoration of AFC. Although not statistically significant, administration of MSC MV increased Ang1 levels in the BAL fluid at 6 h compared to the Control (486 [232;1707] pg/mL for Control vs. 707 [297;769] pg/mL for MSC MV 100 μL at T6 for Ang1 level, p = 0.184 by t-test).

Discussion

The main findings of these studies can be summarized as follows: (1) Intravenous administration of MSC MV improved AFC rate in a dose dependent manner (Figure 2) and reduced lung weight gain (Figure 3) after 6 h of EVLP compared to perfusion alone. Administration of NHLF MV had no therapeutic effects. (2) MSC MV prevented the elevation of tracheal pressure, increased lung compliance (Figure 4), and decreased pulmonary artery pressure and artery resistance (Tables 2 and 3) following perfusion. These effects were associated with increased perfusate NO levels. (3) MSC MV decreased perfusate pH (Figure 6A)

and reduced the elevation of lactate levels following perfusion, showing a correlation between MSC MV administration and improved lung metabolism. (4) CD44 neutralizing antibody eliminated the benefits of MSC MV on AFC (Figure 2), lung weight (Figure 3), compliance (Figure 4), and pulmonary artery pressure and resistance (Tables 2 and 3), demonstrating the critical role of CD44 in the therapeutic effects of MSC MV (Figure 7). (5) And, although not statistically significant, MSC MV administration was associated with a numerical reduction of Syndecan-1 levels, a marker of endothelial glycocalyx breakdown, in the perfusate and a numerical elevation of Ang1 levels in the injured alveolus, suggesting a partial restoration of lung endothelium.

This is the first study demonstrating the therapeutic effects of MSC MV in human lungs rejected for transplantation. We previously reported that human MSC given during EVLP increased AFC at 4 h after intravenous administration compared to a control group receiving perfusion only (7). In the current study, we demonstrated that MSC MV administration were as effective as MSC in restoring AFC. This is an essential point, since impaired AFC contributes to primary graft dysfunction (19,20), a major cause of morbidity and mortality after lung transplantation in the

Table 3: Time dependent values of mean pulmonary vascular resistance

Time (hours)	Perfusion only (n = 6)	MSC MV 100 μL (n = 6)	MSC MV 200 μL (n = 6)	MSC MV 200 μL + anti-CD44 Ab (n = 4)	MSC MV 200 μL + IgG (n = 4)
0			44.7 ± 26.3		
1	48.9 [17.9;83.4]	39.8 ± 28.7	25.5 ± 9.1*	38.3 ± 17.6	22.5 ± 8.2
2	24.1 [16.0;89.2]	35.9 ± 32.1	22.2 ± 8.7*	36.2 ± 25.7	20.1 ± 7.2*
3	33.3 [11.0;105.5]	49.2 ± 36.8	20.8 ± 8.9*	37.7 ± 32.0	18.9 ± 5.7*
4	28.5 [13.8;94.4]	38.8 ± 37.5	21.0 ± 11.0*	38.7 ± 34.4	18.7 ± 5.3*
5	32.3 [8.9;105.9]	41.9 ± 37.1	21.8 ± 12.1*	40.1 ± 36.1	19.2 ± 5.0*
6	27.7 [10.0;100.2]	40.4 ± 25.2	22.3 ± 12.1*	42.9 ± 35.5	19.8 ± 5.0*

Ab, antibody; MSC MV, microvesicles derived from mesenchymal stem cells.
Data are expressed as mean ± SD when normally distributed and as median [Q1;Q3] if not (in mmHg.L^{-1}.min^{-1}). Pulmonary resistances at T0 correspond to the mean value of each group pooled together.
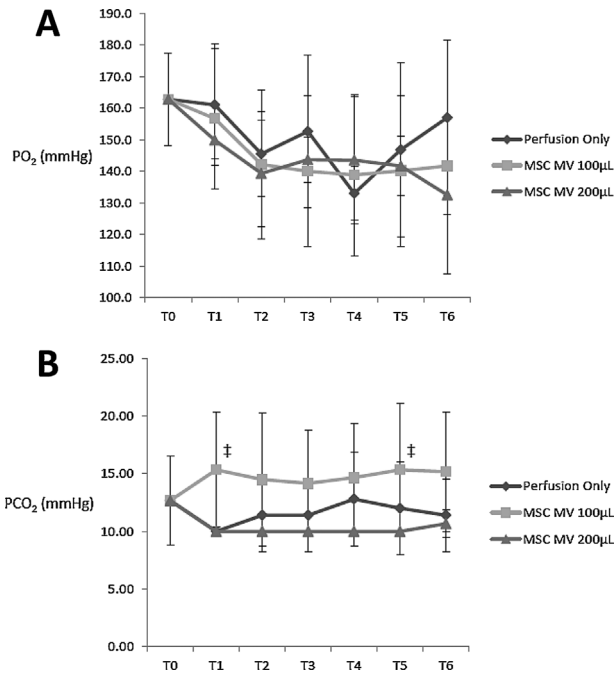*p significant by Student t-test vs T0.

Figure 5: Effect of microvesicles derived from human mesenchymal stem cells on PO₂ and PCO₂ in the perfusate during *ex vivo* lung perfusion. (A) In all lung groups, the PaO_2 decreased with lung perfusion and ventilation. There was no significance difference in PaO_2 levels at T6. (B) There was no significant difference in $PaCO_2$ between the Treatment Groups and the Control. However, at several time points (T1-T5), the $PaCO_2$ was higher in the MSC MV 100 μL vs. the 200 μL group. Data are presented as mean ± SD, N = 4-6 per group. ‡ p significant by ANOVA vs. T6 MV-MSC 200 μL.

Figure 6: Effect of microvesicles derived from human mesenchymal stem cells on perfusate pH during *ex vivo* lung perfusion. (A) Compared to T0 and the Control (Perfusion only) at T6, administration of either dose of MSC MV reduced the perfusate pH to more physiological levels. (B) In addition, there was a strong correlation with the change in pH with the AFC, perhaps suggesting that there were increased metabolic activity in the lungs that received MSC MV. Spearman's rank correlation coefficient, $r_s = -0.7657$. Data are presented as mean ± SD, N = 4–6 per group. *p significant by ANOVA versus T0, †p significant by ANOVA versus Perfusion Group at the corresponding time point. Abbreviations: MSC, mesenchymal stem cells; MV, microvesicles; NHLF, normal human lung fibroblast.

form of noncardiogenic pulmonary edema (21,22). Preserved lung barrier properties with intact AFC have been associated with better clinical outcomes in patients with posttransplant reperfusion edema or acute respiratory distress syndrome (8,19).

Although, the mechanisms underlying the effects of MSC MV on AFC in marginal donor lungs needs to be studied further, we have previously demonstrated that (1) MSC restored AFC rate in marginal donor lungs through KGF secretion following EVLP (7); (2) MSC restored protein permeability across injured human alveolar epithelial type II cells through the prevention of actin stress fiber formation by Ang1 secretion (17); and (3) MSC MV restored lung fluid balance following endotoxin induced acute lung injury in mice in part through the transfer of KGF mRNA to the injured alveolar epithelium with subsequent expression of the growth factor (14). KGF is an epithelial specific growth factor known to up-regulate alveolar fluid transport, and Ang1 is an anti-permeability factor which has been shown to prevent lung protein permeability in part through the restoration of endothelial glycocalyx (23,24). In addition, multiple preclinical studies have demonstrated the benefi-
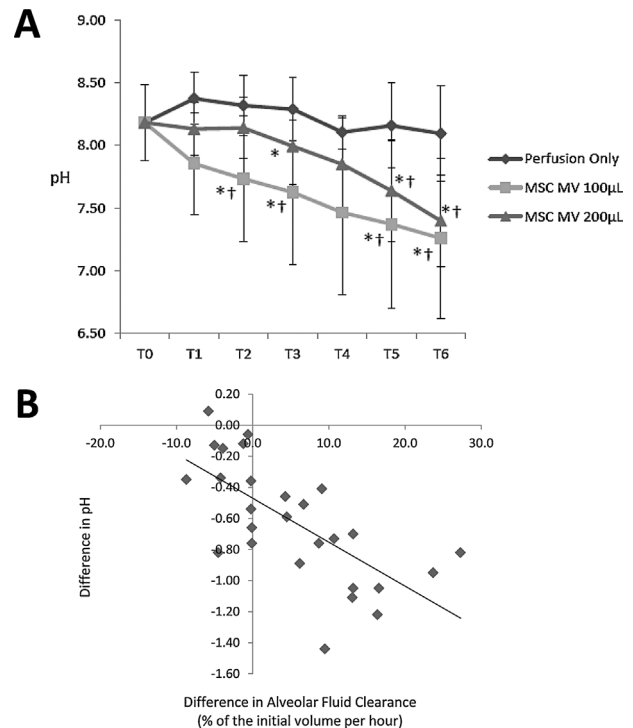
cial effect of MSC MV on tissue metabolism. In a model of myocardial infarction, MSC MV increased ATP levels and decreased oxidative stress, enhancing myocardial viability after ischemia/reperfusion injury (25). Several authors demonstrated the transfer of metabolic enzymes such as pyruvate kinase and glyceraldehyde 3-phosphate dehydrogenase from MSC MV to the injured myocardial tissue (25–27). Recently, Islam et al also demonstrated that MSC protect against acute lung injury by restoring alveolar bioenergetics in part through mitochondrial transfer through connexin43 channels (28). ATP restoration may be an additional mechanism for the restoration of AFC induced by MSC MV following ischemia/reperfusion injury in human lungs.

Administration of MSC MV decreased PAP and PVR following perfusion (Tables 2 and 3), which was associated with increased perfusate NO levels. Gao et al recently showed enhanced endothelial NO synthase (eNOS) activity
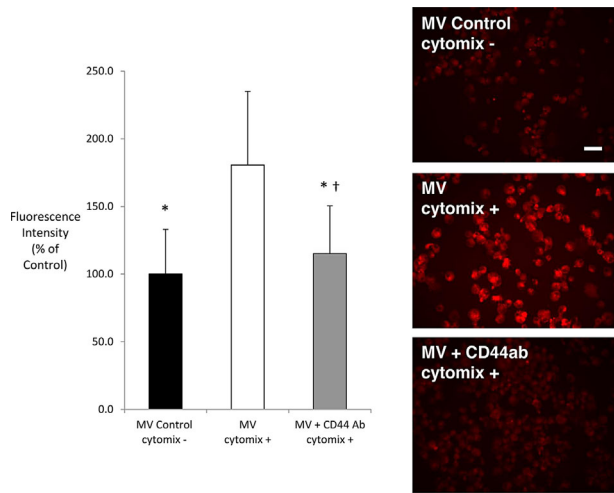
Figure 7: Role of CD44 in MSC MV uptake into primary cultures of human alveolar epithelial type 2 cells. In human alveolar epithelial type 2 cells, MSC MV uptake was dependent on CD44, the cell surface receptor for hyaluronic acid, following injury. Stimulation by an inflammatory injury (cytomix) increased the uptake of fluorescent-labeled MSC MV into alveolar epithelial type 2 cells. Fluorescence intensity was expressed as mean (% of control) $\pm$ SD for each condition, N = 179–262 cells for all groups. *p < 10^{-4} versus MV/cytomix+, †p = 0.001 versus Control Group by ANOVA (Bonferroni). Photomicrographs display the pattern of fluorescence levels observed in each experimental condition. Scale bar is 20 μm.

in pulmonary microvascular endothelial cells following coculture with adipose tissue-derived stem cells (29). In a model of acute lung injury, the authors also demonstrated that intravenous instillation of adipose tissue-derived stem cells increased eNOS protein expression, which reduced the severity of acute lung injury (29). Interestingly, MSC also produced NO (30), which may be critical for their immunomodulatory properties (31). Thus, increased NO concentrations in the perfusate following MSC MV instillation may therefore be responsible for endothelial vasodilatation, decreasing PAP and PVR.

Perfusate pH decreased in the Treatment groups over 6 h of EVLP compared to the Control group (Figure 6A). However, perfusate lactate levels decreased in the Treatment groups compared to the Control group. In addition, there was a strong correlation between the decrease in pH and the increase in alveolar fluid clearance (Figure 6B). Although seemingly contradictory, we believe that MSC–MV instillation may account for these findings through several mechanisms: (1) Through an increase in perfusate NO levels, MSC MV may vasodilate cold, ischemic distal lung tissue as apposed to perfusion alone decreasing PAP and leading to the release of acids, accounting for the low pH; and (2) simultaneously, MSC MV may restore the energetics of the alveolar epithelium (25,26) whether through restoration of metabolism or prevention of apoptosis, leading to improved AFC and decreased lactate levels.

Zhu et al demonstrated that the adhesion molecule CD44, expressed on the MSC plasma membrane, was essential for the migration of MSC into the extracellular matrix (32). Bruno et al showed the presence on MSC MV of several adhesion molecules such as CD44, CD29, α4-, and α5-integrins (13). Anti-CD44 and anti-CD29 blocking antibodies inhibited MSC MV uptake in tubular epithelial cells. More importantly, the authors showed that the biological effects of MSC MV were dependent on CD44, which was responsible for the transfer of the content of the MSC MV (mRNA, miRNA, and protein) following internalization (13). In the current study, we found that CD44 expression on MSC MV was also critical for the biological effects of MSC MV. Inhibition of CD44 by anti-CD44 blocking antibody reduced the therapeutic effect of MV on AFC, lung weight, compliance, and pulmonary artery pressure and vascular resistance (Figure 7).

There are some limitations to the current study. (1) The primary endpoint was the AFC rate at 6 h, not improvement in oxygenation; instillation of large volumes of normal saline to determine AFC will lead to inaccurate blood gas measurements (i.e. PaO$_2$) (33). (2) Although CD44 is critical for the therapeutic effects, the actual mechanisms leading to improved AFC remains to be determined further. MSC MV did not decrease alveolar inflammation as assessed by BAL TNFα levels, but there was a nonsignificant increase in Ang1 levels associated with a decrease in syndecan-1 levels in the perfusate. Shedding of the syndecan-1, a component of the endothelial glycocalyx, is associated with endothelial injury and increased protein permeability (34,35). We speculate that one of the mechanisms is through the transfer of mRNA from the MV to the injured alveolar epithelium such as possibly Ang1 with subsequent expression of the protein as we have previously shown with KGF secretion (14).

In conclusion, MSC MV administration enhanced the rate of alveolar fluid clearance in marginal donor lungs in a dose-dependent manner. This effect was associated with a decrease in lung weight gain, an increased in lung compliance, and a decrease in PAP and PVR, which was associated with increased perfusate NO levels. Lactate levels were also decreased with MSC MV treatment, suggesting an improvement in aerobic metabolism. CD44 expression on MSC MV was critical for MV uptake and the therapeutic effects. Similar to the parent stem cell, MSC MV administration may become a method of improving marginal donor lungs during EVLP for transplantation taking advantage of the beneficial attributes of mesenchymal stem cells without the inherent limitations of cell-based therapy.

Acknowledgments

Disclosure

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