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Mesenchymal Stem Cells Enhance Lung Recovery After Injury, Shock, and Chronic Stress

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Abstract

Background—Normal lung healing is impaired when lung contusion (LC) is followed by hemorrhagic shock (HS) and chronic stress (CS). Mesenchymal stem cells (MSCs) are immunomodulatory, pluripotent cells that are under investigation for use in wound healing and tissue regeneration. We hypothesized that treatment with MSCs can reverse the impaired healing seen after LC combined with HS and CS (LCHS/CS).

Methods—Male Sprague-Dawley (SD) rats (n=6/group) underwent LCHS with or without a single iv dose of 5×10^6 SD rat MSCs following resuscitation. Thereafter, rats were subjected to two hours of CS daily on days 1–6 and were killed on day 7. Lung histology was scored according to a well-established lung injury score (LIS) that included interstitial and pulmonary edema, alveolar integrity, and inflammatory cells. Scoring ranges from 0 (normal lung) to 11 (most severely injured). Whole blood was analyzed for the presence of CD4⁺CD25⁺FoxP3⁺ T regulatory cells (Treg) by flow cytometry.

Results—Seven days after isolated LC, LIS had returned to 0.8 ± 0.4 , however, after LCHS/CS healing is significantly delayed (7.2 ± 2.2 ; $p < 0.05$). Addition of MSC to LCHS/CS decreased LIS to 2.0 ± 1.3 ($p < 0.05$) and decreased all subgroup scores (inflammatory cells, interstitial and pulmonary edema, and alveolar integrity) significantly as compared to LCHS/CS ($p < 0.05$). The percentage of Tregs found in the peripheral blood of animals undergoing LCHS/CS did not significantly change from LC alone ($10.5 \pm 3.3\%$ vs $6.7 \pm 1.7\%$; $p > 0.05$). Treatment with MSCs significantly increased the Treg population as compared to LCHS/CS alone ($11.7 \pm 2.7\%$ vs $6.7 \pm 1.7\%$; $p < 0.05$).

Conclusion—In this model, the severe impairment of wound healing observed one week after LCHS/CS is reversed by a single treatment with MSCs immediately after resuscitation. This improvement in lung healing is associated with a decrease in the number of inflammatory cells and lung edema and a significant increase in peripheral Tregs. Further study into timing of

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administration and mechanisms by which cell-based therapy using MSCs modulate the immune system and improve wound healing is warranted.

INTRODUCTION

Impaired wound healing has long been recognized after traumatic injury and hemorrhagic shock¹⁻³. The systemic inflammatory response induced by injury is necessary to initiate healing, however, when injury is complicated by hemorrhagic shock the upregulation of inflammatory mediators becomes excessive. This inflammatory storm contributes to end-organ dysfunction after severe injury⁴. Furthermore, chronic exposure to stress prolongs wound healing in both animal models and humans⁵⁻⁸. This stress-induced impairment in healing is believed to be secondary to excessive production of glucocorticoids and resultant immunosuppression²⁰. Clinically, impaired healing increases complication rates and prolongs hospital stay⁹.

Mesenchymal stem cells (MSCs) are a promising therapy for cell-based therapy to enhance wound healing. These multipotent cells are found in the bone marrow as well as other tissues. MSCs possess both paracrine and immunomodulatory properties and have been shown to be therapeutic in multiple disease states, including traumatic brain injury, myocardial infarction, and autoimmune disease¹⁰⁻¹². One of the many immunomodulatory effects MSCs have been shown to exert is expansion of the circulating population of immunosuppressive T regulatory cells (Tregs), a subset of T cells involved in immune homeostasis¹³⁻¹⁴.

We hypothesized that administration of MSCs will reverse the impairment of wound healing observed in our severe model of injury in rats: lung contusion combined with hemorrhagic shock and chronic stress. Furthermore, we hypothesized that this improvement will be correlated with an expansion of the systemic population of T regulatory cells.

METHODS

Experimental Groups

Male Sprague-Dawley Rats (n=6/group) were assigned to the three following experimental groups: 1) unilateral lung contusion (LC); 2) LC followed by a 45-minute period of hemorrhagic shock (HS) and then daily chronic restraint stress (CS) for six days after injury (LCHS/CS); and 3) LCHS/CS plus mesenchymal stem cells (LC/CS+MSC). Rats were sacrificed on day seven after injury; blood was collected for flow cytometry, and lungs were examined for histologic evidence of healing. Plasma was taken from an additional group (n=6) of naïve, uninjured rats for flow cytometry.

Lung Contusion and Lung Contusion Hemorrhagic Shock Model

As previously described¹⁶, rats were anesthetized using intraperitoneal sodium pentobarbital (50mg/kg) and unilateral LC was induced. Rats then underwent cannulation of the right internal jugular vein and right femoral artery immediately after LC with polyethylene (PE-50; Becton Dickinson and Co., Sparks, MD) and Silastic (Dow Corning Corp., Midland, MI) tubing, respectively. All tubing was flushed with 10 units/ml of heparinized saline. A

continuous blood pressure monitoring device was connected to the femoral artery catheter for the duration of the shock period to measure mean arterial pressure (MAP) and heart rate. Animals were bled to a MAP of 30–35mmHg and maintained at that pressure for 45 min. Temperature was maintained at approximately 37 °C with the use of an electric heating pad placed under the surgical platform. After the completion of the shock period, shed blood was re-infused at a rate of 1mL/min.

Chronic Stress Model

Rats underwent daily periods of restraint stress on days one through six after LC prior to sacrifice on day seven. As described previously¹⁵, for a two-hour period between 8am and 12pm, rats were placed in 16.5cm by 7.5cm restraint containers. Rats were repositioned and exposed to two min of continuous alarming at 30, 60, and 90 min in order to prevent acclimation. Animals were returned to their housing at the completion of the stress period.

Mesenchymal Stem Cell Culture

As described previously¹⁵, Sprague-Dawley rat mesenchymal stem cells (MSCs) (Cyagen Biosciences, Santa Clara, CA) were cultured and expanded. Briefly, after thawing, cells were transferred into 15mL OriCell MSC Growth Medium (Cyagen Biosciences, Santa Clara, CA), washed, resuspended, and seeded into T25 flasks with additional growth medium. Cells were incubated at 37°C with humidified 5% CO₂. According to protocol, growth medium was changed one day after initial incubation and then every three days. To re-seed during expansion, once MSCs reached 80–90% confluence, cells were dissociated with Trypsin-EDTA seeded at $3 \times 10^3/\text{cm}^2$. On the day of injection, MSCs were quantified and aliquoted into 5×10^6 cells/vial. Cells were washed twice with Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, CA) and a volume of 5×10^6 cells in one mL IMDM was incubated at 37°C until injection.

Mesenchymal Stem Cell Injection

Within 10 min of completion of resuscitation, the right internal jugular vein cannula was accessed for MSC injection. A single dose of 5×10^6 cells in one mL IMDM was given into the jugular vein over five min.

Lung Histology and Lung Injury Score

After killing the rats, lungs were stored in 10% buffered formalin prior to dehydration and embedding in paraffin. 4-micrometer thick sections were stained with hematoxylin and eosin (H&E). After slides were coded to a blinded reader, standard light microscopy was used to read slides and the degree of injury was scored according to a modified quantitative lung injury score (LIS)³¹ composed of scores for alveolar integrity, degree of pulmonary and interstitial edema, and number of inflammatory cells. Scores range between 0 (uninjured) and 11 (most severely injured). In each sample, 30 random high power fields were read and score reported as an average.

Flow Cytometry for T-regulatory Cells

Whole blood was collected at the time of killing the rats by direct cardiac puncture using a heparinized syringe and analyzed for Treg by flow cytometry using T regulatory Cell Staining Kit from eBioscience, Inc (San Diego, CA). Briefly, 100 μ L whole blood aliquots (one million cells) were placed in 5mL polystyrene tubes then stained with 10 μ L of both BD Pharmingen™ (BD Biosciences, Franklin Lakes, NJ) mouse anti-rat CD4 antibody conjugated with fluorescein isothiocyanate (FITC) and BD Pharmingen™ (BD Biosciences, Franklin Lakes, NJ) rat antimouse CD25 antibody conjugated to phycoerythrin (PE). Cells were then incubated in the dark for 30 min, centrifuged at 300 $\times$ g for 5 min, and washed twice with stain buffer. One mL of FoxP3 fixation/permeabilization working solution (eBioscience, Inc., San Diego, CA) was then added to each sample and samples were pulse vortexed. This was followed by a 2-h dark incubation period. Without washing, 2mL fixation/permeabilization working solution was added to each tube. Cells were then centrifuged, supernatant discarded, and resuspended in 100 μ L 1X permeabilization buffer; 10 μ L eBioscience mouse anti-rat FoxP3 conjugated to APC (San Diego, CA) was then added. Cells were then incubated in the dark for 30 min, washed twice with permeabilization buffer, and resuspended in stain buffer prior to analysis using BD FACSCalibur flow cytometer (BD, Franklin Lakes, NJ) equipped with CellQuest software (BD, Franklin Lakes, NJ) with an event count of 300,000 for each run.

Animals

250–350g male Sprague-Dawley rats (Charles River, Wilmington, MA), were maintained in a barrier-sustained animal facility according to the recommendations of the Guide for the Care and Use of Laboratory Animals after approval by the Rutgers New Jersey Medical School Animal Care and Use Committee. Rats were housed at 25° C with a 12-h light/dark cycle and free access to water and chow (Teklad 22/5 Rodent Diet W-8640; Harlen, Madison WI).

Reagents

Bovine serum albumin (BSA), and 2-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO). Methylcellulose was purchased from Stemcell Technologies (Vancouver, Canada). Fetal bovine serum (FBS), Iscove's Modified Dulbecco's Medium (IMDM), glutamine, penicillin/streptomycin, and trypan blue were obtained from Invitrogen (Carlsbad, CA). All cytokines rhEpo, rhIL-3, rhGM-CSF were purchased from R&D Systems (Minneapolis, MN). Sodium pentobarbital was purchased from B&B Pharmacy (Bellflower, CA) and heparin was obtained from Hospira Inc. (Lakefront, IL).

Statistical Analysis

Data presented as mean $\pm$ SD. Statistical analysis using GraphPad Prism (Version 4.0, San Diego, CA) consisted of one-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparison post-test. *p <0.05 vs. LC or **p <0.05 vs LCHS/CS considered significant.

RESULTS

Effect of Mesenchymal Stem Cells on Total Lung Injury Score

At day seven, LIS in rats undergoing unilateral LC was 0.8 ± 0.4 and grossly the lungs appeared healed. Wound healing is significantly impaired following LCHS/CS with LIS 7.2 ± 2.2 ($p < 0.05$ vs LC). A single infusion of MSCs immediately after resuscitation improved wound healing and LIS compared to rats that did not receive MSCs ($p < 0.001$ vs LCHS/CS), with a LIS no longer statistically different from LC alone (Figure 1)

Effect of Mesenchymal Stem Cells on Lung Injury Subgroup Scores

Effect of Mesenchymal Stem Cells on Pulmonary and Interstitial Edema

Scores—Seven days after isolated LC the LIS sub-scores for pulmonary and interstitial edema scores were 0 ± 0 and 0.8 ± 0.5 respectively. When animals underwent LCHS/CS, both pulmonary and interstitial edema scores were increased (1.3 ± 0.5 and 2.0 ± 0.5 respectively, $p < 0.05$ vs LC). Treatment with MSCs decreased pulmonary and interstitial edema scores as compared to LCHS/CS, returning them to levels seen following LC alone (0 ± 0 and 0.7 ± 0.8 , $p < 0.001$ vs LCHS/CS) (Table 1).

Effect of Mesenchymal Stem Cells on Alveolar Integrity—Rats undergoing unilateral LC had an alveolar integrity score of 0 ± 0 seven days after injury. This score remained increased in animals undergoing LCHS/CS at 1.3 ± 0.5 ($p < 0.001$ vs LC). In those animals receiving MSCs, alveolar integrity score had returned to levels seen after LC alone by seven days at 0.3 ± 0.5 ($p < 0.01$ vs LCHS/CS) (Table 1).

Effect of Mesenchymal Stem Cells on Inflammatory Cells—The inflammatory cell/high power field (hpf) score in rats undergoing unilateral LC was 0 ± 0 one week after injury. In rats undergoing LCHS/CS, this score remained increased at 2.2 ± 0.8 after one week ($p < 0.001$ vs LC). Those rats that underwent LCHS/CS + MSC had a score of 1.0 ± 0 , significantly less than those animals not receiving MSCs, although still increased when compared to LC ($p < 0.001$ vs LCHS/CS, $p < 0.01$ vs LC) (Table 1).

Effect of Mesenchymal Stem Cells on T Regulatory Cell Population

LC alone did not significantly change the number of peripheral Tregs compared to naïve animals ($10.5 \pm 3.3\%$ vs. 7.8 ± 1.5 ; $p > 0.05$). Rats subjected to LCHS/CS had similar numbers of Tregs as compared to naïve ($6.7 \pm 1.7\%$ vs. 7.8 ± 1.5 ; $p > 0.05$). Treatment with MSCs resulted in a 53% increase in the Treg population as compared to LCHS/CS alone ($11.7 \pm 2.7\%$ vs $6.7 \pm 1.7\%$; $p < 0.05$ vs LCHS/CS) (Figure 2). Note that secondary to incomplete intracellular staining of FoxP3 in 3 samples in the LC alone group, $n=3$ in that group; staining was complete in all 6 rats in the naïve, LCHS/CS, and LCHS/CS + MCS groups. While this may affect the significance comparing LC to naïve, it does not affect the comparison between LCHS/CS and LCHS/CS+MSC.

DISCUSSION

In the current study we examined the role of MSCs in improving the impairment in wound healing seen one week following LCHS followed by six days of CS. Whereas lungs display near complete histologic recovery within seven days after unilateral LC¹⁶, exposure to the additional insults of HS and CS significantly delay healing. We have previously shown that MSCs enhance healing after LC, resulting in complete healing at five days post-injury¹⁷. More recently, we reported the benefit of MSCs in healing when LC was combined with either HS or CS^{15,18}, however, it was unknown if MSCs retained their therapeutic effect in this most severe injury model, LCHS/CS. We demonstrated that a single dose of MSCs given immediately after the resuscitation period reversed this HS- and CS-induced healing impairment. This improved healing is associated with an increase in the peripheral population of T regulatory cells, implicating the immunomodulatory properties of MSCs in their ability to enhance healing.

Wound healing impairment has long been recognized after both trauma with clinically relevant hemorrhage^{1,3} and exposure to chronic psychologic stress⁵⁻⁷. In our model, when animals undergoing LC are exposed to HS and/or CS, they have a synergistic effect, with the most significant healing impairment seen in animals exposed to LCHS/CS. While these phenomena appear discrete at first glance, they have been shown to affect healing by many similar mechanisms. Angele et al found an increase in the pro-inflammatory cytokines IL-1 β and IL-6 as well as a decrease in anti-inflammatory TGF- β after combined trauma and HS³. Similarly, investigations into the mechanisms behind stress-induced impairment of healing have implicated a glucocorticoid-mediated suppression of the inflammatory phase of healing, with disrupted levels of IL-1, IL-6, IL-8, and TNF- α ^{19,20}. While many studies have examined the potential of MSCs to enhance wound healing, few studies have examined their effect in either lung injury or the setting of trauma. Xu et al showed a decreased influx of neutrophils and pulmonary edema as well as decreased production of pro-inflammatory IFN- γ and IL-1 β within the air spaces after MSC administration in a model of endotoxin-induced acute lung injury²¹. MSCs enhanced lung recovery after ventilator-induced injury in a study by Curley et al with an increased alveolar concentration of anti-inflammatory mediators TNF- α and IL-10²². In the current study, we demonstrated improvement in wound healing associated with marked decreases in pulmonary and interstitial edema scores after treatment with MSCs, consistent with previous findings of the anti-inflammatory effect of MSCs.

Perhaps the most important mechanism by which MSCs enhance wound healing on a systemic level is their ability to modulate the immune system²³. While interaction between MSCs and multiple cells of the innate and adaptive immune systems has been described, of particular interest is their interaction with T lymphocytes, particularly T regulatory cells (Treg). The initial pro-inflammatory response after injury is driven by activation of T helper 1 (Th1) lymphocytes, which, in the case of shock and stress, may be even further potentiated²⁴. The corresponding anti-inflammatory response occurring later after injury is mediated by T helper 2 (Th2) cells²⁴. T regulatory cells (Treg) are an immunosuppressive subclass of T cells that, after injury, function to restore the Th1/Th2 balance, bringing an earlier shift to the anti-inflammatory, Th2 phenotype²⁵. A decrease in Tregs has been shown in autoimmune diseases and early after traumatic injury²⁶⁻²⁷, with a shift toward the pro-

inflammatory Th1 phenotype. MSCs have been shown to increase the Treg population in both the peripheral blood and lung 5 days after LC¹³. Furthermore, these cells are necessary for MSC-mediated healing; when Tregs are bound by an anti-CD4 antibody, and their action blocked, the therapeutic effect of MSCs is lost²⁸. In a model of allergic airway inflammation, Kavanagh et al demonstrated a MSC-mediated expansion of the Treg population and a loss of MSC-mediated protection after Treg depletion²⁹. Consistent with these findings, we show a significant increase in the peripheral Treg population in rats receiving MSCs after undergoing LCHS/CS. We showed a concomitant decrease in the number of inflammatory cells within the lung parenchyma in animals receiving MSCs compared to those animals undergoing LCHS/CS alone, implicating the immunomodulatory effects of MSCs, likely through their interactions with Tregs in their protection against the wound healing impairment seen after shock and stress.

In the current study, we demonstrated enhanced healing after LCHS/CS with the administration of a single iv dose of MSCs given immediately after resuscitation. This healing was associated with decreases in all components of the lung injury score, most notably pulmonary and interstitial edema and the number of inflammatory cells found within the lung parenchyma. MSCs protect against the deleterious effects of shock and stress in part through their immunomodulatory properties of expanding the systemic T regulatory cell population. While this study provides proof of concept for the potential benefit of MSCs in healing after severe injury and stress, our study has several limitations. The optimal dosing of MSCs remains unknown as does the most efficacious therapeutic window for administration. We only studied the effects of MSCs on healing in male rats and acknowledge that females may behave differently. Furthermore, characterization of the mechanisms by which of MSCs exert their effects is needed, including where these cells home to once injected into the body, and how they interact with injured tissues and immune effector cells, including T regulatory cells.

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Figure 1. Lung Histology Seven Days After Injury

Lung sections seven days after LC alone demonstrate almost full histologic recovery (A). Lungs from rats undergoing LCHS/CS demonstrate incomplete healing at seven days (B), with continued presence of inflammatory cells and edema. When animals received MSCs following LCHS/CS, lung healing was almost complete at seven days (C). Slides prepared with hematoxylin and eosin. 20× magnification. LC=lung contusion, HS=hemorrhagic shock, CS=chronic stress, MSC=Mesenchymal stem cells.

Figure 2. T Regulatory Cells in Peripheral Blood

7 days following injury those animals undergoing LCHS/CS have no change in peripheral T Regulatory cells as compared to naïve. Those animals receiving MSCs have a significant increase in peripheral T Regulatory cells as compared to LCHS/CS alone. Dotted line represents naïve. LC= lung contusion; HS= hemorrhagic shock, CS = Chronic Stress; MSC= mesenchymal stem cells. Data presented as mean score $\pm$ standard deviation; n= 3 in LC group, n=6 in all other groups; *p<0.05 vs LCHS/CS.

Table 1

Lung Injury Score Total and Subgroup Score Seven Days Following Injury

Group	Inflammatory cells/hpf	Interstitial Edema	Pulmonary Edema	Alveolar Integrity	Total LIS
LC	0 ± 0	0.8 ± 0.5	0 ± 0	0 ± 0	0.8 ± 0.4
LCHS/CS	2.2 ± 0.8 *	2.3 ± 0.5 *	1.3 ± 0.5 *	1.3 ± 0.5 *	7.2 ± 2.2 *
LCHS/CS+MSC	1.0 ± 0 **	0.7 ± 0.8 **	0 ± 0 **	0.3 ± 0.5 **	2.0 ± 1.3 **

Data presented as mean ± standard deviation;

* p<0.05 vs LC
** p<0.05 vs LCHS/CS