Published in final edited form as:

J Trauma Acute Care Surg. 2015 April ; 78(4): 767–772. doi:10.1097/TA.0000000000000592.

Can Mesenchymal Stem Cells Reverse Chronic Stress-Induced Impairment of Wound Healing Following Traumatic Injury?

Amy V. Gore, MD, Letitia E. Bible, MD, David H. Livingston, MD, Alicia M. Mohr, MD, and Ziad C. Sifri, MD

Department of Surgery, Division of Trauma, Rutgers – New Jersey Medical School

Amy V. Gore: goreav@njms.rutgers.edu; Letitia E. Bible: biblele@njms.rutgers.edu; David H. Livingston: livingst@njms.rutgers.edu; Alicia M. Mohr: ammohr721@gmail.com

Abstract

Intro—One week following unilateral lung contusion (LC), rat lungs demonstrate full histologic recovery. When animals undergo LC plus the addition of chronic restraint stress (CS), wound healing is significantly delayed. Mesenchymal stem cells (MSC) are pluripotent cells capable of immunomodulation that have been the focus of much research in wound healing and tissue regeneration. We hypothesize that the addition of MSCs will improve wound healing in the setting of CS.

Methods—Male Sprague-Dawley rats (n=6-7/group) were subjected to LC/CS with or without the injection of MSCs. MSCs were given as a single IV dose of 5×10^6 cells in 1mL IMDM media at the time of LC. Rats were subjected to two hours of restraint stress on days 1-6 following LC. Seven days following injury, rats were sacrificed and lungs examined for histologic evidence of wound healing using a well-established histologic lung injury score (LIS) to grade injury. LIS examines inflammatory cells/high power field (hpf) averaged over 30 fields, interstitial edema, pulmonary edema, and alveolar integrity with scores ranging from 0 (normal) to 11 (highly damaged). Peripheral blood was analyzed by flow cytometry for the presence of T-regulatory ($C4^+CD25^+FoxP3^+$) cells. Data analyzed by ANOVA followed by Tukey's multiple comparison test, expressed as mean $\pm$ SD.

Results—As previously shown, seven days following isolated LC, LIS has returned to 0.83 ± 0.41 , with a subscore of zero for inflammatory cells/hpf. The addition of CS results in a LIS score of 4.4 ± 2.2 , with a subscore of 1.9 ± 0.7 for inflammatory cells/hpf. Addition of MSC to LC/CS decreased LIS score to 1.7 ± 0.8 with a subscore of zero for inflammatory cells/hpf. Furthermore, treatment of animals undergoing LC/CS with MSCs increased the %T-regulatory cells by 70% in animals undergoing LC/CS alone ($12.9 \pm 2.4\%$ vs $6.2 \pm 1.3\%$)

Conclusion—Stress-induced impairment of wound healing is reversed by addition of MSCs given at the time of injury in this rat lung contusion model. This improvement in lung healing is

Address correspondence to: Ziad C. Sifri, MD, Rutgers New Jersey Medical School, Department of Surgery, 150 Bergen Street M232, Newark, NJ 07101, Tel: (973) 972-3808, FAX: (973) 972-7441, sifrizi@njms.rutgers.edu.

Authorship Statement: The role of each author is as described below. Amy Gore was involved in experimental design, data acquisition, analysis and interpretation of data, and manuscript preparation. Letitia Bible in data acquisition and analysis. David Livingston, Alicia Mohr, and Ziad Sifri in design, data analysis and interpretation, and critical revision.

The authors have nothing to disclose or have any conflicts to report.

associated with a decrease in the number of inflammatory cells and an increase in the number of T regulatory cells. Further study into the mechanisms by which MSCs hasten wound healing is warranted.

INTRODUCTION

Exposure to chronic stress following injury has been shown to prolong wound healing in both animal models and humans¹⁻⁴. This prolonged healing is associated with increased rate of complications and prolonged hospital stay⁵. While the mechanisms of chronic stress-induced healing impairment remain unknown, it is believed that stress stimulates the HPA-axis, resulting in production of glucocorticoids and suppression of immune cells⁶. Mesenchymal stem cells (MSC) are multipotent cells found in the bone marrow, as well as elsewhere, that have great potential for cellular therapy. These cells exert many paracrine and immunomodulatory effects and have been shown to have potential therapeutic effects in various clinical applications including autoimmune disease, myocardial infarction, and traumatic brain injury⁷⁻⁹. T-regulatory cells (Treg) are a subset of T cells involved in immune homeostasis and self-tolerance¹⁰. Depletion of Treg cells have been implicated in a number of autoimmune and inflammatory diseases¹¹⁻¹². MSCs have been shown to expand the Treg population following traumatic injury and this may be one mechanism by which they exert their protective effects¹³. We hypothesize that administration of MSCs given at the time of injury to rats undergoing unilateral lung contusion (LC) followed by daily chronic restraint stress (CS) can reverse chronic stress-induced impairment of wound healing and that this will be associated with an expansion of the systemic Treg population.

METHODS

Experimental Groups

Rats (n=6/group) were assigned to the following experimental groups: unilateral lung contusion (LC), lung contusion followed by daily chronic restraint stress (CS) for six days after injury (LC/CS), and lung contusion followed by daily restraint stress plus mesenchymal stem cells (LC/CS+MSC). Rats were sacrificed on day seven following injury; blood was collected for flow cytometry and lungs were examined for histologic evidence of healing. Plasma was taken from an additional group (n=7) of naïve, uninjured rats for flow cytometry.

Lung Contusion Model

As previously described¹⁶, rats were anesthetized using intraperitoneal sodium pentobarbital (50mg/kg) prior to undergoing unilateral lung contusion (LC). LC was induced after securing a 12mm metal plate to the right axilla, using the blast wave of a percussive nail gun (Craftsman 968514 Stapler, Sears Brands, Chicago IL).

Chronic Stress Model

Animals underwent daily periods of restraint stress for the six days following LC, prior to sacrifice on day 7. Rats were placed in 16.5cm by 7.5cm restraint containers for a two-hour period between 8am and 12pm daily. In order to prevent acclimation, rats were stimulated at

30, 60, and 90 minutes by repositioning as well as two minutes of continuous alarming (80-85 decibels). Animals were returned to normal housing at the completion of the two-hour restraint period. As animals undergoing CS were denied access to water or chow during the stress period, animals not undergoing CS were also not permitted water or chow during this period.

Animals

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

Mesenchymal Stem Cell Culture

Sprague-Dawley rat mesenchymal stem cells (MSCs) (Cyagen Biosciences, Santa Clara, CA) were cultured and expanded. Briefly, cells were thawed and transferred into 15mL OriCell MSC Growth Medium then centrifuged at 250×g for 5 minutes. Supernatant was aspirated and cells were re-suspended in 2-3mL of growth medium and seeded into T25 flasks with additional growth medium. Cells were incubated at 37°C with humidified 5% CO₂. Medium was changed on day one after incubation and then every three days. Cells were dissociated with Trypsin-EDTA and re-seeded at $3 \times 10^3/\text{cm}^2$ once they reached 80-90% confluence. Expansion continued until cells were harvested for injection. 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) and a volume of 5×10^6 cells in one mL IMDM was incubated at 37°C until injection.

Mesenchymal Stem Cell Injection

As above, animals were anesthetized with intraperitoneal sodium pentobarbital (50mg/kg). The right internal jugular vein was cannulated with polyethylene tubing (PE-50; Becton Dickinson and Co., Sparks, MD) prior to animals undergoing LC. Within ten minutes of injury, injection of 5×10^6 cells in one mL IMDM was given into the jugular vein over five minutes.

Lung Histology and Lung Injury Score

After sacrifice, lungs were placed in 10% buffered formalin. Samples were then dehydrated and embedded in paraffin blocks. Sections of 4-micrometer thickness were cut and stained with hematoxylin and eosin (H&E). Slides were read using standard light microscopy and degree of injury was scored according to a quantitative lung injury score (LIS) ranging between 0 and 11, with 11 representing the most severe injury³⁴ (Table 1). Slides were coded to blind reader to their origin and evaluated with grading of 30 random high power fields/sample.

Flow Cytometry for T-regulatory Cells

Whole blood was collected 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 aliquots (one million cells) of whole blood was placed in 5mL polystyrene tubes. Samples were then stained with 10 μ L of both BD Pharmingen™ mouse anti-rat CD4 antibody conjugated with fluorescein isothiocyanate (FITC) and BD Pharmingen™ rat anti-mouse CD25 antibody conjugated to phycoerythrin (PE) (BD Biosciences, Franklin Lakes, NJ) and incubated in the dark for 30 minutes. Cells were then centrifuged at 300 $\times$ g for 5 minutes and washed with stain buffer for a total of two washes. Following washes, 1mL FoxP3 fixation/permeabilization working solution was added to each sample and pulse vortexed. Samples were then incubated for two hours in the dark. Without washing, 2mL fixation/permeabilization working solution was added to each tube. Cells were then centrifuged and resuspended in 100 μ L 1X permeabilization buffer. 10 μ L eBioscience mouse anti-rat FoxP3 conjugated to APC was added prior to incubation in the dark for 30 minutes. Cells were then washed twice with permeabilization buffer and resuspended in stain buffer prior to analysis by Cells were analyzed using BD FACSCalibur flow cytometer (BD) equipped with CellQuest software (BD) with an event count of 300,000 was for each run.

Reagents

Bovine serum albumin (BSA), and 2-mercaptoethanol were purchased 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 the Kruskal-Wallis test followed by Mann-Whitney U test or one-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparison post-test as appropriate. *p <0.05 vs. LC or **p <0.05 vs LC/CS considered significant.

RESULTS

Effect of Mesenchymal Stem Cells on Total Lung Injury Score

As previously shown, one week following injury, the lung injury score (LIS) in rats undergoing unilateral lung contusion is 0.8 ± 0.4 indicating complete healing. The addition of daily restraint stress results in impaired healing, with the LIS remaining elevated at 4.4 ± 2.2 (p=0.0012 vs LC). When MSCs are given at the time of injury, rats undergoing LC/CS LIS is no longer statistically different from LC alone, with a total LIS of 1.7 ± 0.8 (p=0.014 vs LC/CS)(Figure 1).

Effect of Mesenchymal Stem Cells on Subgroup Scores

Effect of Mesenchymal Stem Cells on Edema Scores—Seven days following injury, LIS individual score for pulmonary and interstitial edema scores are 0 ± 0 and 0.75 ± 0.5 respectively. While the addition of chronic stress increases interstitial edema scores (1.4 ± 0.5 vs 0.75 ± 0.5 ; $p=0.035$), there was no effect on pulmonary edema (0.4 ± 0.5 vs 0 ± 0 ; $p>0.05$). Treatment with MSCs did not statistically affect either edema score when compared to either LC alone or LC/CS (0 ± 0 and 1.2 ± 0.4 , $p>0.05$). (Table 2).

Effect of Mesenchymal Stem Cells on Alveolar Integrity—Rats undergoing unilateral LC have an alveolar integrity score of 0 ± 0 seven days following injury. There is no significant change in alveolar integrity score in rats undergoing LC/CS or LC/CS+MSC (0.5 ± 0.5 and 0.7 ± 0.8 , respectively; $p>0.05$)(Table 2).

Effect of Mesenchymal Stem Cells on Inflammatory Cells—The inflammatory cell/high power field (hpf) score in rats undergoing unilateral LC is 0 ± 0 one week after injury. In rats undergoing LC/CS, this score remains elevated at 1.9 ± 0.7 ($p=0.0002$). Those rats that underwent LC/CS + MSC had a score of 0 ± 0 ($p=0.0002$)

Effect of Mesenchymal Stem Cells on T Regulatory Cell Population

SD rats undergoing LC alone had $10.5 \pm 3.3\%$ T regulatory cells in their peripheral blood, not significantly different from naïve (7.8 ± 1.5 ; $p>0.05$). When rats were subjected to CS following LC, their T regulatory cell population had a trend toward suppression at $6.2 \pm 1.3\%$. The addition of MSCs to rats undergoing combined LC/CS resulted in a 70% increase in %T-regulatory cells as compared to LC/CS alone ($12.9 \pm 2.4\%$) ($p<0.001$ vs LC/CS, $p<0.01$ vs Naïve) (Figure 2). Note that secondary to incomplete intracellular staining of FoxP3 in 3 samples in the LC alone group, $n=3$ in that group. While this may affect the significance in comparing LC alone to either of the other groups, this does not affect the comparison between LC/CS and LC/CS+MSC.

DISCUSSION

In the current study we demonstrate that a single dose of intravenous MSCs given shortly following injury can improve wound healing seen in the setting of chronic stress. Our data also suggests that this improved healing may be due to a MSC-mediated increase in the T-regulatory cell population. Our lab has previously investigated the role of MSCs in wound healing in both the setting of isolated unilateral LC and LC in combination with lymph duct ligation (LDL)^{14,15}. Hannoush et al. showed that following unilateral LC, rat lungs return to normal histology seven days following injury¹⁶ and that the addition of MSCs after injury can hasten this healing, resulting in histologic recovery five days after injury¹⁵. Furthermore, when rats are subjected to LDL, and bone marrow-derived cells, including MSCs, are prevented from mobilizing to the periphery, wound healing is delayed. This impairment in wound healing is abolished by the addition of exogenous MSCs at the time of injury¹⁵, indicating that these cells are important for normal healing. Here we examine the effect of MSCs on wound healing in a more clinically relevant model of disordered wound healing resulting from chronic stress.

Psychological stress has been shown to negatively impact wound healing and is related to increased complication rate and length of hospitalization following surgery⁵. Padgett et al demonstrated that chronic restraint stress slows healing of cutaneous punch wounds in mice and that this is associated with an increase in serum corticosterone levels; when stressed animals were treated with a glucocorticoid receptor antagonist, healing rates returned to those of control animals¹⁷. Detillion et al examined the effect of the hypothalamic-pituitary-adrenal (HPA) axis in hamsters undergoing chronic restraint stress following cutaneous wounding and demonstrated that stress resulted in impaired wound healing and increased cortisol levels in socially isolated hamsters and treatment with oxytocin facilitated wound healing¹. Psychological stress has been implicated in delayed wound healing in humans as well. Kiecolt-Glaser et al demonstrated attenuated wound healing in the elderly primary caregivers of spouses suffering from Alzheimer's dementia wherein, on average, wound healing was delayed nine days as compared to age-matched controls³. When dental students were given oral mucosal wounds either before an exam or during a vacation, wound healing was slowed by approximately 40% during periods of academic stress⁴. While the mechanisms behind chronic stress-induced healing impairment are not well characterized, it is thought that a glucocorticoid-mediated immunosuppression interferes with the inflammatory phase of wound healing. Multiple cytokines have been implicated in this dysfunction, including IL-1, IL-6, IL-8, and TNF- α ⁶. Here we show an increase in the number of inflammatory cells within the lung parenchyma in those animals undergoing LC/CS and a decrease in these cells in those animals receiving MSCs with no significant change in edema scores. It may be that while these inflammatory cells are recruited to the injured tissue, their function is compromised by the stress state. Kiecolt-Glaser et al demonstrated a decrease in proinflammatory cytokines including IL-1 β , IL-6, and TNF- α in the wounds of stressed individuals, indicating that stress may dampen the local inflammatory response within the wound.

There has been much interest in MSCs as a cellular therapy to enhance wound healing and tissue regeneration. There is a broad array of clinical disorders in which MSCs have been studied as potential therapy including myocardial infarction, traumatic brain injury, diabetes mellitus, hepatic failure, and acute renal failure^{8, 19-22}. While there is a paucity of studies examining the effect of MSCs on traumatic lung injury, multiple studies have looked at the effect of MSCs on acute lung injury induced by barotrauma or bleomycin or indirect lung injury following hemorrhage and septic challenge²³⁻²⁷. Early studies on MSCs focused on engraftment and differentiation as potential mechanisms, however, the current belief is that the paracrine and immunomodulatory effects of MSCs are much more important in their therapeutic efficacy²⁸. Curley et al. found that MSCs enhance lung recovery in rats following ventilator-induced injury and that this was associated with an increased alveolar concentration of TNF-alpha and IL-10²³. Xu et al demonstrated that MSCs given to mice undergoing endotoxin-induced ALI resulted in a decreased influx of neutrophils to the airspaces as well as decreased pulmonary edema and that this was associated with a decreased production of the proinflammatory mediators IFN-gamma, IL-1beta, macrophage inflammatory protein (MIP)-1 alpha and IL-8²⁴ as well as an even greater increase in G-CSF. In the setting of acute traumatic injury followed by chronic restraint stress, MSC may

function by dampening the pro-inflammatory effects of chronic stress and bringing order to this disrupted system through the use of paracrine mediators.

MSCs have been shown to interact with several cells of the both the innate and adaptive immune systems including dendritic cells, NK cells, T cells, and B cells²⁹. Of particular interest is how MSCs interact with T-regulatory cells, which are actively involved in immune homeostasis. Treg dysfunction has been implicated in autoimmune and immunopathological diseases¹² and may be implicated in the suppression of Th1 response in post-traumatic immunosuppression³⁰. Venet et al investigated the lymphocyte subpopulations involved in indirect acute lung injury, finding that CD4+ T cells were specifically recruited to the lung, with a specific increase in the Treg population³¹. Cook et al showed that the Treg population was increased 5 days following unilateral LC and even further elevated with the addition of MSCs¹³. Additionally, when Tregs were blocked, MSCs no longer had a therapeutic effect on healing, indicating that Treg are necessary for MSC-mediated healing³². Kavanagh et al examined the effect of MSCs on allergic airway inflammation and demonstrated that MSCs expanded the Treg population in both lung and spleen following exposure to sensitized allergen and that when animals that had undergone Treg depletion were exposed to allergen, MSCs no longer conferred protection³³. In the current study, we show a significant increase in the Treg population in animals undergoing LC/CS who received MSCs as compared to those that did not, implicating this expanded population of immunosuppressive cells in the mechanism by which MSCs exert their protective effect against chronic stress-induced impairment of wound healing.

This is one of the first studies to examine the effects of MSCs on chronic stress-induced impairment of wound healing following traumatic injury. While chronic stress delays wound healing and decreases Treg population, MSCs mitigate the deleterious effects of stress, in part by decreasing the number of inflammatory cells found within the lung parenchyma and modulating the immune system by expanding the T regulatory cell population. Further study into the timing and dosing of MSCs as well as the mechanisms by which MSCs interact with immune cells and promote healing is warranted.

Acknowledgments

This research was supported by the National Institutes of Health grants K08 NIH GM078304 and T32 GM069330.

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Figure 1. Lung Histology Seven Days Following Injury

Lung sections seven days following LC alone demonstrate full histologic recovery (A).

Lungs from rats undergoing LC/CS demonstrate incomplete healing at seven days (B), with continued presence of inflammatory cells. When animals received MSCs following LC/CS, lung healing was complete at seven days (C). Slides prepared with hematoxylin and eosin. 20X magnification. LC=lung contusion, CS=chronic stress, MSC=Mesenchymal stem cells.

Figure 2. Effect of Mesenchymal Stem Cells on Peripheral T Regulatory Cell Population

One week following injury there is no significant difference in T regulatory cell population in peripheral blood of those animals undergoing LC alone (n=3) as compared to naïve (n=7). When animals undergo CS following injury, there is a trend toward depression that did not reach statistical significance (LC/CS, n=6). Those animals receiving MSCs (LC/CS+MSC, n=6) had a significant increase in the peripheral T regulatory cell population as compared to both naïve ($p<0.01$) and animals undergoing LC/CS ($P<0.001$). Dotted line represents naïve; LC= lung contusion; CS = chronic stress; MSC = mesenchymal stem cell. * $p<0.001$ vs LC/CS.

TABLE 1**Lung Injury Score**

Parameter	Points
Interstitial Edema	
None	0
Minimal	1
Moderate	2
Severe	3
Pulmonary Edema	
<5%	0
5-25%	1
>25%	2
Alveolar Integrity	
Normal	0
Abnormal moderate	1
Abnormal severe	2
Inflammatory Cells/HPF	
<5	0
6-10	1
11-15	2
16-20	3
>20	4

Table 2

LIS Total and Subgroup Score Seven Days Following Injury

Group	Inflammatory cells/hpf	Interstitial Edema	Pulmonary Edema	Alveolar Integrity	Total LIS
LC	0 ± 0	0.75 ± 0.5	0 ± 0	0 ± 0	0.8 ± 0.4
LC/CS	1.9 ± 0.7 *	1.4 ± 0.5 *	0.4 ± 0.5	0.7 ± 0.8	4.4 ± 2.2 *
LC/CS+MSC	0 ± 0 **	1.2 ± 0.4	0 ± 0	0.5 ± 0.5	1.7 ± 0.8 **

Data presented as mean score ± standard deviation;

* p<0.05 vs LC
** p<0.05 vs LC/CS, n=9/group