Mesenchymal stem cells reverse trauma and hemorrhagic shock-induced bone marrow dysfunction

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

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

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Abstract

Background: Lung contusion (LC) followed by hemorrhagic shock (HS) causes persistent bone marrow (BM) dysfunction lasting up to 7 d after injury. Mesenchymal stem cells (MSCs) are multipotent cells that can hasten healing and exert protective immunomodulatory effects. We hypothesize that MSCs can attenuate BM dysfunction after combined LC-HS.

Materials and methods: Male Sprague–Dawley rats (n = 5–6 per group) underwent LC plus 45 min of HS (mean arterial pressure of 30–35). Allogeneic MSCs (5 × 10^6 cells) were injected intravenously after resuscitation. At 7 d, BM was analyzed for cellularity and growth of hematopoietic progenitor cell (HPC) colonies (colony-forming unit–erythroid; burst-forming unit–erythroid; and colony-forming unit–granulocyte, erythrocyte, monocyte, megakaryocyte). Flow cytometry measured %HPCs in peripheral blood; plasma granulocyte colony-stimulating factor (G-CSF) levels were measured via enzyme-linked immunosorbent assay. Data were analyzed by one-way analysis of variance followed by the Tukey multiple comparison test.

Results: As previously shown, at 7 d, LCHS resulted in 22%, 30%, and 24% decreases in colony-forming unit–granulocyte, erythrocyte, monocyte, megakaryocyte; burst-forming unit–erythroid, and colony-forming unit–erythroid colony growth, respectively, versus naive. Treatment with MSCs returned all BM parameters to naive levels. There was no difference in %HPCs in peripheral blood between groups; however, G-CSF remained increased up to 7 d after LCHS. MSCs returned G-CSF to naive levels. Plasma from animals receiving MSCs was not suppressive to the BM.

Conclusions: One week after injury, the persistent BM dysfunction observed in animals undergoing LCHS is reversed by treatment with MSCs with an associated return of plasma G-CSF levels to normal. Plasma from animals undergoing LCHS plus MSCs was not suppressive to BM cells in vitro. Treatment with MSCs after injury and shock reverses BM suppression and returns plasma G-CSF levels to normal.

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1. Introduction

Persistent injury-associated anemia and increased susceptibility to infection [1,2] are manifestations of the bone marrow (BM) dysfunction observed after severe injury with significant clinical ramifications. In a rodent model of lung contusion (LC) alone, this dysfunction is manifested as a reduction in BM cellularity and growth of hematopoietic progenitor cells (HPCs) within the BM itself and mobilization of HPCs to the periphery with an associated rise in plasma granulocyte colony-stimulating factor (G-CSF) early after injury; however, rats recovered within 7 d [3,4]. When animals undergo lung contusion following hemorrhagic shock (LCHS), BM dysfunction is both more acutely marked and persists for >1 wk [3,4]. Mesenchymal stem cells (MSCs) are a type of multipotent cell with multiple immunomodulatory and paracrine functions that are essential to maintain the BM microenvironment and support hematopoiesis [5–7]. They have been investigated in several disease states, such as graft-versus-host disease, aplastic anemia, and myocardial infarction, as a potential cellular therapy to expedite healing and restore homeostasis [8–10].

We have previously investigated the role of these cells in lung healing [11]; however, we have not examined their role in treating the BM dysfunction that ensues after injury. We hypothesize that treatment of rats undergoing combined LCHS with MSCs given early after resuscitation can reverse injury-associated BM dysfunction. Furthermore, we hypothesize that protection occurs via a systemic effect by altering the plasma in shocked animals such that it is no longer suppressive to BM cells in vitro.

2. Materials and methods

2.1. Experimental groups

Male rats (n = 5–6 per group) were assigned to experimental groups as follows: LC alone, LC plus MSCs (LC + MSCs), LCHS, and LC followed by HS plus MSCs (LCHS + MSCs). A control naive group of uninjured animals undergoing daily handling was also used. On day 7, rats were sacrificed and BM and blood collected.

2.2. Animals

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

2.3. LC model

After intraperitoneal administration of sodium pentobarbital (50 mg/kg), a 12-mm metal plate was secured to the rat’s right axilla and a percussive nail gun (Craftsman 968514 Stapler; Sears Brands, Chicago, IL) was used to induce a unilateral LC.

2.4. HS model

As previously described [12], immediately after LC, rats in the LCHS and LCHS + MSC groups underwent cannulation of the right internal jugular vein and right femoral artery with polyethylene (PE-50; Becton Dickinson and Co, Sparks, MD) and Silastic (Dow Corning Corp, Midland, MI) tubing, respectively. To prevent clotting, all tubing was flushed with 10 U/mL of heparinized saline. The femoral artery catheter was then connected to a continuous blood pressure monitoring device for measurement of mean arterial pressure (MAP) and heart rate. Animals were bled to an MAP of 30–35 mm Hg for 45 min; additional blood was withdrawn or reinfused as needed to maintain MAP within range. 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 reinfused at a rate of 1 mL/min.

2.5. MSC culture

As previously described [13], Sprague–Dawley rat MSC cultures (Cyagen Biosciences, Santa Clara, CA) were established and expanded. Briefly, cells were thawed and transferred into 15 mL OriCell MSC Growth Medium (Cyagen Biosciences, Santa Clara, CA). Cells were washed and resuspended in 2–3 mL of fresh growth medium. Cells were incubated in a 37°C humidified 5% CO2 incubator after seeding into T25 flasks containing additional growth medium. Medium was changed at 24 h and then every 72 h. Cells were dissociated with trypsin–EDTA when 80–90% confluent and reseeded at 3 × 103/cm2. This continued until adequate cell counts were available for harvest. On the day of injection, 5 × 10^6 MSCs were quantified and aliquoted into individual vials. Cells were washed with Iscove’s Modified Dulbecco’s Medium (IMDM) and resuspended in 1-mL IMDM before injection.

2.6. MSC injection

A previously prepared suspension of 5 × 10^6 MSCs in 1-mL of IMDM was infused via the previously cannulated right internal jugular vein for >5 min, beginning within 10 min of injury in the LC and LC + MSC groups or within 10 min of return of shed blood in the groups undergoing HS.

2.7. BM cellularity

BM cells were harvested from the right femur to determine cellularity and establish cell culture. An 18-gauge needle was inserted into the femur and BM was aspirated into 1-mL IMDM. Cells were suspended, stained with 0.4% trypan blue, and hemocytometer was used to determine total viable cell count.

2.8. BM HPC cultures

Growth of HPCs from the BM was assessed by culturing colony-forming unit–granulocyte, erythrocyte, monocyte,
chloride in a 37°C solution of IMDM supplemented with 30% fetal bovine serum, 2% bovine serum albumin, rat growth factor, 1% methylcellulose, 2 × 10^{-4} mol/L 2-mercaptoethanol, penicillin, streptomycin, and glutamine was prepared and 1.5 × 10^6 cells added to it. All cultures were plated in duplicate. Those plates growing BFU-E and CFU-E were further supplemented with 1.3 U/mL rhEpo and 6 U/mL rhIL-3. Plates growing CFU-GEMM were supplemented with 3 U/mL recombinant human granulocyte-macrophage colony-stimulating factor. Cultures were incubated at 37°C in 5% CO2 incubator. CFU-E colonies were counted on day 7, BFU-E on day 14, and CFU-GEMM on day 17 by an observer blinded to sample origin.

2.9. Cultures with experimental plasma

BM was collected from naïve rats, and cellularity and stock solutions were prepared as described previously. Again, cells were plated in duplicate. Although all BM cells came from naïve rats, cells were plated with the addition of 5% vol/vol of plasma from rats in one of the following experimental groups: unmanipulated control, LCHS, or LCHS + MSC. An additional control group consisting of naïve BM cells incubated with media alone was used for comparison (naïve). Plasma was obtained after sacrifice on day 7 after injury. As previously mentioned, cultures were counted after the appropriate number of days of incubation according to colony type by a blinded observer.

2.10. Flow cytometry for HPCs

Blood samples were obtained via direct cardiac puncture using a 10-mL heparinized syringe at the time of sacrifice. A well-established, single-platform enumeration method was used to determine the frequency of CD71+, CD117+ HPCs within unfractionated blood samples. Briefly, 100 μL whole blood was labeled with 10 μL of BD Pharmingen mouse anti-rat CD71 antibody conjugated with fluorescein isothiocyanate and labeled with 10 μL of BD Pharmingen rat anti-mouse CD117 (c-Kit) antibody conjugated with phycoerythrin (BD Biosciences, Franklin Lakes, NJ) for 30 min. Erythrocytes were then lysed with ammonium chloride in a 37°C humidified 5% CO2 incubator for 10 min. After lysis, cells were centrifuged at 300 × g for 5 min and supernatant was discarded before washing three times. After fixation with BD Cytofix solution (BD Biosciences), cells were analyzed using BD FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuest software (BD Biosciences) with an event count of 50,000 each run.

2.11. Plasma G-CSF levels

A commercial enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) run in duplicate was used to determine plasma G-CSF levels. Briefly, 50 μL of assay diluent RD1-54 and 50 μL of standard, control, or sample were added to wells precoated with polyclonal anti-mouse G-CSF. Plate was covered and after 2 h of incubation at room temperature, wells were aspirated and washed a total of five times. One hundred microliters of mouse G-CSF conjugate was then added to each well before another 2 h of covered incubation. Plate was again washed a total of five times before adding100 μL substrate solution to each well and a 30 min of dark incubation. One hundred microliters of stop solution was then added to each well, mixed gently, and plate was read using 450–540 nm wavelength correction.

2.12. Reagents

Bovine serum albumin and 2-mercaptoethanol were purchased from Sigma–Aldrich (St. Louis, MO). Methylcellulose was purchased from Stemcell Technologies (Vancouver, Canada). Fetal bovine serum, IMDM, glutamine, penicillin, streptomycin, and trypan blue were obtained from Invitrogen (Carlsbad, CA). All cytokines rhEpo, rhIL-3, and recombinant human granulocyte-macrophage colony-stimulating factor 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).

2.13. Statistical analysis

All data are expressed as the mean ± standard deviation. Statistical analyses were performed using one-way analysis of variance followed by the Tukey–Kramer multiple comparison posttest or the Student t-test with GraphPad Prism (version 4.0; GraphPad Software, Inc, La Jolla, San Diego, CA). Results were considered significant if *P < 0.05 versus LC or **P < 0.05 versus LCHS.

3. Results

3.1. Effect of MSCs on BM cellularity

There is no statistically significant difference in BM cellularity in those animals undergoing either unilateral LC alone or LC + MSC compared with naïve levels at 7 d. Seven days after LCHS there is a 13% decrease in BM cellularity compared with naïve (197 ± 19 versus 225 ± 6; P < 0.05). The addition of MSCs to animals undergoing LCHS resulted in a 9.7% increase in cellularity compared with LCHS alone, returning cellularity to naïve levels (217 ± 4 versus 197 ± 19; P < 0.05; Fig. 1A).

3.2. Effect of MSCs on BM HPC cultures

There is no significant change in growth of any of the HPC types examined 7 d after either LC alone or LC + MSCs. There is a 22% decrease in CFU-GEMM 7 d after LCHS (28 ± 1 versus 35 ± 1; P < 0.05). The addition of MSCs at the time of resuscitation results in a 16% increase in CFU-GEMM growth, returning it to naïve levels (33 ± 1 versus 28 ± 1; Fig. 1B). At 7 d after LCHS there are 30% and 24% decreases in BFU-E and CFU-E, respectively (50 ± 3 versus 68 ± 3 and 60 ± 3 versus 77 ± 4, respectively; P < 0.05). When animals undergoing LCHS were given MSCs, there were 33% and 30% increases in BFU-E and CFU-E, respectively, again returning colony growth to naïve levels (70 ± 1 versus 50 ± 3 and 81 ± 1 versus 60 ± 3, respectively; P < 0.05; Fig. 1C and D).
3.3. Effect of MSCs on mobilization of HPCs and plasma G-CSF

Seven days after either LC or LCHS there was no statistically significant difference in HPC mobilization (Fig. 2A). Furthermore, the addition of MSCs to LC or LCHS groups did not significantly alter the percentage of HPCs circulating in the peripheral blood. However, plasma G-CSF levels remained increased in those animals undergoing LCHS (12.3 ± 3.6 versus 4.7 ± 2.1; P < 0.05). The addition of MSCs returned G-CSF to naive levels (5.4 ± 1.7 versus 4.7 ± 2.1; P > 0.05; Fig. 2B).

3.4. Effect of experimental plasma on naive BM HPC growth in vitro

There was no difference in CFU-E colony counts in naive BM incubated with media alone and naive BM incubated with 5% vol/vol naive plasma (unmanipulated control). When naive BM cells were incubated with 5% plasma from 7-d animals undergoing LCHS, there was a 30% decrease in CFU-E colony growth compared with naive cells incubated with media alone (57.3 ± 2.6 versus 77.4 ± 4.0; P < 0.001). When naive BM cells were incubated with plasma from animals undergoing LCHS with MSCs after resuscitation, suppression was no longer observed (76.1 ± 2.3 versus 77.4 ± 4.0; P > 0.05; Fig. 3).

4. Discussion

BM dysfunction has long been described after severe injury and HS [1,2]. In human trauma patients, this dysfunction manifests as both disordered growth of HPCs within the BM itself and increased mobilization of these cells to the periphery [2]. In critically injured patients, this dysfunction...
manifests as a persistent posttraumatic anemia [14]. In the present study, we examine the effects of MSCs in a well-established rat model of combined LCHS resulting in BM dysfunction [3,4]. We demonstrate that treatment with MSCs immediately after resuscitation results in a return of HPC colony growth within the BM and plasma G-CSF levels to naive within 7 d after injury. Furthermore, treatment with MSCs alters the composition of the plasma such that it is no longer suppressive to HPC colony growth in culture.

MSCs are multipotent cells that have been shown to have myriad paracrine and immunomodulatory functions [15]. These cells are considered to constitute an essential part of the BM microenvironment and are important in supporting hematopoietic homeostasis via the secretion of various cytokines and expressing adhesion molecules involved in hematopoietic stem cell homing [5–7]. Although MSCs have not previously been examined in the treatment of posttraumatic BM dysfunction, their effects on the BM have been studied in the context of aplastic anemia, lupus-associated anemia, and in cotransplantation with MSCs as part of a BM transplant [16–18]. Wu et al. [19] treated a cohort of 21 patients with severe aplastic anemia with MSCs cotransplanted with haploidentical hematopoietic stem cells and demonstrated sustained hematopoietic engraftment without an increase in graft-versus-host disease. MSCs have also been shown to improve cell counts in patients with refractory cytopenia secondary to systemic lupus erythematosus [17]. Although these studies report dramatic results in severely ill patient populations, the exact mechanism of action of MSCs largely remains unknown. Our data demonstrate that a single dose of intravenous MSCs given immediately after resuscitation in LCHS animals resulted in increase in BM cellularity and HPC growth to naive levels by 7 d after injury. The ability of MSCs to modulate and support the BM microenvironment may be protective against the dysfunction observed in the setting of combined LCHS.

Other manifestations of BM dysfunction after LCHS at 7 d include HPCs returning from the periphery to the BM, despite modest increase in plasma G-CSF levels. Increases in plasma G-CSF have been shown in severely injured trauma patients.

**Fig. 2** — Effect of MSCs on HPC mobilization and G-CSF 7 d after injury and shock. (A) There is no change in the percentage of HPCs found in the peripheral blood after LC or LCHS with or without MSCs. (B) The addition of MSCs to LCHS causes a significant decrease in the plasma G-CSF level, returning plasma G-CSF to naive levels. n = 5–6 per group (dotted line represents naive). *P < 0.05 versus naive, **P < 0.05 versus LCHS. G-CSF = granulocyte colony-stimulating factor; HPC = hematopoietic progenitor cell; HS = hemorrhagic shock; LC = lung contusion; MSC = mesenchymal stem cell.

**Fig. 3** — Effect of experimental plasma on CFU-E colony growth. Naive BM cells cultured with media plus the addition of 5% plasma from 7-d animals undergoing LCHS suppresses CFU-E colony growth. When incubated with plasma from animals receiving MSCs suppression is no longer observed. Dotted line represents naive BM cells incubated with media alone. Unmanipulated control (UC) represents naive BM cells incubated with 5% naive plasma (n = 5–6 per group; *P < 0.001 versus naive, **P < 0.001 versus LCHS). BM = bone marrow; CFU-E = colony-forming unit–erythroid; HS = hemorrhagic shock; LC = lung contusion; MSC = mesenchymal stem cell.
and this increase is associated with a significant increase in peripheral HPCs [20]. Petit et al. [21] reported that G-CSF within the BM modulates the release of HPC from their stromal attachments via elastase-dependent degradation, allowing mobilization of these cells to the periphery. Clinically, G-CSF is often given to mobilize both hematopoietic and MSCs to the periphery in preparation for BM transplantation. The current data shows a relationship between exogenous MSC administration and an endogenous decrease in G-CSF. It appears that treatment with MSCs hastens the return of G-CSF to naive levels, possibly indicating an earlier return of HPCs from the periphery and swifter recovery of BM homeostasis after injury.

Although it is possible that MSCs have local effects within the BM niche, we hypothesized that their ability to protect the BM against posttraumatic dysfunction is because of their systemic effect after a global ischemia–reperfusion event. Plasma from trauma patients has been shown to inhibit HPC colony growth in vitro up to 2 wk after injury [22]. Consistent with prior findings, we show that plasma from animals that had undergone LCHS significantly suppressed growth of CFU-E, whereas plasma from animals receiving MSCs did not. Although these data indicate that MSCs modulate the plasma, reversing this in vitro suppressive effect, it is unknown what components of the plasma are altered. Nevertheless, this finding indicates that the effects of MSCs go beyond the local BM niche.

Our data establish the protective role of MSCs in BM dysfunction observed after trauma and HS. MSCs given immediately after resuscitation reverse BM dysfunction observed 7 d after injury by returning BM cellularity and HPC colony growth to naive levels. This protection is associated with a decrease in plasma G-CSF and the return of HPCs from the periphery. Furthermore, we demonstrate that MSCs modulate the suppressive effect of plasma on BM cells in vitro, indicating a systemic effect of these cells. Furthermore, studies are necessary to elucidate the mechanisms by which MSCs function on both a local level within the BM niche and systemically. Additionally, the optimal dosing of MSCs and therapeutic window for administration remain to be defined. The use of MSCs as a cellular therapy after severe trauma with HS may provide great benefit in the treatment of BM dysfunction and its resultant anemia.

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Author contributions: A.V.G. was involved in experimental design, data acquisition, analysis and interpretation of data, and manuscript preparation. L.E.B. was involved in data acquisition and analysis. D.H.L., A.M.M., and Z.C.S. were involved in data analysis and critical revision.

Disclosure

The authors have no further disclosures to report.

REFERENCES

[20] Cook KM, Sifri ZC, Baranski GM, Mohr AM, Livingston DH. The role of plasma granulocyte colony stimulating factor and
