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Bone Marrow Mesenchymal Stromal Cells Attenuate Organ Injury Induced by LPS and Burn

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Abstract

Bone marrow mesenchymal stromal cells (MSCs) suppress immune cell responses and have beneficial effects in various inflammatory-related immune disorders. A therapeutic modality for systemic inflammation and its consequences is not available yet. Thus, this work investigates the therapeutic effects of MSCs in injury-models induced by Lipopolysaccharide (LPS) or burn. Gene expression was analyzed in MSCs when exposed to inflammatory serum from injured animals and it showed remarkable alterations compared to normal culture. In addition, injured animals were transplanted intramuscularly with MSCs. Forty eight hours after cell transplantation, kidney, lung, and liver were analyzed for infiltration of inflammatory cells and TUNEL expressing cells. Results showed that MSCs attenuate injury by reducing the infiltration of inflammatory cells in various target organs and by reducing cell death. These data suggest that MSCs emerge as key regulators of immune/inflammatory responses *in vivo* and as attractive candidates for cell-based treatments for systemic inflammatory-based disorders.

Keywords

anti-inflammation; anti-apoptosis; sepsis; burn; lipopolysaccharide (LPS); systemic inflammatory response syndrome (SIRS); and multiple organ injury

Introduction

Trauma, sepsis and burn related syndromes are the fifth leading cause of death for all age groups, ranking just behind heart diseases, cancer, stroke and chronic diseases (20). All these disorders have been identified to have a severe inflammatory response to injury (10,20). Over one third of all in-hospital patients, including more than 50% of all Intensive care units (ICU) patients, and greater than 80% of surgical patients show a certain degree of systemic inflammation. Although systemic inflammation is a normal response to injury, it may facilitate the development of serious complications added to the underlying injury (19,29). Even with improved treatments of trauma and critical care patients, and the reduction in mortality in those

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patients that develop multiorgan dysfunction syndrome (MODS), there are still >50% of patients with MODS who die (3,8,39).

There have been many clinical attempts to control the inflammatory response using specific anti-cytokine or anti-mediator therapies (9,12). Recently, MSCs have been studied in several clinical settings of immune/inflammatory disease, which includes graft versus host disease (GVHD) (28), ischemic heart disease (17), ischemic kidney injury (27), type 2 diabetes mellitus (14), ischemic tissues (4) and Crohn disease (15,30,31). Our group has described the immunomodulatory functions of MSCs and their secreted molecules in immune-related animal models of acute liver failure and enteropathy (35,41). These results to date indicate that the secreted bioactive molecules of MSCs provide a regenerative microenvironment for a variety of injured adult tissues which limits the area of damage and allows a self-regulated regenerative response. Since the complexity of the mechanism of fine control between immunity and tolerance, which are involved not only in preventing infection or inflammation but also in limiting collateral immune-mediated tissue damage, underlines the critical nature of immunomodulatory activity, MSCs could represent a viable innate regulatory unit by this dynamic response. Although all the immune modulators, secreted molecules, and bioactive functions are yet to be described, the data available clearly supports the concept that the paracrine function of MSCs has a therapeutic effect during sepsis, a condition characterized by a whole-body inflammatory state (32).

Here we demonstrate the therapeutic potential of MSCs in two different animal models of injury induced by LPS and 30% burn, both of which are known to initiate an increase in the proinflammatory state with multiple organ injury (21,22,24). We show the positive impact of the transplantation of MSCs by histological alterations in multiple vital organs and with the up-regulation of anti-inflammatory and anti-apoptotic gene expression in MSCs exposed to inflammatory conditions.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 270–320g (Charles River Laboratories, Boston, MA) were used for burn and sepsis induction. The animals were cared for in accordance with the guidelines set forth by the Committee on Laboratory Resources, National Institutes of Health, and Subcommittee on Research Animal Care and Laboratory Animal Resources of Massachusetts General Hospital. All animals had free access to food and water, both before and after the operation.

MSC Culture

Human MSCs were kindly provided by the Tulane Center for Gene Therapy (New Orleans, LA). MSCs were cultured and characterized for surface marker expression and adipocytic and osteogenic differentiation capacity as previously reported (1). Cells were used for experiments during passages 3–7. To quantify the gene expression of the activated MSCs, 0.5×10^6 MSCs were cultured 24 hours in 10% bovine serum using 6 well plates and after washing by phosphate buffered saline (PBS) cells were exposed to 10% serum from burn (burn-derived serum) or LPS (LPS-derived serum) induced rats as described below. After culturing in the serum for 24 hours, medium was collected and stored at -80°C until analyzed by PCR.

Quantitative Real Time RT-PCR

Quantitative real time RT-PCR was performed in Strategene MXPro 3005p (La Jolla, CA) using human NF κ B signaling pathway PCR arrays from SABiosciences (Frederick, MD) following manufacturer's recommendations.

Burn Induction

Each rat received a 30% total body surface area (TBSA) full-thickness scald burn under general anesthesia (intra-peritoneal injections of ketamine and xylazine at 110 and 0.4 mg/kg body weight) and analgesia (buprenorphine 1 mg/kg body weight) following a modified procedure as previously described (38). Rats were anesthetized, shaved, and received a 30% TBSA scald burn (100°C water; water contact 10 seconds to the back). After thermal injury, rats were immediately resuscitated by intraperitoneal injection of Ringer's lactate (50 ml/kg body weight).

LPS Induction

Lipopolysaccharide (LPS: extracted from *Escherichia coli* 0111: B4) was purchased from Sigma (St. Louis, MO). To induce endotoxemia in animal 10mg/kg of LPS was injected intra-peritoneum.

MSC transplantation

2×10^6 of MSCs were suspended in 500 μ l of PBS with 2.5mg/ml of rat type I collagen (Trevigen, Gaithersburg, MD) and administered to the left thigh muscle of the animals immediately after the induction of burn or LPS.

Serum Collection from Burn and LPS induced Animals for Cell Culture Study

Rat's carotid arteries were cannulated 24 hours before treating under general anesthesia (intra-peritoneal injections of ketamine and xylazine at 110 and 0.4 mg/kg body weight) prior to the transplantation of MSC. Serum of injured animals was collected at 12 hours and 24 hours after the induction, blood was drawn from carotid artery and the serum was stored at -80°C until used for cell culture studies.

Histological studies

Kidney, lung and liver from each experimental group (control burn rats, control LPS rats, MSC treated burn rats and MSC treated LPS rats) were collected at 24 hours after the induction of burn or LPS. Formalin-fixed, paraffin-embedded liver samples were sectioned at 4 μm thickness and stained with hematoxylin-eosin (H&E). For terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining of the liver, we used the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) according to the vendor's instructions. Quantification of cell numbers in stained tissue sections was performed in 10 random 40 $\times$ images per animal using the public software ImageJ (<http://rsb.info.nih.gov/ij/>). Quantitative evaluation of the kidney injury was performed based on histological parameters; tubular necrosis, interstitial edema, loss of brush border, and casts formation. The scoring system used was 0, absent; 1, present; and 2, marked as described previously (40). Quantitative evaluation of the lung injury was performed via a grading system by inflammatory cell infiltration, interstitial edema, intra-alveolar edema, and alveolar integrity as described previously (11). Inflammatory cell infiltration in liver samples was quantified by counting mononuclear inflammatory cells in portal and peri-portal areas. TUNEL-positive cells were quantified using appropriate criteria for a specific threshold of staining intensity as well as corresponding sizes of the nuclei. Blind analysis of the histological samples was performed by two independents.

Statistical Analysis

Data represent the mean of each experiment $\pm$ SD. Statistical significance was determined by a student's t-test analysis, in which each value was compared with the control values performed with Prism (GraphPad Software, La Jolla, CA).

Results

MSCs Alter Gene Expression in Response to Inflammatory Serum Stimulation

To determine if MSCs can be activated and alter dynamically their gene expression, we first studied the reciprocal response of MSCs to inflammatory signals found in the serum of injured animals. To this end, we performed quantitative RT-PCR for four genes in MSCs after 24 hours of stimulation with LPS-derived serum or burn-derived serum, namely, interleukin (IL)-10, V-akt murine thymoma viral oncogene homolog 1 (Akt1), Mitogen-activated protein kinase kinase 1 (MAP3K1) and V-raf-1 murine leukemia viral oncogene homolog 1 (RAF1). Although IL-10 is one of the key factors of the anti-inflammatory potential of MSCs, its production by MSCs is controversial (33,36). Gene expression analysis showed approximately 3-fold higher gene expression of IL-10 in MSCs, which were exposed to either inflammatory serum, compared to normal culture control (Figure 1A). Akt1 is also known as an important regulator of cell survival (5) and indeed MSCs overexpressing Akt1 have been successfully administered in ischemic heart disease (17). Moreover, MSCs showed up-regulation of Akt1, when exposed to the inflammatory conditions (Figure 1B). Two other genes, which also play important roles in cell survival and are relate to IL-10 signaling pathway (23,26), were similarly up-regulated in MSCs when exposed to inflammatory conditions cause by LPS, however MAP3K1, was down-regulated in MSCs when exposed to burn-derived serum (Figure 1C, D). These findings clearly demonstrate that MSCs can alter their gene expression profile, associated with inflammation and cell survival, once they are stimulated with inflammatory signals.

Intramuscular Transplantation of MSCs Improves Serology and Organ Injury in Endotoxemic Rats

Although many reports have described the therapeutic potential of MSC transplantation in different clinical settings (17,28,34), only a few have shown their capacities in inflammatory-derived injuries (32). Based on our previous studies (36,42) and gene expression profiling, we hypothesized that MSCs can attenuate organ injury via secretion of anti-inflammatory and anti-apoptotic molecules when they are activated by an inflammatory environment. To evaluate the systemic effect of transplanted MSCs in multiple organs, we sampled three vital organs at 24 hours after the transplantation, namely, lung, kidney and liver, which are known to present alterations after exposure to LPS (6).

First we administered MSCs in endotoxemic animals using LPS-injection. Kidney injury after treatment was quantified by serum BUN level (Figure 2A), inflammatory scoring (Figure 2B, C, D, E) and counting of apoptotic cell numbers via TUNEL positive cells (Figure 2F, G). BUN levels were significantly reduced by MSC treatment (35.5 ± 7.64 mg/dl) compared to control animals (50.2 ± 7.82 mg/dl; $P=0.0158$) (Figure 2A). Histological analysis demonstrated a significant reduction of tissue injury ($P<0.0001$) (Figure 2B) with less apoptotic cells ($P=0.0009$) (Figure 2F) in MSC treated animals compared to controls. Lung injury was also quantified by inflammatory scoring and counting of apoptotic cell numbers. Histological analysis demonstrated a significant reduction of tissue injury ($P=0.0002$) (Figure 2B, D) with less apoptotic cells ($P=0.0001$) (Figure 2G) in MSC treated animals compared to controls. Histological analysis of liver sections showed significantly less inflammatory cell infiltration (20.67 ± 7.81 /field of view) compared to control animals (80 ± 30.25 /field of view; $P=0.0009$) (Figure 2B, E). These histological appearances are consistent with the decreased serological levels of AST in animals treated with MSCs (Figure 2H). These results indicate that transplantation of MSCs could effectively reduce inflammatory infiltration and cell death in several organs after LPS induced-injury in rats.

Transplantation of MSCs Improves Serology and Organ Injury in Burned Rats

Burn injury is also known to cause organ injury subsequent to a state of general inflammation (21). To determine if MSCs have a therapeutic effect in organ injury caused by burn injury, we administered MSCs to burn-induced rats and analyzed their serological and histological appearances.

Kidney injury after treatment was quantified by serum BUN level (Figure 3A), inflammatory scoring (Figure 3B, C, D, E) and counting of apoptotic cell numbers via TUNEL positive cells (Figure 3F, G, H, I). The BUN level was significantly reduced by MSC treatment (26 ± 7.07 mg/dl) compared to control animals (63.8 ± 16.9 mg/dl; $P=0.0043$) (Figure 3A). Histological analysis demonstrated the significant reduction of tissue injury ($P<0.0001$) (Figure 3B, C, D, E) with less apoptotic cells ($P<0.0001$) (Figure 3F, G, H, I) in MSC treated animals compared to controls. These results of reduced kidney injury were more apparent than those observed in LPS treated rats. Lung injury was also quantified by inflammatory scoring and counting of apoptotic cell numbers. Histological analysis demonstrated a significant reduction of tissue injury ($P<0.0001$) (Figure 3B, D) with less apoptotic cells ($P=0.0018$) (Fig. 3F, H) in MSC treated animals compared to controls, which was similar to the result in LPS-endotoxemic animals. Histological analysis of liver sections showed significantly less inflammatory cell infiltration (13.17 ± 6.74 /field of view) compared to control animals (38.83 ± 14.26 /field of view; $P=0.0026$) (Fig. 3B, E). However these histological results did not lead to a significant decrease in serological levels of AST in animals treated with MSCs (Fig. 3J). These results confirm that MSC could effectively reduce inflammatory infiltration and cell death in multiple organs after burn injury, however the targeted organ was slightly different in burn animals compared to LPS-endotoxemic animals.

Discussion

This study demonstrates that transplantation of MSCs can attenuate the effects of systemic inflammation and organ injury in two different animal models of injury. This therapeutic effect was observed in all three vital organs in LPS-endotoxemic animals and particularly in kidney and lung in burn-induced animals, demonstrating the anti-inflammatory and anti-apoptotic effects of MSCs. These results suggested that the effect of MSCs on each disease was slightly different depending on the injury induced, which is supported by previous reports that have described different mechanisms of injury caused by LPS or burn injury (13,25). Although the mechanism of injury is different in each disease, the MSCs provided a significant therapeutic benefit at the same critical time point. This outcome observed in these two different animal models demonstrating a fine adjustment of MSCs to the surrounding environment *in vivo* is significant to investigate a clinically feasible modality for the treatment of injuries that result in severe systemic inflammation or even MODS.

The result of gene expression analysis supports the hypothesis that MSCs act as dynamic cells (42) that can be activated by an inflammatory environment and alter their characteristics (genome and secretome) while providing immunomodulation. These results would facilitate the characterization of the dynamics, duration and trajectory of the systemic inflammatory response and would permit the identification of possible targets for therapies and possible predictive biomarkers.

IL-10 is one of the key cytokines with anti-inflammatory capacities and it has been demonstrated that MSCs can affect the secretion of IL-10 in macrophages or dendritic cells (2,32). However, it is still controversial if MSCs can secrete therapeutic levels of IL-10 by themselves (33,36). Based on our current results, it's possible that the differences in IL-10 expression seen in these reports is due to differences in the local cellular environment (i.e., level of inflammation) surrounding the MSCs.

It has been reported that genetically engineered MSCs expressing Akt could induce an anti-apoptotic effect to cells via subsequent signal transduction in an ischemic heart disease model (16). Our results support the notion that the activation of Akt1 signaling pathway of transplanted MSCs can provide an anti-apoptotic effect in a clinical setting of systemic inflammation. In addition, kidney injury in severe burn, which was seen in our experiment, has been reported to be controlled by Mitogen-activated protein kinase (MAPK) signaling pathway (25). Taken together, these alterations of gene expression would provide a new strategy to define an important therapeutic target of MSC treatment. Although MSCs are known to have immunosuppressive properties affecting T-cells (7) or dendritic cells (31) via secretion of numerous molecules which include various cytokines and chemokines (37), there are few reports describing their therapeutic effect on inflammation (18,32).

In conclusion, we have demonstrated that transplantation of MSCs has a therapeutic benefit in LPS and burn-injured animals by providing anti-inflammatory and anti-apoptotic effects. In addition, these stem cells can genetically react to inflammatory conditions. The acquisition of a mechanistic knowledge is critical to the development of improved therapeutic and predictive clinical outcome strategies caused by different injuries. Moreover, these results would be of considerable importance for the development of novel and much needed approaches to predict and treat injury effectively before critical complications develop.

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Figure 1. Alteration of gene expression in MSCs exposed to inflammatory serum

Relative mRNA expression in 0.5×10^6 MSCs of each experimental group. (A) IL-10, (B) Akt1, (C) MAP3K1 and (D) Raf1. Control (cultured in 10% bovine serum), Burn (cultured in 10% burn-derived serum) and LPS (cultured in 10% LPS-derived serum).

Figure 2. Serological and histological appearance of MSC treated animals after LPS injection
 Serological result of (A) BUN in MSC transplanted rats after LPS injection, while rats without cell transplantation after LPS injection served as a control. (B) H&E staining of tissues from control group; kidney, lung and liver, and from MSC transplanted group. (C) Kidney injury score, (D) lung injury score and (E) liver inflammatory cell infiltration was evaluated based on H&E staining. (F) TUNEL staining of tissues from control group; kidney, lung and liver, and from MSC transplanted group. (G) TUNEL positive cell count was performed for each organ. (H) Serological result of AST in MSC transplanted rats after LPS injection. Scale bars represent 100 μm. *P<0.05

Figure 3. Serological and histological appearance of MSC treated animals after burn induction
 Serological result of (A) BUN in MSC transplanted rats after LPS induction, while rats without cell transplantation after burn induction served as a control. (B) H&E staining of tissues from control group; kidney, lung and liver, and from MSC transplanted group. (C) Kidney injury score, (D) lung injury score and (E) liver inflammatory cell infiltration was evaluated based on H&E staining. (F) TUNEL staining of tissues from control group; kidney, lung and liver, and from MSC transplanted group. TUNEL positive cell count was performed for each organ; (G) kidney, (H) lung and (I) liver. (J) Serological result of AST in MSC transplanted rats after LPS injection. Scale bars represent 100 μ m. * $P<0.05$