Human Bone Marrow Derived Mesenchymal Stem Cells Regulate Leukocyte-Endothelial Interactions and Activation of Transcription Factor NF-Kappa B

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Abstract

Bone marrow derived mesenchymal stem cells (MSCs) have been shown to demonstrate benefit in multiple disease models characterized by inflammation such as sepsis and acute lung injury. Mechanistically we hypothesized that MSCs exhibit these properties through inhibition of leukocyte activation and modulation of leukocyte-endothelial interactions; key interlinked processes involved in the deleterious effects of injury and inflammation. In this paper we found that MSCs co-cultured with a monocytoid line, U937, inhibit U937 binding to pulmonary endothelial cells (PECs) stimulated with the inflammatory cytokine TNFα. Furthermore, we show that these effects on functional adhesion are not due to changes in inflammatory adhesion molecule expression on U937s. No changes were found in CD62L, CD29, CD11b and CD18 expression on U937s co-cultured with MSCs. To determine if the effects of MSCs on leukocyte-endothelial interactions are due to the effects of MSCs on leukocyte activation, we investigated whether MSCs affect functional activation of the transcription factor NF-Kappa B. We found that MSCs significantly inhibit transcriptional activation of NF-kappa B in U937s. We also found that MSCs inhibit DNA binding of NF-kappa B subunits p50 and p65 to putative NF-kappa B DNA binding sites. Concomitant with a decrease in NF-kappa B activation was a significant increase in IL-10, an anti-inflammatory cytokine known to inhibit activation of NF-kappa B. Taken together, these findings show that MSCs have potent effects on leukocyte-endothelial interactions which may be due to the direct effects of MSCs on IL-10 and NF-kB. These findings suggest a potential therapeutic role for MSCs in diseases characterized by inflammation such as acute lung injury or multi-organ failure induced by traumatic injury.

Keywords: Acute lung injury; Progenitor cells; Inflammation

Abbreviations: MSCs: Mesenchymal stem cells; PECs: pulmonary endothelial cells; TBI: traumatic brain injury; ALI: Acute Lung Injury; ARDS: Acute Respiratory Distress Syndrome; PBS: Fetal bovine serum; PMA: phorbol myristate acetate

Introduction

Human mesenchymal stem cells (MSCs) are pluripotent stromal cells that have demonstrated benefit when infused in multiple preclinical models of inflammation and injury, such as myocardial infarction, stroke, traumatic brain injury and lung injury [1-5]. Many of these studies have shown potent systemic and local effects of MSCs. Our past work has shown that MSCs have potent stabilizing effects on the vascular endothelium when administered intravenously after traumatic brain injury [5]. In these studies we found that MSCs inhibit blood brain barrier permeability when administered after TBI, which appears to be due to the presence of soluble factor(s) produced by MSC administration. In addition to these stabilizing effects on the vascular endothelium, a number of groups have shown that MSCs have potent anti-inflammatory effects in models of disease characterized by high levels of inflammation such as Acute Lung Injury and Acute Respiratory Distress Syndrome (ALI and ARDS) [6-8]. Utilizing a mouse model of sepsis and ALI, Nemeth et al. have shown that MSCs improved survival through modulation of the innate immune system, specifically by modulating the release of FGE2 from lung macrophages leading to increased production of the anti-inflammatory cytokine IL-10. [7,9]. MSCs have also been shown to modulate immunological responses via T-cell suppression [10-12]. To date MSCs have been investigated as a new therapeutic strategy for T cell-mediated diseases such as graft-versus-host disease (GVHD), Crohn’s disease, and the prevention of organ transplantation rejection [13]. In all of these disease contexts, limited numbers of MSCs have been observed to migrate to injured sites after systemic administration, but primarily most of the cells administered intravenously become lodged through a first-pass effect in the lung, liver or spleen regardless of the disease model [14,15]. Tissue-specific engraftment is not required and this aspect of MSC therapy in disease suggests a systemic effect of MSCs possibly produced by soluble factors.

Considering these data demonstrating an anti-inflammatory therapeutic effect of MSCs, we sought to find out more about their putative mechanisms of action. We hypothesized that MSCs regulate leukocyte activation and interaction with vascular endothelium, two processes that are highly interlinked and are key mediators of tissue injury in a number of disease states. To investigate these mechanisms of action, we designed in vitro experiments to study the effects of MSCs on leukocyte-endothelial interactions and leukocyte activation.

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Our findings do indeed suggest that MSCs inhibit inflammation and leukocyte-endothelial interactions through direct interactions with leukocytes.

### Methods

**Primary cells and cell lines:** First passage human MSCs and pulmonary endothelial cells (PECs) were purchased from Lonza (Walkersville, MD). MSCs were cultured in MSC growth media (MSC-GM, Lonza); PECs were maintained in EGM-2MV media from Lonza. MSCs were used at passage 3–7 for all experiments. U937, a monocytoid immortalized cell line, were obtained from ATCC (Bethesda, MD) and passaged in RPMI 1640 with 10% fetal bovine serum. All cell lines were maintained at 37°C and 5% CO₂.

**Leukocyte binding assay:** PECs were grown on 96 well plates. Approximately, 1 x 10⁵ cells/well were seeded and incubated at 37°C for 24 hours or until confluent. U937s were serum starved for 24 hours in RPMI 1640 with 1% FBS before culture in the following groups: (1) U937s alone, and (2) U937s + MSCs (1:10 ratio) in direct contact. Adhesion molecule expression on PECs was stimulated by the addition of TNFα (50ng/ml). Co-cultures were incubated at 37°C for 16 hours. U937s were stimulated with phorbol myristate acetate (PMA, 200ng/ml) for 20 minutes and then removed from co-cultures. Cells were labeled with Calcein-AM (Invitrogen, Carlsbad, CA), added to wells and allowed to adhere for one hour. Non-adherent cells were gently washed away in PBS and labeled cells that remained were quantified by fluorescent reading on the Biotek Synergy II (Biotek, Winooski, VT) at 490nm wavelength excitation and 520nm emission.

**Flow cytometry:** MSCs were characterized by flow cytometry as described previously [15]. U937s were characterized for surface adhesion markers and integrins by flow cytometry (BD Facs Calibur, BD Biosciences, San Jose, CA). U937 cells were cultured in 6 well plates for 24 hours in 2 groups: (1) U937s alone, and (2) U937s + MSCs (10:1 ratio with replicates of 4/group). The U937s were collected and subsequently treated with PMA (200ng/ml). After 20 minute incubation, the cells were collected and stained with fluorochrome conjugated antibodies to CD11b, CD18, CD29, and CD62L (BD Biosciences). The cells were then analyzed on the BD LSR II flow cytometer (BD Biosciences). Expression of adhesion molecules was quantified by mean fluorescence intensity using the FlowJo Program software (Ashland, OR).

**Nuclear NF-kappa B DNA binding activity:** Nuclear extracts were prepared using the Nuclear Extract Kit from Active Motif (Carlsbad, CA) according to the manufacturer’s protocol. Protein concentration was determined by Bradford assay (Pierce Biochemicals). p50 and p65 DNA binding was quantified by Trans-AM NF-kappa B DNA binding ELISA from Active Motif (Carlsbad, CA), according to the manufacturer’s protocol. Nuclear extracts (2-5µg) were combined with a standardized biotinylated oligonucleotide and plated on to a streptavidin coated plate and incubated for one hour at room temperature. The plate was then washed three times with wash buffer and antibodies to NF-kappa B p50 and p50 subunits were added and incubated for one hour. HRP conjugated secondary antibody was then added and incubated for one hour. The plate was then developed and read on a 96 well plate spectrophotometer at 450nm with reference wavelength of 655nm.

**Leukocyte NF-kappa B reporter gene assay:** U937s were co-transfected with NF-kappa B cis-Reporter plasmid (1 µg/5 x 10⁴ cells, Agilent Technologies, Santa Clara, CA) and CMV-Renilla luciferase plasmid (Promega Madison, WI) using the Amaxa electroporation system (Walkersville, MD), according to the manufacturer’s protocol. The cells were then cultured in RPMI-1640 with 10% FBS for 16 hours. U937s were then collected, pooled, counted and plated in 6 well plates either with or without MSCs. The cells were then incubated at 37°C overnight. In the morning either lipopolysaccharide (LPS, 5µg/ml) or PBS (vehicle) was added to the dish. The cells were incubated again at 37°C for 4 hours

**Flow cytometric cytokine bead assay:** U937s were cultured either alone in varying concentrations (1.5 x 10⁵/mL, 3.75 x 10⁵/mL, and 5 x 10⁶), or with MSCs in varying ratios (1:2, 1:5 and 1:10) in 6 well plates for 12 hours. LPS (5µg/mL) or vehicle was then added to the plates to increase the baseline of NF-kappa B. A hour after incubation the supernatants were collected. Cytokines (TNF, IL-1β, IL-6, IL-8, and IL-10) were quantified by flow cytometry by following the manufacturer’s protocol for the human inflammatory cytokines bead assay kit (BD Biosciences Cat. #551811, Franklin Lakes, NJ).

### Statistical analysis

Statistical analysis was performed using Microsoft Excel and Stata 11 (College Station, TX). Analysis was performed by Student’s t-test or one-way ANOVA with post-hoc Bonferroni correction, as needed.

### Results

MSCs inhibit adhesion of U937s to pulmonary endothelial cells (PECs)

Leukocyte adhesion to endothelium is a critical component in the development of an inflammatory response to injury. Injured vasculature and activated leukocytes often upregulate expression of adhesion molecules that mediate binding between the two cell types. Multiple factors are responsible for the compromise of the endothelial barrier in injury. The lungs are often one of the most sensitive organs to inflammatory insults and compromise of the alveolar–capillary barrier is found early after the initial insult whether it is trauma or infection [16]. In addition to factors such as hypoxia and thrombin, inflammatory changes caused by cytokine and chemokine release, leukocyte adhesion, diapedesis and infiltration into the lungs have all been shown to contribute to the clinical severity and outcome in ALI/ARDS [3]. Taking this into account and the demonstrated predominance of IV-administered MSCs in the pulmonary vasculature, we chose to focus our studies upon the effects of MSCs on pulmonary endothelial cells (PECs). We sought to determine if MSCs modulate leukocyte binding to PECs stimulated with the inflammatory cytokatic TNFα. In these studies, U937s were co-cultured with MSCs for 24 hours followed by stimulation with PMA, to stimulate calcium dependent binding (see schematic Figure 1A). PECs were treated with TNFα (10ng/ml) and binding of calcein-labeled cells to treated PECs was quantified by flowimetry Figure 1B. U937 cells are a monocytoid line that we have previously used to study leukocyte-endothelial adhesion [17,18]. Binding studies reveal that PMA stimulated U937-PEC binding and U937s co-cultured with MSCs demonstrate significantly decreased binding to PECs Figure 1B. Representative photographs of calcein-labelled U937s binding to PECs are depicted in Figure 1C. These results suggest that MSCs do indeed modulate leukocyte endothelial cell interactions.

MSCs do not affect U937 adhesion molecule expression

To determine if the binding differences noted were due to changes...
in adhesion molecule expression on the surface of the U937 cells, we analyzed the expression of CD62L, CD29, CD11b and CD18. These adhesion molecules and integrins mediate binding of leukocytes to endothelial cells and the surrounding extracellular matrix. CD11b is a component of LFA-1 which binds to ICAM-1 on endothelial cells and CD18 is a component of VLA-4 which binds to VCAM-1 on endothelial cells. In these studies, U937s were co-cultured with MSCs for 24 hours followed by stimulation with PMA for 20 minutes (See schematic in Figure 2A). Bright field microscopy in Figure 2B shows culture of the U937 cells alone and Figure 2C shows a representative co-culture of the two cell types where the adherent MSCs are apparent in the background with adherent U937s in the foreground. Flow cytometric analysis of the U937 cells after co-culture reveals that there are no significant differences in CD62L, CD29, CD11b and CD18 expression on the U937s. Figure 2D shows no significant changes in mean fluorescent intensity (n=4) for each of the representative markers. Figure 3 shows the lack of a change or shift in both groups in histogram form for each of the surface markers.

MSCs inhibit NF-kappa B DNA binding

Since we did not find any changes in leukocyte adhesion molecules with MSC treatment, we speculated that the potent differences found in leukocyte-endothelial binding may be due to an inhibition of leukocyte activation by MSCs. We chose to look at the effects of MSCs on the transcription factor NF-kappa B, a factor that has been extensively characterized in multiple disease models and has been shown to be involved in the regulation of inflammatory processes in disease [19-22]. Our past work has also shown that MSCs do in fact inhibit NF-kappa B in Kaposi’s Sarcoma cells through modulation of the kinase Akt [15]. Using NF-kappa B activation as a surrogate readout for leukocyte activation, we found that nuclear extracts from U937s co-cultured with MSCs demonstrate decreased NF-kappa B DNA binding activity determined by a DNA binding ELISA for p50 and p65 activity (see materials and methods). We found that NF-kappa B subunit p50 was significantly less active in nuclear extracts from U937s co-cultured with MSCs compared to nuclear extracts from U937s stimulated with PMA alone or U937s co-cultured with PECs as a control cell (0.63 ± 0.03 vs. 0.92 ± 0.14 and 0.63 ± 0.03 vs. 1.7 ± 0.3, respectively, p=0.05, Table 1). DNA binding of the p65 subunit of NF-kappa B was also significantly inhibited in U937s cultured with MSCs versus U937s stimulated with PMA or U937s co-cultured with PECs as a control cell (0.34 ± 0.06 vs. 0.55 ± 0.01 and 0.34 ± 0.06 vs. 0.793 ± 0.07, respectively, p<0.05, Table 1).

MSCs inhibit NF-kappa B transcriptional activation in U937s

Since our data suggest that MSCs inhibit activation of NF-kappa B in U937s, we sought to determine if our findings with NF-kappa B DNA binding would translate into a functional decrease in NF-kappa B mediated transcription. U937s were transfected with a reporter plasmid containing five NF-kappa B DNA binding sites upstream

MSCs inhibit NF-kappa B DNA binding
of a firefly luciferase reporter gene. All cells were also co-transfected with a control Renilla luciferase plasmid (see schematic Figure 4A). All U937 cells were simulated with LPS to increase the baseline of NF-kappa B activation. Data from the reporter gene assay showed that LPS significantly increased nuclear NF-kappa B in U937 cells cultured alone (0.075 ± 0.002 vs. 0.003 ± 0.002, Figure 4B). U937 cells co-cultured with MSCs demonstrated decreased NF-kappa B transcriptional activation (0.050 ± 0.001, p<0.05 Figure 4B). Taken together these data do indeed suggest that MSCs inhibit NF-kappa B DNA binding and transcriptional activation, possibly providing an explanation for our noted effect of MSCs on leukocyte-endothelial interactions.

MSCs induce production of the anti-inflammatory cytokine IL-10

To better understand the mechanism of action of MSCs on the inhibition of NF-kappa B we sought to determine if MSCs modulate the expression of factors that may regulate NF-kappa B. IL-10 has been shown to be a key mediator and regulator of the anti-inflammatory effects of MSCs in multiple disease models [7]. IL-10 has also been clearly shown to regulate the activation of NF-kappa B in inflammatory cells [23]. Using a flow cytometric bead based assay, supernatants from co-cultured cells were analyzed for the production of IL-10. These studies reveal that the MSCs and unstimulated U937s do not produce detectable levels of IL-10, but when co-cultured, there is a significant increase in IL-10 (7.5 ± 0.53pg/mL vs. 0 ± 0 pg/mL, p<0.01, Figure 5), at both 1:2 and 1:5 ratios of MSCs to U937s. Taken together these data suggest that MSCs do increase production of the anti-inflammatory cytokine IL-10, which may contribute to the noted inhibition of NF-kappa B and leukocyte endothelial interactions by MSCs.

Discussion

MSCs have demonstrated benefit in preclinical and clinical models of injury and inflammation. Clinical trials in multiple inflammatory conditions, including graft versus host disease (GVHD), and Crohn’s...
colitis, have demonstrated benefit, with many trials currently still underway [24]. In preclinical models of disease, MSCs have been shown to have potent effects in many models including ALI/ARDS, arthritis, acute hepatic failure, transplant, and diabetes [6,25-29]. Nemeth et al., have shown increased survival associated with MSC therapy in sepsis [7]. While this group and other investigators have examined the immunomodulatory effects of MSCs on inflammatory cells, few have investigated the effects in relation to the vascular endothelium. The stem cell niche and the interactions between cells created by the therapeutic delivery of MSCs are still wholly unknown. In this paper we sought to better understand a defined interaction between MSCs and inflammatory cells and how this interaction affects leukocyte-endothelial cell binding. Since MSCs are often administered for therapeutic purposes intravenously, one of the main cell types they come into contact with is inflammatory cells in the pulmonary vasculature [14]. Our studies aimed to recapitulate some of these interactions in vitro, to better understand mechanistically the anti-inflammatory effects of MSCs.

In this study we show that inflammatory cells (U937s) co-cultured with MSCs display diminished potential to bind to pulmonary endothelial cells. Since we did not see large differences in leukocyte adhesion markers with MSC treatment, we hypothesized that MSCs may be affecting leukocyte-endothelial adhesion through inhibitory effects on leukocyte activation. To study this further, we utilized NF-kappa B activation as a surrogate marker for leukocyte activation. We used two methods to show that MSCs decrease NF-kappa B activity in U937s; DNA binding and transcriptional activation by NF-kappa B. Co-culture of U937s with MSCs significantly diminished both measures of NF-kappa B activity. The role of NF-kappa B on inflammation and injury has been well documented throughout the years. Investigators in Germany isolated neutrophils from severely injured trauma patients and found significantly increased levels of NF-kappa B translocation to the nucleus compared to healthy volunteers [6,30]. Similarly, researchers utilizing an animal model of trauma and sepsis found increases in NF-kappa B activation in macrophages compared to sham. By inhibiting NF-kappa B, the authors were able to demonstrate decreased activation of alveolar macrophages and increased therapeutic benefit [20]. These data as a whole suggest an important role for NF-kappa B in inflammatory disease outcome. It is of interest to note that regardless of changes in leukocyte adhesion markers, there are changes in MSC treated U937 that diminish binding. This data suggests that non-specific changes U937 may be taking place that alter adhesion such as changes in conformation or matrix interactions. Further study in this area is warranted.

To determine if IL-10 may play a role in the effects of MSCs on NF-kappa B, we investigated if IL-10 production was increased in our co-cultures. We indeed found that IL-10 was significantly increased in U937s by co-culture with MSCs suggesting a possible paracrine mechanism of action for the inhibition of NF-Kappa B in leukocytes by MSCs. Interestingly, the influence of MSCs on IL-10 production has been well documented in many models in vitro and in vivo and has been suggested by multiple groups to be a key source of the therapeutic
benefit of MSCs [3,6,7,31]. A number of studies have also shown that MSCs function through soluble factors that produce a local paracrine and systemic effect [32-35]. There are many candidate factors that have been proposed as being critical to the therapeutic effects of MSCs. Our data in traumatically brain injury (TBI) support this view and have shown that intravenously delivered MSCs potently inhibit blood brain barrier permeability, without many cells present in the injured brain, suggesting the presence of soluble factors. These data and the data of others support the view that MSCs modulate inflammation and vascular stability through systemic, rather than local effects [14,36]. Previous work by our group has demonstrated that MSCs stabilize the endothelium and prevent disruption of the blood brain barrier through regulation of endothelial cadherin/beta-catenin signaling [5]. This effect combined with our data demonstrates a robust influence of others support the view that MSCs modulate inflammation and systemic effects [35].

In summary, we have shown that MSCs decrease leukocyte-endothelial binding and NF-kappa B activation in inflammatory cells. Our data indicates that MSCs decrease leukocyte activation in response to stimulus which may be the explanation for the diminished binding to PECs. Decreases in NF-kappa B activity may be due to increased IL-10 production by monocytes and macrophages, resulting in an overall decrease in the inflammatory state. MSCs remain an important area for translational study in treating the immunological response to sepsis, and other inflammatory conditions.

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