Research Report

Nitric oxide-mediated immunosuppressive effect of human amniotic membrane-derived mesenchymal stem cells on the viability and migration of microglia

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Abstract

Human amniotic membrane-derived mesenchymal stem cells (AMSCs) are considered a novel and promising source of stem cells for cell replacement-based therapy. Current research is mostly limited to investigating the cellular differentiation potential of AMSCs, while few have focused on their immunosuppressive properties. This study is aimed at exploring and evaluating the immunosuppressive effect of human AMSCs on the viability and migratory properties of microglia. We found, from results of cell viability assays, that AMSCs can reduce the activity of inflammatory cells by secreting nitric oxide (NO). Also, based on results from wound healing and transwell migration assays, we show that AMSCs can inhibit the migration of human microglia as well as the mouse microglial cell line BV2, suggesting that they have the ability to inhibit the recruitment of certain immune cells to injury sites. Furthermore, we found that NO contributes significantly to this inhibitory effect.

Abbreviations: AMSCs, amniotic membrane-derived mesenchymal stem cells; NO, nitric oxide; CNS, central nervous system; MSCs, mesenchymal stem cells; MHC, major histocompatibility complex; HLA, human leukocyte antigen; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; CM, conditioned medium; AMSC-CM, AMSCs conditioned medium; NOS, nitric oxide synthase; SMT, S-methylisothiourea sulfate; PGE2, prostaglandin E2; TGF-β1, transforming growth factor beta 1; STAT5, signal transducer and activator of transcription 5; PBS, phosphate-buffered saline; DMEM/F-12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; FBS, fetal bovine serum; CCK-8, Cell Counting Kit-8; OD, optical density

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1. Introduction

Mesenchymal stem cells (MSCs) are a promising resource for cell replacement therapy-based clinical applications. They can be isolated from many adult tissues, including bone marrow, adipose tissue, placenta, amnion and cord blood (Shi et al., 2012). However, compared to MSCs derived from adult sources, AMSCs have more useful properties owing to their derivation from an early embryological stage. Their differentiation potential is less restricted, and they display low levels of expression of major histocompatibility complex (MHC) antigens (Miki and Strom, 2006; Manuelpillai et al., 2011). Several studies have reported that AMSCs can differentiate into neurons, cardiomyocytes, alveolar epithelium and pancreatic b-islet cells after transplantation, and can secrete proteins normally produced by hepatocytes (Parolini et al., 2010). AMSCs have been used in the treatment of acute chemical and thermal eye burns, pulmonary fibrosis, critical limb ischemia, inflammatory bowel disease, cardiac ischemia, and liver-based metabolic diseases, without any adverse side-effects (Parolini et al., 2010; Zhao et al., 2005; Meller et al., 2000). Another property of AMSCs that make them an attractive option for potential stem cell-based therapies is their low antigenicity. AMSCs express low levels of the highly polymorphic MHC class I antigens (HLA-A, HLA-B and HLA-C) but almost no MHC class II antigens (HLA-DR, HLA-DQ and HLA-DR) on their surfaces. This property is different from bone marrow-derived MSCs, that always display significant levels of MHC II antigens (Portmann-Lanz et al., 2006). Besides, MSCs are known to have the ability to modulate the function of several major immune cell types involved in alloantigen recognition and elimination, including T cells, B cells, natural killer cells, and antigen presenting cells (Shi et al., 2012).

Microglia are the major immunocompetent cells of the central nervous system (CNS), and strongly implicated in both neuroprotection as well as neurodegeneration. Under normal conditions, microglia act analogous to peripheral macrophages, as they secrete trophic factors and cytokines and remove debris and toxins from the extracellular space (Ni and Aschner, 2010). But during traumatic brain injury (TBI), or neurodegenerative diseases like Parkinson’s disease and Alzheimer’s disease, microglia are activated. These activated microglia respond to focal cerebral ischemic insults by migrating rapidly to the lesion sites, and releasing pro-inflammatory factors such as tumor necrosis factor (TNF)-a and interleukin (IL)-1β, which are neurotoxic (Burguillos et al., 2011; Lamberts et al., 2005). Activated microglia are also involved in facilitating other diseases of the CNS, including ischemia, infectious diseases, inflammatory demyelinating diseases, and neoplastic diseases (Tambuyzer et al., 2009). It has been demonstrated that blockade of microglial activation using anti-inflammatory drugs such as minocycline, can attenuate the pathology of Parkinson’s disease (Wu et al., 2002).

In this study, we have analyzed the interactions of human AMSCs with human microglia and BV2 cells (an immortalized mouse microglial cell line infected with a v-raf/v-myc oncogene carrying retrovirus J2), to determine whether the former can influence the viability and migratory properties of microglia. Additionally, we have also explored possible mechanisms underlying the immunosuppressive effect of AMSCs on microglia.

2. Results

2.1. Characterization of AMSC-morphology and cell surface markers

We observed the morphology of P3 AMSCs under the microscope. At 24 h after seeding, AMSCs formed new colonies and exhibited a typical spindle-shaped cell body (Fig. 1A). Using flow cytometry, we analyzed the expression of different cell surface molecules, CD29, CD45, CD90 and CD11b, and found that while more than 95% of P3 AMSCs express AMSC-specific markers CD29 (99.38%) and CD90 (99.85%), they lacked the expression of hematopoietic surface markers CD45 (0.73%) and CD11b (2.19%) (Fig. 1B).

2.2. AMSCs decrease the viability of human microglia and BV2 cells

Previous studies have shown that MSCs can reduce the viability of a variety of immune cells. Based on these reports, we first tested the ability of AMSCs to influence the survival of the major immune cells of the CNS – the microglia. We used conditioned medium (CM) derived from AMSCs to culture human microglia and BV2 cells, and found that the growth of both these cell types decreased progressively in a time-dependent manner. Specifically, the viability of these cells decreased by 60.64 ± 10.74% (human microglia) and 58.43 ± 9.58% (BV2 cells) after 48 h in AMSCs conditioned medium (AMSC-CM) (Fig. 2A and B). These findings suggest that the immunosuppressive function of AMSCs might be mediated via its effect on the viability of immune cells such as the microglia, most likely by secreting certain extracellular soluble factors.

2.3. AMSCs significantly decrease migration of human microglia and BV2 cells to wound site

Microglia are highly migratory cells, and are known to migrate to lesions and injury sites in vivo. Hence, we next investigated whether AMSCs can affect this important property of microglia using the wound healing assay. As shown in Fig. 3A and C, control human microglia and BV2 cells spontaneously migrated and filled 62.37 ± 10.94% and 72.78 ± 12.64% of the wounded...
region, respectively, within 24 h. In contrast, those cells that were cultured in AMSC-CM displayed a significant reduction in their migratory ability. At 24 h after scratching, the migration of human microglia and BV2 cells in the AMSC-CM group was inhibited by 20.13 ± 4.84% and 34.42 ± 5.96%, respectively. Again, AMSCs seem to secrete extracellular effector molecules that have the ability to inhibit the migration of microglial cells. Furthermore, we detected fewer microglial cells in cultures grown in AMSC-CM when compared to the control group (Fig. 3), suggesting that the small molecules secreted by AMSCs might influence the proliferation of microglia.

2.4. AMSCs reduce the average number of migrating microglial cells in transwell chambers

To further confirm the inhibitory effect of AMSCs on microglial migration, we performed the transwell assay. Microglial cells were seeded into the upper chamber, which was then placed into bottom wells containing either AMSCs (co-culture group) or no cells (control group). The migration of microglial cells from the upper to the lower surface of the membrane was assayed. Alternately, to determine if the migration of microglial cells could be affected by factors secreted by AMSCs into the extracellular medium alone, the bottom wells were filled with AMSC-CM instead of AMSCs (AMSC-CM group). As shown in Fig. 4, fewer cells of the co-culture group could pass through the membrane in comparison to cells of the control group. Interestingly, chemotactic migration toward the serum was also dramatically decreased in cells of the AMSC-CM group compared to control cells.

2.5. Nitric oxide plays an important role in mediating the inhibitory effects of AMSCs

To determine if the NO secreted by AMSCs has an inhibitory effect on the viability of microglial cells, we cultured human...
3. Discussion

In the current study, we demonstrate the immunosuppressive properties of AMSCs by showing their ability to inhibit the activity of human microglia and an immortalized mouse microglia cell line – the BV2 cells. We have shown that AMSCs significantly reduce the viability and migratory ability of human microglia and BV2 cells. To elucidate the underlying mechanism, we investigated whether the secretion of NO by AMSCs contributed to their immunosuppressive properties. We found that when the synthesis of NO is inhibited by the addition of the NOS inhibitor SMT into the culture medium, the human microglia and BV2 cells cultured in AMSC-CM showed significantly better viability and migratory ability. This result provides evidence that NO contributes to the inhibitory effect of AMSCs on immune cells. Our study shows that AMSCs have an immunosuppressive effect not only on human microglia but also on mouse microglial cells, indicating that they could be used as a potential therapeutic tool to ameliorate neuronal injury of the immune system.

Microglia play an important role in the pathogenesis of many neurological disorders, such as cerebral ischemia, Alzheimer’s disease, and Parkinson’s disease (Levesque et al., 2010; Park et al., 2012). Numerous studies have provided evidence that microglia can be activated in response to injuries and diseases of the CNS (Rock et al., 2004; Raivich, 2005). Activated microglia show enhanced viability, a state in which they can produce a large amount of inflammatory cytokines, leading to permanent scarring and fibrosis characteristics of several chronic diseases (Zhang et al., 2009). Hence, attenuation of deleterious microglial viability could have a therapeutic effect on neuronal damage and degeneration. The migration of microglia to the site of damage/injury is a central consequence of inflammation in the brain (Han et al., 2014). There is increasing evidence that microglia respond to focal cerebral ischemic insults by migrating rapidly toward the lesion sites. Consequently, a reduction in microglial migration through stem cell-based therapeutic approaches might contribute significantly to inhibiting inflammation and associated neuronal cell death. Many studies have focused on assessing the efficacy of such therapies. In our study, we proved that AMSCs have immunosuppressive properties. Specifically, we show that they can reduce the viability and migration of both human and mouse microglia. After culturing in CM derived from AMSCs, both cell types showed a significantly reduced viability. Since microglia are known to be neurotoxic in their highly active state (Burguillos et al., 2011), we believe that AMSCs could perform a neuroprotective function by negatively influencing the viability of microglia. We have also shown, through the transwell migration assay, that when cultured in AMSC-CM or co-cultured with AMSCs, microglia show weakened migratory abilities. This suggests that AMSCs have the capacity to prevent neuronal cell death caused by microglia migrating toward lesion sites.

MSCs were recently shown to have potent anti-inflammatory and anti-proliferative properties. For example, MSCs can inhibit the proliferation of monocytes and lymphocytes (Jones et al., 2007; Spaggiari et al., 2006; Jiang et al., 2005; Nauta et al., 2006). Although the underlying mechanisms are not fully understood,
some researchers have suggested that prostaglandin E2 (PGE2) and transforming growth factor beta 1 (TGF-β1) might facilitate the anti-proliferative effects of MSCs (Groh et al., 2005; Bouffi et al., 2010). In the current study, we have shown that the NO secreted by AMSCs into the extracellular space contributes significantly to latter’s immunosuppressive properties, especially on human and mouse microglial cells. Other studies have reported the inhibitory properties of NO, although the precise mechanisms remain unclear (Bogdan, 2001). At high concentrations, NO is known to suppress the phosphorylation of STAT5 (signal transducer and activator of transcription 5) in T cells and promote immune cell apoptosis in vitro (Sato et al., 2007). Because the secretion of NO by AMSCs is abundant and persistent, we believe that when transplanted into the brain, these cells will continue to play an immunosuppressive role.

In summary, our study shows that the immunosuppressive effect of AMSCs is facilitated through its ability to reduce the viability and migration of both human as well as mouse microglial cells. Our results also suggest that the anti-survival and anti-migratory effect of AMSCs is mediated by the secretion of NO. Based on these data, we believe that AMSCs play a major role in maintaining immune homeostasis during tissue injury. More importantly, they offer a promising therapeutic option for some CNS diseases associated with deleterious activation of immune cells.

4. Experimental procedures

4.1. Generation of human AMSCs and preparation of conditioned medium

Human AMSCs were acquired, with informed consent, from full-term placentae of six healthy women after vaginal delivery or cesarean section. Briefly, the amnion was separated from the chorion, rinsed with phosphate-buffered saline (PBS) buffer three times, and minced with a pair of microsurgical scissors. The shredded tissue was then treated with 0.25% trypsin (Gibco, USA) thrice, to remove epithelial cells. It was then incubated with 0.5% collagenase IV (Sigma, USA) for 60 min at 37°C. The dissociated cells were filtered through a 100 μm cell strainer, pelleted by centrifugation at 300g for 5 min, resuspended in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and plated into 25 cm² culture flasks at a density of 2 x 10⁶ cells/mL. After culturing at 37°C in a 5% CO₂ humidified atmosphere for 3 days, the medium was changed in order to
remove non-adherent cells. The remaining adherent cells (primary AMSCs) were passaged every 2 days. Cells from 3 to 5 generations were used in our experiments. The purity of the population was assessed by cytofluorometric analysis, based on the expression of AMSC-specific markers CD29 and CD90, and the absence of hematopoietic markers CD11b and CD45. Briefly, after the cells were harvested and transferred to adherent culture, they were fixed in 4% paraformaldehyde for 15 min. Next, they were incubated with 3% bovine serum albumin and primary antibodies (AbD Serotec, UK) against CD29, CD90, CD45, and CD11b for 1 h at room temperature, washed with PBS, and incubated with the secondary antibody for 45 min. Finally, the stained cells were washed three times and analyzed using a flow cytometer (FACS Calibur, BD Biosciences, USA).

To prepare the AMSC-CM, 5 × 10^5 MSCs were plated in a 25-cm^2 culture flask in 5 mL DMEM/F-12 containing 10% FBS. Twenty-four hours later, the media was replaced with 5 mL DMEM/F-12 without FBS and the culture was continued for another 24 h. Subsequently, the culture medium was collected and centrifuged at 300g for 3 min to remove any cell debris before using in culture. This medium has been defined as AMSC-CM in our study.

### 4.2 Microglia culture

Primary human microglia (Sciencell, Carlsbad, California, USA) were cultured in DMEM/F-12 supplemented with 10% FBS, and maintained at 37 °C and 5% CO2 in a humidified atmosphere. We used cells at P3–P8 following recovery, to perform experiments. BV2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China, and cultured the same way as the human microglia.

### 4.3 Viability assay

We used the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay to determine the effect of AMSCs on the viability of human microglia and BV2 cells. Briefly, these cells were plated into 96-well plates at a concentration of 5000 cells per well and cultured in DMEM/F-12 or AMSC-CM in the presence of 10% FBS at 37 °C for 12 h, 24 h, and 48 h. All experiments were done in triplicate and repeated three times independently. In some groups, SMT (Beyotime, China) was added to the culture media to inhibit the secretion of NO from AMSCs. The CM harvested from these cells was considered the reduced NO containing AMSC-CM. To determine the concentration of cells, optical density (OD) was measured using the Thermo multiscan MK3 ELISA plate reader (Thermo, USA) at a wavelength of 450 nm, according to the CCK-8 assay kit protocol. Cell viability was calculated using the following equation:

\[
\text{Cell inhibitory rate} = \left[ 1 - \frac{(\text{OD experiment} - \text{OD blank})}{(\text{OD control} - \text{OD blank})} \right] \times 100\%.
\]
4.4. Wound healing assay

Human microglia and BV2 cells were seeded at a density of $5 \times 10^5$ cells per well in six-well plates and allowed to grow to 80% confluency in DMEM/F-12 with 10% FBS. The monolayer was scratched using a 10 μL plastic pipette tip to create a uniform wound. After wounding, the culture was washed with PBS to remove debris, then incubated in DMEM/F-12 or AMSC-CM containing 10% FBS for another 24 h. The distance migrated by the microglia was measured under a microscope equipped with a camera. Images were procured from six random fields, and the wound closure rate was calculated by measuring the space remaining in the wounded areas, as seen in the microscopic images. All experiments were done in triplicate and repeated three times independently.

4.5. Transwell migration assay

Transwell membrane filters (8 μm pore size; Corning, Lowell, MA) were used to examine the effect of AMSCs on migration of microglial cells. First, we plated $2 \times 10^4$ human microglial cells or BV2 cells in the upper chamber with 200 μL serum-free medium. This upper chamber was then placed within the bottom wells containing 600 μL complete medium either with $3 \times 10^4$ AMSCs (Co-culture group) or without any AMSCs (Control group). Cells were also cultured in AMSC-CM containing...
10% FBS in the bottom wells to determine whether migration was affected by factors secreted by AMSCs and hence found in the CM (AMSC-CM groups). Following incubation at 37 °C for 24 h, non-migrating cells on the upper surface of the membrane were carefully removed with a cotton swab. Cells on the lower surface of the membrane were first fixed in 4% formaldehyde for 20 min, then stained with 0.2% crystal violet for 5 min. For quantification, six randomly chosen fields on the lower membrane surface were imaged using computer-assisted microscopy. In some experimental groups, AMSC cultures were treated with SMT to inhibit the secretion of NO, so we could investigate the effect of AMSC-secreted NO on the migration of microglia. All experiments were done in triplicate and repeated three times independently.

4.6. Statistical analysis

All data are represented as mean ± SD. Comparison between multiple groups was performed using one-way ANOVA followed by Student’s unpaired t-test. A p value < 0.05 was considered statistically significant. All calculations were performed using the Statistical Package for the Social Sciences, version 13.0 (SPSS, Chicago, IL).

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