Skin-Derived Mesenchymal Stem Cells Alleviate Atherosclerosis via Modulating Macrophage Function

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Key Words. Skin-derived mesenchymal stem cells • Macrophages • Atherosclerosis • Nuclear factor-κB

ABSTRACT

Mesenchymal stem cells (MSCs) exhibit immunosuppressive efficacy and significantly inhibit the formation of the atherosclerosis (AS) plaque in apolipoprotein E-knockout (apoE−/−) mice. Of note, the largest lymphoid organ, the skin, provides a readily accessible and ideal source of tissue for the isolation of MSCs: skin-derived MSCs (S-MSCs). However, the effect and the mechanism of the therapeutic properties of S-MSCs in the progression of AS are unclear. We therefore investigated a direct effect of S-MSC treatment in the formation of atherosclerotic plaque in apoE−/− mice. Fifty apoE−/− mice were divided into four groups: the control group (AS), the S-MSC treatment group (S-MSC treatment), the nuclear factor-κB (NF-κB)−/−S-MSC treatment group (KO-S-MSC treatment), and the additional S-MSC migration group. Brachiocephalic artery ultrasonic biomicroscope (UBM) analysis showed that S-MSC treatment significantly reduced lesion size compared with the control groups (p < .01). Histological studies demonstrated that the plaque area of the mouse aortic arch was significantly decreased after S-MSC treatment. All alterations were dependent on NF-κB activation. After tail-vein injection, S-MSCs were capable of migrating to atherosclerotic plaque and selectively taking up residence near macrophages. S-MSC treatment reduced the release of the proinflammatory cytokine tumor necrosis factor (TNF)-α and increased the expression of the anti-inflammatory factor interleukin (IL)-10 in the atherosclerotic plaque, which was also dependent on NF-κB activation. In vitro, we found lipopolysaccharide (LPS) induced NF-κB-dependent expression of cyclooxygenase-2 (COX-2) in S-MSCs. Prostaglandin E2 (PGE2) expression was markedly increased after LPS-stimulated S-MSCs were cocultured with macrophages. LPS-stimulated macrophages produced less TNF-α/IL-1β and more IL-10 when cultured with S-MSCs, and although both were dependent upon NF-κB, the release of IL-10 was diminished if the S-MSCs were pretreated with a COX-2 inhibitor or an EP2/EP4 antagonist. Our data demonstrated that S-MSCs inhibited the formation of the atherosclerotic plaque in apoE−/− mice by modulating the functionality of macrophages, suggesting that S-MSCs may potentially have a role in stem cell-based therapy for AS. Stem Cells Translational Medicine 2015;4:1294–1301

SIGNIFICANCE

A combination of in vitro and in vivo experiments showed that skin-derived mesenchymal stem cells (S-MSCs) can attenuate the plaque size of atherosclerosis. This is probably because S-MSCs beneficially modulate the response of macrophages through an increased release of prostaglandin E2 acting on the EP2 and EP4 receptors of the macrophages, stimulating the production and release of the anti-inflammatory cytokine interleukin-10, and decreasing the production of proinflammatory cytokine tumor necrosis factor-α. S-MSCs inhibited the formation of the atherosclerotic plaque in apolipoprotein E-knockout mice by modulating the functionality of macrophages, and the suppressive property of S-MSCs is dependent on NF-κB signaling. This study provides direct evidence that S-MSCs have a potent immunosuppressive effect in the development of atherosclerosis in mice, suggesting that S-MSCs can easily be cultured and have similar function to bone marrow-derived MSCs, a promising cell source for stem cell-based therapies of atherosclerosis, and possibly also in transplantation.

INTRODUCTION

Mesenchymal stem cells (MSCs), also known as multipotent mesenchymal stromal cells, exist in almost all tissues, such as bone marrow, fat, the dermis, arteries, and the umbilical cord [1]. Intravenously injected bone marrow-derived MSCs (BM-MSCs) preferentially home to a variety of organs, with injured or damaged tissues taking priority [2, 3], and they play an important role in regulating the inflammatory response to achieve their immunosuppressive properties [4]. A recent study has characterized a novel population of MSCs derived from skin (S-MSCs), with functional similarities to BM-MSCs, which are a promising cell source for stem cell-based therapies of chronic
inflammatory diseases and possibly transplantation, and revealed an important immunomodulatory activity to markedly suppress the development of experimental allergic encephalomyelitis in mice, a mouse model of multiple sclerosis [5].

Inflammation is central to all stages of atherosclerosis (AS). Macrophages produce the majority of the inflammatory cytokines in atherosclerotic plaques and play a critical role in the pathophysiology of AS [6, 7]. Previous studies suggest that MSCs are capable of regulating the function of macrophages and dendritic cells (DCs), either directly through cell-cell contact or indirectly via the production of soluble mediators [8-11]. BM-MSCs (activated by lipopolysaccharide [LPS] or tumor necrosis factor [TNF-α]) reprogram macrophages by releasing prostaglandin E2 (PGE2), which acts on macrophages to release interleukin (IL)-10 through the prostaglandin EP2 and EP4 receptors, and attenuate sepsis [9]. IL-10 is a prototypic anti-inflammatory cytokine; the most recent discoveries suggest that it exerts its antiatherogenic effects on plaque development of AS by influencing the local inflammatory process within the atherosclerotic lesion [7], such as inhibiting macrophage activation as well as matrix metalloproteinase, pro-inflammatory cytokines, and cyclooxygenase-2 (COX-2) expression during the process within the atherosclerotic lesion [7].

Thus far, no study has reported the effect of S-MSCs on macrophages or in the treatment of AS. In this study, we demonstrated that S-MSCs inhibited the formation of the atherosclerotic plaque in apoE−/− mice by modulating the functionality of inflammatory macrophages, suggesting that S-MSCs may have therapeutic potential for the treatment of AS.

**Materials and Methods**

**Mice**

C57BL/6J, male, apoE−/− mice were purchased from the Animal Center of Peking University, Beijing, China (approval number: SCXK [jing] 2006-0008). Starting from 8 weeks of age, 50 male apoE−/− mice were randomly divided into 4 groups: AS (control group, n = 15), fed a high-fat diet that contained 21% fat from lard and 10% neutralized formalin at a constant pressure of 100 mmHg. The aortic arch was removed and embedded in paraffin. Five sections (5 μm) were taken 35 μm apart from the aortic arch of every mouse and were stained with hematoxylin and eosin (H&E) and Masson’s trichrome. The brachiocephalic arteries were removed and cryosectioned; the 8-μm cryosections were fixed in ice-cold acetone for 10 minutes before staining. The following antibodies were used for immunofluorescence staining: anti-mouse CD11b-PE [catalog no. 553311; BD Biosciences, San Jose, CA, http://www.bdbiosciences.com], anti-mouse TNF-α [catalog no. AF-410-NA; R&D Systems, Minneapolis, Minnesota, http://www.rndsystems.com], and Alexa Fluor 555 donkey anti-goat anti-phos-IκBα (catalog no. 9246; Cell Signaling, Danvers, MA, http://www.cellsignal.com). Alexa Fluor 555 donkey anti-goat IgG (H+L) (catalog no. A21432; Life Technologies, Waltham, MA, http://www.thermo Fisher.com), and Alexa Fluor 555 donkey anti-goat IgG (H+L) (catalog no. A21432; Life Technologies) as secondary antibodies. 4’,6-Diamidino-2-phenylindole (DAPI) (Fulka, Sigma-Aldrich Co., St. Louis, MO, http://www.sigmaaldrich.com) was used for staining the nuclei.

**Gene Expression Analysis**

The properties were analyzed by Western immunoblot and enzyme-linked immunosorbent assay (ELISA). For the Western immunoblot experiments, the following antibodies were used: anti-phos-IκBα (catalog no. 9246; Cell Signaling, Danvers, MA, http://www.cellsignal.com), anti-COX-2 (catalog no. 160106; Cayman Chemical Co., Ann Arbor, MI, http://www.caymanchem.com) and anti-actin antibodies. Chemically pure acetyl-11-keto-b-boswellic acid (AKBJ8A) was used as an NF-κB inhibitor [17]. Lipopolysaccharide was purchased from Sigma-Aldrich Co. Quantitative analyses of PGE2 and IL-10 protein levels were performed by ELISA using commercially available kits (Biolegend, San Diego, CA, http://www.biolegend.com). The COX-1 inhibitor (indomethacin, 5 μM; catalog no. 70270), the EP2 receptor antagonist (A6809, 10 μM; catalog no. 14050) and the EP4 receptor antagonist (GW627368, 10 μM; catalog no. 10009162) were purchased from Cayman Chemical Company. The supernatants from the stimulated cell cultures were...
frozen and analyzed according to the manufacturer’s instructions. Finally, Stop Solution was added, and the optical density of each well was determined within 30 minutes using a microplate reader (450-nm wavelength).

Cytokine mRNA was quantified by real-time polymerase chain reaction (PCR). Total RNA was extracted from cultured cells with TRIzol Reagent (Invitrogen/Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com). Reverse transcription was performed using the PrimeScript RT Reagent kit (TaKaRa, Shiga, Japan, http://www.takara.com). Quantitative PCR was carried out using the SYBR Green PCR Master Mix (TaKaRa) in the ABI Prism 7900 (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, http://www.appliedbiosystems.com). All gene expression results were normalized to the expression of the housekeeping gene GAPDH. Primer sequences were as follows: TNF-α (forward: GAACTGGCCAGAGAGGCCACT; reverse: AGGGTCTGGGC-CATAGAACT); IL-1β (forward: ACCTTTCAGGA GAGGACATGA; reverse: CTATGGAAGCTGACACACCA); IL-10 (forward: CAGCCGG-ACCATCATTAAT; reverse: GGACAGAACTGACACACCA); GAPDH (forward: TGTGTCGGTCGTTGATCTG; reverse: CTTGCTTC-ACCACCTTCTTG).

Statistical Analysis

Values shown represent the mean ± SEM, where applicable. Statistical significances were calculated with the Newman-Keuls test. Differences were considered significant at \( p < .05 \).

RESULTS

Effect of S-MSC Treatment on the Progression of Atherosclerotic Lesions

Cells with phenotypic and functional characteristics of MSCs, besides bone marrow MSCs, have been identified [18]. The latest study has characterized a novel population of MSCs, skin-derived MSCs, which reveal in vivo immunosuppressive properties in autoimmunity [5]. This study was the basis for our investigation of the role of S-MSCs in the treatment of AS. We isolated the cells from the dissociated dermis of 1- to 3-day-old wild-type or NF-κB KO-MSC treatment groups (supplemental online Table 1). All gene expression results were normalized to the expression of the housekeeping gene GAPDH. Primer sequences were as follows: TNF-α (forward: GAACTGGCCAGAGAGGCCACT; reverse: AGGGTCTGGGC-CATAGAACT); IL-1β (forward: ACCTTTCAGGA GAGGACATGA; reverse: CTATGGAAGCTGACACACCA); IL-10 (forward: CAGCCGG-ACCATCATTAAT; reverse: GGACAGAACTGACACACCA); GAPDH (forward: TGTGTCGGTCGTTGATCTG; reverse: CTTGCTTC-ACCACCTTCTTG).

Table 1. Effects of S-MSC treatment assessed by UBM on established plaque progression in brachiocephalic artery

| Treatment group | Plaque area, mm² | \( \nu_{\text{max}} \) mm/second |
|-----------------|------------------|-------------------------------|
| AS              | 0.183 ± 0.051    | 508.82 ± 161.65              |
| MSC treatment   | 0.067 ± 0.015*   | 189.96 ± 57.73*              |
| KO-MSC treatment| 0.146 ± 0.0493 (ns) | 417.96 ± 173.87 (ns) |

\( *p < .05 \) vs. AS.

Abbreviations: AS, atherosclerosis; KO-MSC; NF-κB KO-MSC treatment group; MSC, mesenchymal stem cell; ns, nonsignificant; \( \nu_{\text{max}} \), maximal flow velocity.

S-MSC Migration

We next determined the fate of injected S-MSCs in vivo in AS mice. The mice were fed a high-fat diet for 21 weeks and were also injected i.v. with S-MSCs derived from enhanced green fluorescent protein (EGFP) transgenic mice (Fig. 2A) in the last week. Immunofluorescence staining revealed that the injected S-MSCs labeled with EGFP preferentially trafficked to the plaque (Fig. 2B), and seemed to migrate toward macrophages (Fig. 2C). These data indicate that the injected S-MSCs have the ability to migrate toward inflamed tissues and possibly to interact with activated macrophages within the atherosclerotic plaque.

S-MSCs Modulated Cytokine Expression in AS Mouse Model

To assess the inhibitory function of S-MSCs in vivo, immunofluorescence microscopy of sections from atherosclerotic arteries of three groups mice (AS groups, S-MSC treatment group, and NF-κB KO-MSC treatment group) were stained for the proinflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10. In contrast to the AS groups, TNF-α expression was markedly decreased (Fig. 3A), whereas IL-10 expression was increased in the S-MSC treatment group but not in the NF-κB KO-MSC treatment group (Fig. 3B). Because TNF-α and IL-10 play essential roles in the pathogenesis of AS, our results reveal that S-MSCs could specifically inhibit proinflammatory cytokines and increase the anti-inflammatory cytokine expression in vivo.

Molecular Basis of S-MSC and Macrophage Interaction

In vivo, it remains unclear which factor(s) attracts S-MSCs to migrate toward infiltrating macrophages and whether these two different types of cells interact with each other when they meet. To understand the molecular basis of the interaction between S-MSCs and macrophages, we performed a series of experiments in vitro. We stimulated WT-S-MSCs and NF-κB KO-MSCs with LPS and found that NF-κB signaling is activated only in WT-S-MSCs 15 minutes after LPS stimulation (Fig. 4A). In a number of cell types, NF-κB has been shown to induce prostaglandin production and release through a pathway involving COX-2 [9, 19]. Herein, we found a significant increase in the expression of COX-2 in S-MSCs after LPS stimulation for 3 hours and 5 hours (Fig. 4B); however, when the S-MSCs were collected from NF-κB KO-MSC mice (Fig. 4B) or pretreated with NF-κB inhibitor AK278A (10 μM) for 30 minutes, there was no change in COX-2 expression when S-MSCs were stimulated with LPS (Fig. 4C). Estimation of P-IκBα or COX-2 protein band intensity normalized to actin (Fig. 4A–4C). Thus, these data...
suggest that the COX-2 expression was dependent on the activation of the NF-κB signaling pathway in S-MSCs, and COX-2 could produce the substrate for prostaglandin synthase enzymes [9]. For this reason, using ELISA, we measured the amount of PGE2 in the supernatants of cultures of S-MSCs isolated from wild-type or NF-κB2/2 mice in the presence of macrophages for an additional period of 3 or 5 hours with LPS stimulation (100 ng/ml). We observed a significant increase of PGE2 release in the supernatants of cultures with wild-type S-MSCs but not NF-κB2/2 S-MSCs, suggesting that the PGE2 release was dependent on NF-κB signaling (Fig. 4D). Interestingly, a recent study has determined that PGE2 produced from BM-MSCs could affect other immune cells via EP1 and EP4 receptors [20]. Furthermore, macrophages expressing PGE2 receptors EP2 and EP4 were identified [21]. Together, our findings indicate that PGE2 released from S-MSCs might, indeed, be responsible for reprogramming the infiltrated macrophages in atherosclerotic lesions.

**Effect of S-MSCs on Cytokine Production in LPS-Induced Macrophages**

To further examine the modulatory effect of S-MSCs on macrophages in detail, we cultured macrophages alone or with S-MSCs isolated from either wild-type or NF-κB2/2 mice and stimulated them for an additional 6 hours with LPS. We then measured TNF-α, IL-1β, and IL-10 expression by real-time-polymerase chain reaction...
We consistently found that TNF-α and IL-1β expression were decreased and IL-10 expression was increased after coculture, and both alterations for TNF-α and IL-10 were dependent on NF-κB activation of S-MSCs (Fig. 5A). These results suggested that S-MSCs could regulate the profile of cytokines produced by activated macrophages in vitro.

In addition, we pretreated the S-MSCs and macrophages with EP2 and EP4 receptor antagonists or a COX-2 inhibitor for 30 minutes before LPS stimulation for 12 hours, and the IL-10 release in coculture supernatants was investigated by ELISA. The result displayed blockades of EP2 and EP4, or that COX-2 prevented the increase of IL-10 secretion in LPS-stimulated S-MSCs cocultured with macrophages (Fig. 5B). The data suggested that S-MSCs regulated the profile of cytokines produced by activated macrophages in vitro, which may be due to the COX-2 or PGE2 released from S-MSCs.

The combination of our in vivo and in vitro experiments showed that the interaction between S-MSCs and macrophages was likely due to an increased release of COX-2 or PGE2 from the S-MSCs acting on the EP2 and EP4 receptors of the macrophages. In turn, this stimulated the release of anti-inflammatory cytokine IL-10 and decreased release of inflammatory cytokines TNF-α and IL-1β to perform their immunosuppressive effects that eventually led to reduced atherosclerotic lesions in apoE−/− mice (Fig. 6).

**DISCUSSION**

The development of atherosclerotic plaques is a multistep inflammatory process [22]. Macrophages are ubiquitous in all stages of AS and possess crucial effector functions in the process of AS [6, 23, 24]. Specifically, macrophages are the main source of cytokines in atherosclerotic plaques [25]. Proinflammatory cytokines such as TNF-α may act as autocrine or paracrine molecules on the cells to promote the development of AS [26]. Consistently, apoE/TNF-α double-knockout mice show a significant decrease in relative atherosclerotic lesion size [27]. Elevated levels of TNF-α are associated with an elevated risk for recurrent myocardial infarction and cardiovascular death after a first myocardial infarction [25]. A prototypic anti-inflammatory cytokine, IL-10, produced primarily by the macrophages and Th2 subtype T lymphocytes [28], has been shown to attenuate atherogenesis by inhibition of the release of several proinflammatory cytokines (including IL-1β and TNF-α) and matrix metalloproteinase [12], and alter...
**Figure 4.** Examination of molecular alterations underlying the effect of S-MSCs on macrophages. (A, B): Wild-type S-MSCs or KO-S-MSCs (1 × 10^6 per assay) were stimulated with LPS (100 ng/ml) for the indicated time. Whole-cell lysates were separated and analyzed by Western immunoblots with antibodies phosphor-IκBα (A) and COX-2 (B). (C): Effects of NF-κB on the LPS-induced COX-2 expression. S-MSCs pretreated with 10 µmol/l AKβBA for 30 minutes were stimulated with LPS (100 ng/ml) for the indicated time on the immunoblot with staining of actin-loading controls. Densitometric scanning of three Western blots is quantified in the bar graphs. The results shown are representative of at least three independent experiments. (D): S-MSCs isolated from wild-type or NF-κB^-/-^ mice were grown to confluence and cultured or cocultured with macrophages for an additional period of 3 or 5 hours with LPS (100 ng/ml) stimulation. Supernatants were collected, and the levels of PGE2 were determined by enzyme-linked immunosorbent assay (n = 4). Error bars represent mean ± SEM. ***, p < .01. Abbreviations: AKβBA, acetyl-11-keto-β-boswellic acid; COX-2, cyclooxygenase-2; KO-S-MSCs, nuclear factor-κB^-/-^ skin-derived mesenchymal stem cell treatment group; LPS, lipopolysaccharide; PGE2, prostaglandin E2; S-MSC, skin-derived mesenchymal stem cell.
lipid metabolism in macrophages in AS [7]. A recent publication showed that IL-10 induces macrophage polarization toward the M2 phenotype, which reduces murine AS [29, 30]. The atheroprotective role of IL-10 was confirmed by IL-10-deficient mice on atherosclerosis in apoE−/− mice due to reprogramming macrophages for their IL-10 production.

S-MSCs seem to be potent immunomodulators, but their mechanism of action is largely unknown. S-MSCs are reported to inhibit the polarization of pathogenic T-helper type 1 (Th1) and Th17 cells and markedly suppress the development of experimental allergic encephalomyelitis in mice [5]. In the present study, we show that S-MSCs can be used to improve AS in mice. We demonstrate that, in S-MSCs, NF-κB signaling is required to induce prostaglandin production through a pathway involving COX-2, which produces the substrate for prostaglandin synthase enzymes [19]. BM-MSCs have been shown to produce PGE2 and possibly affect other immune cells via EP1-EP4 receptors [20]. In peritoneal macrophages, PGE2 was shown to modulate inflammatory reactions via the EP2 and EP4 receptors [21]. In agreement with the previous studies described in this article, we reveal a significant increase in the expression of COX-2 in S-MSCs and PGE2 in cocultures of S-MSCs and macrophages, and these actions occur in an NF-κB-dependent manner after LPS stimulation. Additionally, we note that the increased IL-10 release from the cocultures is dependent on the COX-2 protein.

In conclusion, we provide direct evidence that S-MSCs have a potent immunosuppressive effect in the development of AS in mice. The combination of our in vitro and in vivo experiments shows that S-MSCs can attenuate the plaque size of AS, which is likely because S-MSCs beneficially modulate the response of macrophages through an increased release of PGE2 acting on the EP2 and EP4 receptors of the macrophages, stimulating the production and release of the anti-inflammatory cytokine IL-10 and decreasing the production of proinflammatory cytokine TNF-α. Our results suggest that S-MSCs can easily be cultured and have similar function to BM-MSCs, and they are a promising cell source for stem cell-based therapies of AS and, possibly, transplantation.

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Figure 5. S-MSCs inhibit the production of inflammatory cytokines and enhance the production of IL-10 by LPS-induced MACs via PGE2. MACs were cultured overnight in the absence or presence of S-MSCs or KO-MSCs (MACs: S-MSCs or KO-MSCs ratio = 10:1). (A): Cells were then washed and incubated for 6 hours with or without LPS (100 ng/ml), and cytokines were analyzed in cells by real-time polymerase chain reaction (RT-PCR). Effect of the indomethacin (COX-1/2 inhibitor; 5 μM), A6809 (EP2 receptor antagonist; 10 μM), and GW627368X (EP4 receptor antagonist; 10 μM) on the LPS-induced IL-10 release in MAC and S-MSC cocultures. Cell cocultures pretreated with COX-2 inhibitor or EP2/EP4 antagonist or medium (none) for 30 minutes were stimulated with or without LPS (100 ng/ml) for an additional 12 hours. (B): The production of IL-10 was analyzed in supernatants of cocultures by enzyme-linked immunosorbent assay. All results are shown as representative of the arithmetic mean ± SEM of six independent experiments. * p < .05, ** p < .01, versus MACs plus LPS. Abbreviations: COX-2, cyclooxygenase-2; Ctrl, control; IL-1β, interleukin 1-β; IL-10, interleukin 10; MAC, macrophage; KO-MSC, NF-κB−/−-skin-derived mesenchymal stem cell treatment group; LPS, lipopolysaccharide; MAC, macrophage; MSC, skin-derived mesenchymal stem cell; ns, nonsignificant; TNF-α, tumor necrosis factor-α.

Figure 6. Scheme for the S-MSCs suppressing the progression of AS via regulating macrophage activation. Abbreviations: AS, atherosclerosis; COX-2, cyclooxygenase-2; IL-1β, interleukin 1-β; IL-10, interleukin 10; LPS, lipopolysaccharide; S-MSC, skin-derived mesenchymal stem cell; TNF-α, tumor necrosis factor-α.
AUTHOR CONTRIBUTIONS

Q.L.: study design, collection and/or assembly of data, data analysis, manuscript writing; W.S.: collection and/or assembly of data, manuscript writing; X.W., K.Z., and W.X.: collection and/or assembly of data; P.G.: manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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