Mesenchymal Stem/Stromal Cells Protect the Ocular Surface by Suppressing Inflammation in an Experimental Dry Eye

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Dry eye syndrome (DES) is one of the most common ocular diseases affecting nearly 10% of the US population. Most of the currently available treatments are palliative, and few therapeutic agents target biological pathway of DES. Although DES is a multifactorial disease, it is well-known that inflammation in the ocular surface plays an important role in the pathogenesis of DES. Mesenchymal stem/stromal cells (MSCs) have been shown to repair tissues by modulating excessive immune responses in various diseases. Therefore, we here investigated the therapeutic potential of MSCs in a murine model of an inflammation-mediated dry eye that was induced by an intraorbital injection of concanavalin A. We found that a periocular administration of MSCs reduced the infiltration of CD4⁺ T cells and the levels of inflammatory cytokines in the intraorbital gland and ocular surface. Also, MSCs significantly increased aqueous tear production and the number of conjunctival goblet cells. Subsequently, corneal epithelial integrity was well-preserved by MSCs. Together, the results demonstrate that MSCs protect the ocular surface by suppressing inflammation in DES, and suggest that MSCs may offer a therapy for a number of ocular surface diseases where inflammation plays a key role.

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INTRODUCTION

Dry eye syndrome (DES) is one of the most common ocular disorders. The prevalence of DES ranges from 7% to 33% worldwide,1–8 and studies suggest that approximately nine million people in the United States suffer from advanced effects of DES.1–8 Also, DES results in functional and occupational disability in patients with Sjögren’s syndrome or ocular graft-versus-host disease.10–13 Unfortunately, most of the treatments to date are based on topical administration of tear substitutes, and are only palliative. Thus, efforts are being made to develop novel therapies for DES by targeting the underlying causes of the disease.

The causes of DES are multifactorial. However, inflammation in the ocular surface plays a main role in the pathogenesis of DES.14,15 In fact, an accumulating body of evidence supports the notion that DES is a localized autoimmune disease involving both innate and adaptive immunity such as CD4⁺ T cells in the development and progression of the disease.14,15 Accordingly, therapies that inhibit immune response may be useful for treating DES.

One strategy for modulating excessive immune response is administration of mesenchymal stem/stromal cells (MSCs). MSCs were first found as resident cells forming a niche for hematopoietic cells in the bone marrow of mammals, and have been further explored as reparative cells that limit tissue destruction and enhance repair in various diseases.16 The mechanisms of tissue repair by MSCs are largely attributed to their immune-modulatory effects.17,18 Therefore, MSCs have been widely tested in clinical trials for a number of immune-mediated diseases with encouraging results.

Here, we investigated the effects of MSCs on the ocular surface in an inflammation-mediated dry eye model in mice.

RESULTS

Establishment of an inflammation-induced dry eye in mice

To create the inflammation-induced dry eye model, we injected 10 or 20 μl concanavalin A (ConA; 1, 5, or 10 mg/ml), that is the prototypic T-cell mitogen,19 into the intraorbital gland in mice. For control, the same volume of phosphate-buffered solution (PBS) was injected. One week later, aqueous tear production was measured, and the ocular surface was observed for epithelial integrity. Also, intraorbital glands and ocular surface including the cornea and conjunctiva were analyzed by histology and assayed for levels of inflammatory cytokines (Figure 1a). We found that 10 mg/ml ConA induced severe infiltration of CD3⁺ T cells in the intraorbital gland (Figure 1b), and tear production was markedly decreased as measured by a cotton thread test (Figure 1c). Also, the levels of IL-2 and IFN-γ that are derived from activated T cells20 were significantly increased in the intraorbital gland and ocular surface (Figure 1d–f), whereas the levels of TNF-α, IL-1β, and IL-6 were not affected by ConA (Figure 1f). 20 μl injection of ConA was more

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effective in inducing inflammation than 10 μl ConA. The integrity of corneal epithelium was significantly disturbed by ConA as indicated by increased corneal dye staining (Figure 1g). Together, the results demonstrate that an intraorbital injection of ConA (20 μl, 10 mg/ml) induced DES in mice by causing inflammation, reducing tear secretion, and disrupting corneal epithelium.

**MSCs increased tear production and suppressed inflammation**

To determine whether MSCs have therapeutic effects in an inflammation-mediated dry eye, we administered human or mouse bone marrow-derived MSCs (hMSCs, mMSCs; 1 × 10^6 or 1 × 10^5 cells/20 μl balanced salt solution; BSS), human dermal fibroblasts (hFbs; 1 × 10^6 cells/20 μl BSS), or the same volume of BSS periorbitally after ConA injection into the intraorbital gland (Figure 2a). For negative control, PBS was injected into the intraorbital gland instead of ConA. As expected, aqueous tear production was markedly reduced on day 7 by ConA injection (Figure 2b). The tear production was significantly increased in the eyes that were treated with hMSCs or mMSCs (Figure 2b). However, the administration of hFbs was not effective in increasing tear production (Figure 2b).

The levels of T-cell–derived cytokines, IL-2 and IFN-γ, were significantly reduced in the intraorbital gland and ocular surface by hMSCs or mMSCs (Figure 2c,d). Both 1 × 10^5 and 1 × 10^6 hMSCs were similarly effective, and the effects of mMSCs were similar to those of hMSCs. However, hFbs markedly increased the levels of IL-2 and IFN-γ in the intraorbital gland and ocular surface (Figure 2c,d).

Corneal staining score, which is indicative of corneal epithelial defects, was significantly lower in the hMSCs- or mMSCs-treated eyes, compared to the BSS-treated eyes, suggesting that corneal epithelial integrity was preserved by MSCs (Figure 2c).

**MSCs decreased IFN-γ-secreting CD4+ cell infiltration in the intraorbital gland**

We further analyzed for the cell population infiltrating the intraorbital gland by ConA. Hematoxylin-eosin staining showed that the intraorbital gland was severely disrupted with extensive acinar atrophy and periductal infiltration of inflammatory cells on day 7 after ConA injection (Figure 3a). Most of the inflammatory cells infiltrating the intraorbital gland were CD3+ cells (Figure 3a). The treatment with hMSCs or mMSCs markedly decreased the infiltration of CD3+ cells and preserved the structure of the
intraorbital gland (Figure 3a). Flow cytometry revealed that the percentage of CD4+ cells or IFN-γ+CD4+ cells was increased in the intraorbital gland after ConA injection, and significantly reduced by treatment with hMSCs (Figure 3b). However, the percentage of IFN-γ+CD8+ cells or IL-17+CD4+ cells in the intraorbital gland was not increased by ConA and not affected by hMSCs (Supplementary Figures S1 and S2). Also, the percentage of CD11b+ , CD11c+, NK1.1+, or B220+ cells was not altered by hMSCs (Supplementary Figure S3). In addition, hMSCs did not increase the percentage of CD4+Foxp3+ regulatory T cells (Tregs) (Supplementary Figure S4).

MSCs restored goblet cells in the conjunctiva

Periodic Acid Schiff (PAS) staining of the conjunctiva showed that the number of goblet cells which are responsible for mucous tear production was markedly decreased by ConA injection (Figure 4a). The conjunctival goblet cell counts were significantly higher in the hMSCs- or mMSCs-treated eyes, compared to the BSS-treated controls (Figure 4b). The mMSCs were more effective in increasing goblet cells than hMSCs.

MSCs did not engraft

To evaluate the engraftment of MSCs, we carried out real-time RT PCR assays for human-specific GAPDH (hGAPDH) in the intraorbital gland from mice that received peri-orbital infusion of 1×10⁵ hMSCs at day 0 of ConA injection. Results demonstrated that less than 10 hMSCs were present in the intraorbital gland on days 1 and 7 after injection. Therefore, the data indicate that MSCs suppressed immune response and improved DES without the long-term engraftment.

MSCs inhibited T-cell proliferation and Th1 differentiation in vitro

We next tested whether MSCs might directly inhibit CD4+ cell proliferation and differentiation into IFN-γ-secreting Th1 cells. To evaluate proliferation, we isolated CD4+ cells from the peripheral blood of mice, and labeled the cells with 5-carboxyfluorescein diacetate succinimide ester (CFSE). The CFSE-labeled CD4+ cells were stimulated by ConA (5 µg/ml), and co-cultured with hMSCs (Figure 5a). To rule out the possibility that hMSCs might inhibit CD4+ cells by directly blocking ConA, we activated CD4+ cells with anti-CD3/CD28, and co-cultured with hMSCs. Similarly, hMSCs suppressed the anti-CD3/CD28-induced proliferation of CD4+ cells (Figure 5b). However, human fibroblasts (hFbs) did not suppress the proliferation of CD4+ cells, indicating that the effects of hMSCs on CD4+ cell suppression were not due to the media exhaustion during co-culture of the cells (Figure 5b). We also evaluated the effects of hMSCs on Th1
differentiation by co-culturing CD4+ cells with hMSCs in the presence of IL-1β and IL-23 for 5 days. Flow cytometry demonstrated that hMSCs significantly reduced the percentage of IFN-γ+CD4+ cells (Figure 5c).

Inhibition of tryptophan metabolism did not negate the effect of hMSCs

Previous studies have shown that hMSCs inhibit T-cell proliferation by secreting indoleamine 2,3-dioxygenase (IDO) that catabolizes tryptophan required for T-cell proliferation.\(^{21-23}\)

Based on these reports, we tested whether IDO secretion by hMSCs might mediate the inhibitory effects of hMSCs on CD4+ cell proliferation. However, the addition of an IDO inhibitor 1-methyl-D-tryptophan (1-MT) did not ablate the effects of hMSCs in suppressing ConA-induced CD4+ cell proliferation in vitro or in increasing tear production or goblet cell counts in ConA-induced dry eye in mice (Supplementary Figure S5). The data, therefore, indicate that the immune-modulatory effects of MSCs in the Con A-mediated dry eye might be independent of IDO.

DISCUSSION

Data demonstrate that a local infusion of MSCs protected the ocular surface in an inflammation-mediated dry eye. The beneficial effects of MSCs were largely mediated by suppression of inflammation and by restoration of tear production and conjunctival goblet cells. Especially, MSCs decreased the number of IFN-γ-secreting CD4+ cells \(^{15,20}\) and suppressed CD4+ cell proliferation and IFN-γ+CD4+ cell differentiation in vitro. Given that CD4+ cells play an important role in the ocular surface inflammation including simple DES and devastating ocular GVHD,\(^{15,20}\) our results suggest that MSCs may be a useful therapy for a number of ocular surface diseases which are accompanied by inflammation.

In this study, we injected ConA into the intraorbital gland to create an inflammation-mediated dry eye in mice. ConA is a prototypic T-cell mitogen that expands and activates T cells.\(^{19}\) Therefore, the model made it possible to follow the inflammatory response of the intraorbital gland and ocular surface and the immune-modulating effects of MSCs on the ocular inflammation. As a result, we observed that an intraorbital injection of ConA induced CD4+ T-cell–mediated inflammation in the intraorbital gland.
animal models that mimic different mechanisms of DES would be necessary to find out the immune-modulatory mechanisms of MSCs in DES.

In conclusion, we here demonstrate that a periorbital infusion of human or mouse bone marrow-derived MSCs protected the ocular surface by suppressing inflammation in an experimental dry eye which was mediated by CD4+ T-cell–mediated response. Our study has an implication for further exploration of MSCs as a novel therapy for patients with severe DES and other inflammatory ocular diseases.

MATERIALS AND METHODS

Animals. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital Biomedical Research Institute (IACUC No. 12–0360). Eight-week-old female BALB/c mice were purchased from Orient Bio (Seongnam, Korea), and maintained in a specific pathogen-free environment with continuously available water and food. Animals were treated in strict accordance with the ARVO statement for the use of animals in ophthalmic and vision research.

Preparation of cells. The hMSCs were obtained from the Center for the Preparation and Distribution of Adult Stem Cells (http://medicine.tamhsc.edu/irm/msc-distribution.html) that supplies standardized preparations of MSCs enriched for early progenitor cells to over 300 laboratories under the auspices of an NIH/NCRR grant (P40 RR 17447-06). Animal experiments were performed with passage two hMSCs from one donor. The cells consistently differentiated into three lineages in culture, were negative for hematopoietic markers (CD34, CD36, CD117, and CD45), and were positive for mesenchymal markers CD29 (95%), CD44 (>93%), CD49c (99%), CD49f (>70%), CD99 (>99%), CD90 (>99%), CD105 (>99%), and CD166 (>99%).

The hMSCs were cultured in complete culture medium (CCM) containing 17% fetal bovine serum (FBS; Gibco/Life Technologies, Grand Island, NY), 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen/Life Technologies, Carlsbad, CA) until 70% confluence was reached, and harvested with 0.25% trypsin/1 mmol/l EDTA at 37 °C for 2 minutes for injection. After washing, the cells were resuspended in BSS (BioWhittaker, Walkersville, MD) for injection.

gland and ocular surface, and thereby decreased tear production, reduced conjunctival goblet cells, and disrupted corneal epithelial integrity, all of which are characteristic findings of DES. The periorbital administration of MSCs suppressed activation of CD4+ T cells, and enhanced tear production and ocular surface integrity. However, the causes of DES are diverse, and DES can be induced by different pathophysiologic mechanisms as well as T-cell–mediated inflammation. Thus, further studies using other animal models that mimic different mechanisms of DES would help elucidate the effects of MSCs in dry eye.

Although our data convincingly indicate that MSCs suppressed T-cell–mediated inflammation in the ocular surface and prevented the progression of DES, the mechanism is not yet clear. In this study, the effects of hMSCs were similar to mMSCs. Of note was the finding that hFBs did not improve DES, and rather elicited strong immune response in the intraorbital gland and the ocular surface. These findings indicate that the therapeutic effects of hMSCs on inflammatory DES are not the result of xenogeneic reaction of human cells injected in mice, but the unique effects that MSCs possess.

We did not detect MSCs in the intraorbital gland on days 1 and 7 after injection into mice. This finding is consistent with previous reports that MSCs rapidly disappear in vivo after injection, and repair tissues without the long-term engraftment and through suppression of early immune response.

As for the mechanism of T-cell suppression, several reports suggested that hMSCs inhibit T cells by inducing Tregs. We did not observe an increase in Tregs by hMSCs in this study. However, it is possible that MSCs might recruit other immunoregulatory cells than Tregs. Other studies suggested that inhibition of tryptophan metabolism by IDO is responsible for T-cell suppression by hMSCs. However, 1-MT, an IDO inhibitor, was not effective in reversing the effects of hMSCs in vivo and in vitro in our study. Moreover, some of the immune-modulatory effects of MSCs are species-specific. For instance, inducible nitric oxide synthase (iNOS) is known to mediate the immune-suppressive activity of mMSCs, whereas IDO is involved in the immunosuppressive activity of hMSCs. Hence, although we observed similar beneficial effects of hMSCs and mMSCs on DES, the mechanism(s) might be different between hMSCs and mMSCs. Further studies would be necessary to find out the immune-modulatory mechanisms of MSCs in DES.

In conclusion, we here demonstrate that a periorbital infusion of human or mouse bone marrow-derived MSCs protected the ocular surface by suppressing inflammation in an experimental dry eye which was mediated by CD4+ T-cell–mediated response. Our study has an implication for further exploration of MSCs as a novel therapy for patients with severe DES and other inflammatory ocular diseases.

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Figure 4 PAS staining of the conjunctiva. (a) Representative photograph of Periodic Acid Schiff (PAS) staining of the conjunctiva showed that the number of goblet cells was markedly reduced by concanavalin A (ConA) injection (10 mg/ml, 20 μl), and improved by treatment with human or mouse bone marrow-derived mesenchymal stem/stromal cells (hMSC, mMSC). (b) The goblet cell counts in the conjunctiva were significantly higher in the hMSC- or mMSC-treated eyes, compared to the BSS-treated controls. The data are presented as the mean ± SEM. **P < 0.01; ***P < 0.001.
The hFbs were primary cultured fibroblasts from human skin, and obtained from CELLINETEC (Bern, Switzerland). The cells were cultured and prepared for injection in the same manner as hMSCs.

The mMSCs were prepared from the marrow of long bones of hind limbs of 8-week-old female BALB/c mice using the previously described method with modification. Briefly, the bone marrow extracts were obtained by flushing the cut ends of the bones, and cell suspension was filtered through a 70 μm strainer to remove bone spicules. After centrifugation, the cells were resuspended in CCM, and distributed into culture plates at density of 1 × 10^6 cell/cm². The plates were cultured undisturbed at 37 °C with 5% CO₂ in a humidified chamber (normoxia condition) for 3 days. After 3 days, the nonadherent cells were removed by gentle swirling, and adherent cells were replaced with CCM. After additional 4 days of culture, the plates were washed with serum-free medium, and harvested with 0.25% trypsin/1 mmol/l EDTA at 37 °C for 2 minutes before injection.

Animal models and treatment. To create dry eye, mice were anesthetized with intraperitoneal injection of zolazepam-tiletamine (Zoletil, Virbac, Carros, France), and 10 or 20 μl ConA (Sigma-Aldrich, St Louis, MO), that was diluted in PBS at concentrations of 1, 5, and 10 mg/ml, was injected into the intraorbital gland through transconjunctival approach using a Hamilton syringe with a 33 gauge needle (Hamilton, Reno, NV) under an operating microscope (Carl Zeiss, Jena, Germany). The same volume of PBS was injected and served as negative control.

Immediately after ConA injection, hMSCs (1 × 10^5 or 1 × 10^6 cells/20 μl BSS), mMSCs (1 × 10^6 cells/20 μl BSS), or the same volume of BSS were injected into the periorbital space using 30 gauge needle-syringe (Becton Dickinson, Franklin Lakes, NJ). In some experiments, 1-MT (10 mg; Sigma-Aldrich) was injected periorbitally along with hMSCs. The experiments were repeated in three independent sets, and each set included at least five mice per group.

Measurement of tear production. Aqueous tear production was measured with standardized phenol red-impregnated cotton threads (FCI Ophthalmics, Pembroke, MA). The threads were applied to the ocular surface in the lateral can thus for 60 seconds, and wetting of the thread was measured in millimeters.

Corneal dye staining. To evaluate the corneal epithelial integrity, corneal staining was performed by administering one drop of 3% Lissamine Green B (Sigma-Aldrich) to the inferior lateral conjunctival sac. The corneal surface was observed, and photographed with a digital camera fitted with a macro lens (Canon, Melville, NY). The total area of fluorescein staining of the cornea was scored in a blinded manner as follows: score 0 for no punctuate staining; score 1 when one fourth or less was stained; score 2...
when one half or less was stained; and score 3 when one half or more was stained. The average corneal staining score was calculated for each mouse.

**Histopathology.** The whole eyeball and intraorbital gland were excised, and fixed in formalin. The samples were sliced in 4 μm sections and subjected to hematoxylin–eosin, PAS, or CD3 immunohistochemical staining. To count goblet cells, four different sections through superior and inferior conjunctival fornices were selected in the PAS-stained slides, and the average counts of PAS-stained cells were calculated under a microscope using ×10 object. For CD3 immunohistochemical staining, a rabbit anti-mouse CD3 (ab5690, Abcam, Cambridge, MA) was used as a primary antibody.

**Real-time RT-PCR.** For RNA extraction, the intraorbital gland or ocular surface including the cornea and conjunctiva was cut into small pieces with microscissors, lysed in RNA isolation reagent (RNA Bee, Tel-Test, Friendswood, TX), and homogenized using a sonicator (Ultrasonic Processor, Cole Parmer Instruments, Vernon Hills, IL). Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA) and used to synthesize double-stranded cDNA by reverse transcription (SuperScript III; Invitrogen/Life Technologies). Real-time amplification was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA): IL-2 (TaqMan Gene Expression Assays ID, Mm00434256_m1), IFN-γ (TaqMan Gene Expression Assays ID, Mm01168134_m1), TNF-α (TaqMan Gene Expression Assays ID, Mm00433260_g1), IL-1β (TaqMan Gene Expression Assays ID, Mm00434228_m1), and IL-6 (TaqMan Gene Expression Assays ID, Mm00446196_m1). An 18s rRNA (TaqMan Gene Expression Assays ID, Hs99999905). The values were normalized to total eukaryotic 18s and used for normalization of gene expression. For all the PCR probe sets, TaqMan Gene Expression Assay kits were purchased from Applied Biosystems. The assays were performed in dual technical replicates for each biological sample.

**Standard curve for human GAPDH.** A standard curve was generated by adding serial dilutions of hMSCs to mouse tissue as previously described. Briefly, 0, 10, 100, 1,000, 10,000, or 100,000 hMSCs were added to each mouse intraorbital gland, respectively. Following RNA extraction, cDNA was generated using 1 μg total RNA, and real-time amplification was performed using hGAPDH (TaqMan Gene Expression Assays ID, Hs99999905_05). The values were normalized to total eukaryotic 18s rRNA. The standard curve was made based on hGAPDH expression from a known number of hMSCs added to one mouse intraorbital gland.

**Flow cytometry.** Intraorbital gland was placed and minced between the frosted ends of two glass slides in RPMI media (WelGENE, Daegu, Korea) containing 10% FBS and 1% penicillin–streptomycin. Cell suspensions were collected, and incubated for 30 minutes at 4 °C with fluorescein-conjugated anti-mouse antibodies: CD4, CD8, CD11b, CD11c, CD25, B220, NK1.1, IFN-γ, Foxp3 (eBioscience, San Diego, CA), and IL-17A (BD Pharmingen, San Diego, CA). For intracellular staining, the cells were stimulated for 4h with 50 ng/ml phorbol myristate acetate and 1 μg/ml ionomycin in the presence of GolgiPlug (BD Pharmingen), and incubated with the fluorescence-conjugated anti-mouse IFN-γ antibody (eBioscience) after fixation and permeabilization. The cells were analyzed by FACSCanto flow cytometer (BD Biosciences) and Flowjo program (Tree Star). All the experiments were performed six times independently.

**Statistical analysis.** Values were compared between the groups using the one-way ANOVA or two-tailed Student’s t test (Prism, GraphPad Software, La Jolla, CA), and shown as the mean value ± SEM or SD. Differences were considered significant at P < 0.05.

**SUPPLEMENTARY MATERIAL**

**Figure S1.** An intravitreal injection of concanavalin A (ConA) did not induce infiltration of CD8+ cells or IFN-γ CD8+ cells in the intraorbital gland.

**Figure S2.** The percentage of IL-17 CD4+ cells in the intraorbital gland was not increased by ConA injection.

**Figure S3.** The percentage of CD11b+, CD11c+, NK1.1+, or B220+ cells in the intraorbital gland was not different between BSS (balanced salt solution)- and MSC (human bone marrow-derived mesenchymal stem/stromal cells)-treated groups.

**Figure S4.** Treatment with human bone marrow-derived mesenchymal stem/stromal cells (MSC; 1 × 106 or 1 × 105 cells) did not induce Foxp3-expressing CD4+ regulatory T cells in the intraorbital gland.

**Figure S5.** Inhibition of tryptophan metabolism by an IDO inhibitor 1-methyl-D-tryptophan (1-MT) did not negate the effects of human bone marrow-derived mesenchymal stem/stromal cells (MSC).

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