Effects of Mesenchymal Stem/Stromal Cells on Cultures of Corneal Epithelial Progenitor Cells With Ethanol Injury

Joo Youn Oh,[1,2] Jung Hwa Ko,[2] Mee Kum Kim,[1,2] and Won Ryang Wee[1,2]

[1]Department of Ophthalmology, Seoul National University Hospital, Seoul, Korea
[2]Laboratory of Ocular Regenerative Medicine and Immunology, Biomedical Research Institute, Seoul National University Hospital, Jongno-gu, Seoul, Korea

Correspondence: Joo Youn Oh, Department of Ophthalmology, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul 110-744, Korea;
jooyounoh77@gmail.com.

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Purpose. Mesenchymal stem/stromal cells (MSCs) facilitate the regeneration of injured tissue. Our group has previously shown that human MSCs (hMSCs) or hMSC-derived factors suppress excessive inflammatory response in the cornea following chemical injury in vivo. We here investigated direct effects of hMSC-derived factors on cultures of chemically injured human corneal epithelial progenitor cells (hCEP), independent of systemic anti-inflammatory effects that hMSCs have been shown to have in vivo.

Methods. We injured hCEP by incubation in 20% ethanol for 30 seconds, and cultured the cells in fresh medium or in medium derived from cultures of human dermal fibroblasts (hFbs), hMSCs, or TNF-α-activated hMSCs. After 24 hours, we evaluated the survival, proliferation, and apoptosis of the cells.

Results. The hMSC-conditioned medium enhanced survival and proliferation and inhibited apoptosis of chemically injured hCEP. In addition, the conditioned medium accelerated the wound healing of corneal epithelium in tissue cultures of rabbit corneas following injury. The effects of the hMSC-conditioned medium were increased by preincubating hMSCs with TNF-α. The increased effectiveness of the medium from the preactivated hMSCs was in part explained by increased concentration of the multifunctional protein stanniocalcin-1 that inhibits apoptosis and promotes survival of cells.

Conclusions. Together, the data account for beneficial effects of hMSCs on tissue-endogenous stem cells involving hCEP, and provide a basis for using MSCs or MSC-derived factors to treat diseases of the cornea and other tissues.

Keywords: mesenchymal stem/stromal cells, corneal epithelial cells, apoptosis, proliferation, survival

Mesenchymal stem/stromal cells (MSCs) have beneficial effects in a number of models for human diseases. Some of the therapeutic effects are explained by the cells' protecting tissues from damage by modulating excessive inflammatory responses.[1,2] Other effects of MSCs involve the promotion of wound healing by inducing proliferation of tissue-endogenous stem/progenitor cells or by inhibiting apoptosis of injured cells.[2,3] Recent evidence suggests that the key therapeutic mechanism of MSCs is the paracrine production of therapeutic factors.[4,5]

In view of these observations, our group has performed experiments to test the hypothesis that MSCs and MSC-derived factors are beneficial for treating corneal surface disease where inflammation and wound healing defect are the main problem. Consistent with this hypothesis, we previously observed that either human MSCs (hMSCs) or hMSC-derived factors promote corneal regeneration by suppressing inflammation in the cornea after chemical and mechanical injuries in vivo.[6,7] The beneficial effects of hMSCs were largely explained by the cells' upregulation of expression of the multipotent anti-inflammatory protein TNF-α-stimulated gene/protein 6 (TSG-6).[6,8]

In addition, other studies demonstrated that hMSCs protect cells from apoptosis by secreting the multifunctional antiapoptotic protein stanniocalcin-1 (STC-1).[9,10] Stanniocalcin-1 is a 247-amino acid protein that was originally discovered as an essential hormone for regulating calcium and phosphate metabolism in fish.[11] Recently, it has been shown that STC-1 reduces reactive oxygen species and increases survival of injured cells such as alveolar epithelial cells, photoreceptors, or retinal ganglion cells.[10,12,13]

In the present study, we investigated the effects of hMSC-derived factors on the survival, proliferation, and apoptosis of corneal epithelial cells after ethanol injury using in vitro cultures of human corneal epithelial progenitor cells (hCEP) and tissue cultures of rabbit corneas. The use of these ex vivo systems enabled us to test direct effects of MSCs on corneal cells independently of any systemic effects that MSCs have been shown to have on the inflammatory and immune systems.[1,2] Also, we tested the effects of hMSCs that were preactivated by a key proinflammatory cytokine, TNF-α, because the production of therapeutic factors by MSCs is known to be enhanced in an inflammatory microenvironment.[14,15]

Methods

The experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic Vision

and Research, and the protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

Cell Culture and Reagents

Primary hCEP from one donor at passage 2 were obtained from CELLnTEC (Bern, Switzerland) and cultured in serum-free CnT-20 medium (CELLnTEC). Passage 4 cells were used in all experiments. For injury induction, the cells were seeded at a density of 25,000 cells/cm^2 and incubated in 20% ethanol for 30 seconds. We previously showed that exposure of hCEP to 20% ethanol for 30 seconds significantly suppressed proliferation and induced apoptosis in the cells.[16] After incubation, the cells were thoroughly washed with PBS three times and cultured in one of the following media for 24 hours: (1) fresh medium (CnT-20; CELLnTEC), (2) medium conditioned from cultures of human fibroblasts (hFbs), (3) medium from cultures of hMSCs, or (4) medium from cultures of TNF-α-treated hMSCs. In order to obtain hFb-conditioned medium, primary human dermal fibroblasts at passage 3 (CELLnTEC) were plated at low density (100 cells/cm^2) and cultured in complete culture medium (CCM) with 16% fetal bovine serum (FBS) for 4 days. Then the medium was removed, and the cells were washed with PBS three times and cultured in serum-free medium (CnT-20; CELLnTEC) for 24 hours, and the cell-free supernatant was obtained. For hMSC-conditioned medium, a frozen vial of passage 1 human bone marrow–derived MSCs was obtained from the Center for the Preparation and Distribution of Adult Stem Cells (Institute for Regenerative Medicine, Texas A&M Health Science Center, Temple, TX, USA; http://medicine.tamhsc.edu/irm/msc-distribution.html), which supplies standardized preparations of MSCs enriched for early progenitor cells to over 300 laboratories under the auspices of a National Institutes of Health/National Center for Research Resources grant (P40 RR 17447-06). All of the experiments were performed with hMSCs from one donor (No. 7075). Passage 3 hMSCs were plated at low density (100 cells/cm^2) and incubated in CCM with 16% FBS for 4 days. Then, the medium was replaced with serum-free medium (CnT-20; CELLnTEC) with or without 10 ng/mL recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) after washing with PBS, and the cells were cultured for 24 hours. The cell-free supernatant was obtained for use in further experiments. For a blocking experiment, the anti-STC-1 or goat normal IgG (1 μg/mL; R&D Systems) was added to cultures of hCEP simultaneously with the hMSC-conditioned medium and maintained for 24 hours. For an additional experiment, recombinant human (rh) STC-1 protein (BioVender, Brno, Czech Republic) was added to hCEP right after injury and maintained for 24 hours.

Cytotoxicity, Viability, and Proliferation Assays

Cytotoxicity was evaluated by measuring activity of lactate dehydrogenate (LDH) released from the cells damaged by ethanol following the manufacturer's protocol (LDH-Cytotoxicity Assay Kit; Abcam, Cambridge, MA, USA). Cell viability and proliferation were measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay according to the manufacturer's protocol (Vybrant MTT Cell Proliferation Assay Kit; Invitrogen, Carlsbad, CA, USA). Cell proliferation was quantitated by measuring BrdU (bromodeoxyuridine) incorporation in cells using colorimetric immunoassay (Cell Proliferation ELISA, BrdU; Roche, Indianapolis, IN, USA).

Apoptosis Assay

To evaluate apoptosis, cells were stained with a combination of propidium iodide (PI)-phycoerythrin (PE) (Molecular Probes, Inc., Leiden, The Netherlands) and annexin V (ANX)-FITC (Molecular Probes, Inc.) and analyzed by flow cytometry. The populations of early apoptotic cells (PI$^-$/ANX$^+$) and late apoptotic cells (PI$^+$/ANX$^+$) were compared between treatment and control groups.

Real-Time RT-PCR

For RNA extraction, cells were lysed in RNA isolation reagent (RNA Bee; Tel-Test, Inc., Friendswood, TX, USA), and total RNA was then extracted using RNeasy Mini kit (Qiagen, Valencia, CA, USA). Double-stranded cDNA was synthesized by reverse transcription (SuperScript III; Invitrogen). Real-time amplification was performed (Taqman Universal PCR Master Mix; Applied Biosystems, Carlsbad, CA, USA) and analyzed on an automated instrument (7900HT Fast Real-Time PCR System; Applied Biosystems). Polymerase chain reaction probe sets for p63 (transformation-related protein 63), ABCG2 (ATP-binding cassette subfamily G member 2), and K12 (cytokeratin 12) were commercially purchased (Taqman Gene Expression Assay Kits; Applied Biosystems). For assays, reactions were incubated at 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute. For normalization of gene expression, 18S rRNA probe was used as an internal control.

Immunocytochemical Staining

For immunocytochemical staining, the cells were fixed with 4% paraformaldehyde for 10 minutes and incubated in blocking buffer (3% BSA, 0.2% Triton X-100, 0.02% azide in PBS) for 1 hour. Primary antibodies were added in blocking buffer for 1 hour and secondary antibodies for 30 minutes. Goat polyclonal anti-human p63 (sc-25039; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and goat polyclonal anti-human cytokeratin 3 (sc-49179; Santa Cruz Biotechnology, Inc.) were used as primary antibodies. The anti-goat IgG (1:1000) (Jackson Immunoresearch, West Grove, PA, USA) was used as secondary antibody. The slides were visualized with fluorescent microscopy (Eclipse 80i; Nikon, Melville, NY, USA).

Tissue Culture of Rabbit Cornea and Determination of Epithelial Wound Healing

For organ culture of the whole cornea, fresh corneoscleral rims were obtained from New Zealand white rabbits (Charles River Laboratories International, Wilmington, MA, USA); each cornea was exposed to 20% ethanol for 30 seconds, and the epithelium was scraped off using the blade. The corneas were incubated in fresh or hMSC-conditioned medium for 48 hours at 37°C in an incubator with 5% CO_2. At 24 and 48 hours following injury, the corneas were stained with 5 μg/mL PI for 20 minutes in an incubator, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 4 hours, and then observed with fluorescence microscopy with a rhodamine filter as previously described.[17] The size of corneal epithelial defects was quantified and compared by the ratio of the PI-stained area to the total corneal area using an image analyzer (Image Pro Plus; Media Cybernetics, Bethesda, MD, USA).

ELISA

The amount of STC-1 in the cell-free supernatant from cultures of hMSCs or TNF-α-treated hMSCs was determined using an ELISA kit for STC-1 (R&D Systems).

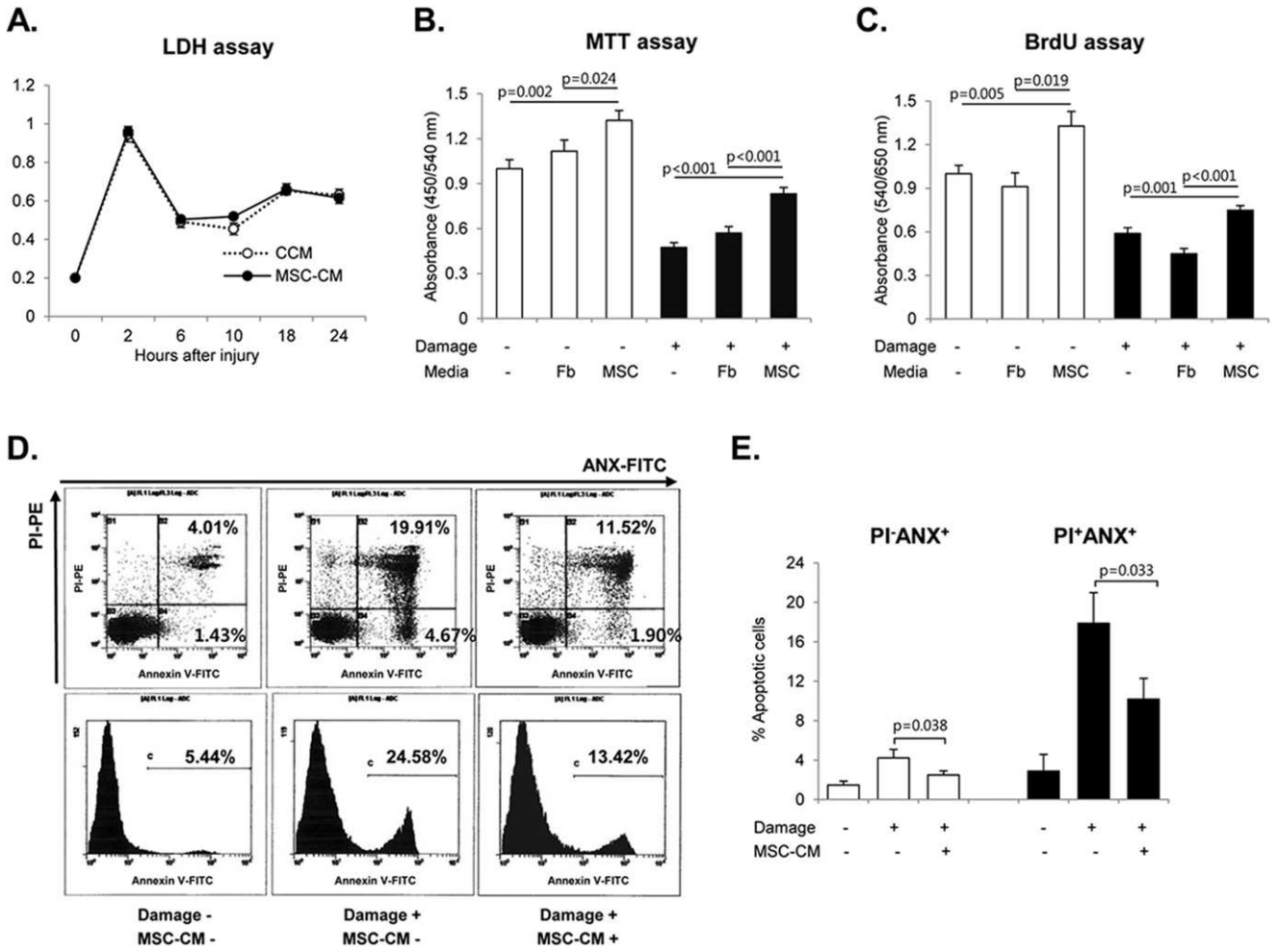

Figure 1. Effect of MSC-derived factors on lysis, survival, proliferation, and apoptosis of hCEP. Human corneal epithelial progenitor cells (hCEP) were injured by incubation in 20% ethanol for 30 seconds and cultured for 24 hours in fresh complete culture medium (CCM), medium derived from cultures of human fibroblasts (Fb), or medium from cultures of human mesenchymal stem/stromal cells (MSC-CM). (**A**) Analysis for lactate dehydrogenate (LDH) in the supernatant of cultures of hCEP showed that LDH was released from the cells immediately after injury, indicating cell lysis by ethanol. MSC-CM had no effect on the amount of LDH released from injured cells. (**B**) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay showed that the number of metabolically active cells was significantly increased in both uninjured and ethanol-injured hCEP by MSC-CM. (**C**) Bromodeoxyuridine (BrdU) uptake assay showed that the cell proliferation was also increased by MSC-CM. (**D**, **E**) Flow cytometry showed that the percentage of either PI^-/annexin V^+ (early apoptotic cells) or PI^+/annexin V^+ cells (late apoptotic cells) was markedly reduced by MSC-CM in both uninjured and ethanol-injured hCEP. Data are represented as mean ± SEM from at least six independent experiments.

Statistical Analysis

Comparisons of parameters among the groups were made by unpaired and two-tailed Student's *t*-tests using SPSS software (SPSS 12.0; Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

Results

Human MSC-Derived Factors Increased Proliferation and Decreased Apoptosis of hCEP

Incubation of hCEP in 20% ethanol for 30 seconds markedly decreased the survival of the cells by inhibiting proliferation and inducing cell lysis and apoptosis (Fig. 1). The hMSC-conditioned medium did not affect the lysis of hCEP following injury as determined by LDH amount in the supernatant of cell cultures (Fig. 1A). However, the number of metabolically active cells and proliferating cells was significantly higher in the cells cultured in the hMSC-conditioned medium compared to those cultured in fresh or hFb-conditioned medium as determined by MTT assay and BrdU uptake (Figs. 1B, 1C). The hMSC-conditioned medium was also effective in promoting proliferation of uninjured normal hCEP in cultures (Figs. 1B, 1C).

We next evaluated the effect of hMSC-derived factors on apoptosis of ethanol-injured hCEP. The hMSC-conditioned medium decreased apoptosis of hCEP as indicated by flow cytometry of either PI^-/ANX^+ cells or PI^+/ANX^+ cells (Figs. 1D, 1E).

Human MSC-Derived Factors Induced Expression of Stem Cell–Specific Markers in hCEP

We further assayed hCEP for the expression of putative markers for epithelial stem cells, p63 and ABCG2, and for a marker for fully differentiated epithelial cells, K12.[18] As expected, real-time RT-PCR and immunofluorescent staining revealed that the expression of p63, ABCG2, and K12 markedly decreased in hCEP 24 hours after ethanol injury (Figs. 2, 3). Interestingly, incubation of injured hCEP in the hMSC-

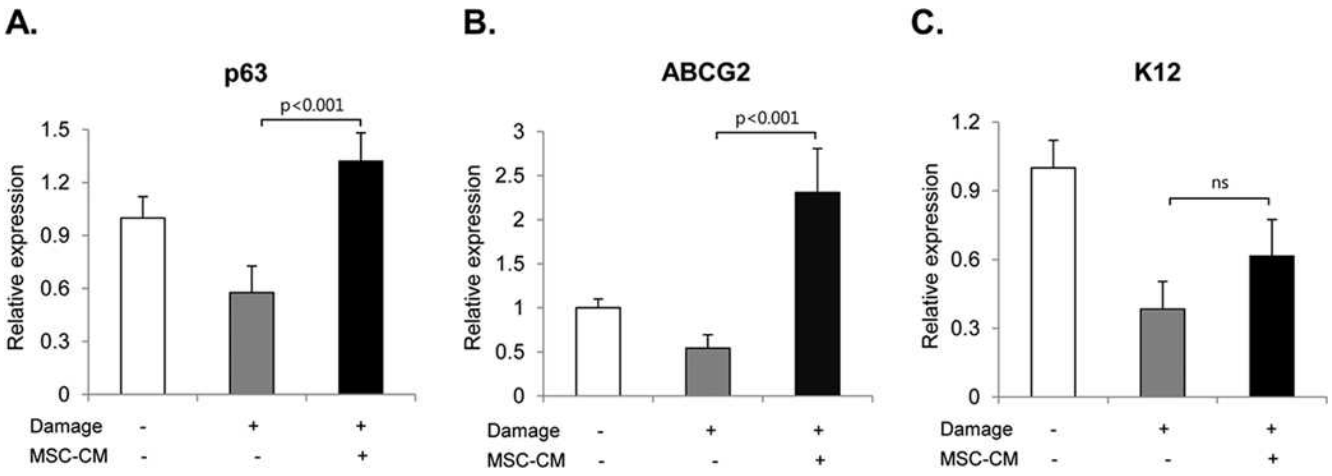

Figure 2. Effect of MSC-derived factors on gene expression of epithelial stem cell markers (p63, ABCG2) and differentiated corneal epithelial cell marker (K12) in hCEP. Real-time PCR analysis demonstrated that ethanol injury markedly reduced the levels of p63, ABCG2, and K12 transcripts in human corneal epithelial progenitor cells (hCEP). The incubation of injured cells in the medium conditioned from human mesenchymal stem/stromal cell culture (MSC-CM) significantly increased the transcript levels of p63 and ABCG2, while the expression of K12 remained unaltered. Data are represented as mean + SEM from three independent experiments.

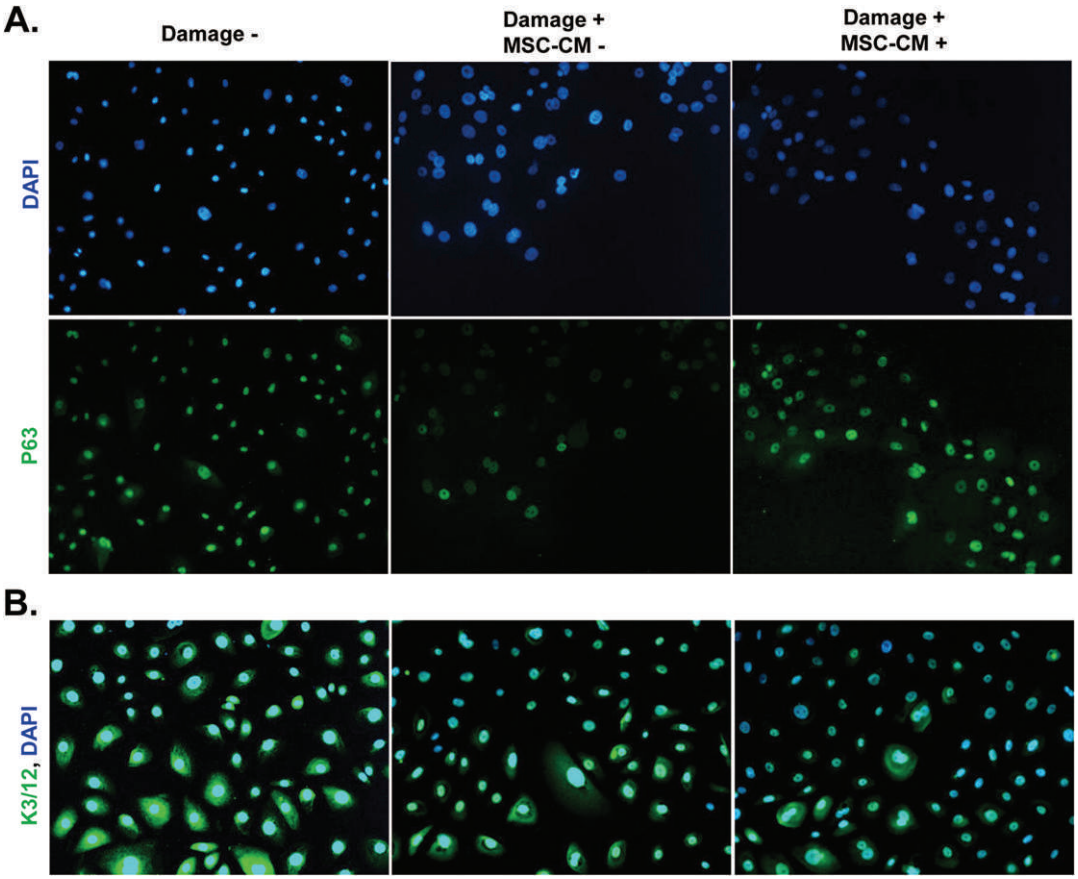

Figure 3. Effect of MSC-derived factors on corneal epithelial cell marker expression. (**A**) Immunostaining for epithelial stem cell marker p63 showed that the number of p63-expressing cells was markedly decreased in human corneal epithelial progenitor cells (hCEP) at 24 hours after ethanol exposure. The conditioned medium from human mesenchymal stem/stromal cells (MSC-CM) significantly increased the number of p63-expressing cells. (**B**) The number of hCEP expressing K3/12, a differentiated corneal epithelial cell marker, was significantly reduced by ethanol and not affected by MSC-CM.

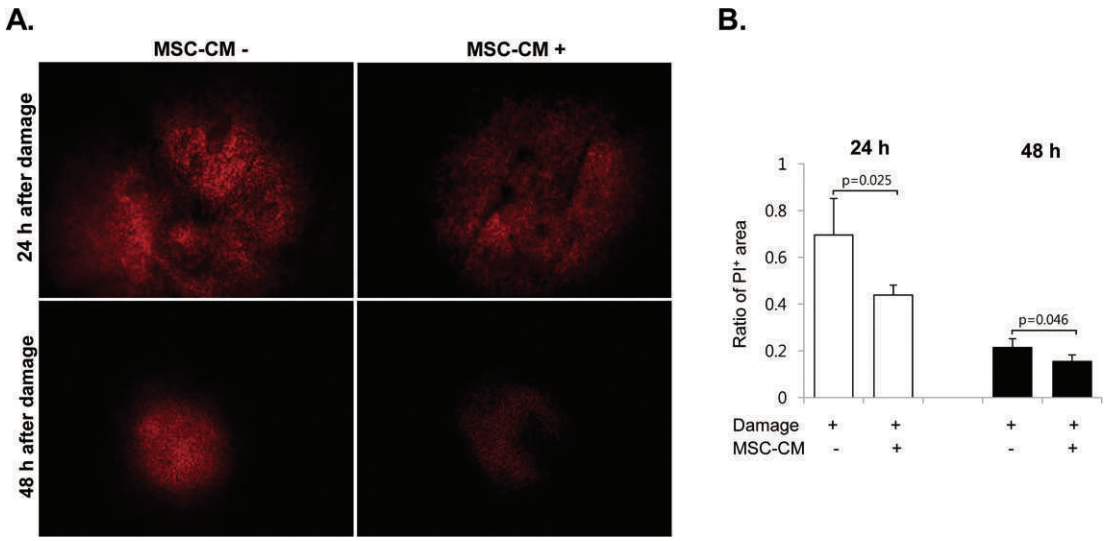

FIGURE 4. Effect of MSC-derived factors on epithelial wound healing of the rabbit cornea. (**A**) Ex vivo cultures of rabbit corneas were injured by exposure to 20% ethanol for 30 seconds and removal of the whole corneal epithelium including the limbal area. Twenty-four and 48 hours later, the corneal surface was stained with propidium iodide (PI) and photographed. The PI staining showed that corneal epithelial wound healing was more rapid in the corneas incubated in the medium conditioned from human mesenchymal stem/stromal cell culture (MSC-CM). (**B**) The degree of epithelial defects as measured by the ratio of the PI-stained area to the whole cornea was significantly lower in the corneas incubated in MSC-CM compared to controls. Data are represented as mean + SEM from three independent experiments, each with three rabbits per group.

conditioned medium significantly increased the expression of p63 and ABCG2 (Figs. 2A, 2B, 3A). However, the expression of K12 was not altered by the hMSC-conditioned medium (Figs. 2C, 3B).

hMSC-Derived Factors Promoted Corneal Epithelial Wound Healing After Chemical Injury in Rabbit Cornea

In order to determine whether the protective effects of hMSC-derived factors on corneal epithelial cells observed led to the wound healing of the cornea, we tissue cultured rabbit corneas and injured the cornea by applying 20% ethanol to the surface for 30 seconds and removing the whole epithelium including the limbus. Then, the corneas were incubated either in the hMSC-conditioned medium or in fresh medium for 48 hours. At 24 and 48 hours following injury, corneal epithelial wound healing was assessed using PI staining (Fig. 4A). The PI-stained area that defines the de-epithelialized lesion was rapidly reduced in the corneas incubated in hMSC-conditioned medium compared to those in fresh medium, indicating that the wound healing was accelerated by hMSC-derived factors (Figs. 4A, 4B).

Preactivation of hMSCs by TNF-α Was More Effective in Rescuing hCEP by Secreting STC-1

Several studies previously demonstrated that preactivation of MSCs by TNF-α or IFN-γ increases their beneficial effects in vivo because the production of additional therapeutic factors by MSCs is increased by TNF-α or IFN-γ.[14,15] These observations are consistent with the notion that MSCs upregulate the expression of therapeutic factors through cross-talk with injured tissues.[1,2] Therefore, we next investigated the effects of the medium derived from hMSCs preactivated by TNF-α. We found that the conditioned medium from TNF-α-activated hMSCs was more effective in increasing survival and proliferation of ethanol-damaged hCEP than the medium from normal cultures of hMSCs (Figs. 5A, 5B).

In order to identify the factor responsible for the effects of hMSC-conditioned medium, we focused on an antiapoptotic molecule, STC-1, that is known to be produced by hMSCs upon coculture with apoptotic cells.[9,10] We found that the level of STC-1 was approximately 100 times higher in the medium from cultures of TNF-α-activated hMSCs compared to cultures of normal hMSCs (Fig. 5C; note log scale). Addition of an antibody to STC-1 to the cultures significantly reduced the effects of the preactivated hMSC-conditioned medium on the survival and proliferation of damaged hCEP, whereas addition of control IgG did not affect the action of hMSC-conditioned medium (Figs. 5D, 5E). Also, addition of rhSTC-1 to the cultures significantly increased the survival and proliferation of damaged hCEP in a dose-dependent manner (Figs. 5D, 5E). Similarly, the antiapoptotic effect of the preactivated hMSC-conditioned medium was significantly attenuated by addition of the anti-STC-1 to cultures, while addition of rhSTC-1 reproduced the effects of hMSC-conditioned medium in reducing the number of apoptotic hCEP (Figs. 5F, 5G). Therefore, the data collectively indicate that hMSCs activated by TNF-α were more effective in protecting hCEP from injury at least in part by producing a large amount of STC-1.

DISCUSSION

The results demonstrate that hMSC-derived factors increased the survival of hCEP by promoting proliferation and inhibiting apoptosis. These beneficial effects were enhanced by preincubating hMSCs with the proinflammatory cytokine TNF-α. The increased effectiveness of the preactivated hMSCs was mediated in part by an increased concentration of the multifunctional protein STC-1.

The corneal epithelium serves as a biological and physical barrier by protecting the interior structures of the eye from environmental insults. The transparency of the cornea and therefore visual acuity are both dependent upon the integrity and functionality of corneal epithelium. Most of the diseases occurring in corneal epithelium are accompanied by inflammation and wound healing defect. Our group previously

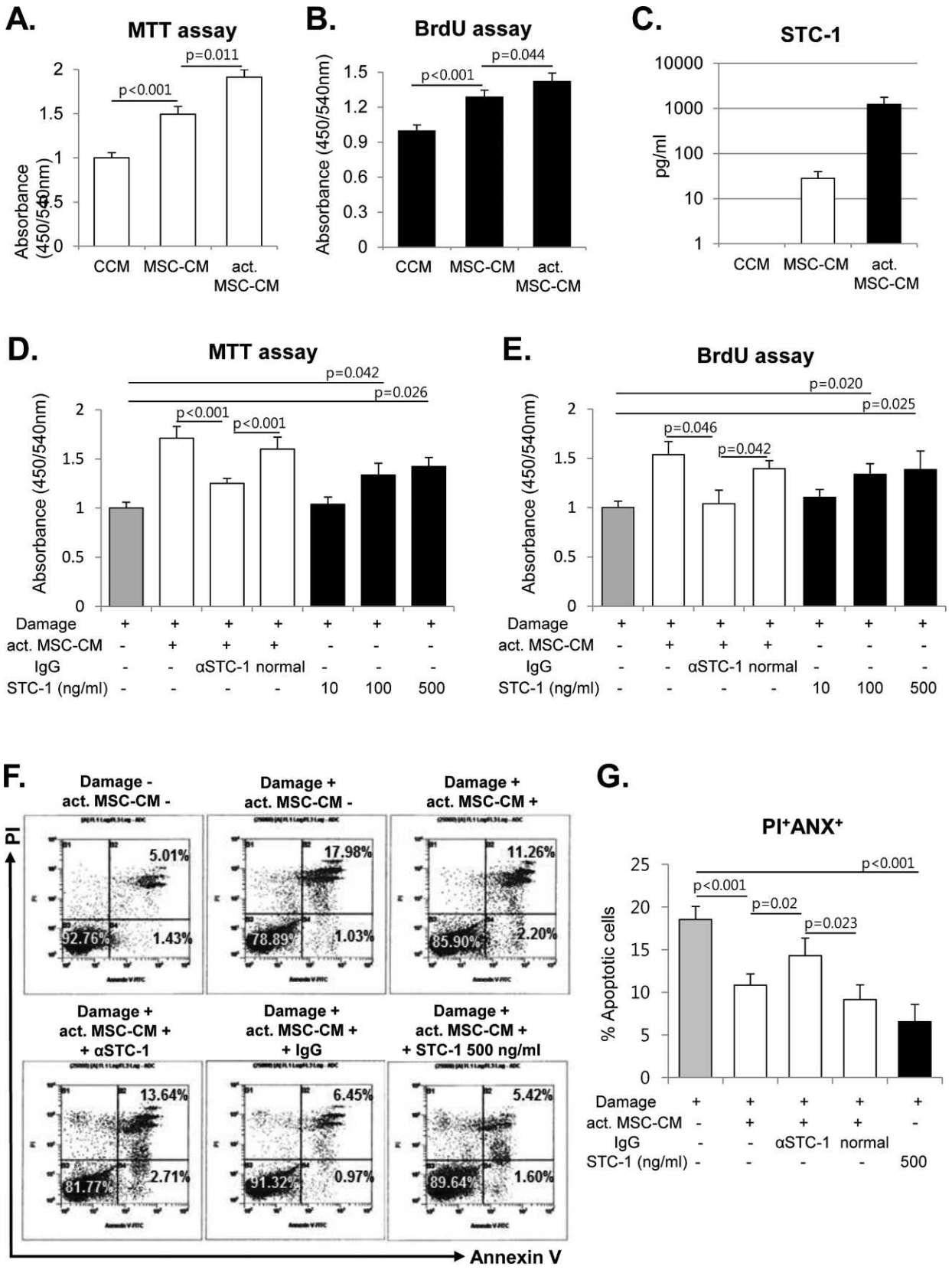

FIGURE 5. Effect of TNF-α-activated MSC on survival, proliferation, and apoptosis of hCEP. (**A**, **B**) Conditioned medium from cultures of human mesenchymal stem/stromal cells (MSC) that were preactivated by TNF-α (act. MSC-CM) was more effective in increasing the survival and proliferation of ethanol-injured human corneal epithelial progenitor cells (hCEP) than the medium derived from normal cultures of MSC (MSC-CM). (**C**) The protein level of stanniocalcin (STC)-1 was approximately 100-fold higher in act. MSC-CM than in MSC-CM. Note log scale. (**D**, **E**) Blocking of STC-1 using neutralizing antibody (αSTC-1) abrogated the effects of act. MSC-CM on the survival and proliferation of damaged hCEP, while addition

of recombinant human STC-1 dose dependently increased the survival and proliferation of the cells. (**F**, **G**) Act. MSC-CM markedly decreased the apoptosis of ethanol-injured hCEP as measured by the number of PI^+/ANX^+ cells. However, the antiapoptotic effect of act. MSC-CM was significantly attenuated by αSTC-1. Also, the addition of STC-1 reduced the number of apoptotic hCEP following injury in a dose-dependent manner. Data are represented as mean + SEM from at least six independent experiments.

demonstrated that either MSCs or MSC-derived factors modulate inflammatory responses in the cornea after injuries in vivo.[6,7] In the present study, we used ex vivo culture systems of hCEP and rabbit corneas in order to evaluate the effects of hMSCs solely on hCEP independent of their effects on the immune cells or other cell types in vivo. As a result, we found that the hMSC-derived factors had direct protective effects on hCEP by increasing proliferation and inhibiting apoptosis, and thereby accelerated wound healing of the cornea following injury. These findings suggest that MSCs restrict damage and promote regeneration in the cornea by protecting hCEP as well as by inhibiting collateral damage from excessive inflammation.

Importantly, our results indicate that conditioned medium from hMSCs preactivated by TNF-α is more effective in protecting hCEP from injury than medium from standard cultures. These findings support the notion that MSCs would have more potent effects in vivo by producing more therapeutic factors through cross-talk with injured tissues.[1,2,14,15] One of the candidate factors secreted by MSCs in response to injury is STC-1. Previous studies reported that STC-1 is secreted by hMSCs in response to signals from apoptotic cells and mediates an antiapoptotic action of hMSCs.[9,10] In fact, STC-1 has been shown to have multiple biological effects in mammals, involving protection of cells against ischemia,[19,20] suppression of inflammatory responses,[21,22] or reduction of reactive oxygen species[21–23] and the subsequent apoptosis in alveolar epithelial cancer cells, photoreceptors, or retinal ganglion cells.[10,12,13] Similarly, our data demonstrate that TNF-α-activated hMSCs secreted a large amount of STC-1, and STC-1 was responsible at least in part for the protective effects of hMSCs on corneal epithelial cells. However, in addition to STC-1, there are myriad therapeutic factors that MSCs produce through their broad range of responses to different microenvironments of injured tissues. Further studies may identify additional MSC-derived factors that can be used therapeutically.

Taken together, our results suggest that hMSCs, independently of the effects of the cells on the systemic immune system, can directly promote regeneration of injured corneal epithelium. The results add to previous knowledge about the beneficial effects of MSCs on tissue-endogenous stem cells and regeneration in various disease models, and provide a further basis for using MSCs or MSC-derived factors to treat diseases of the cornea and other tissues that involve inflammation and wound healing defect.

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