

Anti-inflammatory protein TSG-6 reduces inflammatory damage to the cornea following chemical and mechanical injury

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Previous reports demonstrated that adult stem/progenitor cells from bone marrow (multipotent mesenchymal stem cells; MSCs) can repair injured tissues with little evidence of engraftment or differentiation. In exploring this phenomenon, our group has recently discovered that the therapeutic benefits of MSCs are in part explained by the cells being activated by signals from injured tissues to express an anti-inflammatory protein TNF- α -stimulated gene/protein 6 (TSG-6). Therefore, we elected to test the hypothesis that TSG-6 would have therapeutic effects in inflammatory but non-infectious diseases of the corneal surface. We produced a chemical and mechanical injury of the cornea in rats by brief application of 100% ethanol followed by mechanical debridement of corneal and limbal epithelium. Recombinant human TSG-6 or PBS solution was then injected into the anterior chamber of the eye. TSG-6 markedly decreased corneal opacity, neovascularization, and neutrophil infiltration. The levels of proinflammatory cytokines, chemokines, and matrix metalloproteinases were also decreased. The data indicated that TSG-6, a therapeutic protein produced by MSCs in response to injury signals, can protect the corneal surface from the excessive inflammatory response following injury.

inflammation | TNF- α -stimulated protein 6 | mesenchymal stem cells | neutrophil | matrix metalloproteinase

The cornea is both an important component of the refractive system of the eye and a physical barrier that protects the interior structures of the eye from environmental insults. The transparency of the cornea depends upon the integrity of the outermost layer, the corneal epithelium. Some of the most severe diseases of the cornea are a result of chemical or thermal burns that cause intractable inflammation and limbal epithelial stem cell (LESC) deficiency (LSCD) (1). The current treatments of LSCD include anti-inflammatory therapies such as steroids or transplants of amniotic membrane in the acute phase and transplantation of LESCs in the chronic phase (2). However, the anti-inflammatory drugs currently available are often unable to prevent subsequent loss of LESCs. Also, there are many drawbacks (3–5) associated with transplanting LESCs from a patient's healthy eye (i.e., limbal autograft), a living relative, or cadaveric donors (i.e., limbal allograft). Limbal autografts risk creating LSCD in the donor eye (6) and cannot be used in patients with bilateral LSCD. Limbal allografts require long-term systemic immunosuppression, and even with immunosuppression, rejection occurs in 42.9% to 64.0% of patients (7, 8). One therapeutic strategy for corneal inflammation is the use of the adult stem/progenitor cells from bone marrow referred to as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs). Previous reports showed that application of MSCs or conditioned medium from cultures of MSCs to chemically injured cornea suppressed inflammation and neovascularization and promoted corneal wound healing in rats (9–11). However, the therapeutic factors produced by MSCs were not defined. In parallel observations, our group (12) recently reported that i.v. infusion of human MSCs in a mouse model of myocardial in-

farction reduced inflammatory damage and improved cardiac function, largely because the cells were trapped in the lung as microemboli and activated to express the anti-inflammatory protein TNF- α -stimulated protein 6 (TSG-6) (13, 14).

Here we demonstrated that injection of TSG-6 into the anterior chamber of the eye markedly reduced the inflammatory damage to the cornea produced by chemical and mechanical injury.

Results

Effects of TSG-6 on Corneal Opacity and Neovascularization. To test the therapeutic effects of TSG-6 in the chemically injured cornea, 100% ethanol was briefly applied to the cornea of Lewis rats, followed by mechanical debridement of the corneal and limbal epithelium (9). Immediately after injury, 2 μ g recombinant human TSG-6 (rhTSG-6) in 5 μ L of PBS solution or the same volume of PBS solution was injected into the anterior chamber of the eyes. The corneal surface was then evaluated for transparency and neovascularization. The corneas of control eyes developed severe opacity by day 3 (Fig. 1 *A* and *B*) and severe neovascularization by day 21 after injury (Fig. 1 *A* and *C*). In contrast, corneal opacity was significantly reduced in TSG-6-treated corneas, and the eyes retained optical clarity through day 21 after injury (Fig. 1 *A* and *B*). Additionally, corneal neovascularization was less in TSG-6-treated group compared with PBS solution-treated corneas (Fig. 1 *A* and *C*).

Effects of TSG-6 on Corneal Inflammation. To explore the mode of action of TSG-6, we examined the corneas for inflammation. Histology of the PBS solution-treated corneas demonstrated extensive infiltration of neutrophils on day 3 following an injury (Fig. 2*A*). On day 21, the corneal stroma in PBS solution-treated controls was severely disoriented with thickened lamellae and extensive invasion of new vessels. Also, the PBS solution-treated corneas were marked by thickened epithelium with goblet cell infiltration, suggesting conjunctivalization of the corneal surface, an indicator of LSCD. In contrast, although denudation of the epithelium from injury was apparent on day 3, there was significantly less neutrophil infiltration in the corneas from TSG-6-treated eyes (Fig. 2*A*). Normal-appearing epithelium was restored on the TSG-6-treated corneas by day 21, and the stromal lamellae were well preserved and regularly aligned. Also, new vessel invasion was not observed.

For a quantitative measure of neutrophil infiltration, the corneas were assayed for the myeloperoxidase (MPO) concentration, an enzyme stored in cytoplasmic azurophilic granules of neutrophils and released extracellularly by activated neutrophils (15).

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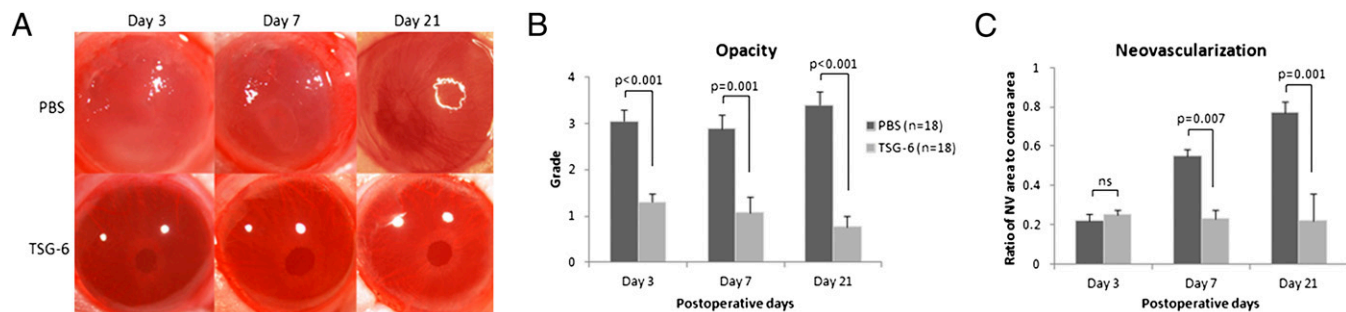


Fig. 1. TSG-6 reduced corneal opacity and neovascularization in the cornea following injury. (A) Representative photographs demonstrated the corneal surface on days 3, 7, and 21 after injury. Marked corneal opacity was present by day 3 after injury, and significant neovascularization developed by day 21 after injury in PBS solution-treated cornea. Corneal opacity and neovascularization were significantly decreased by TSG-6 injection. Note clear pupillary margin and well demarcated light reflex in TSG-6-treated cornea. (B) Quantification of corneal opacity following a clinical grading system on a scale from 0 to 4. (C) Quantification of neovascularization. Values are expressed in mean \pm SD ratio of the neovascularized area to the whole corneal area.

MPO was expressed at high levels on day 3 in the corneas from control eyes (Fig. 2B). As expected, treatment with TSG-6 significantly decreased the levels of MPO in the cornea. In addition, the levels of total MMP-9 and active MMP-9 were markedly increased on day 3 in the PBS solution-treated control corneas as assayed by gel zymography (Fig. 2C) and ELISA (Fig. 2D). Treatment with TSG-6 significantly decreased the levels of MMP-9 in the corneas.

A similar pattern was observed in data obtained by assays of the tissue for expression of proinflammatory cytokines and chemokines. Quantitative real-time RT-PCR revealed increases in mRNA levels for proinflammatory cytokines and chemokines on day 3 in the injured cornea: IL-6, IL-1 β , CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CXCL1/CINC-1, MT-MMP, and CSF3 (Fig. S1). The expression of proinflammatory cytokines and chemokines was markedly reduced by TSG-6 treatment. Consistent with the real-time RT-PCR results, the protein levels of IL-6, IL-1 β , CXCL1/CINC-1, and CCL2/MCP-1 were significantly decreased in the TSG-6-treated cornea as assayed by ELISA (Fig. 3). Of note was that significant levels of TNF- α were not detected in treated or untreated corneas by ELISA or real-time RT-PCR analysis. The changes in the expression of cytokines and chemokines were also reflected in microarray data (Fig. 4 and Table S1).

Dose Dependence of the Effects of TSG-6. To obtain quantitative data, a standard curve was developed to compare clinical evaluations of the cornea with the MPO concentration. As indicated in Fig. S2, clinical grade of corneal opacity on day 3 showed a significant positive correlation with the MPO concentration ($r^2 = 0.907$).

To study a dose response to the therapy, varying doses of TSG-6 (0.0002–2 μ g) were injected into the anterior chamber of the eyes immediately after injury. The anti-inflammatory effects of TSG-6 were dose-dependent as evaluated by corneal visualization of opacity (Fig. 5A), MPO ELISA (Fig. 5B), MMP-9 zymography (Fig. 5C), and MMP-9 ELISA (Fig. 5D). Evaluation of corneas by both clinical examination and MPO assays on day 3 indicated that a significant improvement in the corneas was achieved with injection of as little as 2 ng TSG-6 (Fig. 5A and B). A dose as low as 2 ng TSG-6 also produced a significant decrease in MMP-9 expression (Fig. 5D). However, superior effects were consistently obtained with 2 μ g TSG-6, the maximal dose that could be injected because of the solubility limit of the protein and the maximal volume that could be safely injected into the anterior chamber of the rat eye (16).

Time Course of Inflammatory Reaction and Effects of TSG-6. To examine the early inflammatory response to the injury, corneal tissue and systemic blood were evaluated at 2 to 72 h after injury. Opacity

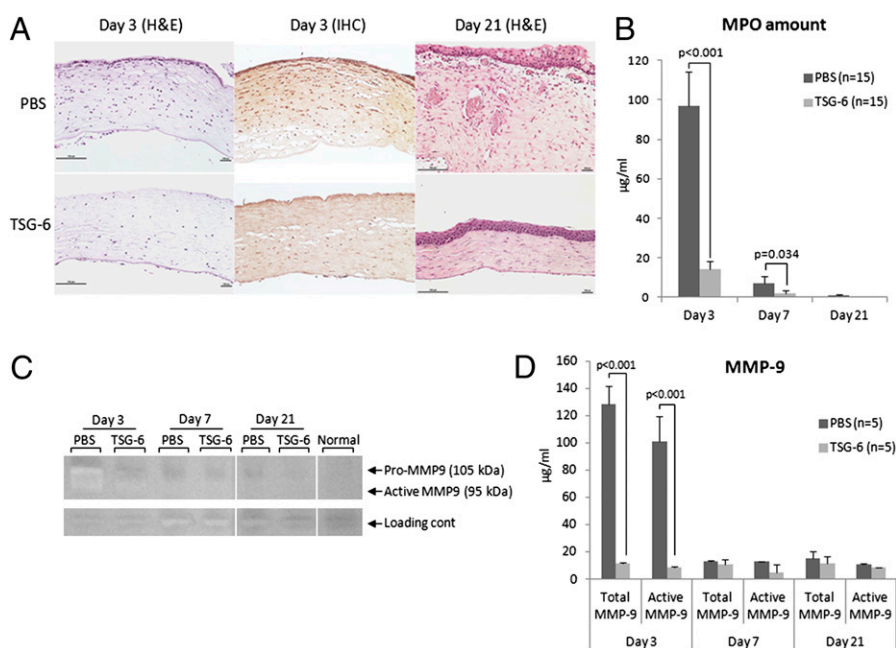


Fig. 2. TSG-6 reduced inflammation in the cornea following injury. (A) Representative images of H&E staining and immunohistochemistry (IHC) for neutrophil elastase showed severe neutrophil infiltration on day 3 after injury in the PBS solution-treated cornea. Also, thickened fibrovascular corneal stroma was observed on day 21. In contrast, neutrophil infiltration was much decreased in the TSG-6-treated cornea on day 3, and normal-appearing epithelium and stroma were restored by day 21. Note that corneal epithelium was denuded from injury on day 3 in PBS- and TSG-6-treated corneas. (B) The MPO concentration, as quantified by ELISA, was markedly increased in the cornea on day 3 after injury. TSG-6 significantly decreased the levels of MPO on day 3 and day 7. (C) Gelatin zymography demonstrated an increase in the expression of pro-MMP-9 and active MMP-9 on day 3, which was markedly decreased by TSG-6 injection. (D) The levels of total and active MMP-9 in the whole cornea were also significantly decreased by treatment with TSG-6 as assayed by ELISA. Values are mean \pm SD.

and inflammatory infiltrates in the cornea became apparent at 24 h (Fig. 6A). Neutrophil infiltration as reflected by histology and MPO levels was increased slightly before 4 h and then increased markedly with a peak at 24 to 72 h (Fig. 6B–D). The neutrophil counts in the systemic blood were also increased after injury and reached the peak at 24 h (Fig. S3). Thereafter, the neutrophil counts in the systemic blood returned to normal at 48 and 72 h. An increase in corneal infiltration of neutrophil and neutrophil counts in the blood of untreated rats was markedly suppressed in TSG-6-treated rats (Fig. 6D and Fig. S3A). In contrast to the MPO levels, the level of IL-6 in the cornea increased to a maximum at approximately 4 to 8 h (Fig. 6D). The levels of other cytokines and chemokines (IL-1 β , CXCL1/CINC-1, and CCL2/MCP-1) reached peak levels at 24 h (Fig. 6D). As expected, treatment with TSG-6 lowered the levels of MPO, proinflammatory cytokines, and chemokines in the cornea at the time points examined.

Window for TSG-6 Administration. One critical question for acute therapies of corneal injuries is how soon after the injury the therapy must be administered. Hence, to explore the window of opportunity for treatment of corneal inflammation with TSG-6, we injected TSG-6 (2 μ g) or PBS solution at different time points (0–24 h) after injury. As indicated in Fig. 7, injection of TSG-6 within 4 h after injury significantly decreased inflammation in the cornea. Notably, injection of TSG-6 2 h after injury was as effective in reducing corneal inflammation as administration immediately after injury. However, TSG-6 was not effective if administered 8 to 24 h after injury, by which time neutrophils had intensely infiltrated the cornea (Fig. 6B and C).

Discussion

Severe diseases of the cornea that produce LSCD can be heritable, but they are more commonly caused by acquired conditions such as chemical or thermal burns, systemic autoimmune disease, contact lens keratopathy, recurrent ocular surgeries, or Stevens–Johnson

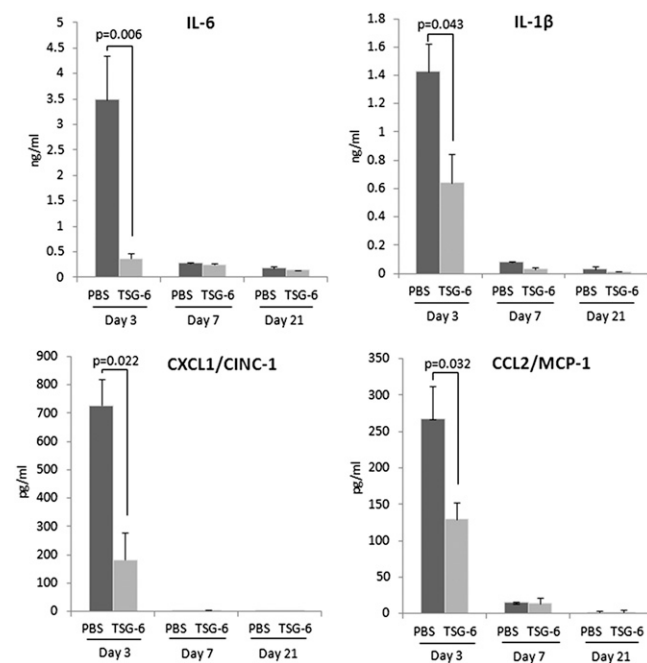


Fig. 3. TSG-6 reduced protein concentration of proinflammatory cytokines and chemokines. ELISAs showed that the levels of proinflammatory cytokines (IL-6 and IL-1 β) and chemokines (CXCL1/CINC-1 and CCL2/MCP-1) were markedly increased on day 3 after injury in the PBS-treated corneas. In contrast, the production of proinflammatory cytokines and chemokines was significantly decreased in the TSG-6-treated corneas. Values are mean \pm SD; $n = 5$ for each group.

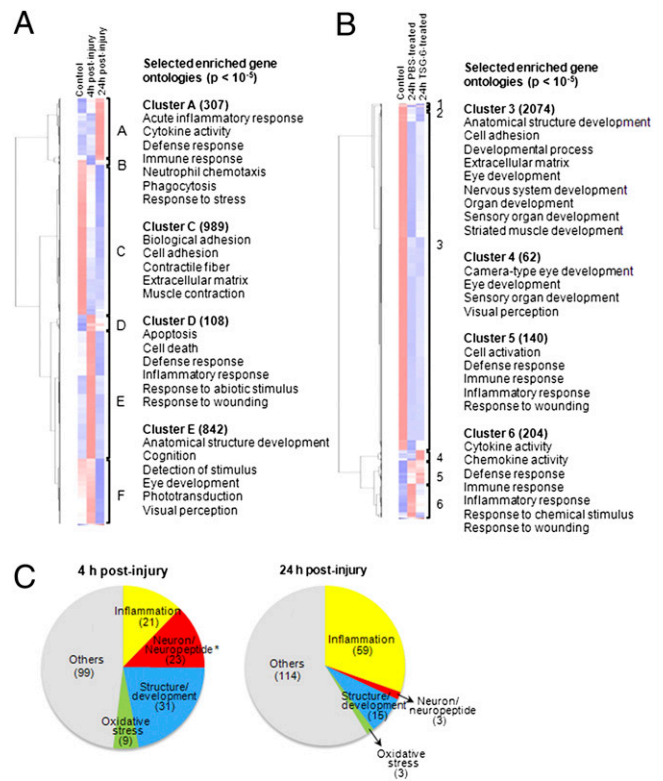


Fig. 4. Microarray analysis of the cornea following injury. (A) Microarray heat map of genes from the corneas 4 h and 24 h after injury is shown. (B) Heat map of genes from PBS- and TSG-6-treated corneas at 24 h after injury is shown. Gene ontology categories and the number of genes up-regulated (red) or down-regulated (blue) greater than twofold are indicated. Genes expressed differentially in PBS- versus TSG-6-treated corneas at 24 h are shown in Table S1. (C) Diagrams of genes up-regulated greater than fivefold in the corneas 4 h and 24 h after injury. The number of genes in each ontology category is indicated. Among genes that were temporarily up-regulated after injury (asterisk), secretogranin II showed the largest change: the gene was up-regulated 15.8-fold at 4 h and returned to normal at 24 h. In the TSG-6-treated corneas, up-regulation of the secretogranin II gene was 1.5-fold at 4 h.

syndrome. Patients with severe chemical injuries usually have a poor prognosis because of a severe inflammatory response that frequently produces a profound loss of LSCs (1). The annual incidence of LSCD from chemical injuries of the cornea is approximately 500,000 in the United States and accounts for 7% to 18% of the 2.5 million cases of ocular trauma seen in emergency departments each year in the United States (17, 18). However, currently available anti-inflammatory therapies are not curative in many cases and are unable to prevent development of LSCD following chemical injuries. Therefore, chemical injuries of the cornea impose a severe burden on the patient and the health care system (19).

The results here demonstrated that a single intraocular injection of TSG-6 protected the cornea from opacification and neovascularization following injury. Administration of TSG-6 dramatically decreased cytokine and chemokine production, neutrophil infiltration, and levels of MMP-9 during the first few hours and days after the injury. Therefore, TSG-6 limited destruction of the tissue by the normal but excessive inflammatory response to a sterile injury. Subsequently, normal reepithelialization of the cornea was achieved. It is of interest that the intraocular injection of TSG-6 was effective even though the aqueous fluid turns over in approximately 40 min in the rodent eye (20). A similar sequence of events, with a reduction in the early inflammatory response, was observed after the systemic infusion of TSG-6 into mice with myocardial infarcts (12).

TSG-6 is a 35-kDa secreted protein composed mainly of contiguous Link and CUB modules (21, 22). The protein is not

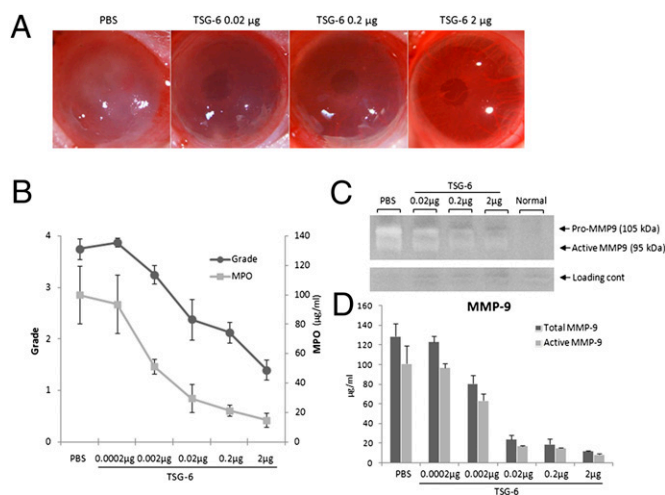


Fig. 5. TSG-6 reduced corneal inflammation and opacity in a dose-dependent manner. (A) Representative corneal photographs on day 3 after injury demonstrated that TSG-6 suppressed development of corneal opacity after chemical injury in a dose-dependent manner. (B) The anti-inflammatory effects of TSG-6 were dose-dependent as reflected in clinical grade of corneal opacity and MPO concentration. Values are mean \pm SD; $n = 3$ for each group. (C) Gelatin zymography for pro- and active MMP-9. (D) Total and active MMP-9 concentration in the cornea as quantified by ELISA. Values are mean \pm SD; $n = 5$ for each group. Significant improvements were observed with dose of 0.002 μ g but maximal effects were obtained with 2 μ g.

expressed in normal cells or tissues, but is expressed after cells are exposed to TNF- α and other proinflammatory cytokines. TSG-6 has been reported to participate in ECM remodeling by interacting with the glycosaminoglycan hyaluronan, chondroitin sulfate, aggrecan, and the serine protease inhibitor inter- α inhibitor (21, 23, 24). Also, TSG-6 was shown to have anti-inflammatory activities in several different models (14, 25). In transgenic mice, inactivation of the gene increased inflammatory responses, and overexpression of the gene decreased inflammation and joint destruction following induced arthritis (26–28). Also, systemic or local administration of recombinant protein dramatically improved the arthritis (29). The anti-inflammatory activity of TSG-6 was attributed to its ability to inhibit components in the inflammatory network of proteases and to suppress neutrophil migration into the site of inflammation (13, 14, 30). In the burned cornea, neutrophil infiltration is particularly important, and it is an early event. If uncontrolled, the release of various proteases by neutrophils can result in stromal degradation and ulceration, eventually leading to permanent vision-impairing corneal opacification and neovascularization (31). The present study showed that intraocular TSG-6 decreased the infiltration of neutrophils into the cornea and the production of proteases following injury. This finding was also supported by our observation that TSG-6 injected after 8 h after injury (the time point when a significant number of neutrophils had already infiltrated the cornea) did not suppress corneal inflammation. However, the mechanism by which TSG-6 inhibits neutrophil invasion has not been fully defined. Several reports suggested that TSG-6 inhibited the migration and extravasation of neutrophils by down-regulating the protease network (27, 30, 32). However, in the experiments presented here, local injection of TSG-6 suppressed the plasma levels of neutrophils as well as the neutrophil infiltration into the cornea. Therefore, the effects of TSG-6 on inhibiting neutrophil infiltration shown in the present study cannot be explained solely by the action of TSG-6 on neutrophil migration and extravasation. Rather, in conjunction with the finding of marked reduction of proinflammatory cytokines and chemokines by TSG-6 treatment, our data support the hypothesis that TSG-6 acts on tissue-resident cells for anti-inflammatory effects via modulating chemokines/cytokines produced by injured tissue.

The effectiveness of injection of TSG-6 into the anterior chamber may explain the previous report that an injury to the cornea was improved by application to the corneal surface of either MSCs or conditioned medium from cultures of MSCs (9). However, MSCs may secrete a large number of additional factors in response to signals from injured tissues with a therapeutic potential that may have contributed to beneficial effects of MSCs in the cornea. Some of the factors in addition to TSG-6 may be critical to the tissue repair observed with conditioned medium from MSCs or systemically administered MSCs (9–11).

In conclusion, our data demonstrate that TSG-6, a therapeutic protein produced by MSCs in response to injury signals, prevented opacity and neovascularization in the cornea following chemical and mechanical injury by suppressing inflammation. Intraocular TSG-6 administration may offer significant promise for patients with intractable corneal inflammatory diseases.

Materials and Methods

Animals and Reagents. The experimental protocols were approved by the institutional animal care and use committee of Texas A&M Health Science Center. Six-week-old male Lewis rats (LEW/CrI; Charles River Laboratories) weighing 180 to 200 g were used in all experiments. rhTSG-6 was purchased from R&D Systems. The rhTSG-6 used in this study was pretested for anti-inflammatory activity in a mouse model of zymosan-induced peritonitis (33).

Animal Model of Injury and Treatment. Rats were anesthetized by isoflurane inhalation. To create the chemical burn, 100% ethanol was applied to the whole cornea including the limbus for 30 s, followed by rinsing with 10 mL of balanced salt solution. Then, the whole corneal and limbal epithelium was mechanically scraped using a surgical blade. Upon completion of the procedure, the eyelids of a rat were closed with one 8-0 silk suture at the lateral third of the lid margin. At predetermined time points after injury, rhTSG-6 (0.0002–2 μ g in 5 μ L of PBS solution) or the same volume of PBS solution was injected into the anterior chamber of the rat eye using a 32-gauge needle and syringe.

Ocular Surface Evaluation and Clinical Outcome Analysis. After injury and treatment, the rat corneas were examined for corneal opacity and neovascularization under a dissecting microscope and photographed. Corneal opacity was assessed and graded by a blinded investigator, who was an ophthalmologist, as described by Sonoda and Streilein (34): grade 0, completely transparent cornea; grade 1, minimal corneal opacity, but iris clearly visible; grade 2, mild corneal opacity, iris vessels still visible; grade 3, moderate corneal opacity, pupil margin but not iris vessels visible; and grade 4, complete corneal opacity, pupil not visible. Corneal neovascularization was quantified by calculating the wedge-shaped area of vessel growth using the following equation (35): $A = C / 12 \times 3.1416 [r^2 - (r - l)^2]$, where A is the area, C is a fraction of circumference based on 12-h clock, l is the radius from the center to the border of vessel growth, and r is the radius of the cornea (35). The amount of neovascularization was compared between the groups using the ratio of the neovascularized area to the whole corneal area. An image analyzer (Image Pro Plus; Media Cybernetics) was used for measuring the radius and area.

Histopathology The cornea was excised after the rat was killed and fixed in 10% paraformaldehyde. The cornea was cut into 4- μ m sections and stained with H&E or subjected to immunohistochemistry. Immunohistochemistry was performed to confirm the presence of neutrophils in the corneas. The formalin-fixed corneal section was deparaffinized with ethanol and antigen was retrieved using a steamer. The polyclonal rabbit antibody against the rat neutrophil elastase (1:200; Abcam) was used as primary antibody and anti-rabbit IgG (1:5,000; Abcam) as secondary antibody. The DAB staining was performed using the DAB Peroxidase Substrate kit (Vector Laboratories). The slides were counterstained with hematoxylin.

Real-Time RT-PCR. Total RNA from the cornea was extracted (RNeasy Mini kit; Qiagen) and about 10 μ g of total RNA per one cornea was used to synthesize double-stranded cDNA by reverse transcription (SuperScript III; Invitrogen). Real-time amplification was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) and analyzed on 7900HT fast real-time PCR system (Applied Biosystems). For assays, reactions were incubated at 50 $^{\circ}$ C for 2 min, 95 $^{\circ}$ C for 10 min, and then 40 cycles at 95 $^{\circ}$ C for 15 s followed by 60 $^{\circ}$ C for 1 min. For normalization of gene expression, 18S rRNA probe (TaqMan gene expression assays, ID Hs03003631_g1) was used as internal control. For all of the PCR probe sets, TaqMan gene expression assay kits were purchased from Applied Biosystems.

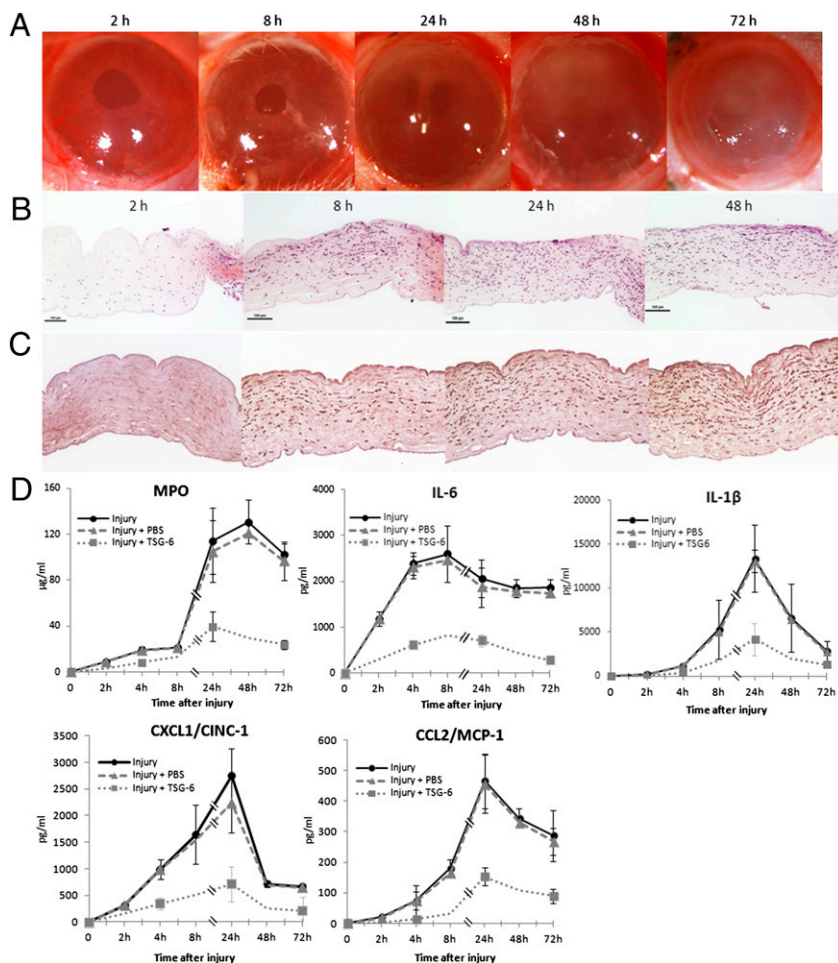


Fig. 6. TSG-6 reduced the early inflammatory response. Representative corneal photographs (A), H&E staining (B), and immunohistochemical staining for neutrophil elastase (C) demonstrated the time-dependent infiltration of leukocytes and development of opacity in the cornea following injury. (D) The temporal changes in the expression of MPO, cytokines, and chemokines in the cornea are shown. The MPO concentration increased markedly between 8 h to 24 h. The level of IL-6 increased to a maximum at approximately 4 to 8 h. The levels of IL-1 β , CXCL1/CINC-1, and CCL2/MCP-1 reached peak at 24 h. TSG-6 treatment lowered the levels of MPO, cytokines, and chemokines at examined time points. Values are mean \pm SD; $n = 3$ for each group.

ELISAs. For protein extraction, the cornea was sectioned into small pieces and lysed in 150 μ L of tissue extraction reagent containing protease inhibitors (Invitrogen). The samples were sonicated on ice and centrifuged twice (15,000 \times g at 4 $^{\circ}$ C for 20 min). The supernatant was assayed for levels of proinflammatory cytokines and chemokines with commercial ELISA kits for IL-6, IL-1 β , and CXCL1/CINC-1 (Quantikine kit; R&D Systems); for CCL2/MCP-1 (immunoassay kit; Invitrogen); and for MPO (rat MPO ELISA kit; HyCult Biotech). For quantification of total and active MMP-9, the corneas were lysed in EDTA-free buffer (Complete Lysis-M, EDTA-free; Roche) before assay (Amersham MMP-9 Biotrak activity assay system; GE Healthcare).

Gelatin Zymography. Corneal tissue was lysed in EDTA-free lysis buffer (Roche). Five-microliter aliquots of corneal extracts were analyzed by zymography using precast gelatin gels (10% Zymogram gelatin gels; Invitrogen/Novex). After electrophoresis, the gel was renatured by washing the gel twice in 0.25% Triton X-100 (30 min per wash) at room temperature with constant gentle agitation. The gel was developed overnight at 37 $^{\circ}$ C in developing buffer (Invitrogen). Then the gel was stained with colloidal blue (Invitrogen) and extensively washed (>20 h) to yield the uniform background signal. Digital images of stained wet gels were captured using a scanner. Gelatinase activity in the gel was visible as a clear area on a blue background indicating where the gelatin had been digested. The molecular weights of the gelatinases in the samples were determined from protein markers.

Microarrays. RNA target for microarrays was prepared using the 3' IVT Express Kit (Affymetrix) according to manufacturer's instructions. Briefly, 200 ng of total RNA was used to synthesize first-strand cDNA. The cDNA was then converted into double-stranded cDNA and used in *in vitro* transcription to

synthesize biotinylated cRNA. The cRNA was purified with magnetic beads, fragmented, and 12.5 μ g was used in the hybridization onto RG-230 2.0 arrays. The arrays were stained, washed, and scanned for fluorescence. Microarray

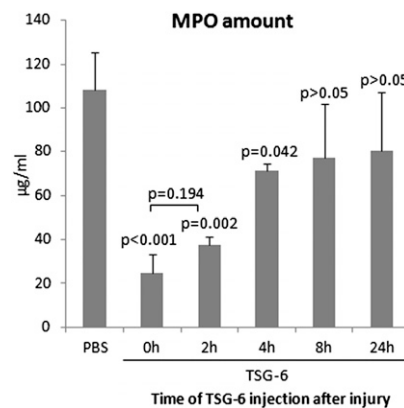


Fig. 7. TSG-6 administration within 4 h after injury decreased corneal inflammation. Injection of TSG-6 within 4 h after injury significantly decreased the infiltration of neutrophils as examined by MPO assay. Note that injection of TSG-6 2 h after injury suppressed corneal inflammation as effectively as administration immediately after injury. TSG-6 injection at 8 h was not effective. Values are mean \pm SD; $n = 3$ for each group.

data were normalized and analyzed using the Partek Genomics Suite 6.4 (Partek) and dChip software. For comparative analysis, data were filtered based on fold changes of two or more (either up- or down-regulated). For the hierarchical clustering analysis data were filtered using coefficient of variation higher than 0.6 and presence call of at least 33%. This filtering reduced the number of genes for clustering 1 (comparison among control, PBS solution 4-h, and PBS solution 24-h samples) to 2,809 and for clustering 2 (comparison among control, PBS solution 24-h, and TSG-6 24-h samples) to 2,499. The expression levels of the filtered genes were standardized and used in hierarchical clustering. A total of six clusters were selected in each hierarchical clustering on the similar level of hierarchy and studied for enriched Gene Ontology tags based on hypergeometric distribution.

Leukocyte Count and Differential. The differential count of leukocytes in the systemic blood was evaluated. Approximately 1 mL of blood was collected by cardiac puncture while the rat was under anesthesia with isoflurane inhalation.

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