Induction of Fibroblast Senescence During Mouse Corneal Wound Healing

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PURPOSE. To investigate the presence and role of fibroblast senescence in the dynamic process of corneal wound healing involving stromal cell apoptosis, proliferation, and differentiation.

METHODS. An in vivo corneal wound healing model was performed using epithelial debridement in C57BL/6 mice. The corneas were stained using TUNEL, Ki67, and α-smooth muscle actin (α-SMA) as markers of apoptosis, proliferation, and myofibroblastic differentiation, respectively. Cellular senescence was confirmed by senescence-associated β-galactosidase (SA-β-gal) staining and P16Ink4a expression. Mitogenic response and gene expression were compared among normal fibroblasts, H2O2-induced senescent fibroblasts, and TGF-β-induced myofibroblasts in vitro. The senescence was further detected in mouse models of corneal scarring, alkali burn, and penetrating keratoplasty (PKP).

RESULTS. The apoptosis and proliferation of corneal stromal cells were found to peak at 4 and 24 hours after epithelial debridement. Positive staining of SA-β-gal was observed clearly in the anterior stromal cells at 3 to 5 days. The senescent cells displayed P16Ink4a+/vimentin+α-SMA++, representing the major origin of activated corneal resident fibroblasts. Compared with normal fibroblasts and TGF-β-induced myofibroblasts, H2O2-induced senescent fibroblasts showed a nonfibrogenic phenotype, including a reduced response to growth factor basic fibroblast growth factor (bFGF) or platelet-derived growth factor-BB (PDGF-BB), increased matrix metalloproteinase (MMP)1/3/13 expression, and decreased fibronectin and collagen I expression. Moreover, cellular senescence was commonly found in the mouse corneal scarring, alkali burn, and PKP models.

CONCLUSIONS. Corneal epithelial debridement induced the senescence of corneal fibroblasts after apoptosis and proliferation. The senescent cells displayed a nonfibrogenic phenotype and may be involved in the self-limitation of corneal fibrosis.

Keywords: corneal wound healing, senescence, apoptosis, proliferation, nonfibrogenic

Cellular senescence is a highly stable cell cycle arrest that is triggered by different forms of stress, such as DNA damage, oncogene activation, oxidative and genotoxic stress, irradiation, and chemotherapeutic agents.1–5 Senescent cells undergo morphologic change, chromatin remodeling, and metabolic reprogramming, and secrete multiple proinflammatory factors, cytokines, growth factors, and matrix metalloproteinases (MMPs), termed the senescence-associated secretory phenotype (SASP).6–8 Cellular senescence is involved in developmental processes, tissue remodeling, disease development, aging, and tissue homeostasis.6,7 The harmful roles of senescent cells in aging and age-related diseases, such as sarcopenia, adiposity, atherosclerosis, diabetes, and Parkinson’s and Alzheimer’s diseases have been well described.7–9 Therefore, selective killing of senescent cells, so-called senolytic therapy, has been recently reported to improve the health span of mice and ameliorate the consequences of age-related disease or cancer therapy.10–15

Cellular senescence also plays a beneficial role in tumor suppression by halting the growth of premalignant cells.16–18 Moreover, senescent cells have been identified in the transient anatomic structures during embryonic development, including apical ectodermal ridge, neural roof plate, mesonephric tubules, and endolymphatic sac. Loss of cellular senescence leads to developmental abnormalities.19,20 In normal wound healing, transient appearance of senescent cells might contribute to wound resolution21,22 and fibrosis limitation.23 For instance, the senescence of skin fibroblasts restricts fibrosis during cutaneous wound healing23; the senescence of hepatic stellate cells limits liver fibrosis during liver injury.24–26 and premature senescence of cardiac myofibroblasts restrains cardiac fibrosis.27 Moreover, senescent cells have been proven to accelerate wound closure by inducing myofibroblast differentiation in cutaneous wound healing.22 The cornea is a specialized transparent tissue and vulnerable to various injuries, such as infection, trauma, and surgery. Corneal wound healing is a complex process involving cell apoptosis, proliferation, migration, differentiation, and extra-cellular matrix (ECM) remodeling.28 Early apoptosis and proliferation of keratocytes have been reported in human and...
animal corneas after injury. After corneal wound healing, quiescent keratocytes are activated into fibroblasts in the area adjacent to the wound site, and fibroblasts may further differentiate into myofibroblasts, which represent a contractile cell phenotype characterized by the presence of α-smooth muscle actin (α-SMA) and secretion of ECM proteins. Myofibroblasts promote wound contraction, but persistent existence leads to corneal stromal fibrosis, also termed stromal scarring or haze, often causing decline in vision and even blindness.

Our previous studies have reported that senescent fibroblasts accumulate and aggravate neovascularization in alkali-burned mouse cornea. However, the presence and role of senescent cells in normal corneal wound healing and scarring remain elusive. In the present study, we demonstrated that cellular senescence occurred after keratocyte apoptosis and proliferation during the dynamic process of epithelial debridement-induced corneal wound healing. Moreover, we identified the phenotypic characteristics of senescent cells and confirmed their presence in the mouse models of corneal scarring, alkali burn, and corneal transplantation.

**MATERIALS AND METHODS**

**Animals**

C57BL/6 and BALB/c mice (male, 6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All experiments were carried out in accordance with the Committee guidelines of Shandong Eye Institute and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The mouse corneal epithelial debridement model was prepared according to a previous report. In brief, C57BL/6 mice were anesthetized by intraperitoneal injection of 0.6% pentobarbital sodium followed by topical application of 2% xylocaine. Corneal central epithelium (2.5-mm diameter) was removed with an AlgerBrush II Rust Ring Remover (Alger, Lago Vista, TX, USA) and subsequently ofoxacin eye drops were applied to avoid infection. Mouse corneas were harvested at the indicated time points for analysis of apoptosis, proliferation, and senescence.

The mouse corneal alkali burn model was performed as described previously. In brief, a 2-mm-diameter filter disc with 2 μL 1 M sodium hydroxide (NaOH) was placed on the central cornea of anesthetized C57BL/6 mice for 40 seconds and then irrigated with physiological saline (0.9% NaCl) for another 40 seconds. Corneas were harvested after 14 days for senescence-associated β-galactosidase (SA-β-gal) staining and real-time quantitative PCR (qPCR).

The mouse corneal scarring model was prepared as described previously. In brief, under systemic and topical anesthesia, mouse corneal epithelium (3-mm diameter) and anterior stroma were removed with the AlgerBrush II Corneal Rust Ring Remover and a razor blade, respectively. Corneas were harvested after 7 days for SA-β-gal staining and real-time qPCR.

**Human Corneal Fibroblast Culture and Treatment**

Human corneal fibroblasts (HCFs) were isolated, cultured, and treated as in our previous description. For the induction of stress-induced premature senescence, HCFs were treated with 200 μM hydrogen peroxide (H2O2) 2 hours daily for 3 days, and the cells were cultured for another 3 days after the last H2O2 treatment. For the induction of myofibroblasts, HCFs were exposed to 10 ng/mL TGF-β (R&D, Minneapolis, MN, USA) for 3 days.

**TUNEL Assay**

Mouse eyes were enucleated and the corneal epithelium was removed at the indicated time points, embedded in Tissue-Tek optimum cutting temperature compound (OCT) (Sakura Finetechical, Tokyo, Japan), and sectioned into 10-μm thickness. To detect cell apoptosis, TUNEL staining was performed using In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the supplier’s recommendations. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and the staining was imaged using a Nikon (Eclipse e800; Tokyo, Japan) fluorescence microscope.

**SA-β-gal Staining**

SA-β-gal staining was performed following the manufacturer’s instructions (Beyotime, Haimen, China). Briefly, mouse corneal epithelium was firstly removed through the Dispase II (Roche) treatment. The mouse corneas and cultured HCFs were washed with PBS, fixed for 15 minutes at room temperature, incubated in the SA-β-gal staining solution (pH 6.0) overnight at 37°C, and viewed under a Nikon microscope. The corneas stained with SA-β-gal were further embedded in OCT, cut into 10-μm sections, counterstained with DAPI, and captured using a Nikon microscope.

**Immunofluorescence Staining**

Corneal sections and cultured cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After permeabilization, all samples were blocked with 5% BSA (Solarbio, Beijing, China) and incubated with the primary antibodies of Ki67, vimentin, P16, α-SMA, CD45, CD11b, P21, γH2AX (phospho S139), collagen I (COL I), and fibronectin (FN) (Abcam, Cambridge, UK) at 4°C overnight; they were then washed and incubated with donkey Alexa Fluor 488- or 594-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 1.5 hours at room temperature and washed again with PBS. For F-actin staining, the cultured cells were fixed, permeabilized, and stained with FITC-conjugated phalloidin (Molecular Probes, Carlsbad, CA, USA). All staining was examined under a Nikon fluorescence microscope. Tunel, Ki67, and SA-β-gal cells within the corneal stroma were counted in five to seven serial sections from each cornea using ImageJ software (National Institutes of Health, Bethesda, MD, USA). At least three corneas per group were analyzed.

**Flow Cytometry Assay**

To sort senescent cells by flow cytometry, the cells were isolated from mouse corneal stroma with collagenase treatment and labeled with the cellular senescence detection kit SPIDER-β-gal (Dojin Lab, Kumamoto, Japan), as described previously. Briefly, SPIDER-β-gal was added and incubated with the cells for 30 minutes at 37°C under protection from light. After incubation, the cells were colabeled with the fibroblast marker vimentin-Alexa Fluor 647 (Abcam), washed, and sorted immediately using a FACScan flow cytometer (BD FACSAria3; BD Biosciences, San Jose, CA, USA) for SPIDER-β-gal and vimentin+ cells. Sorted SPIDER-β-gal+ vimentin+ cells and...
Fibroblast Senescence in Cornea

**TABLE.** List of Primers Used for mRNA Expression Analyses

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer, 5′→3′</th>
<th>Reverse Primer, 5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-p16</td>
<td>GGAGTCCGCTGGACAGACAGA</td>
<td>GAATCGGGTTAGCCGGCAA</td>
</tr>
<tr>
<td>m-p21</td>
<td>TGCCACAGAATATGAAGGTTG</td>
<td>TTGCCTCTGTCGGGAAAC</td>
</tr>
<tr>
<td>m-β-actin</td>
<td>GGCACACACTTTCTACAATGT</td>
<td>GTGTTGTTGAGCTTGAGCC</td>
</tr>
<tr>
<td>m-z-SMA</td>
<td>TGCGAGCTGAGATTGTTC</td>
<td>CGTTGTTTCTCAATGGTAC</td>
</tr>
<tr>
<td>b-p16</td>
<td>GAGGCAGCTGGAGCCCTTT</td>
<td>CGGCTTCTGGCAGCTAATA</td>
</tr>
<tr>
<td>b-p21</td>
<td>GCCCTGACTGTTTCTTCTG</td>
<td>ATTGACATTTGGGAGGAG</td>
</tr>
<tr>
<td>b-SMA</td>
<td>GTGGAGACAGACAGACCA</td>
<td>CACCAGTGACTGCTGAAATC</td>
</tr>
<tr>
<td>b-collagen I (Col I)</td>
<td>CTGAGTCTCATCAAAATGCTCTTG</td>
<td>CCGGATCTGGCAGCTGAAATC</td>
</tr>
<tr>
<td>b-fibronectin (Fn)</td>
<td>GGGACCTCTAGGGAAGAAAAA</td>
<td>CGGATATTTCCCTGTCACCTGT</td>
</tr>
<tr>
<td>b-Mmp4</td>
<td>AAGATGGAAGGTTGACCAACA</td>
<td>ATCAACCCCGGCATACAGTAG</td>
</tr>
<tr>
<td>b-Mmp5</td>
<td>TGATGGACAAGCTGGGTTTGG</td>
<td>AGGAGTGCCCACATTTCAGT</td>
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<tr>
<td>b-Mmp13</td>
<td>GGGGCTGCTTTTAACTCTGGTC</td>
<td>CATTGGTCTCGGCTTHTTGGA</td>
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<tr>
<td>b-Mmp14</td>
<td>CAACACCTGCTCAGGAGAGGA</td>
<td>TCATCAAAACACCAGTTCTT</td>
</tr>
<tr>
<td>b-Gapdh</td>
<td>AGGGCTGCTTCTTATACCTCTGTG</td>
<td>CCCGACTGTTTGGAGGAA</td>
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SPIDER-β-gal− vimentin+ cells were used for analysis of real-time qPCR. Likewise, after incubation, the cells were labeled with vimentin-Alexa Fluor 647 (Abcam) or the fibrocyte marker CD45-APC/Cy7 (BioLegend, San Diego, CA, USA) to identify the origin of senescent cells. To determine the response of cells to growth factor, CFSE (carboxyfluorescein diacetate succinimidyl ester) assay was performed. H2O2- and TGF-β−treated HCFs and normal HCFs were suspended in Dulbecco’s modified Eagle’s medium (DMEM)/F12 media and labeled with 5 and 20 ng/mL platelet-derived growth factor-BB (PDGF-BB) (R&D, Minneapolis, MN, USA) for 3 days and analyzed by flow cytometry. There were three replications per group.

**Western Blot**

Mouse corneal stromal tissues were harvested at the indicated time points after the epithelium and endothelium were removed by gentle scraping with a scalpel blade. Cultured HCFs were harvested after various treatments. Cell lysates were prepared using radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors (Roche). The primary antibodies used were as follows: P16, P21, P53 (phospho S15), P53 (total), γH2A.X (phospho S139), α-SMA, FAK (phospho Y397), FAK, FN, COL I, MMP5, MMP13, MMP14 (Abcam), MMP1, β-actin, and GAPDH (Proteintech, Wuhan, China). After incubation with a horseradish peroxidase-conjugated secondary antibody (Proteintech), the blots were visualized via enzyme-linked chemiluminescence using the ECL kit (Chemicon, Temecula, CA, USA). The levels of proteins were quantified using ImageJ software.

**Real-Time Quantitative PCR**

Total RNA of corneal stromal tissues or cultured cells was extracted using Nucleospin RNA kits (BD Biosciences, Palo Alto, CA, USA). Complementary DNA was synthesized using an AMV first-strand cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer’s instructions. Real-time qPCR was performed using SYBR Green reagents and analyzed by the Applied Biosystems 7500 Real-Time System (Applied Biosystems, Foster City, CA, USA) according to our previous descriptions. The specific primers used in this assay are listed in the Table. Mouse β-actin and human Gapdh were used as internal control.

**Statistical Analysis**

All the experiments were performed at least three times. The data are presented as mean ± SD (standard deviation). Statistical significance was evaluated using the unpaired 2-tailed Student’s t-test and among more than two groups by 1-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001.

**RESULTS**

**Apoptosis, Proliferation, and Senescence of Corneal Stromal Cells After Epithelial Debridement**

Mouse corneal wound healing model with epithelial debridement was used to explore the dynamic fate changes of keratocytes. Cellular apoptosis and proliferation were evaluated by fluorescence staining of TUNEL and Ki67 on corneal sections at the indicated time points. The results showed that the number of TUNEL+ apoptotic cells peaked at 4 hours (Figs. 1A, 1D), while the Ki67+ proliferating cells peaked at 24 hours (Figs. 1B, 1E). To detect the senescent cells, SA-β-gal staining was performed in the whole-mount corneas and sections with the epithelium removed previously. The results showed that positive staining accumulated in the anterior stroma, but not in the posterior stroma (Fig. 1C). Through the counting of SA-β-gal+ cells, the percentage of senescent cells was found to peak at 3 days (approximately 6.57% ± 0.46% of cells in corneal stroma). Thereafter, senescent cells declined gradually, but were still visible until 7 days after epithelial debridement (Figs. 1C, 1F). Quantitative PCR analysis confirmed that the mRNA transcripts of senescence-associated genes p16 and p21 peaked at 3 days after epithelial debridement (Fig. 1G). Western blot results showed that the protein level of P16, P21, P53-Ser15, and γH2A.X was increased by 2.44 ± 0.47-, 1.93 ± 0.47-, 2.95 ± 0.56-, and 1.05 ± 0.46-fold, respectively (Figs. 1H, 1I). These results suggest that keratocytes experience apoptosis and proliferation in the early stage of corneal wound healing, while becoming senescent in the late stage.

**Phenotype Identification of Senescent Corneal Stromal Cells In Vivo**

Our previous study confirmed that senescent cells mainly originate from activated fibroblasts in the alkali-burned mouse cornea. To identify the senescent cells in the corneal wound healing of epithelial debridement, immunofluorescence staining of fibroblast marker vimentin and senescence marker P16...
FIGURE 1. Dynamic changes of corneal stromal cells after epithelial debridement. Apoptosis, proliferation, and senescence of corneal stromal cells at the indicated time points were analyzed after corneal epithelial debridement. (A) Representative image of TUNEL staining at 4 hours. (B) Representative image of Ki67 staining at 24 hours. (C) Representative images of SA-β-gal staining of whole-mount and sectioned corneas. Arrows show SA-β-gal+ cells. Dashed lines show the position of epithelial basement membrane. The frames are shown in an enlarged view. (D–F) Quantification of TUNEL+, Ki67+, SA-β-gal+ cells in all stromal cells. (G) Quantitative PCR analysis of senescence-associated gene expression. (H, I) Western blot analysis of senescence-associated protein expression after 3 days of epithelial debridement. De. epi, epithelial debridement. **P < 0.01, ***P < 0.001. Scale bars: 50 μm.
was performed in the sections following whole-mount SA-β-gal staining. The results showed that the SA-β-gal cells were vimentin+ and P16+ in the anterior stroma, although a number of vimentin+ SA-β-gal− P16− cells were found in the posterior stroma (Figs. 2A, 2B). Previous reports have found that the fibrocytes (vimentin+ and CD45+, a representative marker of fibrocytes44,45) can migrate into the stroma during corneal wound healing following injury.44 We further sorted the senescent cells with SPiDER-β-gal and CD45. The results showed that only ~0.59% SPiDER-β-gal− CD45− cells but ~5.96% SPiDER-β-gal+ CD45− cells were detected in the corneal stromal cells after 3 days of epithelial debridement (Supplementary Fig. S1). These results suggest that the fibrocytes and their progeny may be also an origin of senescent cells, while activated resident fibroblasts represent the major source of senescent cells in the corneal stroma after epithelial debridement.

As fibroblasts differentiate into myofibroblasts during wound healing, we performed the dual immunofluorescence staining of P16 and myofibroblast marker α-SMA. The staining indicated that most P16+ cells expressed α-SMA in the corneal stroma (Fig. 2C). To further identify the gene expression of senescent cells, we sorted the SPiDER-β-gal− and SPiDER-β-gal+ cells in all vimentin+ cells. Quantitative PCR analysis showed that robust upregulation of p16, p21, and α-Sma was found in the SPiDER-β-gal+ vimentin+ cells when compared with the SPiDER-β-gal− vimentin+ cells (Fig. 2D). Overall, our results confirmed that the senescent cells were mainly derived from
the corneal resident fibroblasts and assumed the myofibroblast characteristic of α-SMA expression.

Comparison of Normal and Senescent Fibroblasts, and Myofibroblasts

To compare the characteristics of normal fibroblasts, senescent fibroblasts, and myofibroblasts, HCFs were treated with hydrogen peroxide (H₂O₂) to induce senescence, or treated with TGF-β to induce myofibroblastic differentiation as in our previous studies. The enhanced SA-β-gal staining, flat and enlarged cell morphology, and increased expression of P16, P21, and γH2A.X were observed in the H₂O₂-induced senescent fibroblasts when compared with normal fibroblasts (Figs. 3A–D). However, TGF-β-induced myofibroblasts showed only upregulation of P16 and P21 expression, and no apparent SA-β-gal staining or γH2A.X expression (Figs. 3A–D). Moreover, TGF-β-induced myofibroblasts showed a remarkable accumulation of F-actin, and significant upregulated expression of α-SMA and p-FAK, compared with normal fibroblasts (Figs. 3E–G).
G) Notably, senescent fibroblasts displayed similar features to TGF-β-induced myofibroblasts, although the senescent cells had mild accumulation of stress fibers (Fig. 3E) and weaker upregulation of α-SMA (Figs. 3E–G) and p-FAK (Fig. 3G) expression. In addition, we observed that only a few α-SMA+ cells in the corneal stroma assumed the positive staining of desmin, a marker of fully developed myofibroblasts (Supplementary Fig. S2). The results indicated that the senescent fibroblasts presented some characteristics of the developing myofibroblasts, but not the fully developed myofibroblasts.

No Response of Senescent Corneal Fibroblasts to Growth Factors

Growth factors bFGF and PDGF-BB have been reported to be directly associated with corneal scarring by modulating fibroblast proliferation during wound healing.46,47 Given that growth arrest is the typical characteristic of senescent cells, we measured the differences of mitogenic activity in response to bFGF or PDGF-BB among normal and senescent fibroblasts and myofibroblasts by CFSE labeling. In the process of cell division and proliferation, CFSE-labeled fluorescence intensity decreases step-by-step, as shown in Figures 4A and 4C; that is, the stronger fluorescence intensity represents less cell division. The analysis of CFSE mean values demonstrated that both fibroblasts and myofibroblasts proliferated markedly in response to bFGF or PDGF-BB, whereas senescent fibroblasts were resistant to the mitogenic stimulation (Fig. 4). Therefore, considering the different responses to growth factors, senescent fibroblasts represented a distinct population that differed from both normal fibroblasts and myofibroblasts.

Nonfibrogenic Myofibroblast Phenotype of Senescent Corneal Fibroblasts

To explore the potential role of senescent fibroblasts in corneal wound healing, we further examined the difference in ECM-associated gene expression among normal fibroblasts, senescent fibroblasts, and myofibroblasts. Immunofluorescence results showed that the senescent fibroblasts assumed weaker staining intensity of COL I and FN than either normal fibroblasts or myofibroblasts (Fig. 5A). Consistently, qPCR and Western blot showed that the expression of COL I and FN was markedly downregulated in the senescent fibroblasts but predominantly upregulated in myofibroblasts (Figs. 5B–D). Furthermore, we tested the mRNA and protein expression of MMP1, MMP3, MMP13, and MMP14 (major matrix metalloproteinases targeting ECM proteins). Conversely, the expression of MMP1, MMP3, and MMP15 was dramatically upregulated in senescent fibroblasts, but downregulated or unchanged in myofibroblasts (Figs. 5B–D). These results suggest that senescent fibroblasts exhibit a nonfibrogenic phenotype, which may restrain corneal scarring through reduced ECM synthesis and deposition.
Presence of Senescent Cells in Multiple Corneal Injury Models

In the cornea, the senescence of epithelial and endothelial cells has been reported in human samples, animal models, and cultured cells. To explore whether senescent cells commonly occur in the stroma after corneal injury, we further established corneal scarring, alkali burn, and PKP mouse models. The whole-mount and section staining showed that the SA-b-gal® cells accumulated in the corneal stroma, with varied numbers and locations in different models (Figs. 6A, 6B). In the corneal scarring and alkali burn models, the senescent cells were diffused in the mechanical injury or alkali-burned area while localized mainly near the suture sites and donor edges in the PKP model (Fig. 6A). The upregulated expression of a-Sma and p16 was further confirmed by qPCR (Fig. 6C). These results confirmed that senescent cells existed in the stroma of multiple corneal injury models.

DISCUSSION

Apoptosis, proliferation, migration, and differentiation of stromal cells have been reported in the dynamic process of corneal wound healing. In the present study, we confirmed for the first time the presence of stromal cell senescence after the peak of apoptosis and proliferation in mouse corneal epithelial wound healing. Moreover, we identified that the senescent cells mainly originated from the activated corneal resident fibroblasts and displayed a nonfibrogenic myofibroblast phenotype.

Wound healing is a tightly controlled process, as its dysregulation may result in fibrosis, scarring, and loss of tissue function. In multiple tissues and organs, such as liver, skin, and heart, the myofibroblasts initially proliferate and produce ECM components and eventually go into senescence, which limits their overproliferation and converts them into matrix-degrading cells to prevent tissue fibrosis or scar formation. In the present study, we showed that senescent corneal fibroblasts were unresponsive to the stimulation of growth factors, and displayed decreased expression of ECM proteins and increased expression of matrix-degrading enzymes. Surprisingly, the senescent corneal fibroblasts were found to be a-SMA®, and represent a nonfibrogenic myofibroblast phenotype. Our results seem to be consistent with a recent study that found that the nonfibrogenic, a-SMA® myofibroblasts could be directly generated by senescence induction in cancer-associated fibroblasts. Therefore, we propose that the senescence of corneal resident fibroblasts is a programmed wound healing response that functions as a self-limiting mechanism for the prevention of corneal fibrosis.
Whether the injured cornea heals with transparency or with scarring depends on the type and level of injury, epithelial basement membrane (EBM) injury, and regeneration.

When the EBM is rapidly repaired (such as in epithelial debridement), the growth factors including TGF-β and PDGF are cut off with the halted myofibroblastic development. However, when EBM regeneration is impaired (such as in corneal scarring, alkali burn, and PKP), TGF-β and PDGF penetrate the stroma at high levels, leading to myofibroblast development and maturation, finally causing corneal scarring or fibrosis.

In this study, we found that activated corneal resident fibroblasts and invading fibrocytes, both having been reported to develop into myofibroblasts, underwent senescence after epithelial debridement. These senescent cells expressed vimentin and α-SMA, but a few cells expressed desmin, characteristic of a fully developed myofibroblast. Moreover, apoptosis of myofibroblasts has been widely reported in corneal and dermal wound healing. Thus, we speculate that in the epithelial debridement model, both corneal resident fibroblasts and invading fibrocytes may begin to differentiate toward the myofibroblast phenotype but halt that development once the EBM is repaired, and then more cells undergo senescence and apoptosis. However, in other models, defective EBM regeneration may cause more stromal cells to become mature myofibroblasts, although they finally undergo senescence and apoptosis. Therefore, it is likely that both senescence and apoptosis are involved in the myofibroblasts' disappearance during mouse corneal wound healing. Further research is needed to demonstrate the final fate of corneal myofibroblasts in corneal wound healing.

FIGURE 6. Senescent cells are commonly found in the stroma after corneal injury. Corneas were analyzed after 7 days of corneal scarring, 14 days of alkali burn, and 14 days of PKP. (A) Representative images of SA-β-gal staining of whole-mount corneas. Scale bar in second image: 200 μm; Scale bar in third image: 50 μm. (B) Representative images of SA-β-gal staining of sectioned corneas. Arrows show senescent cells in the stroma. Scale bar: 50 μm. (C) Quantitative PCR analysis of α-Sma and p16 gene expression. *P < 0.05, **P < 0.01, ***P < 0.001.
Extensive research has shown that p16 is a cyclin-dependent kinase inhibitor that promotes cell cycle arrest via the Rb tumor suppressor pathway, and P53 is a tumor suppressor that promotes cell cycle arrest via its target gene p21. In skin, liver, and heart, the cellular senescence program is dependent on both the P53/p21 and P16/Rb pathways, and genetic ablation of p53/p21 and p16/p19 inhibits senescence, leading to more ECM deposition and aggravated fibrosis. In the corneal suture model, we observed that the number of corneal myofibroblasts was significantly increased in p16/p19 knockout mice compared to control mice, whereas the number of senescent cells was not markedly decreased by p16/p19 knockout mice (data not shown). The results suggest that p16/p19 partially modulates cell cycle arrest, and other signaling pathways may be necessary for the induction of cellular senescence in the cornea.

In summary, our findings revealed that senescent resident fibroblasts commonly existed in corneal wound healing. The senescent cells displayed a nonfibrogenic phenotype and may be involved in the self-limitation of corneal fibrosis.

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