The Senescence Difference Between the Central and Peripheral Cornea Induced by Sutures

Suxia Li

Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences

Qiaoqiao Dong

Shandong Eye Hospital, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences

Muchen Dong

Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences

Mingli Qu

Shandong Eye Hospital, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences

Yao Wang

Shandong Eye Hospital, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences

Qingjun zhou

Shandong Eye Hospital, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences

Weiyun Shi (✉ wys@sdmhu.edu.cn)

Shandong Eye Hospital, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences

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Abstract

Introduction: Cell senescence plays a regulatory role in tissue fibrosis, and corneal scarring is usually more severe in the central cornea based on clinical observation. We attempted to explore the senescence difference between the central and peripheral cornea in an in vivo mouse model of suture-induced senescence and an in vitro model of senescence with hydrogen peroxide (H₂O₂)-induced rabbit corneal fibroblasts.

Methods: Male Balb/c mice (6-8 weeks) received sutures in the central, superior, inferior, nasal, and temporal cornea, and the sutures were removed on day 14. Corneal neovascularization (CNV) was observed under a slit lamp microscope with a digital camera. Fibroblasts isolated from central and peripheral rabbit corneas were induced with H₂O₂ to establish an in vitro model of senescence. The senescence was evaluated with SA-β-gal staining and gene expression analysis of p21, p27, and p53.

Results: Senescent cells accumulated in the corneal stroma from day 3 to day 14 after the operation and peaked on day 14, later than CNV. More senescent keratocytes were observed in the peripheral cornea in the mouse model. The peripheral corneal fibroblasts were more likely to become senescent related to H₂O₂ in vitro. The PCR results revealed that the senescence-related genes, p21, p27, and p53, were all highly expressed in the peripheral corneal fibroblasts. **Conclusions:** As senescent fibroblasts can limit tissue fibrosis, the senescence difference between the central and peripheral cornea may contribute to the difference in scarring.

Introduction

The cornea in the anterior part of the eye is important for clear vision with its transparent nature and tissue integrity. Keratocytes as the main cells in the corneal stroma usually remain quiescent between the collagen layer and secrete essential extracellular matrix (ECM) proteins[1–3]. When eye injuries such as trauma, infection, and inflammation occur, keratocytes may be transformed to fibroblasts and myofibroblasts, which rapidly secrete overmuch ECM proteins and result in corneal scarring during wound healing[4]. Along with keratocyte activation, new blood vessels arise gradually from the peripheral cornea to the central cornea[5]. Our previous study described senescent fibroblasts could promote the alkali-induced corneal neovascularization (CNV)[6]. In clinical practice, scarring is usually found to be disk-shaped in the central cornea and severer than that in the periphery. The recovery of corneal transparency after injury is important for vision restoration[7].

Cellular senescence refers to the irreversible cell cycle arrest caused by cellular pressures, such as DNA damage, oncogene activation, and oxidative stress[8]. Senescence cells show a large and flattened morphology, with positive senescence-associated β-galactosidase (SA-β-gal) staining. In addition to arrested growth, senescence cells upregulate numerous secreted proteins including cytokines and growth factors termed the senescence-associated secretory phenotype (SASP) or the senescence messaging secretome (SMS)[9–11]. Except suppressing tumorigenesis, the senescence response participates in

several tissue pathologies via the SASP/SMS[12]. Recent reports showed that senescence cells inhibited the fibrosis of liver, skin, and heart damage[12–14], indicating that cellular senescence acts as a potent regulator of fibrosis limitation, although this has not been examined in the cornea. Based on these studies, we hypothesized that the senescence of corneal fibroblasts may contribute to the difference in scarring between the central and peripheral cornea.

Herein, we examined the cellular senescence in the central and peripheral cornea, finding that senescent corneal fibroblasts were more frequently presented in the peripheral cornea in mice, which was coincident with the results of the in vitro experiment. Senescent corneal fibroblasts obtained from the peripheral cornea exhibited a higher level of senescence-related genes of p21, p27, and p53.

Materials And Methods

The suture-induced mouse model of corneal senescence. Balb/c mice (male, 6–8 weeks; Beijing Pharmacology Institute, Beijing, China) were used for the study. All animal experiments were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the ethics committee of Shandong Eye Institute, and all animal experiments were performed accordance with ARRIVE guidelines. After systemic and topical anesthesia, five interrupted stitches of the 11 – 0 polypropylene suture (Mani, Togichi, Japan) were placed in the central, superior, inferior, nasal, and temporal cornea, respectively. Ofloxacin eye ointments were administered immediately after the injury and once daily for 1 week to avoid infection. Only one eye was operated in each mouse for all experiments. The sutures were removed on day 14. At 12 h, and 3, 5, 7, 14, and 21 days after the operation, corneal edema, neovascularization, and scarring were observed under a slit lamp microscope with a digital camera.

Cell culture and senescence induction. New Zealand white rabbits (male, 2–4 months; Kangda, Qingdao, Shandong, China) were used for corneal fibroblasts culture. The peripheral and central corneal tissues were isolated using a trephine of 8 mm and 5 mm in diameter, respectively. The corneal epithelium and endothelium were digested with 2.4 U/ml dispase II (Roche, Basel, Switzerland) overnight at 4°C. The corneal stroma was cut into pieces and incubated with 2 mg/ml collagenase I (Invitrogen, Carlsbad, CA) for 2–4 hours at 37°C. Then the cells were collected and cultured in DMEM/F-12 medium supplemented with 10% FBS. A hydrogen peroxide (H₂O₂)-induced in vitro model of senescent corneal fibroblasts was established as previously described.⁶ Briefly, the cells were exposed three times to 200 µM H₂O₂ for 1 h daily. After each treatment, the cells were washed with PBS and incubated in complete medium for 24 h.

Senescent cell assay. The mouse eyeballs were embedded in the Tissue-Tek optimum cutting temperature (OCT) compound, and 8-µm sections were made (n = 5). The cryosections were fixed with ice methanol at -20°C for 10 min. The rabbit keratocytes were fixed with 4% paraformaldehyde at room temperature for 10 min. Cellular senescence was identified by SA-β-gal staining (Beyotime, Haimen, China). In brief, the samples were washed with PBS, fixed at room temperature for 15 min, and then

washed 2–3 times again, before they were incubated in the SA- β -gal staining solution (pH 6.0) overnight at 37°C and observed under a Nikon microscope.

Real-time RT-PCR. Total RNA was extracted from the H₂O₂-treated peripheral and central rabbit corneal fibroblasts using Nucleospin RNA kits (BD Biosciences, Palo Alto, CA). cDNA was acquired from total RNA using the first strand cDNA synthesis kit (TaKaRa, Dalian, China). The real-time PCR analysis was then performed with the SYBR Green PCR reagent (Invitrogen, Carlsbad, CA) in an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The cycling system was an initial denaturation cycle at 95°C for 10 sec, followed by 45 cycles at 95°C for 15 sec and 60°C for 1 min. The nucleotide sequences of primers used in this assay are listed in Table 1, and GAPDH was used as an endogenous control gene.

Statistical analysis. Data in this study were representative of more than three different experiments and were presented as mean $\pm$ SEM. The differences between control and treated groups were compared with the Student's t test. $P < 0.05$ was considered significant.

Results

Corneal neovascularization after suturing. Corneal neovascularization was detected in the suture-induced senescence mouse model. The new vessels grew gradually from the limbal region to the central cornea after sutures were placed on the cornea. They emerged on day 3, became dense and rough on day 7, and faded to the surrounding sutures on day 14. After the sutures were removed on day 14, the new vessels regressed rapidly and could not be observed on day 21 (Fig. 1).

Senescent cell accumulation in the corneal suture model. In the mouse model of senescence induced by localized corneal sutures, SA- β -gal staining revealed the senescent cell distribution on the whole corneal mount. No positive SA- β -gal staining was observed in the cornea at 12 h after the operation, mild staining of SA- β -gal activity was detected in the cornea on day 3, and strong positive staining was consistently observed on day 5 and day 7 and peaked on day 14. After suture removal on day 14, the SA- β -gal staining weakened rapidly until the senescent cells disappeared completely on day 21 (Fig. 2A). The SA- β -gal staining of the corneal sections showed that the senescent cells were mainly localized in the corneal stroma, especially around the sutures, so the possibility of corneal epithelial or endothelial cell senescence was excluded (Fig. 2B).

Senescence difference between the central and peripheral cornea. To compare the relative percentage of senescent cells in the central and peripheral cornea, we placed sutures at five different regions (Fig. 2A). The whole flat mount which contained tissues from both the central and peripheral suture regions was prepared for SA- β -gal staining. The staining was graded by optical density measured by Image J software. On day 5 after the operation, positive senescence expression was found in all the five locations. The density of SA- β -gal staining positivity was higher in the peripheral area than in the central area

(Fig. 3A). The findings were consistent at different timepoints, and the most significant difference appeared on day 14 (Fig. 3).

Induction of senescent corneal fibroblasts in vitro. To further understand the difference in senescence ability between the central and peripheral corneal fibroblasts, rabbit corneal fibroblasts were obtained and induced for senescence by H_2O_2 *in vitro*. Cellular senescence was identified by SA- β -gal staining. The senescent fibroblasts were found to be enlarged and flattened with accumulated SA- β -gal staining. Similar to the *in vivo* experimental results of the mouse corneal model, the density of senescent cells in the peripheral cornea was much higher than that in the central cornea under the same experimental condition (Fig. 4A). The real time RT-PCR results showed that the level of senescence-related genes, p21, p27, and p53, was higher in the peripheral corneal fibroblasts than in the central ones, which also proved that peripheral corneal fibroblasts were more likely to become senescent (Fig. 4B).

Discussion

In the present study, we observed that in the mouse model of cellular senescence induced by corneal sutures, the senescent corneal fibroblasts were more frequently found in the peripheral cornea than in the central cornea. *In vitro*, the rabbit corneal fibroblasts from the peripheral cornea were more likely to have senescence-associated β -galactosidase staining, and the senescence-related gene level was higher in the peripheral corneal fibroblasts. Since senescent fibroblasts could act as important regulators of tissue fibrogenesis[13], we believed that the senescence difference was one of the reason for the corneal scarring after trauma, infection or inflammation.

Ocular trauma such as suture on the cornea may cause severe damage such as opacification and neovascularization of the corneal stroma during wound healing[15–17]. Our previous study have described senescent cells derived from activated corneal fibroblasts could promote the alkali-induced CNV[6], in this study, senescent fibroblasts were also found to participate in CNV although appeared later than CNV development.

During wound healing, quiescent keratocytes were activated by inflammation-induced transforming growth factor β (TGF β) and transformed into fibroblasts and myofibroblasts, which rapidly synthesized and secreted redundant ECM proteins and repaired the wound[18]. After completion of wound repair, myofibroblasts become senescent or transformed to scar keratocytes[19]. Recent studies have demonstrated that one contribution of senescent fibroblasts during tissue repair is to limit fibrosis, which is commonly observed in chronic wounds. The mark of cellular senescence is the cell cycle arrest, and the enforced cell cycle arrest of senescent fibroblasts *in vivo* provides a brake on the fibrogenic response to damage by limiting the expansion of the cell type responsible for producing the fibrotic scar[14]. A few MMPs, including MMP2, MMP3, and MMP9, are part of the SASP and can degrade excessive collagens and maintain tissue homeostasis during wound healing[20]. The stable cell cycle arrest and SASP of senescent fibroblasts may also be the mechanism for corneal scarring.

The central cornea being thinner than the peripheral cornea in keratoconic eyes and the central cornea being more likely to form scars suggest a difference in keratocytes between the corneal center and periphery[21]. Our results showed that the level of senescence-related genes, p21, p27, and p53, was all higher in the peripheral keratocytes. The expression of p21 and p27 was tightly controlled by the tumor suppressor protein p53, which plays a role in apoptosis, senescence, genomic stability, and inhibition of angiogenesis[22]. P21 and p53 together held the cell cycle at the G1/S stage and arresting growth. The higher expression of these genes may partially contribute to the peripheral keratocyte senescence.

Conclusion

Our study indicates that the senescence difference between the central and peripheral cornea may contribute to the difference in scarring as senescent fibroblasts can limit tissue fibrosis.

Declarations

Statements

Statement of Ethics

This study protocol was reviewed and approved by the Laboratory Animal Ethics Review Committee and Laboratory Animal Welfare Committee of Shandong Provincial Key Laboratory of Ophthalmology , approval number SDSYKYJS.No20130001.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Weiyun Shi and Qingjun Zhou were involved in study design, data analysis, and interpretation of data for the work . Suxia Li and Muchen Dong were involved in data collection, data analysis, manuscript writing and editing. Mingli Qu, Qiaoqiao Dong and Yao Wang were involved in data collection, data analysis. All authors have approved the final version of the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

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Tables

Table 1. Nucleotide sequences of primers used for RT-qPCR

Genes	Forward primer	Reverse primer	Gene accession
P21	AGCGCTGGAAC TTCGACTTT	TGAGGTGCTCGGCCGCTT	XM_002714669.3
P27	CCGCCTGCAGAAACCTCTT	CCACCTCCTGCCATTCGTA	XM_017343370.1
P53	TACTCCCCCTGCCTCAACAA	GCGTCTGACAACTTCCGTCAT	NM_001082404.1
GAPDH	CGCCTGGAGAAAGCTGCTAA	CCCCAGCATCGAAGGTAGAG	NM_001082253.1

Figures

Figure 1

The cornea neovascularization appeared after suture. The CNV emerged on day 3, peaked on day 7, and faded to the surrounding of the sutures on day 14. After the sutures were removed on day 14, the new vessels regressed rapidly and could not be observed on day 21.

A **B**

Figure 2

The senescent cells were stained blue by SA-β-gal in the cornea after suture induction. (A) Mild staining of SA-β-gal activity was detected in the cornea on day 3. Strong positive staining was consistently observed on day 5, day 7, and peaked on day 14. The sutures were removed on day 14, and the senescent cells disappeared completely on day 21. (B) The senescent cells were mainly localized in the corneal stroma.

Figure 3

The difference in senescent cell density in the central and peripheral cornea. (A) At each timepoint after the suture operation, more senescent cells were found in the peripheral cornea than the central cornea. (B) The density of senescent cells in the central and peripheral cornea. * $P < 0.05$, versus the central cornea group. Data are shown as means $\pm$ SEM.

A **B**

Figure 4

The senescent difference between peripheral and central corneal fibroblasts induced by H₂O₂. (A) The rabbit corneal fibroblasts from the peripheral cornea assumed more positive senescence-associated β -galactosidase staining (red arrow). (B) The peripheral corneal fibroblasts showed a higher level of senescence-related genes, p21, p27 and p53, after induced by H₂O₂. *P < 0.05, versus the central cornea group. Data are shown as means $\pm$ SEM.