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Corneal stroma PDGF blockade and myofibroblast development

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Abstract

Myofibroblast development and haze generation in the corneal stroma is mediated by cytokines, including transforming growth factor beta (TGF β), and possibly other cytokines. This study examined the effects of stromal PDGF- β blockade on the development of myofibroblasts in response to -9.0 diopter photorefractive keratectomy in the rabbit. Rabbits that had haze generating photorefractive keratectomy (PRK, for 9 diopters of myopia) in one eye were divided into three different groups: stromal application of plasmid pCMV.PDGFRB.23KDEL expressing a subunit of PDGF receptor b (domains 2-3, which bind PDGF-B), stromal application of empty plasmid pCMV, or stromal application of balanced salt solution (BSS). The plasmids (at a concentration 1000 ng/ μ l) or BSS was applied to the exposed stroma immediately after surgery and every 24 hours for 4-5 days until the epithelium healed. The group treated with pCMV.PDGFRB.23KDEL showed lower α SMA+ myofibroblast density in the anterior stroma compared to either control group ($P \leq 0.001$). Although there was also lower corneal haze at the slit lamp at one month after surgery, the difference in haze after PDGF-B blockade was not statistically significant compared to either control group. Stromal PDGF-B blockade during the early postoperative period following PRK decreases stromal α SMA+ myofibroblast generation. PDGF is an important modulator of myofibroblast development in the cornea.

Keywords

Corneal stroma; myofibroblasts; platelet derived growth factor; platelet derived growth factor beta receptor (PDGFR- β); wound healing; soluble cytokine receptors

1. Introduction

Myofibroblasts participate in corneal responses to injury produced by corneal surgery, trauma and infection (Masur et al., 1996; Jester et al., 1999b; Jester et al., 2002; Funderburgh et al., 2001). Extracellular matrix and other components produced by these cells, in addition to the cells themselves, alter corneal transparency and may lead to severe opacity, termed haze, after some injuries—including photorefractive keratectomy (PRK) when it is used to correct high

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levels of myopia (Jester, et al., 1999a; Moeller-Pedersen, et al., 1998; Mohan, et al., 2003). Recent studies have suggested that epithelial-stromal interactions are critical determinates of myofibroblast generation (Netto, et al., 2006).

Transforming growth factor-beta (TGF- β) has long been appreciated as one of the critical modulators of corneal myofibroblast development *in vitro* (Masur, et al., 1996; Jester, et al., 1999a, Jester, et al., 1999b; Fini, 1999; Petridou, et al., 2000; Funderburgh, et al., 2001; Jester, et al., 2002) and *in vivo* (Jester, et al., 1997). The likely *in vivo* sources for the TGF- β that modulates myofibroblast development are the corneal epithelium and tears (Wilson, et al., 1994; Vesaluoma, et al., 1997a; Netto, et al., 2006). The epithelial basement membrane limits the access of TGF- β to the stroma in the normal unwounded cornea, but once injury occurs, structural and functional defects in the basement membrane allow prolonged penetration of the cytokine into the stroma (Netto, et al., 2006).

Platelet-derived growth factor (PDGF) is another cytokine that has been shown to be an important modulator of myofibroblast development in several non-ocular tissues (Tang, et al., 1996; Boström, et al., 1996; Powell, et al., 1999; Nedeau, et al., 2008). Although the role of PDGF in the development of myofibroblasts in the cornea has not been well characterized, studies have suggested a role for this cytokine in corneal myofibroblast generation (Jester, et al., 2002; Stramer and Fini, 2004).

PDGF is expressed in the corneal epithelium (Kim, et al., 1999) and has been detected in tears (Vesaluoma et al., 1997b). PDGF binds with high affinity to the intact basement membrane and, therefore, its penetration into the corneal stroma is probably limited in the normal unwounded cornea (Kim, et al., 1999). After wounding and damage to the epithelial basement membrane, however, large quantities of PDGF likely gain access to the corneal stroma, where they can bind to PDGF receptors expressed on stromal cells to regulate functions such as cell proliferation and migration (Kamiyama, et al., 1998).

The present study was designed to examine the effects of blockade of PDGF-B in the stroma on the development of myofibroblasts after PRK corneal injury in rabbits through the introduction of a plasmid vector (pCMV.PDGFRB.23KDEL) that expresses a subunit of PDGF receptor β (domains 2-3, which bind PDGF-B), along with KDEL, which is an endoplasmic reticulum retention signal. The expressed PDGFRb23-kdel recombinant protein is retained in the endoplasmic reticulum and sequesters PDGF-B within cells and reduces signaling (Ambati et al., 2007).

2. Methods

2.1 Animals and surgery

The Animal Control Committee at the Cleveland Clinic Foundation approved the animal studies included in this work. All animals were treated in accordance with the tenets of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. A total of eighteen 12-to15 week old female New Zealand white rabbits weighing 2.5-3.0 kg each were included in this study. Anesthesia was achieved by intramuscular injection of ketamine hydrochloride (30mg/kg) and xylazine hydrochloride (5mg/kg). In addition, topical proparacaine hydrochloride 1% (Alcon, Ft. Worth, TX, USA) was applied to each eye just before surgery. One eye of each rabbit, selected at random, had PRK with a 6.0 mm ablation zone using an Apex Summit Laser (Alcon, Fort Worth, TX, USA). Euthanasia was performed after 4 weeks with an intravenous injection of 100 mg/kg pentobarbital while the animal was under general anesthesia.

2.2. Plasmids vector design

Domains 2 and 3 of platelet-derived growth factor receptor beta (PDGFR- β), that are responsible for binding platelet-derived growth factor (PDGF-B), with endoplasmic reticulum retention signal KDEL tag sequence on 3' end, were amplified using PCR, as previously reported (Ambati, et al., 2007). A human PDGF- β cDNA clone was used as the template DNA for the PCR reactions (Open Biosystems, Huntsville, AL). Domains 2 and 3 of PDGF- β with KDEL were amplified with flanking EcoRI and Hind III restriction sites on 5' and 3' end respectively using the primers 5'- TCA GAA TTC ATG GTG GGC TTC CTC CCT AAT GAT GCC GA -3' and 5'- TCG GCA TCA TTA GGG AGG AAG CCC ACC ATG AAT TCT GA -3'. The amplified PCR products were ligated into CMV Script (Invitrogen Carlsbad, CA) vector using EcoRI and Hind III restriction sites. The pCMV.PDGFRB.23KDEL was transfected into competent *Escherichia coli* (DH5- α) cells and selected by using kanamycin. Colonies were screened for the presence of the insert using PCR with T3 and T7 primers. Finally, the orientation of the insert was verified by DNA sequencing (3730 XL 96-capillary sequencer; Applied Biosystems, Foster City, CA). The clone was cultured in Luria's broth (containing kanamycin) and maxiplasmid preparations were made (Eppendorf, Westbury, NY). Puc19 was used as a positive control throughout the transformations.

2.3 In vitro confirmation of expression of PDGF receptor fragment in corneal cells

Primary rabbit corneal fibroblasts were transfected with either empty pCMV vector or pCMV containing the PDGF β receptor-expressing sequence (pCMV.PDGFRB23K) using the Lipofectamine PLUS (Invitrogen, Carlsbad, CA) system according to the manufacturer's recommendations. Briefly, cells were grown in fibronectin coated glass plates to 50-70% confluency and transfected with 2 μ g of plasmid DNA in serum-free media for 3 hours. Immediately after incubation, cells were supplemented with media containing 1% FBS for 24 hours. The following day, cells were fixed in Histochoice MB Fixative (Electron Microscopy Services, Hatfield, PA) for 30 minutes and stained with goat polyclonal to PDGF receptor β (ab10848, Abcam, Cambridge, MA) at 1:20 concentration for 90 minutes at room temperature. Alexa Fluor 594 donkey anti-goat IgG (H+L) (A11058, Invitrogen) at 1:100 was used as secondary antibody.

2.4 Animal treatment groups

Animals were divided into three treatment groups, with six animals in each group: Group 1 animals received pCMV.PDGFRB.23KDEL plasmid, Group 2 animals received empty pCMV plasmid and Group 3 animals had only BSS. Only one eye was treated in each animal. The epithelium was removed by scraping over a 7 mm diameter zone of the central cornea and the 6.0 mm diameter excimer laser PRK ablation was performed on the exposed stromal surface. Then 100 μ l of treatment solution (plasmid at a concentration 1000 ng/ μ l or BSS) was applied and spread over the exposed stroma. The application for each group was repeated every 24 hours for 4-5 days, until the epithelium was completely healed.

2.5 Biomicroscopic grading of corneal haze

The level of opacity (haze) in the cornea was measured with a slit lamp at four weeks after PRK using the scale reported by Fantes and coworkers (1990). Briefly, with animals under anesthesia the opacity in the central cornea was graded. Grade 0 was a completely clear cornea; grade 0.5 had trace haze seen with careful oblique illumination; grade 1 was more prominent haze, but not interfering with visibility of fine iris details; grade 2 was mild obscuration of the iris details and the lens; and grade 4 was complete opacification of the stroma in the area of the ablation. Haze grading was performed in a masked manner by two independent observers and the results for each eye were averaged to obtain the score for that animal.

2.6 Cornea collection, fixation and sectioning

Rabbits were euthanized and the corneoscleral rims of ablated and unablated contralateral control eyes were removed with 0.12 forceps and sharp Westcott scissors. For histological analyses, the corneas were embedded in liquid OCT compound (Sakura Finetek, Torrance, CA, USA) within a 24-mm X 24mm X 5mm mould (Fisher, Pittsburgh, PA, USA). Cornea specimens were centered within mould so that the block could be bisected and transverse sections cut from the center of the cornea. The frozen tissue blocks were stored at -80° C until sectioning was performed. Central corneal sections (7µm thick) were cut with a cryostat (HM 505M, Micron GmbH, Walldorf, Germany). Sections were placed on 25-mm X 75 mm X 1mm microscope slides (Superfrost Plus, Fisher) and maintained frozen at -80° C until staining was performed.

2.7 Immunocytochemistry

Immunofluorescent staining for α -smooth muscle actin (α -SMA), a marker for myofibroblasts, was performed using a mouse monoclonal anti-human smooth muscle actin clone1A4 (Dako, M0851, Carpinteria, CA). Tissue sections (7-µm) were incubated for 90 minutes at room temperature with the anti- α -SMA monoclonal antibody at 1:50 dilution in 1% BSA. This was followed by incubation at room temperature with secondary antibody, Alexa Fluor 488 (Invitrogen, A11001, Carlsbad, California) goat anti-mouse IgG (H+L) (green) at a dilution of 1:100 in 1% BSA for 1 hour. Coverslips were mounted with Vectashield containing DAPI (Vector Laboratories Inc., Burlingame, CA) to allow visualization of all nuclei in the tissue sections. Irrelevant isotype-matched primary antibody, secondary antibody alone and tissue sections from naïve eyes were used for negative controls for each immunocytochemistry experiment. The sections were viewed and photographed with a Leica DM5000 microscope equipped with Q-Imaging Retiga 4000RV (Surrey, BC, Canada) camera and ImagePro software.

2.8 Quantification of α -SMA positive cells

Six corneas for each group were used to quantify α -SMA-positive cells in the tissues. The α -SMA-positive cells in the six randomly selected, non-overlapping, full-thickness central corneal columns extending from the anterior stromal surface to the posterior stromal surface were counted following a method reported previously (Mohan et al, 2003). The diameter of each column was a 400X microscopic field.

2.9 Statistical analysis

Statistical comparisons between the groups were performed using one-way or two-way analysis of variance (ANOVA) with Student-Newman-Keuls method test where applicable (Sigma Stat software 3.5).

3. RESULTS

3.1 In vitro expression of the PDGF β receptor fragment

Primary corneal fibroblast cells transfected with the plasmid expressing the PDGF β receptor fragment expressed the fragment in membrane bound organelles in the cytoplasm (Fig. 1 A). Cells transfected with empty plasmid vector did not express detectible PDGF receptor b (Fig. 1B).

3.2 Biomicroscopic evaluation of haze

The PRK treated corneas of the different groups had different levels of corneal haze at 4 weeks after PRK (Fig. 2). Haze was less in rabbit corneas treated with plasmid pCMV.PDGFRB. 23KDEL compared to control group treated with the empty plasmid vector pCMV or the

control group treated with BSS alone. However, the differences did not reach statistical significance (Kruskal-Wallis One Way Analysis of Variance on Ranks, $P = 0.32$).

3.3 Immunofluorescence detection of myofibroblasts

The generation of myofibroblasts in the rabbit corneas at 4 weeks after PRK was also confirmed by immunocytochemical detection of the marker α -SMA. This was previously found to be the time point at which myofibroblast density is greatest after PRK in the rabbit (Mohan et al., 2003). The expression of α -SMA was negligible in most animals in the soluble PDGF- β receptor plasmid treated group, although two corneas had limited α -SMA expression (Fig. 3). Cells expressing α -SMA were present at much higher density in each control group (Fig. 4). The number of α -SMA-positive cells/400X column were determined for each cornea (Fig. 5) and the number of α -SMA-positive cells was significantly less (ANOVA, $P < 0.001$) in the PDGF- β receptor fragment plasmid treated group compared to either control group (PDGF- β receptor fragment plasmid 13 ± 5.5 ; empty plasmid 57 ± 7.7 ; BSS control 49 ± 5.9). The difference between the control groups was not statistically significant.

4. Discussion

The results of this study in rabbits provide evidence that platelet-derived growth factor in the cornea contributes to the development of myofibroblasts in the stroma associated with haze after an injury stimulus that triggers stromal opacification. Interestingly, there is uncoupling between the generation of anterior stromal haze and the appearance of α -smooth muscle actin (α -SMA)-positive myofibroblasts in this model, with the effect of PDGF-B blockade being greater on the generation of α -SMA+ myofibroblasts (Fig. 5) than it is on stromal opacity (Fig. 2).

PDGF is a mitogenic and chemotactic factor for many cells types, including fibroblasts, vascular smooth muscle cells, and glial cells (Kamiyama, et al., 1998; Alvarez, et al., 2006; Kim, et al., 1999). PDGF functions as a homodimer (AA or BB) or heterodimer (A,B) of two related polypeptides coded by distinct genes (Hart, et al., 1995; Heldin and Westermark, 1999). PDGF mediates its effects through tyrosine kinase receptors that are expressed as homodimers or heterodimers (Hart, et al., 1995). The two PDGF receptors, PDGFR- α and PDGFR- β , dimerize after binding ligand. The α -receptor binds both A and B chains of PDGF, whereas the β -receptor binds preferentially to the B chain of PDGF (Hart, et al., 1995). The plasmid vector used in this study expressed a subunit of PDGF receptor β (domains 2-3, which bind PDGF-B), along with KDEL, which is an endoplasmic reticulum retention signal. The expressed PDGFRb23-kdel recombinant protein is retained in the endoplasmic reticulum and sequesters PDGF-B within cells and reduces signaling (Ambati et al., 2007).

PDGF and PDGF receptors are expressed in the cornea, and a role for PDGF in the modulation of corneal wound healing functions, such as stromal cell proliferation and motility, is established (Imanishi et al., 2000; Kim et al., 1999; Jester et al., 2002). Analogous to transforming growth factor- β (TGF β) (Wilson, et al., 1994), PDGF that modulates myofibroblast development and other stromal cellular functions could be derived from several sources. One source is the overlying corneal epithelium (Kim, et al., 1999). In the unwounded cornea, the intact basement membrane serves as a barrier to penetration of both TGF β and PDGF into the corneal stroma (Kim, et al., 1999). After injury to the epithelium and its basement membrane, these cytokines penetrate into the corneal stroma and initiate development of myofibroblasts from precursor cells. Recent studies have shown that persistent structural and functional defects in the epithelial basement membrane are associated with the development of myofibroblasts in the corneal stroma (Netto, et al., 2006). These defects in the basement membrane may be attributable to stromal surface irregularity, genetic abnormalities, or other unidentified factors. We hypothesize that sustained stromal levels of TGF β , PDGF,

and possibly other cytokines, adequate to drive myofibroblast development and maintain myofibroblast viability are achieved when such defects in the basement membrane persist for a prolonged period of time measured in months or years. Repair of the basement membrane likely leads to a drop in the concentration of these cytokines and apoptosis of myofibroblasts (Wilson, Chaurasia and Medeiros, 2007). Another potential source for PDGF is autocrine production of the cytokine by keratocytes when they are stimulated by TGF β derived from epithelium or other sources (Jester et al., 2002). Stimulation of autocrine PDGF production by TGF β has been demonstrated in fibroblastic cells from other tissues (Soma and Grotendorst, 1989). Presumably, once TGF β levels in the corneal stroma drop, autocrine PDGF production in the keratocytes would also diminish.

Keratocytes that subsequently invade the anterior stroma likely reabsorb and reorder matrix materials such as collagen secreted by the myofibroblasts, reestablishing corneal transparency associated with the clinical disappearance of haze (Zieske, 2001). In some eyes, these cellular processes may take years to clear haze spontaneously. In some cases the stromal opacity generated by PRK has been permanent.

In this study, a slit lamp based clinical grading scale for haze was used to compare differences in corneal transparency between the groups treated with the plasmid vector expressing the PDGF β receptor fragment and the empty plasmid. No difference was noted. It is possible that a small, but statistically significant, difference in the transparency between corneas in the two groups could have been detected if a more quantitative method such as clinical confocal microscopy with image analysis had been used. However, such a small difference would not have altered the overall conclusions of this study and would likely not have been clinically meaningful.

At present we cannot provide a definitive explanation for why a decrease in stromal haze doesn't parallel a decrease in α -SMA+ myofibroblast density in the stroma after PDGF-B blockade. It is possible, however, that this observation provides a clue to the myofibroblast differentiation process. Recently, we have noted that α -SMA+ myofibroblasts that appear in the stroma after corneal injury are preceded by Vimentin+, α -SMA-precursor cells (Chaurasia, Medeiros, Kaur, and Wilson. Invest. Ophthalmol. Vis. Sci. 2008 E-Abstract 2942, ARVO annual meeting, 2008). Nothing is known about regulation of the transition from vimentin+, α -SMA- to vimentin+, α -SMA+ myofibroblasts or about the extracellular matrix production of these specific cell types. Based on the results of the present *in situ* study, *in vitro* experiments are underway to examine the possibility that PDGF regulates the vimentin+, α -SMA- to vimentin+, α -SMA+ transition and, therefore, when PDGF B function is inhibited in the stroma, the majority of cells in the myofibroblast lineage are α -SMA-, but still competent to produce extracellular matrix. Alternatively, perhaps other cell types such as corneal fibroblasts contribute to extracellular matrix production associated with haze.

PDGF, along with other cytokines such as TGF β , participates in stromal-epithelial interactions involved in the generation of myofibroblasts associated with corneal stromal opacity. The process of unraveling these complex cytokine-mediated interactions is likely to provide important insights into the biology of myofibroblasts in the cornea.

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Fig.1.

Plasmid driven expression of PDGF receptor fragment in primary corneal fibroblasts in vitro. Corneal fibroblasts were transfected with pCMV.PDGFRB.23KDEL (A) or the empty plasmid vector (B). After 24 hours the cells were fixed and probed for the PDGF receptor fragment using immunocytochemistry. Note that in cells in A express the PDGF receptor fragment (red stain) and it is present in the cytoplasm in membrane bound organelles, as would be expected for a protein product containing the KDEL domain that is an endoplasmic reticulum retention signal. No expression was detected in control cells transfected with empty plasmid vector (B). Magnification 500X

Fig. 2.

Biomicroscopic quantification of haze in the central corneas of the rabbit eyes of different treatment groups at 4 weeks after -9.0 diopter PRK. There was no significant difference in mean haze graded with the Fantes, et al (1990) scale between the group that receive the PDGF β receptor fragment-expressing plasmid and either control group.

Fig.3. α -SMA muscle actin staining in rabbit corneas in the experimental group treated with pCMVPDGFBRB23KDEL plasmid collected at 4 weeks after -9.0D PRK. A, B: Corneas with no α -SMA staining; C: A cornea with a few α -SMA staining myofibroblasts. Cell nuclei were stained blue with DAPI and α -SMA-positive cells were stained green. Magnification 400X. The bar in each panel is 30 μ m.

Fig.4.
 α -SMA muscle actin staining in rabbit corneas in the control groups collected at 4 weeks after -9.0D PRK. A,B: Corneas treated with empty pCMV plasmid; C: Corneas treated with BSS. Cell nuclei were stained blue with DAPI and α -SMA-positive cells were stained green. Magnification 400X. The bar in each panel is 30 μ m.

Fig.5.

Quantification of α -SMA-positive cells/400X in the rabbit corneas of the different groups at 4 weeks after -9.0D PRK. pCMVPDGFRB: Corneas of rabbits treated with pCMV.PDGFRB23KDEL plasmid; Empty pCMV: Corneas of rabbits treated with the empty pCMV plasmid; BSS: Corneas of rabbits treated with balanced salt solution. The error bars represents the mean $\pm$ standard error. **, P=0.001 compared to BSS; * P<0.001 compared to empty pCMV.