Effect of PDGF, IL-1α, and BMP2/4 on Corneal Fibroblast Chemotaxis: Expression of the Platelet-Derived Growth Factor System in the Cornea

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PURPOSE. The purpose of this study was to examine expression of platelet-derived growth factor (PDGF) and PDGF receptors in the human cornea and to study the effects of the PDGF isotypes on proliferation and chemotaxis of human corneal fibroblasts. The effects of interleukin (IL)-1α, bone morphogenetic protein (BMP)2, and BMP4 on chemotaxis of human corneal fibroblasts were also studied.

METHODS. mRNA expression was monitored with reverse transcription-polymerase chain reaction (RT-PCR) in primary cultured cells. Protein expression in fresh-frozen human corneal sections was studied with immunocytochemistry. Chemotaxis was measured using a modified Boyden chamber, and proliferation was quantitated by cell counting.

RESULTS. PDGF A, PDGF B, PDGF receptor alpha, and PDGF receptor beta mRNAs were detected in corneal epithelial cells, fibroblasts, and endothelial cells in culture. The proteins were expressed in each major cell type in human corneal sections, with PDGF A and PDGF B detected at high levels in the epithelial basement membrane. PDGF, BMP2, and BMP4 had attractive chemotactic effects on corneal fibroblasts, with the PDGF BB dimer having a significantly greater positive chemotactic effect than the other PDGF isotypes. Interleukin-1α had a repulsive chemotactic effect on corneal fibroblasts. PDGF AA, AB, and BB stimulated proliferation of human corneal fibroblasts.

CONCLUSIONS. The PDGF growth factor receptor system is expressed in the human cornea. PDGF, BMP2, BMP4, and IL-1α may modulate keratocyte chemotaxis and proliferation during homeostasis and wound healing. (Invest Ophthalmol Vis Sci. 1999;40:1364-1372)

During the early wound healing response that occurs after corneal scrape injury or surgical procedures such as photorefractive keratectomy, the initial disappearance of anterior stromal keratocytes by apoptosis is followed within a few days by repopulation of the anterior stroma with activated keratocytes or myofibroblast-like cells. The regulatory factors controlling stromal cell repopulation have not been characterized, although return of the anterior stromal cells is thought to occur through a combination of proliferation and migration of remaining keratocytes from the peripheral and posterior stroma. It is likely that these responses are regulated by cytokines released locally into the cornea and tear film.

Platelet-derived growth factor (PDGF) is a cytokine that functions as a homodimer (AA, BB) or heterodimer (AB) of two related, but distinct, polypeptides. PDGF mediates its effects through tyrosine kinase receptors that are also expressed as homodimers or heterodimers of two protein chains (PDGF receptor alpha and PDGF receptor beta, respectively). PDGF is a well-characterized modulator of fibroblast cell mitosis and chemotaxis in other organs and has been localized to the tears after corneal wounding. Recent studies have suggested that PDGF can have a mitogenic or positive chemotactic effect on corneal fibroblasts, although the latter study evaluated only the BB homodimer of PDGF. Other cytokines have also been noted to have a chemotactic effect on corneal fibroblasts in vitro (epidermal growth factor, transforming growth factor-α, fibroblast growth factor-1, insulin-like growth factor, and transforming growth factor-β1) or keratocytes in vivo (interleukin [IL]-1α).

It is important to characterize chemotactic effects of cytokines such as PDGF, IL-1α, and bone morphogenetic protein (BMP) expressed in the cornea. No published studies have systematically examined PDGF growth factor and receptor expression in the cornea. To understand the effects of PDGF on corneal cell chemotaxis and other functions it is important that the pattern of expression of the growth factors and receptors be elucidated. The purpose of this study was to probe the expression of the PDGF and PDGF receptor polypeptides in the human cornea; to explore the chemotactic effects of PDGF, IL-1α, BMP2, and BMP4 on corneal fibroblasts; and to exam-
Materials and Methods

Cell Culture and Ex Vivo Epithelium

Human corneal epithelial, fibroblast, and endothelial cells were cultured from donor corneas that were not used for transplantation because of too young donor age, donor sepsis, or other criteria or from donor rims after removal of the donor button at the time of penetrating keratoplasty.

Human corneal epithelial cells were cultured by placing corneal rim explants that had been denuded of endothelium in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% calf serum (all from Life Technologies, Grand Island, NY), and 5 ng/ml hepatocyte growth factor (Genentech, South San Francisco, CA). Within 1 week epithelial cells migrated from the explants. Once the T-25 cultures reached confluence, the explants were removed and the cultures used in experiments, as will be described.

Human cornea stromal fibroblasts were cultured by incubating stromal explants of eye bank eyes denuded of epithelium and endothelium in Eagle's minimum essential medium (EMEM) with 10% FBS (Hazelton Biologies, Lenexa, KS). First-passage cells from two or three donors were combined in 2.0 ml EMEM with 10% FBS and 5% calf serum. Corneal fibroblasts used in proliferation or chemotaxis experiments were cultured in an identical manner but were passed by trypsinization to passage two or three, as described in individual experiments.

To culture human endothelial cells the Descemet's membrane-endothelial complex was stripped from the stroma of each half button using fine forceps, rinsed twice with Hank's balanced salt solution, and cut into approximately 10 strips with a scalpel blade. All strips from one cornea were transferred to a flask (Primaria T-25; Falcon; Oxnard, CA) containing 1.5 ml EMEM with 10% FBS and 5% calf serum.

Ex vivo corneal epithelium was collected (without culture) by scraping with a scalpel blade to Bowman's layer over 7 mm of central cornea at the time of photorefractive keratectomy. The Investigational Review Board at the Cleveland Clinic Foundation approved these studies.

Reverse Transcription–Polymerase Chain Reaction for the Detection of mRNA

Total cellular RNA was isolated from ex vivo human corneal epithelium removed at the time of photorefractive keratectomy and human primary cultures of corneal epithelial, fibroblast, and endothelial cells using previously described methods.12 cDNA was synthesized, and the quality of each cDNA sample was monitored through the amplification of β-actin.12 Only cDNA yielding amplifications of the expected size of 350 bp for β-actin mRNA, without contamination with the 790-bp genomic β-actin amplification product, was used for experimental amplification.12 PCR primers (Table 1) for PDGF A, PDGF B, PDGF receptor alpha, and PDGF receptor beta were designed from the sequences previously reported to GenBank (Bethesda, MD) using the Oligos 5.0 software program (San Diego, CA).13–16 PCR reactions were performed with a temperature cycler (MJ Research, Watertown, MA) according to a previously described hot-start method using anti-Taq polymerase antibody (Clontech, Palo Alto, CA).12 PCR was performed with the following cycle profile: denaturation 2 minutes at 94°C followed by 35 cycles of annealing 30 seconds at 55°C, extension 1 minute at 72°C, and denaturation 30 seconds at 94°C and terminating with 7 minutes at 72°C. PCR products were cut from agarose gels, cloned into the PGR II Cloning Vector (Invitrogen, San Diego, CA), and sequenced (Sequenase 2.0, United States Biochemical, Cleveland, OH) according to the manufacturers’ protocols.
Immunocytochemistry

Corneoscleral rims were excised from eyes of patients with tumors not involving the cornea or choroidal melanoma. Corneas were excised taking care not to damage the epithelium or endothelium more than 5 minutes after enucleation, respectively, embedded in Histo Prep (Fisher, Fairlawn, NJ), snap frozen in liquid nitrogen, and stored at −85°C. The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from each patient before surgery after the nature and the possible consequences of the study were explained. Seven-micrometer-thick sections were prepared with a cryostat. (Reichert-Jung; Leica, Deerfield, IL) placed on slides (Superfrost Plus; Fisher, Pittsburgh, PA), and frozen at −85°C until staining.

The SC-128 polyclonal antibody to PDGF A (PDGF A SC-128P peptide for preabsorption control), SC-127 polyclonal antibody to PDGF B (PDGF B SC-127P peptide for preabsorption control), SC-338 polyclonal antibody to PDGF receptor alpha (PDGF receptor alpha SC-338P peptide for preabsorption control), and SC-339 polyclonal antibody to PDGF receptor beta (PDGF receptor beta SC-339P peptide for preabsorption control) that have been shown by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA) to be useful for immunocytochemistry with fresh-frozen tissues were used for this study. Each of the antibodies was used at a final concentration of 1 μg/ml. For each experiment, a control was performed in which the primary antibody was omitted. Also, a preabsorption control was performed in parallel with staining for each protein by combining control peptide at a final concentration of 10 μg/ml to the antibody at 1 μg/ml and incubating at 37°C for 24 hours before using the mixture in place of primary antibody alone.

Tissue sections were fixed in acetone at −20°C for 10 minutes and air dried for 5 minutes before immunocytochemistry. Immunocytochemistry for each cytokine or receptor was performed using standard methods involving biotinylated secondary antibodies and streptavidin-conjugated peroxidase (Universal LSAB + Kit, peroxidase; Dako, Carpinteria, CA) according to the manufacturer’s instructions, except that sections were incubated overnight at 37°C with primary antibody or the primary antibody-control peptide mixture. Light micrograph photographs were taken with an ocular lens microscope (Optiphot-2; Nikon, Melville, NY).

Cell Proliferation Assay

Second- or third-passage corneal fibroblast cells were seeded at the density of 15,000 cells per well in six-well plates containing stroma is present in this view. (F) PDGF receptor alpha protein was detectable in the epithelium (e) and keratocytes (arrows) in the stroma (s). The source of higher staining in the basement membrane is unknown. (G) Endothelial cells (arrowheads) express PDGF receptor alpha protein. (H) PDGF receptor beta protein was detectable in the epithelium (e). (I) PDGF receptor beta protein was expressed in keratocytes (arrows). (J) PDGF receptor beta protein was detected in the endothelium (arrowheads) and keratocytes (arrows) in the posterior stroma. (K, L) Control stain without primary antibody for comparison with epithelium (e), stroma (s), and endothelium (arrowheads) of other panels. Pink hue (H, I, J) was caused by a different lot of film, but these panels were otherwise photographed using the same techniques. Magnification, ×400; except (C) ×1000.
FIGURE 3. PDGF AA, BB, and AB dimers stimulated proliferation of corneal fibroblasts, as measured by cell counting, in a dose-response manner. ** Result was significantly different from control (0 ng/ml). *** Significantly different from each other.

EMEM supplemented with 10% FBS (both from Gibco, Gaithersburg, MD). The medium was changed to EMEM with 0.5% serum after 24 hours. Each PDGF isoform (AA, BB, AB, R&D Systems, Minneapolis, MN) was tested at final concentrations from 0 ng/ml to 50 ng/ml in six wells for each concentration in each experiment. The medium and growth factors were renewed every 48 hours. After 7 days of exposure to the cytokines, the cells were trypsinized, pelleted, and resuspended in 1 ml medium. The total number of cells per milliliter were counted using a hemocytometer. Variations were expressed as the SEM. Statistical analysis was performed using analysis of variance with the Bonferroni-Dunn adjustment. The assay was repeated three times for each PDGF isotype at each concentration.

Chemotaxis Assays
A 48-well microchemotaxis chamber apparatus (Neuro Probe, Cabin John, MD) was used for chemotaxis assay using polypyrrolidine-free polycarbonate filters of 10 μm thickness with a pore size of 8 μm. The volume of the upper and lower chambers was 50 μl and 30 μl, respectively. The polycarbonate filters were precoated with collagen type I solution (0.1% in 0.1 N acetic acid; Sigma, St. Louis, MO) by immersing the filters overnight at room temperature and drying in a sterile hood. PDGF isotypes (AA, AB, and BB) were diluted with EMEM containing 0.5% FBS at concentrations from 0 ng/ml to 50 ng/ml. In experiments on BMP, BMP2 or BMP4 (Genetics Institute, Cambridge, MA) was diluted with EMEM containing 0.5% FBS at concentrations from 0 ng/ml to 50 ng/ml. In experiments on BMP, BMP2 or BMP4 (Genetics Institute, Cambridge, MA) was diluted with EMEM containing 0.5% FBS at concentrations from 0 ng/ml to 50 ng/ml.
RESULTS

mRNA coding for PDGF A, PDGF B, PDGF receptor alpha, and PDGF receptor beta were detected in two independent cDNA
samples from human primary epithelial cells, ex vivo corneal epithelium, primary stromal fibroblast cells, and primary endothelial cells using RT-PCR (Fig. 1). Each of the cytokine and receptor mRNAs was expressed in each corneal cell type. Each amplified sequence was confirmed by nucleic acid sequencing.

PDGF A protein was detected by immunocytochemistry in the epithelium, keratocytes, and endothelium in human fresh-frozen cornea using immunocytochemistry (Fig. 2A and 2B). PDGF A immunolocalization was most intense in the basement membrane of the epithelium (Fig. 2A), consistent with the heparin-binding property of the polypeptide. PDGF B protein was also detected in the epithelium, keratocytes, and endothelium in human cornea (Figs. 2C, 2D, 2E). Again, heparin-binding PDGF B was detected at high levels in the basement membrane of the epithelium (Fig. 2C). PDGF receptor alpha (Figs. 2F, 2G) and PDGF receptor beta (Figs. 2H, 2I, 2J) proteins were detected by immunocytochemistry in the epithelium, keratocytes, and endothelium in human fresh-frozen cornea using immunocytochemistry. Control immunocytochemistry in which primary antibody was omitted is shown in Figures 2K and 2L. Individual preabsorption control slides were performed for each of the proteins (PDGF A, PDGF B, PDGF receptor alpha, and PDGF receptor beta). Staining for each of these four preabsorption controls was similar to the control in which the primary antibody was omitted (not shown) and showed minimal staining compared with the corresponding primary antibody–stained sections.

PDGF dimers AA, AB, and BB stimulated human corneal fibroblast proliferation in a dose–dependent manner (Fig. 3). At 10 ng/ml, 25 ng/ml, and 50 ng/ml the mitogenic effect of the BB and AB dimers was significantly greater than that of the AA dimer ($P < 0.05$). Results were consistent when the experiment was repeated.

PDGF stimulated positive chemotaxis of human corneal fibroblasts. Figure 4 shows characteristic staining of the polyvinyl pyrrolidone–free polycarbonate filters stained with hematoxylin for one of the experiments. In Figure 5 it can be seen that each PDGF dimer (AA, AB, BB) stimulated positive chemotaxis in a dose–response manner, although in each case the effect decreased or was eliminated at the higher 50-ng/ml concentration. PDGF BB had a statistically significant greater positive chemotactic effect on the corneal fibroblasts than either PDGF AA or AB ($P < 0.05$). Interleukin-1α at 10 ng/ml or 25 ng/ml had a significant ($P < 0.05$) negative chemotactic effect on human corneal fibroblasts (Fig. 6). Interleukin-1α (negative chemotactic effect) in the upper chamber with PDGF BB (positive chemotactic effect) in the lower chamber, stimulated increased migration of corneal fibroblasts from the upper

**FIGURE 5.** Quantitation of positive chemotaxis of corneal fibroblasts in response to PDGF isotypes. Cells per X400 field were counted on the posterior surface of six wells for each isotype at each concentration with PDGF in the lower chamber. Background migration indicated by 0 ng/ml. Each isotype stimulated increased positive chemotaxis with a maximum at 10 ng/ml. PDGF BB had a significantly greater effect than PDGF AA or AB at 1 ng/ml and 10 ng/ml. * * * * * * * * Result was significantly different from the control (0 ng/ml). * * * * * * * * Significantly different from each other.

**FIGURE 6.** Negative chemotactic effect of IL-1α on corneal fibroblasts. Increased numbers of cells passed through pores in the polycarbonate filters to the posterior surface when IL-1α was present in the medium in the upper chamber above the filters. Negative numbers on the abscissa indicate negative chemotaxis. ** * * Result was significantly different from the control (0 ng/ml).
FIGURE 7. Combined positive chemotactic effect of PDGF BB and negative chemotactic effect of IL-1α. With PDGF BB at 10 ng/ml in the lower chamber, there was a positive chemotactic effect on corneal fibroblasts stimulating migration from the upper chamber through the pores to the posterior surface of the filter. When IL-1α was added to the upper chamber at a concentration of 1 ng/ml or greater, there was an increase in chemotaxis toward the lower chamber indicating that the negative chemotactic effect of IL-1α augmented the positive chemotactic effect of PDGF BB. ** Result was significantly different from the control containing neither cytokine. *** Significantly different from each other.

chamber to the lower chamber (Fig. 7). The positive chemotactic effect of PDGF on corneal fibroblasts was antagonized if PDGF BB and IL-1α were both placed in the lower chamber (Fig. 8). BMP2 and BMP4 at 10 ng/ml or 50 ng/ml also had positive chemotactic effects on human corneal fibroblasts (Fig. 9A, 9B). Each of these experiments was repeated at least two times with similar results each time.

DISCUSSION

Although several studies have shown mitogenic or chemotactic effects of PDGF on corneal cells, no detailed studies on the localization of PDGF and PDGF receptors have been reported in the literature. It is important to understand localized expression of PDGF and PDGF receptors if the functions of this cytokine receptor system in the cornea are to be understood. The results of this study suggest that PDGF A and PDGF B and the cognate receptors PDGF receptor alpha and beta mRNAs and proteins are expressed in all three types of human corneal cells. PDGF A and PDGF B were at especially high levels in the basement membrane of the epithelium (Fig. 2). This is consistent with the heparin-binding properties of both isoforms of PDGF. This depot of PDGF in the epithelial basement membrane could be released by injury to the cornea in which there was damage to the basement membrane (such as in photorefractive keratectomy) and modulate epithelial and keratocyte functions through the receptors expressed by these cells.

What functions are regulated by PDGF and PDGF receptors in the cornea? PDGF could serve unknown autocrine cytokine functions in the cornea, inasmuch as these studies show that each of the PDGF isotypes and receptors are expressed in epithelial, keratocyte, and endothelial cells. These autocrine functions modulated by PDGF are unknown. Studies have shown that PDGF can modulate proliferation and chemotaxis of corneal fibroblasts and endothelial cells. Thus, autocrine effects of PDGF on these cells may include these functions. Previous studies did not observe any mitogenic or chemotactic effects of PDGF on primary cultured corneal epithelial cells (Wilson, unpublished data, 1992). The results of the present study confirm that PDGF stimulates proliferation and positive chemotaxis (attractive chemotaxis) of corneal fibroblast cells. This is consistent with studies of fibroblasts from other tissues in which PDGF has been shown to be mitogenic and chemotactic. The PDGF BB isotype appears to have significantly greater effect on both of these functions in corneal fibroblasts than the AA isotype, with the AB heterodimer being similar to BB or intermediate in effect. Thus, PDGF released from the epithelium or basement membrane of the epithelium may modulate keratocyte proliferation and migration. Previous studies have shown that BMP2 and BMP4...
FIGURE 9. Positive chemotactic effect of BMP2 or BMP4 on cornea fibroblasts. BMP2 (A) or BMP4 (B) in the lower chamber increased migration of corneal fibroblasts through pores in the filter to the posterior surface of the filter. Only 10 ng/ml or 50 ng/ml of either cytokine reached statistical significance. ***Means for BMP2 that are significantly different from each other.

stimulate proliferation or apoptosis of corneal fibroblasts, depending on the status of nuclear factor kappa B activation.\(^1\) No effect of IL-1\(\alpha\) on proliferation of human corneal fibroblasts was noted using identical in vitro culture conditions.\(^1\)

Transforming growth factor-\(\beta\) family members BMP2 and BMP4 have recently been shown to be expressed in the human cornea and modulate apoptosis of corneal fibroblasts.\(^2\) These cytokines are also heparin-binding. The results of the present study show that BMP2 and BMP4 also stimulated positive chemotaxis of corneal fibroblasts, although the effect was less than for PDGF.

This study shows that IL-1\(\alpha\) had a negative chemotactic (repulsive chemotactic) effect on corneal fibroblasts. These results confirm the findings of our previous in vivo microinjection studies in mice.\(^1\) Also, the in vitro experiments show that IL-1 antagonized the positive chemotactic effect of PDGF on corneal fibroblasts.

Thus, PDGF, BMP2, and BMP4 had opposite effects from IL-1\(\alpha\) on keratocyte migration. Because IL-1\(\alpha\) protein is detected in the epithelium, but not keratocytes, in unwounded human cornea,\(^2\) attractive chemotactic effects of PDGF, BMP2, and BMP4 present at high levels in the epithelium and basement membrane of the epithelium may be in equilibrium with the repulsive chemotactic effects of IL-1\(\alpha\) released from the epithelium during homeostasis. Basement membrane-associated heparin-binding growth factors would be released during homeostasis by low, but significant, dissociation.\(^2\) Such a chemotactic equilibrium may serve to maintain normal tissue organization in the anterior cornea and could have a role in maintaining the acellular Bowman’s layer in parallel with apoptotic effects of epithelial cytokines such as Fas ligand.\(^2\) Similar interactions modulated by endothelial PDGF, BMP2, BMP4, and IL-1\(\alpha\) and could maintain posterior corneal tissue organization through effects on posterior keratocyte localization.

These studies also suggest PDGF, BMP2, and BMP4 to be good candidates to modulate anterior stromal keratocyte repopulation that occurs during the first few days after keratocyte apoptosis triggered by epithelial injury.\(^1\) Thus, after apoptosis induced by cytokines released from the injured epithelium,\(^3\) PDGF, BMP2, BMP4, and possibly other cytokines released by epithelial cells or the epithelial basement membrane may stimulate migration and mitosis of the remaining keratocytes to re-establish the cell density in the anterior stroma. PDGF, BMP2, and BMP4 released by healing epithelial cells could reach the stroma in higher concentrations than during homeostasis by diffusion into the stroma before regeneration of the basement membrane. Thus, the relative barrier provided by the epithelial basement membrane to heparin-binding growth factors could serve to limit the exposure of keratocytes to epithelial-derived PDGF, BMP2, and BMP4 during homeostasis, while at the same time providing a reservoir of cytokine to be released in the event of anterior corneal injury. Future studies should investigate the role of these cytokines on proliferation and migration of keratocytes in the wounded cornea.

References


