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Rapid Communication

In Situ Injury-induced Release of Basic-fibroblast Growth Factor from Corneal Epithelial Cells

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Basic-fibroblast growth factor (b-FGF) binds to heparan sulfate proteoglycan in Bowman's layer of the cornea. The mechanism by which the molecule is deposited in Bowman's layer is the subject of controversy since b-FGF lacks a signal peptide sequence for extracellular secretion. Using immunofluorescence, the authors studied the presence and distribution of b-FGF in the bovine cornea and the conditions under which it could be released and bound to Bowman's layer. The results indicate that corneal epithelium contains b-FGF but that uninjured corneas do not contain detectable levels of b-FGF in Bowman's layer. Injury to the corneal epithelium results in the binding of b-FGF to Bowman's layer. Removal of the intact corneal epithelium without cell injury does not result in the binding of b-FGF to Bowman's layer. These findings suggest that one mechanism for the release of b-FGF from corneal epithelial cells is passive leakage after cell injury with secondary binding to Bowman's layer. (Am J Pathol 1991, 139:961–967)

Heparin-binding basic-fibroblast growth factor (b-FGF) is an ubiquitous molecule that is produced by a wide variety of cells derived from mesoderm and neuroectoderm.[1] B-FGF can function as a mitogen, morphogen, or terminal differentiation factor in cells bearing specific surface receptors for the molecule.[1] In the normally avascular and optically clear cornea, b-FGF increases the rate of corneal epithelial wound healing.[2]

B-FGF is present in corneal endothelial cells and has been demonstrated in extracellular matrix specifically bound to the proteoglycan heparan sulfate.[3] In normal bovine cornea, bioactive endogenous b-FGF can bind to heparan sulfate proteoglycan in Bowman's layer and Descemet's membrane.[4] Radioiodinated b-FGF can bind to Bowman's layer, Descemet's membrane, and vascular basement membranes in vascularized rabbit corneas.[5]

B-FGF lacks a secretory signal peptide sequence.[6] This suggests that during synthesis, the nascent protein is unable to enter the endoplasmic reticulum of the cell, and thus, the classical cellular pathway leading to active secretion. Conditioned medium from b–FGF-producing cells contains minor amounts of bioactive b-FGF compared with the intracellular stores of the molecule.[7] It is paradoxical that the molecule is so widely distributed and affects so many different cell types, yet it is not actively secreted. Controversy exists as to whether b-FGF is actively secreted basally into the extracellular matrix through a nonclassical pathway,[8] or whether it is passively deposited after cell injury and/or lysis.[7]

Given the known ability of b-FGF to bind to the cornea, and its possible role in corneal wound healing and angiogenesis, we determined where in the cornea b-FGF was produced, and under what conditions it was released and stored in Bowman's layer and Descemet's membrane. Finally, we wanted to further define the roles of Bowman's layer and Descemet's membrane in normal and injured corneas.

The terms "Bowman's layer" and "Bowman's membrane" are often used synonymously with "corneal epithelial cell basement membrane" in the scientific literature. More correctly, Bowman's layer refers to the acellular, anterior-most portion of the collagenous corneal stroma. It is rich in heparan-sulfate proteoglycan and an-

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atomically distinct from the corneal epithelial basement membrane located anterior to it.[9] Descemet's membrane is a true basement membrane that is produced by the corneal endothelial cells on the posterior surface of the cornea.[10]

Materials and Methods

Tissue Preparation of Normal Corneas

Enucleated fresh whole adult bovine eyes purchased from a local slaughterhouse, and stored for a maximum of 2 hours at 4°C, were used to obtain 10-mm corneal buttons. These were bisected and immediately placed in tissue block molds filled with OCT compound (Ames Co., Elkhart, IN). The corneas were frozen by floating the molds on liquid nitrogen for 30 seconds. Eight micrometer frozen, unfixed, cross-sections were cut on a cryostat and placed on gelatin-coated slides.

Immunofluorescent Localization of b-FGF

Sections were incubated with 2% bovine serum albumin in phosphate-buffered saline (PBS; Gibco, Grand Island, NY) for 1 hour to block nonspecific binding. Affinity-purified polyclonal anti-human b-FGF (1:50 in PBS; 100 microliters/slide; Biomedical Technologies, Inc., Stoughton, MA) was applied for 24 hours at 4°C, followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (1:200–1:400 in PBS; 100 microliters/slide, Cappel, West Chester, PA) for an additional 2 hours at 25°C. Incubations took place in a dark, sealed, moist chamber. Sections were rinsed with PBS × 3 between steps. Some sections were treated with pepsin (200 ng/ml in PBS for 15 min at 25°C) before primary antibody incubation to expose potential cryptic antigenic sites.

Sections were incubated with non-immune rabbit serum (1:50 in PBS; 100 microliters/slide) followed by rhodamine-conjugated secondary antibody or with secondary antibody alone for 24 hours at 4°C to identify any nonspecific binding. Negative control sections were preincubated with heparin sodium (1.0 mg/ml in PBS; Fisher Scientific, Fairlawn, NJ) for 1 hour at 25°C to remove native b-FGF before antibody staining. Positive control sections were preincubated with b-FGF (1.0 μg/ml) for 1 hour at 25°C to identify potential binding sites in the cornea.

Experimental Conditions—Non-lytic and Lytic Models

Three experimental conditions were created: 1) nonlytic removal of the corneal epithelium was achieved by incubating fresh corneal buttons in either 5 ml of Dispase II (Boehringer Mannheim, Indianapolis, IN) in PBS (1.2 units/ml, pH 7.4, 25°C) or 5 ml of 2.5 mM EDTA in Hank's Balanced Salt Solution (Gibco, Grand Island, NY) without Ca^{++} or Mg^{++} (pH 7.4, 25°C) for 1 hour, 2) corneal epithelial cell membranes were lysed by scraping the surface of corneal buttons with a #15 Bard-Parker blade (Becton Dickinson and Co, Lincoln Park, NJ) while the corneas were immersed in 5 ml of Medium-199 (pH 7.4, 25°C), and 3) membrane lysis was achieved by incubating corneal buttons in 5 ml of 0.5% Triton X-100 (Sigma, St. Louis, MO) in Medium-199 (pH 7.4, 25°C) for 1 hour. Lactate dehydrogenase (LDH) levels in the incubation solutions were determined enzymatically on a Synchron CX5 analyzer (Beckman Instrument Inc., Brea, CA) and served as a quantitative index of cell injury and cell permeability.

Results

Immunolocalization of Basic-FGF in Bovine Cornea

B-FGF was localized diffusely within the cytoplasm of corneal epithelial cells in all the sections incubated with primary antibody. Sections without pretreatment and with normal anatomical structure (i.e., well-preserved and attached epithelium) did not contain detectable b-FGF in Bowman's layer (Figure 1A). Normal sections treated with pepsin before staining to expose potential cryptic antigenic sites did not reveal detectable b-FGF in Bowman's layer. Control sections incubated with nonimmune serum followed by secondary antibody or with secondary antibody alone showed minimal nonspecific fluorescence. Sections preincubated with soluble heparin did not stain positively for b-FGF (Figure 1C).

B-FGF was localized to Bowman's layer only in areas where real or artifactual detachment of the corneal epithelium with cell injury was evident. In these areas, Bowman's layer subjacent to the site of injury stained positively for b-FGF (Figure 1E). Bowman's layer subjacent to the intact epithelium and proximal to the site of detachment was unstained.

Sections pretreated with b-FGF showed dense localization of b-FGF throughout Bowman's layer, including areas exhibiting normal morphology, demonstrating the ability of the primary antibody to detect b-FGF in Bowman's layer. B-FGF binding to heparan sulfate proteoglycan in Bowman's layer apparently did not change the conformation of the b-FGF molecule or produce cryptic sites that prevented recognition by the primary antibody.

Inconsistent preservation of Descemet's membrane

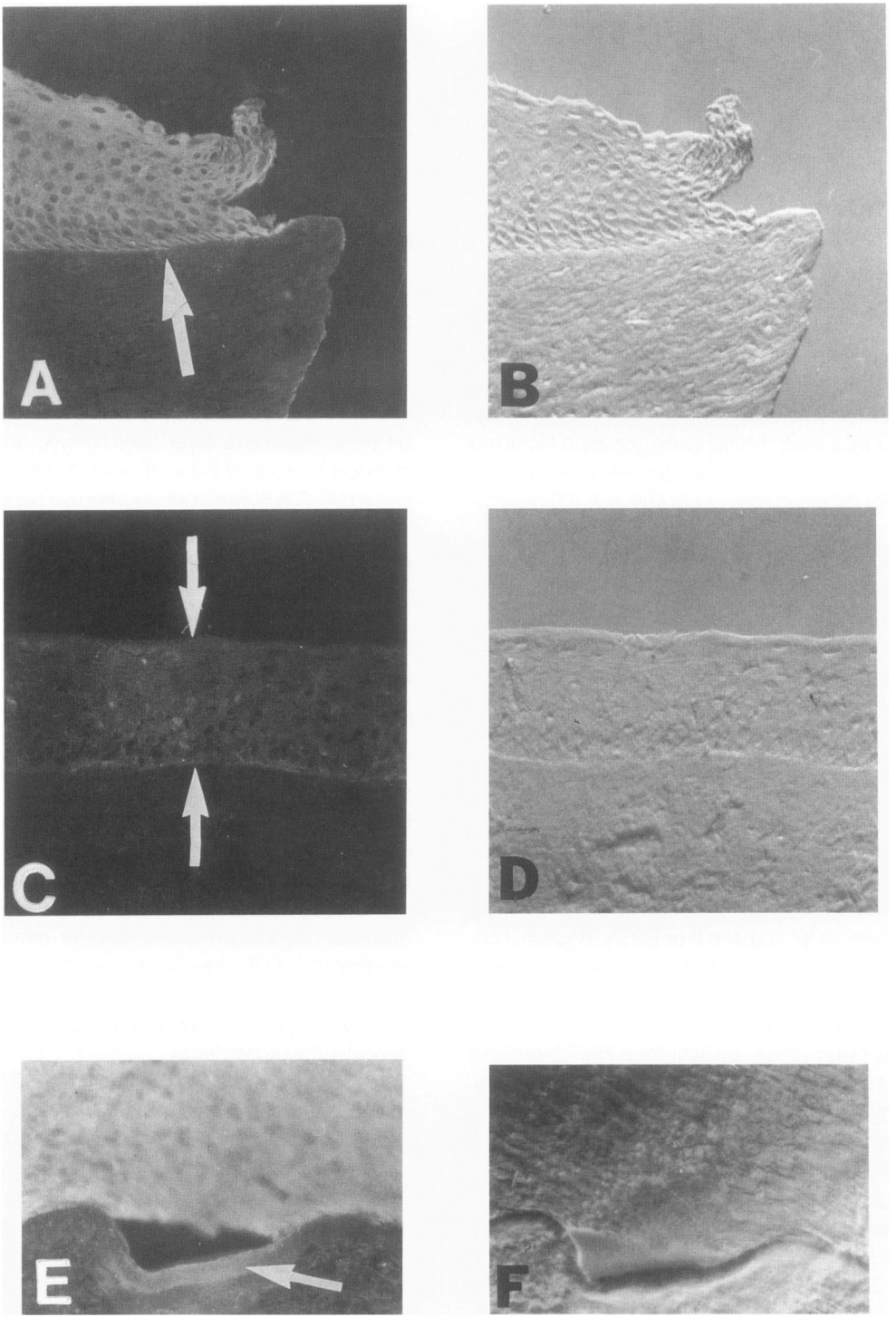

Figure 1. *Immunofluorescence microscopy of bovine cornea stained with anti-b-FGF (left panels:* **A,C,E***) and corresponding phase contrast micrographs of the same sections (right panels:* **B,D,F***),* **(A)** *shows localization of native b-FGF in the corneal epithelium. Arrow points to the unstained Bowman's layer (magnification, ×20),* **(C)** *demonstrates the low level of staining in the corneal epithelium after heparin preincubation (area between arrows) (magnification, ×20),* **(E)** *contains an area of basal epithelial cell detachment with focal localization of native b-FGF to the subjacent Bowman's layer (arrow) (magnification, ×40).*

and the corneal endothelium during tissue processing precluded any reliable analysis of these layers. The level of microscopic resolution was insufficient to consistently determine the presence or absence of b-FGF in the corneal epithelial basement membrane anterior to Bowman's layer.

Immunolocalization in the Non-lytic and Lytic Models

To determine if b-FGF was released passively after epithelial cell injury, conditions were created in which 1) the corneal epithelium was removed in a nonlytic fashion (EDTA or Dispase II preincubation); i.e., the plasma membranes were not ruptured, 2) the corneal epithelial cell membranes were mechanically disrupted (scraping with a blade), or 3) the corneal epithelial plasma membranes were permeabilized *in situ* (Triton-X 100 preincubation).

Sections pretreated with EDTA or Dispase II before staining showed multiple areas of basal epithelial cell detachment. Previous work has shown that Dispase II disrupts the lamina densa of corneal epithelial cell basement membranes, whereas EDTA disrupts cell-to-basement membrane associations.[11] In sections treated with either Dispase II or EDTA, the basal cells appeared intact. The entire length of Bowman's layer in the sections was negative for b-FGF, including areas of focal basal epithelial cell detachment (Figures 2A,C). Sections incubated with b-FGF followed by Dispase II or EDTA treatment showed b-FGF in Bowman's layer (Figure 2E). This confirmed the preservation of the heparan sulfate binding sites after treatment with either Dispase II or EDTA. Heparin, nonimmune, and secondary antibody controls were all negative.

Sections with epithelial cell injury from mechanical scraping showed dense staining for b-FGF throughout the entire thickness of Bowman's layer. Heparin, nonimmune, and secondary antibody controls were all negative after these treatments (Figure 3A). Cell injury to the epithelium with Triton-X 100 solubilization of the cell membrane lipids presumably allowed the passive release of the cytoplasmic contents. This treatment resulted in native b-FGF being deposited in Bowman's layer. Staining was more dense in the anterior-most portion of Bowman's layer adjacent to the basal epithelial cells (Figure 3C).

LDH levels of the incubating solutions for the experimental conditions were as follows: 1) normal uninjured cornea = 222 IU/L; 2) EDTA = 93 IU/L; 3) Dispase II = 26 IU/L; 4) Triton X-100 = 1179 IU/L; and 5) mechanical scraping = 721 IU/L.

Discussion

B-FGF is produced by a wide variety of cell types throughout growth and development and a wide variety of cell types respond to b-FGF through specific FGF cell-surface receptors.[1] The cell biological functions of b-FGF include that of a morphogen, mitogen, and terminal differentiation factor.[1] Given the wide distribution and many important roles of b-FGF during growth and development, it is paradoxical that the molecule lacks a signal peptide sequence for secretion.

Our data supports the hypothesis that one major mechanism by which b-FGF is released and deposited into Bowman's layer is through passive leakage after cell injury. This is based on the following results: 1) the absence of detectable b-FGF in Bowman's layer in morphologically intact normal bovine corneas; 2) the absence of b-FGF in Bowman's layer after nonlytic enzymatic or chemical removal of the epithelium; 3) the presence of native b-FGF in Bowman's layer after mechanical injury to the corneal epithelial cell membranes; and 4) the presence of native b-FGF in Bowman's layer after injury to the corneal epithelial cell membranes with Triton-X 100.

The LDH levels measured during the experimental treatments correlate well with the presumed level of cell membrane injury and support the validity of the experimental models.

Based on these data, and the given level of sensitivity for detecting b-FGF in our system, the following conclusions can also be stated about b-FGF in the cornea: 1) B-FGF is present in the cytoplasm of corneal epithelial cells, 2) b-FGF is not normally present in large amounts in Bowman's layer. This suggests that corneal epithelial cells synthesize but do not actively secrete b-FGF, 3) epithelial cell injury with the passive release of cell-associated b-FGF may be the predominant route by which b-FGF is deposited in Bowman's layer, and 4) Bowman's layer binds b-FGF and may act as a storage depot for the growth factor.

Conclusions about the presence of b-FGF in the corneal epithelial cell basement membrane cannot be drawn from these results. Dispase II and mechanical scraping both disrupt basement membranes. Furthermore, the level of microscopic resolution was insufficient to consistently determine the presence or absence of b-FGF in the corneal epithelial basement membrane. Inconsistent preservation of the corneal endothelium and Descemet's membrane also precluded any definite conclusions regarding the presence and distribution of b-FGF in those layers.

These data are consistent with Bowman's layer acting as a repository of b-FGF.[4] Our work, and the work of others,[2,4,5] serve to confirm this new biochemical function

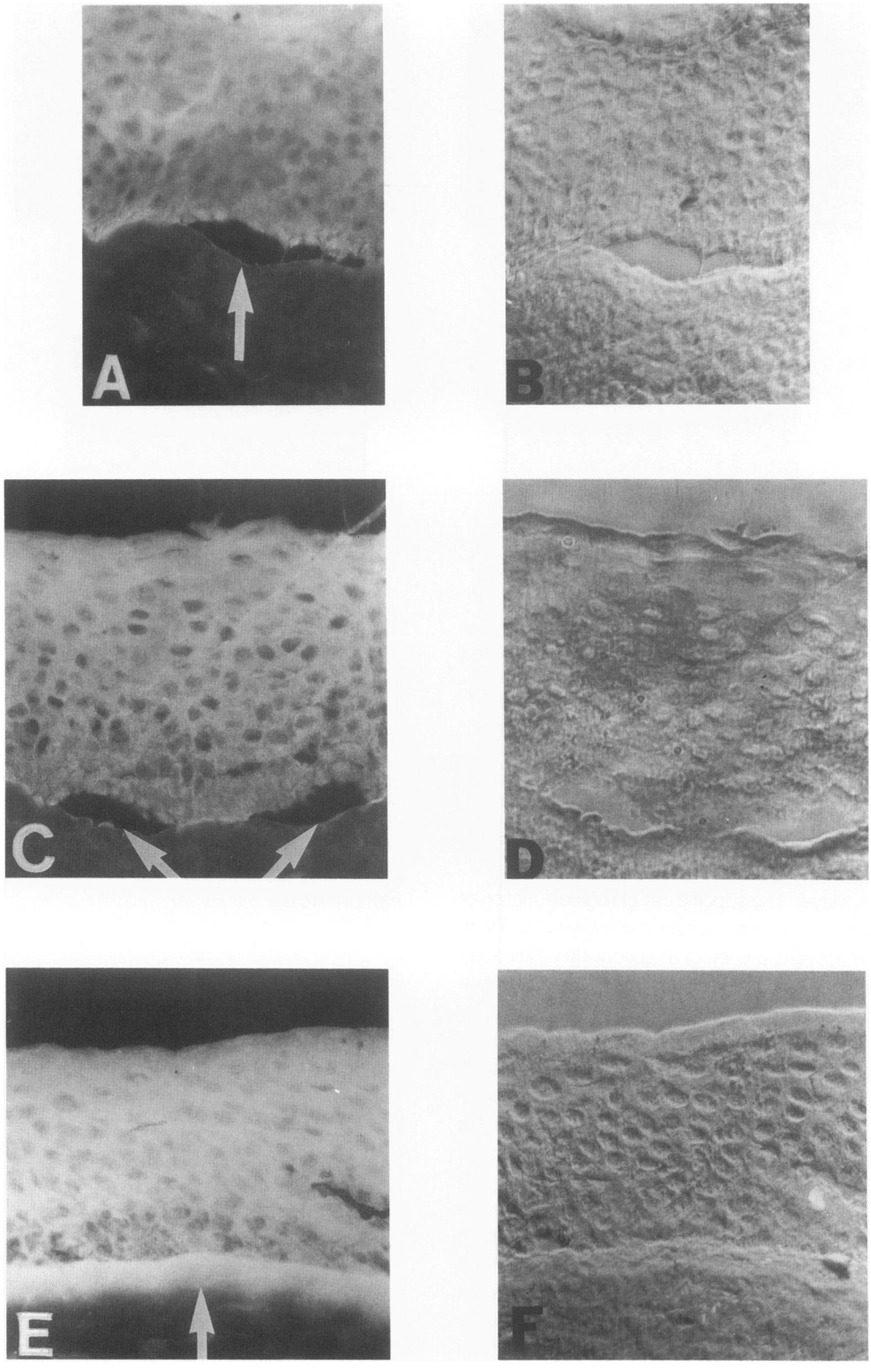

Figure 2. *Non-lytic model—Immunofluorescence mircroscopy of bovine cornea stained with anti-b-FGF (left panels:* **A,C,E***) and corresponding phase contrast micrographs of the same sections (right panels:* **B,D,F***), magnification, ×40,* **(A)** *and* **(C)** *demonstrate the lack of b-FGF in Bowman's layer (arrows) after the non-lytic (non-injurious) detachment of the basal epithelial cell layer with Dispase II and EDTA, respectively. The tissue section in* **(E)** *was first treated with Dispase II and then incubated with exogenous b-FGF. The intense staining demonstrates the ability of Bowman's layer to bind b-FGF after Dispase II protease treatment.*

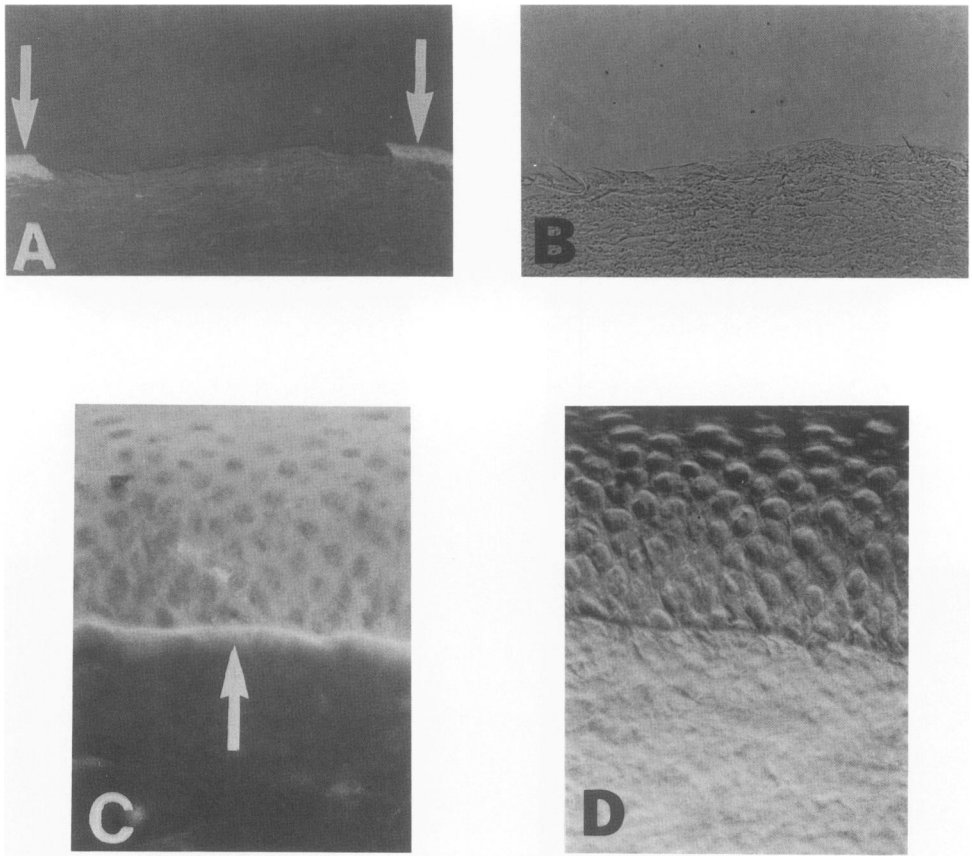

Figure 3. *Lytic model—Immunofluorescence microscopy of bovine cornea stained with anti-b-FGF (left panels:* **A,C***) and corresponding phase contrast micrographs of the same sections (left panels:* **B,D***), magnification, ×40. The epithelium and central portion of Bowman's layer were removed in an injurious manner with a #15 Bard Parker blade,* **(A)** *shows intense localization of b-FGF in the remaining Bowman's layer (arrows),* **(C)** *shows localization of b-FGF to Bowman's layer following solubilization of the epithelial cell membranes with Triton X-100. Note the diffusion gradient from anterior to posterior.*

of Bowman's layer. The specific cell biological function of b-FGF bound to heparan sulfate proteoglycan in Bowman's layer remains a subject of speculation. Released b-FGF after epithelial cell injury may serve to accelerate the healing of corneal epithelial wounds.[5] B-FGF may promote epithelial cell migration and/or mitosis during wound healing. Bowman's layer may also serve a barrier function by sequestering b-FGF from the underlying keratocytes. Clinically, it is known that damage to Bowman's layer results in scarring of the anterior corneal stroma. Excimer laser photoablation leads to scar formation with deposition of type III collagen, a phenotype which is not normally present in the cornea.[12] This corneal scarring may contribute to refractive instability and decreased contrast sensitivity.[13,14] If Bowman's layer is breached or absent, passively released b-FGF may diffuse posteriorly unimpeded and stimulate corneal keratocytes to lay down altered collagen phenotypes. In large injuries, saturation of Bowman's layer b-FGF binding sites may occur. Angiogenesis may then be stimulated leading to corneal neovascularization. These and other hypotheses are the subject of ongoing investigations.

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