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Corneal Wound Healing: Immunohistological Features of Extracellular Matrix Following Penetrating Keratoplasty in Rabbits

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Purpose: To study the distribution and the constituents of the extracellular matrix in the cornea during wound healing following penetrating keratoplasty (PKP).

Methods: Penetrating keratoplasty (PKP) was performed on albino rabbit eyes, and immunohistochemical techniques were used to determine the distribution of types I, III, IV collagens, large proteoglycans, chondroitin 6-sulfate, chondroitin 4-sulfate, and vimentin. The expression of these substances was determined at postoperative times of 3 days, 1 week, 2 weeks, 1 month, and 3 months.

Results: By day 3, staining for type IV collagen was observed along the host-graft junction. By day 7, staining for type III collagen, large proteoglycans, and chondroitin 6-sulfate had increased in the repair region but then decreased with increasing postoperative times. Epithelial wound healing required more than one month, whereas the remodeling of Descemet's membrane did not terminate until 3 months after PKP.

Conclusion: These results suggest that type III collagen, large proteoglycans, and chondroitin 6-sulfate probably play important roles in corneal wound healing after PKP. **Jpn J Ophthalmol 2000;44:334–341**

Key Words: Collagen, corneal wound healing, extracellular matrix, penetrating keratoplasty, proteoglycans.

Introduction

Within the past decade, there has been an explosive growth of investigations on the function of the extracellular matrix (ECM) and the influence of the ECM on cells. Until recently, the ECM was thought to be an inert scaffolding serving to stabilize the structure of tissues. It has recently become apparent that the ECM plays a far more active and complex role in regulating the behavior of cells. The ECM can affect the shape, adhesion, and migration of cells by transmitting extracellular positional information to the intracellular cytoskeletal system via transmembrane receptor molecules.[1,2]

The ECM of the cornea consists primarily of collagens and proteoglycans. New types of collagen have been identified in recent years, and several new molecules, such as type XII collagen, have been identified in the cornea.[3–5] The functional role of these molecules has attracted considerable attention. For the proteoglycans, on the other hand, many points remain to be resolved because of their diverse chemical structure and also because of the complex sulfation structure of the side chain of the glycosaminoglycans. However, recent synthesis of monoclonal antibodies against proteoglycans and the use of high-performance liquid chromatography (HPLC) have made it possible to localize and quantitatively analyze the proteoglycans in the cornea.

Corneal wound healing has been studied extensively, particularly following radial keratotomy[6] and excimer laser[7–9] surgery. In contrast, little information is available on wound healing after penetrating keratoplasty (PKP),[10,11] as far as we are aware.

We performed immunohistochemical experiments to study the mechanism of corneal wound healing after PKP and focused on the alterations of the ECM.

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Materials and Methods

Ten adult albino rabbits weighing 2.5–3.0 kg were used. The experiments were performed in compliance with the ARVO Resolution on the Use of Animal in Research. Rabbits were anesthetized by an intramuscular injection of ketamine hydrochloride (5 mg/kg) and xylazine (5 mg/kg). An autogenous penetrating keratoplasty was performed. The graft was secured with 10-0 nylon interrupted sutures. Tobramycin eye drops were applied twice a day until the rabbits were sacrificed by an overdose of sodium pentobarbital on days 3, 7, and 14, and 1 and 3 months after PKP.

The excised corneas were frozen in Tissue Tek II OCT compound (Baxter Scientific, Columbia, MD, USA). Cryostat sections (7 μm thick) were placed on silane-coated slides, air-dried at room temperature for 2 hours, and then fixed in acetone. Sections were incubated in 0.3% H_2O_2 in phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4) to block endogenous peroxidase activity, and in 1% bovine serum albumin to block nonspecific binding of the antibody. Tissue sections were incubated for 1 hour with the primary antibodies. All the primary antibodies used in this study are listed in Table 1. After washing with PBS, the sections were incubated for 30 minutes with biotinylated anti-mouse or anti-goat IgG antibody (1:100; Dako, Carpinteria, CA, USA) in PBS, rinsed in PBS for 5 minutes, and then incubated with streptavidin-horseradish peroxidase (Dako), diluted in PBS, for 20 minutes. After extensive washing with PBS, bound antibodies were visualized by the diaminobenzidine reaction. The tissue sections were subsequently counterstained with methyl green, washed and mounted in dibutyl phthalate xylene. Negative controls (omitting the primary antibody) were routinely stained with the experimental sections.

The sections were pretreated with chondroitinase ABC (Seikagaku-kogyo, Tokyo) before staining for chondroitin 4-sulfate and chondroitin 6-sulfate. Digestion was done with 0.2 U/mL of the enzyme in 20 mmol/L acetic acid at 37°C for 1 hour.

Results

Three days after PKP, corneal epithelial cells and polymorphonuclear leukocytes had migrated to the host-graft junction (Figures 1A,B). The epithelial cells were distributed over the wound but the basement membrane was not completely formed (Figure 1C). The wound area was edematous and its thickness was twice that of a normal cornea. Keratocytes were also found to have migrated into the wound edge and synthesized new ECM. Positive staining for type IV collagen was observed at the stromal wound edge (Figure 1D). Many activated keratocytes were found around the cut edge of the stroma (Figure 1E). Immunoreactivity for large proteoglycans was seen around the keratocytes adjacent to the wound (Figure 1F). The edge of Descemet's membrane was curled into the stroma. Endothelial cells were not observed in the wound area (Figure 1G).

One week after PKP, the corneal edema had decreased and the corneal thickness was now 1.5 times thicker than normal (Figure 2A). Type IV collagen was preferentially located beneath the epithelial layer, and did not show linear staining but had a diffuse distribution (Figure 2B). The epithelium had returned to normal thickness but staining for vimentin was observed in a part of the basal cells (Figure 2C). The wound at the host-graft junction was filled with newly synthesized ECM consisting of types I, III (Figure 2D), IV (Figure 2B) collagen, large proteoglycans, and chondroitin 6-sulfate (Figure 2E). Staining for chondroitin 4-sulfate was relatively decreased in this new fibrous scar tissue (Figure 2F). The cut edges of Descemet's membrane were not changed, and the defects in the endothelial cells were covered by the migration of adjacent cells (Figure 2A).

Two weeks after PKP, immunoreactivity for type

Table 1. Primary Antibodies Used in This Study

Antibody	Dilution	Antibody Type	Source
Anti-type I collagen	1:200	Mouse MoA	Carbiochem, Cambridge, MA, USA
Anti-type II collagen	1:200	Mouse MoA	Carbiochem, Cambridge, MA, USA
Anti-type IV collage	1:200	Goat MoA	Southern Biotechnology, Birmingham, AL, USA
Anti-large proteoglycan	1:200	Mouse MoA	Seikagaku Kogyo, Tokyo, Japan
Anti-chondroitin 2-sulfate	1:200	Mouse MoA	Seikagaku Kogyo, Tokyo, Japan
Anti-chondroitin 6-sulfate	1:200	Mouse MoA	Seikagaku Kogyo, Tokyo, Japan
Anti-vimentin	1:200	Mouse MoA	Dako, Carpinteria, CA, USA

MoA: monoclonal antibody.

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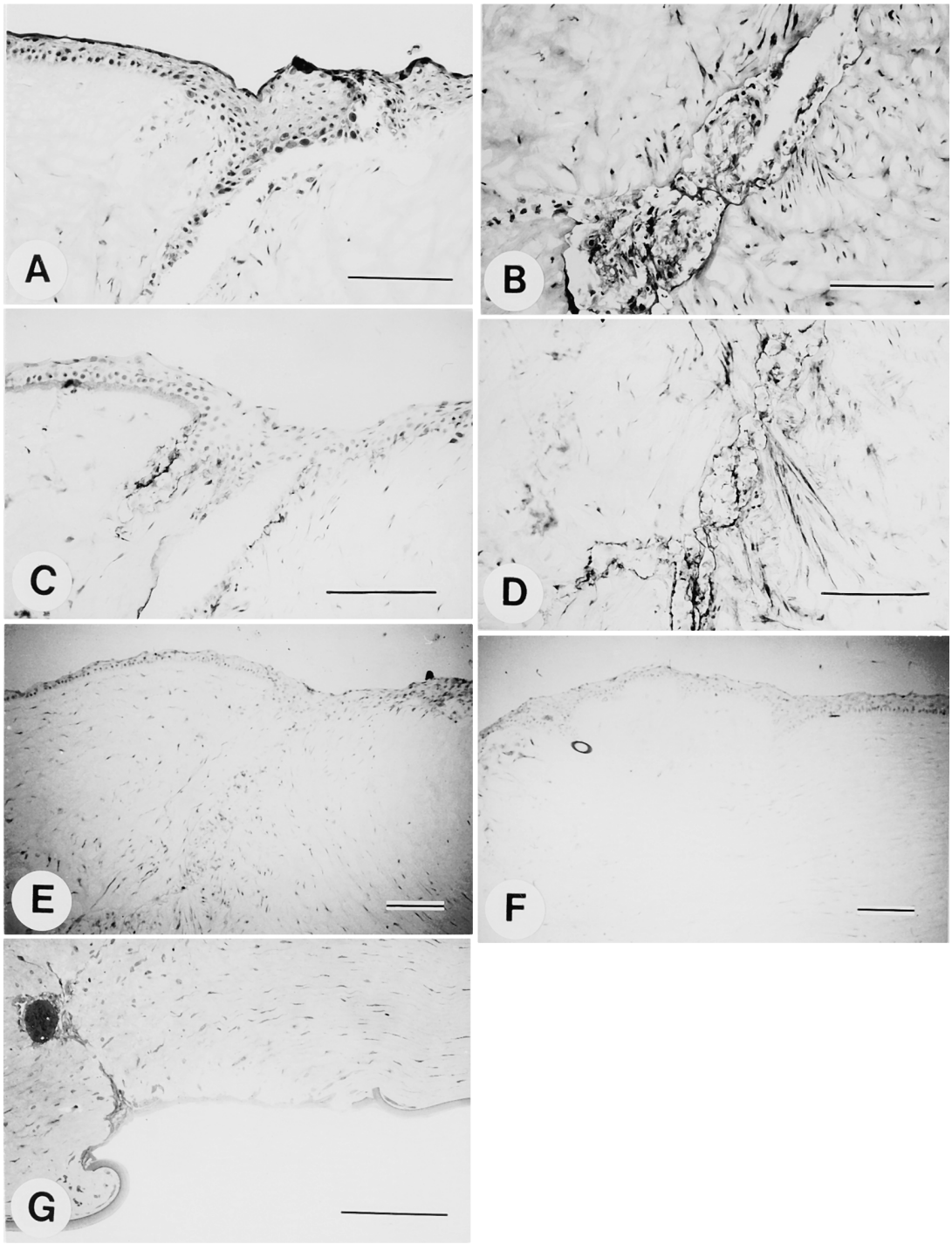

Figure 1. Three days after penetrating keratoplasty. (**A**,**B**) At host-graft junction, epithelial cells have migrated into wound. Hematoxylin-eosin staining. (**A**: epithelial side, **B**: stromal side). (**C**) Immunoreactivity for type IV collagen. A linear structure of basement membrane is lost at the wound. (**D**) Staining for type IV collagen is noted at host-graft junction. (**E**) Immunoreactivity for vimentin. Many activated keratocytes are gathered around wound. (**F**) Faint staining for large proteoglycan is noted around wound. (**G**) Ruptured Descemet's membrane curled into stroma. Toluidine-blue staining. Bars = 100 μm.

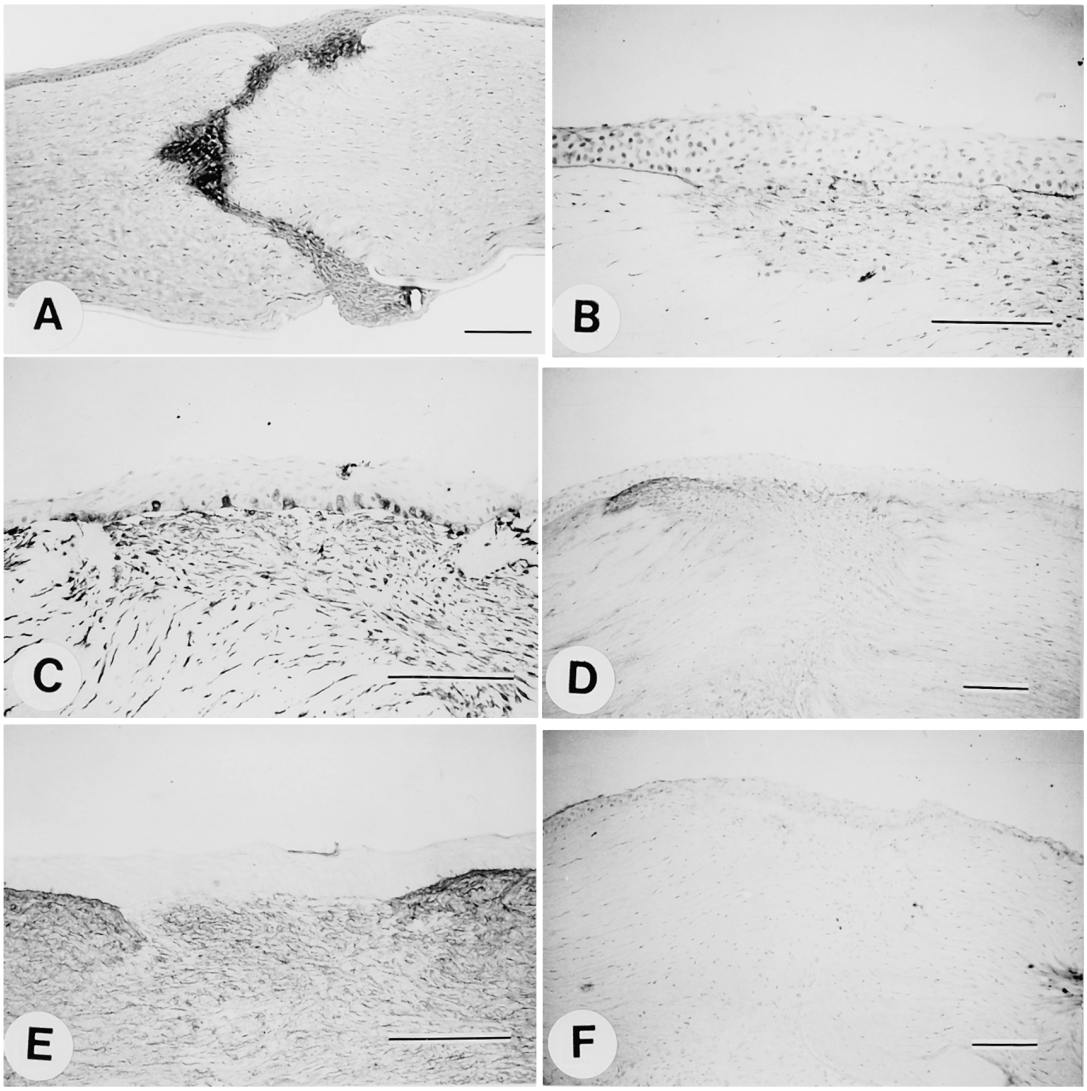

Figure 2. One week after penetrating keratoplasty. (**A**) Host-graft junction is filled with newly synthesized tissue. Toluidine-blue staining. (**B**) Diffuse staining for type IV collagen is noted beneath epithelial layers. (**C**) 1 week after PKP. Intense staining for vimentin appears at stromal wound. Note that some epithelial basal cells show immunoreactivity for vimentin. (**D**) Positive staining for type III collagen is observed at newly synthesized extracellular matrix. (**E**) Staining for chondroitin 6-sulfate. (**F**) Staining for chondroitin 4-sulfate. Bars = 100 μm.

IV collagen still did not show linear staining (Figure 3A). The fibrous scar tissue around the wound included type III collagen (Figure 3B) and large proteoglycans (Figure 3C) as well as those observed 1 week after PKP. Staining for chondroitin 4-sulfate was still decreased from the normal level in this scar tissue (Figure 3D).

At 1 month after PKP, staining for vimentin in the epithelial basal cells had almost dissipated. Labeling for type IV collagen became linear. Immunoreactivity for type III collagen, chondroitin 6-sulfate, and large proteoglycans gradually decreased with increasing postoperative times. In addition, staining for type I collagen and chondroitin 4-sulfate was restored to normal levels. The array of stromal collagen lamellae was disorganized at this time (Figure 4A).

At 3 months after PKP, the stromal collagen lamellae showed a parallel arrangement (Figure 4B). Staining for type IV collagen at the epithelial basement membrane became a linear structure (Figure 4C). The migrated endothelial cells produced a new

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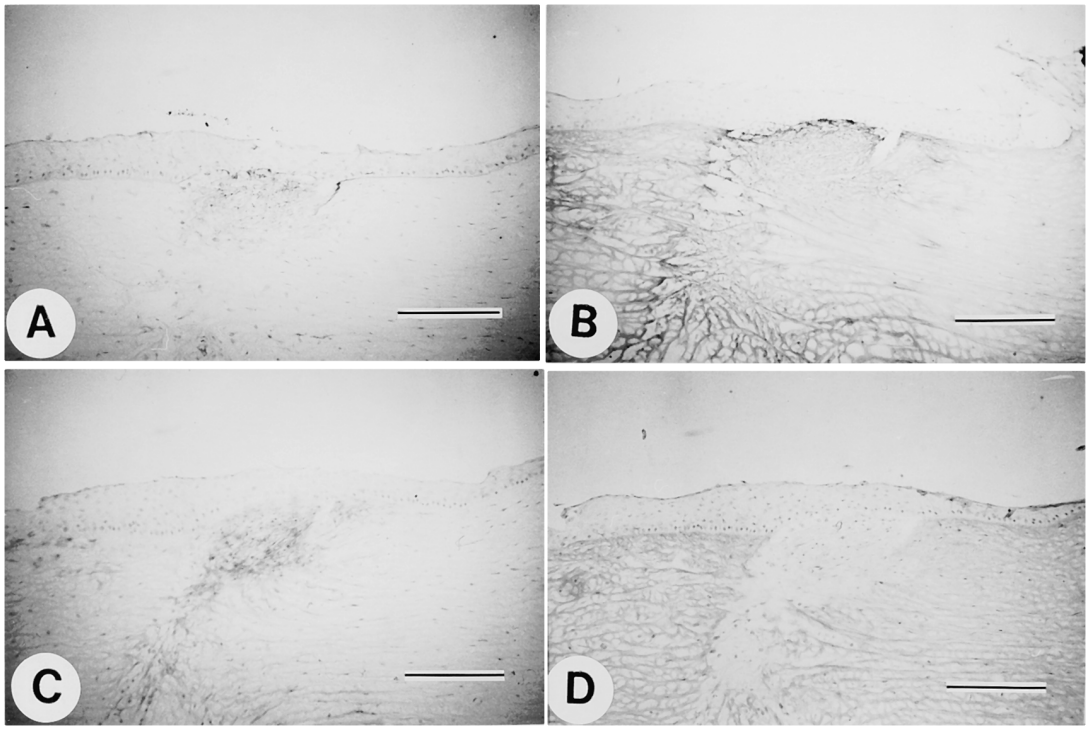

Figure 3. Two weeks after penetrating keratoplasty. (**A**) Staining for type IV collagen still has diffuse distribution. (**B**) Immunoreactivity for type III collagen. (**C**) Intense staining for large proteoglycans is observed at stromal wound. (**D**) Immunostaining for chondroitin 4-sulfate is still decreased at wound area. Bars = 100 μm.

basement membrane, although the thickness of the newly secreted Descemet's membrane was thinner than normal, indicating that the tensile strength was not that of the original tissue (Figure 4D). Staining for large proteoglycans was no longer seen (Figure 4E). Immunoreactivity for chondroitin 6-sulfate in the wound area returned to the normal level (Figure 4F). Stronger staining for type III collagen was still observed at the wound (Figure 4G).

Discussion

The mechanism of corneal wound healing has been extensively studied following keratorefractive surgery, particularly radial keratotomy.[6] Following incisional keratotomy, the corneal epithelial cells migrate rapidly to fill the incisional groove within 1 to 3 days. Keratocytes migrate into the wound and transform into myofibroblast-like cells. These cells then synthesize new ECM materials. The newly synthesized ECM gradually replaces the epithelial plug.

The major difference between the healing of the corneal epithelial wound after radial keratotomy and that after PKP is that the function of the sensory nerve does not recover fully after PKP. This possibly accounts for the delay in healing of the epithelial wound.[12] By using specular microscopy, Tsubota et al[13] demonstrated that the epithelial abnormalities after PKP persisted longer than expected. From the morphological point of view, our results indicated that the epithelial wound healing is accomplished within a week. However, positive immunoreactive staining for vimentin was observed in the epithelial basal cells 2 weeks after PKP. This is of interest as vimentin, an intermediate filament of the mesenchymal system, does not manifest itself in ordinary epithelial cells, but is expressed in epithelial cells whose proliferating activity is increased.[14,15] Thus, the presence of vimentin 2 weeks after PKP indicates that corneal epithelial wound healing was still in progress.

It required more than 1 month for type IV collagen of the epithelial basement membrane to recover to its normal condition. Normalization of the epithelial basement membrane is indispensable for maintaining the adhesiveness of the normal epithelium. Considering these points, the healing of epithelial wounds, including the basement membrane, takes more than 1 month after PKP.

Healing of the stroma is almost completed be-

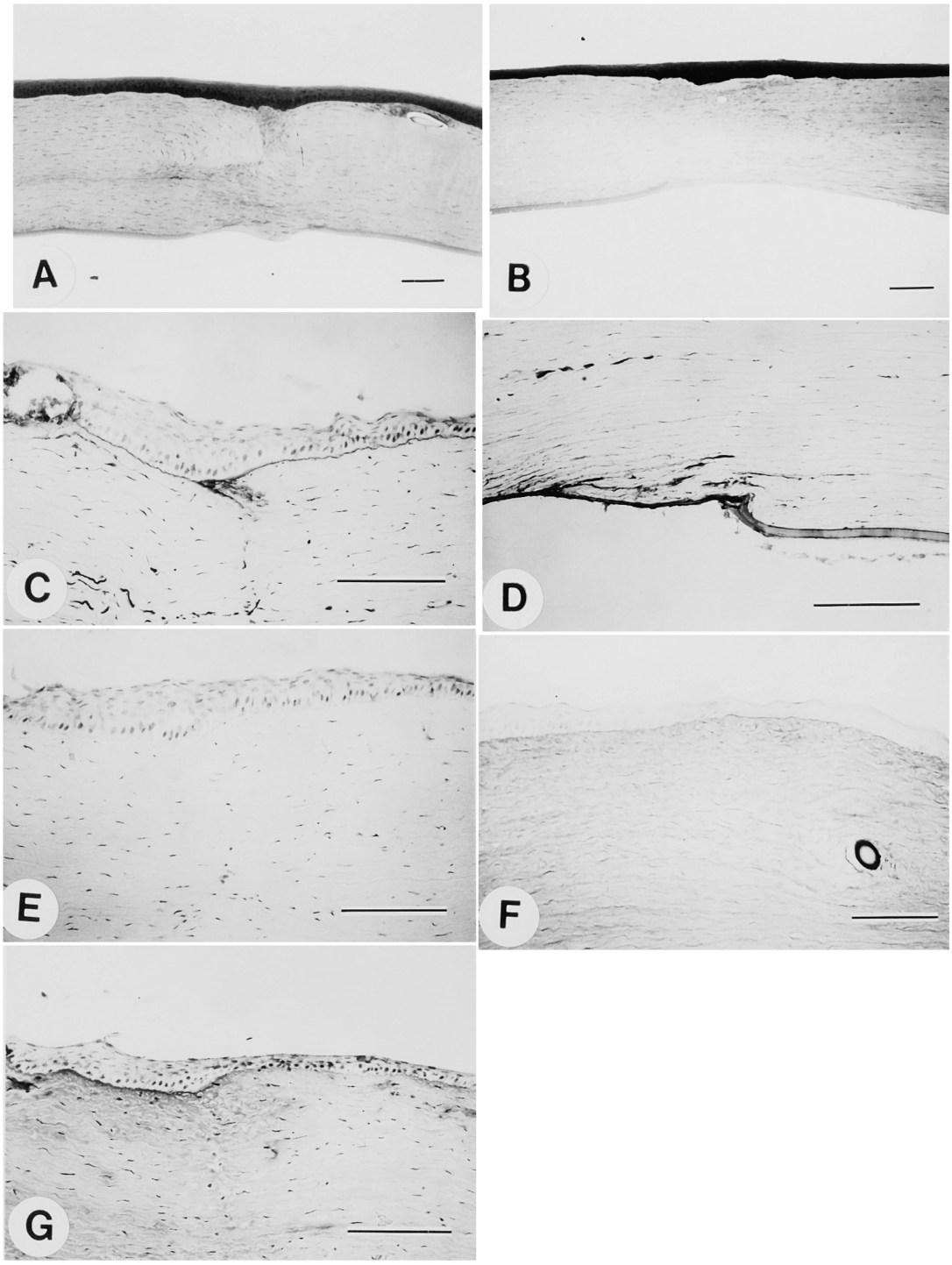

Figure 4. One and 3 months after penetrating keratoplasty (PKP). (**A**) 1 month after PKP. Array of collagen lamellae is still interrupted. (**B**) 3 months after PKP. Array of collagen lamellae is restored to normal order. (**C**) 3 months after PKP. Staining for type IV collagen becomes linear in appearance. (**D**) 3 months after PKP. Thickness of newly synthesized Descemet's membrane is less than half of normal thickness. (**E**) 3 months after PKP. Staining for large proteoglycans. (**F**) 3 months after PKP. There is no difference in immunoreactivity for chondroitin 6-sulfate between wound and uninjured area. (**G**) 3 months after PKP. Staining for type III collagen. Bars = 100 μm.

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tween 1 and 3 months, but reproduction of the Descemet's membrane is still incomplete 3 months after surgery. Therefore, the structural strength of the cornea has probably not returned to normal even 3 months after PKP.

Based on the current study, type III collagen manifests itself intensely at first in the connective tissue produced to fill the gap between the host and graft, but is replaced by type I collagen in a short time. It has already been reported that the deposition of type III collagen increases near the incision of the cornea.[16] The content of type III collagen in the normal cornea is only about 10% of its dry weight.[17] However, type III collagen begins to increase at the site of the wound relatively early after injury and is gradually replaced by type I collagen in the process of wound healing. This phenomenon is observed not only in the cornea but also in the skin.[18] In a study in which fibroblasts were cultured in various three-dimensional collagen gels, type III collagen gel showed the highest contraction rate.[19,20] Therefore, type III collagen is presumed to play an important role in the contraction and closure of the wound in the wound healing process. Type III collagen was also observed in the cornea after excimer laser surgery and probably accounts for the subepithelial haze.[8,9] Thus, an increase in type III collagen is a prominent phenomenon common to various forms of wound healing.

The temporal expression of large proteoglycans in the wound brings up an interesting point. The monoclonal antibody used was produced to react specifically with large proteoglycans, eg, versican.[21–23] Versican is a large chondroitin sulfate proteoglycan isolated from the chondrogenic condensation area of developing chick limb buds. The structural features of versican suggest that this molecule may affect cell-substrate interactions and modulate a variety of cell behavior. Current evidence suggests that versican binds to fibronectin and healuronan.[24] LeBaron et al[25] have shown that versican specifically binds hyaluronan, and this binding is mediated by the N-terminal portion of the versican core protein. Previous studies in our laboratory have indicated that large proteoglycans were expressed during corneal wound healing after excimer laser photoablation.[26] Taken together with our present results, the expression of these large proteoglycans indicates that they probably play a role in corneal wound healing by binding hyaluronan to cell surfaces.

In this study, chondroitin 6-sulfate and large proteoglycans were manifested intensely in the newly formed connective tissue lying between the host and graft, whereas the staining of chondroitin 4-sulfate decreased. Both chondroitin 4-sulfate and chondroitin 6-sulfate are composed of repeated N-acetyl D-galactosamine and D-glucuronic acid, but the sulfuric group is located at C4 of the galactosamine molecule in the former and at C6 in the latter. It is interesting to note such a difference in the tissue distribution and function of two isomers, each differing only by the location of the sulfuric group. Hasty et al[27] reported that chondroitin 6-sulfate showed a significant increase during the wound healing process of rabbit ear cartilage. In addition, Oya et al[28] performed a quantitative analysis of glycosaminoglycans in the tear fluids of rabbits, and they reported chondroitin 6-sulfate in the tears increased during the healing of corneal epithelial wounds. By using HPLC, our previous analysis of glycosaminoglycans in the rabbit cornea demonstrated a significant increase of chondroitin 6-sulfate after excimer laser ablation.[29] In view of these findings, it is conceivable that the transient increase of chondroitin 6-sulfate is an ubiquitous phenomenon of the general healing process of wounds.

In summary, we used immunohistologic techniques to study the mechanisms of corneal wound healing in a penetrating keratoplasty model in rabbits. The immunoreactivity for type IV collagen indicated that the wound healing of the epithelial basement membrane required more than 1 month. On the other hand, stromal wound healing required 3 months, although the remodeling of Descemet's membrane was not completely finished until 3 months after PKP. Our findings of a transient increase of chondroitin 6-sulfate and large proteoglycans suggest that these components may play a crucial role in corneal wound healing.

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