

# Changes in Extracellular Matrix Components After Excimer Laser Photoablation in Rat Cornea

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Purpose: To learn more about corneal wound healing after excimer laser photoablation.

**Methods:** Observations were made on the chronological changes in type I collagen, fibronectin, laminin, and type IV collagen after excimer ablation of rat cornea. Immunohistochemical techniques were used.

**Results:** There was no obvious change in the localization of type I collagen in the ablated area, but the localization of fibronectin, laminin, and type IV collagen changed remarkably. One day after ablation, immunofluorescent staining for fibronectin increased on the ablated surface. Subsequently, the fluorescence of fibronectin, laminin, and type IV collagen increased remarkably; in particular, the localization of these extracellular matrix proteins was sustained in the shallow layer of the stroma until about 24 weeks after ablation. Hematoxy-lin-eosin staining indicated that keratocytes temporarily disappeared 1 day after ablation, and activated keratocytes then migrated to the ablated areas.

**Conclusions:** These results suggest that activated keratocytes might be synthesizing the extracellular matrix components. Therefore sustained responses of keratocytes may be induced by excimer laser photoablation. **Jpn J Ophthalmol 1999;43:348–354** © 1999 Japanese Ophthalmological Society

Key Words: Corneal wound healing, excimer laser, extracellular matrix, keratocytes.

## Introduction

Currently, a 193-nm excimer laser is used for photorefractive and phototherapeutic keratectomies. This laser application removes surface molecules by disrupting molecular bonds with little transfer of heat to the underlying tissue,<sup>1–3</sup> The technique allows more precise and controlled reshaping of the anterior corneal surface than mechanical ablation.<sup>4,5</sup> In 1985, Seiler et al<sup>6</sup> first reported the clinical use of an excimer laser for the correction of astigmatism. Since then, the excimer laser has been used for photorefractive and phototherapeutic keratectomies. However, important problems still remain, such as refractive regression and corneal haze after surgery.<sup>7-10</sup>

The cornea consists of various types of extracellular matrix proteins, and these proteins play important roles in cellular migration, differentiation, and proliferation.<sup>11,12</sup> The corneal stroma is composed mainly of type I collagen, and the corneal epithelial basement membrane is composed mainly of type IV collagen and laminin.<sup>12,13</sup> When the corneal epithelium is debrided or the corneal stroma is incised, fibronectin immediately appears on the surface of the bare stroma and serves as a temporary matrix for epithelial migration. When the corneal epithelial defect is resurfaced, fibronectin disappears from the site of injury.<sup>14-16</sup> By contrast, the basement membrane components, type IV collagen and laminin, have the opposite reaction at the site of corneal injury; base-

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ment membrane components disappear from the wounded area and then reappear in the basement membrane as wound healing advances.<sup>17</sup> Thus, individual extracellular matrix proteins play different roles in response to injury.

To understand corneal epithelial wound healing after excimer laser ablation, we used immunofluorescence histology to investigate the chronological changes in the localization of extracellular matrix proteins (eg, type I collagen, fibronectin, laminin, and type IV collagen), during corneal wound healing after excimer ablation of rat corneas.

## **Materials and Methods**

Twenty-five male Wistar Kyoto rats (Seiwa Animal Research, Fukuoka) weighing 300–400 g were used. Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (30 mg/kg). Excimer laser ablation was performed on both eyes of each rat using Nidek EC-5000 (Nidek, Aichi). The fluence was set at PTK mode, the optical zone was 3 mm, the pulse rate was 30 Hz, and the depth was equivalent to 60  $\mu$ m in humans without epithelial ablation. The use and treatment of rats in this study conformed to the ARVO Resolution on the Use of Animals in Research. Rats were sacrificed by an intraperitoneal injection of sodium pentobarbital on 1, 3, and 7 days, and 4, 8, 16, and 24 weeks after the excimer laser ablation.

The eyes were enucleated, and the right eyes were embedded immediately in OCT compound (Miles, Elkhart, IN, USA) and frozen in acetone dry ice. Sections 8 µm thick were cut with a cryostat microtome (HM 505N; Zeiss, Oberkochen, Germany) and the sections were mounted on a glass slide. The specimens were fixed with 1% periodate lysine paraformaldehyde (PLP) for 5 minutes and rinsed with phosphate-buffered saline (PBS). The specimens were then incubated with 1% casein in PBS for 1 hour at room temperature to block nonspecific binding and again washed with PBS. Sections were then incubated for 1 hour at room temperature in a moist chamber with one of the following primary antisera (LSL Tokyo): rabbit anti-human and bovine type I collagen diluted 1:1000; rabbit anti-mouse type IV collagen diluted 1:1000; rabbit anti-human fibronectin diluted 1:2000; or rabbit anti-mouse laminin diluted 1:2000 with 1% casein in PBS. For control staining, rabbit normal serum (Cappel, West Chester, PA, USA) diluted 1:500 with 1% casein in PBS was used in place of the corresponding primary antiserum.

The specimens were rinsed with PBS four times, 5 minutes per rinse, and then fluorescein-isothiocyanate-labeled goat anti-rabbit IgG (Cappel) diluted 1:1000 with 1% casein in PBS was applied as a secondary antibody. The specimens were incubated for 60 minutes at room temperature in a moist chamber. They were again rinsed with PBS four times for 5 minutes and were mounted in a 1:2 glycerin-PBS solution. Sections were observed with an epifluorescence microscope (Axioskop 50; Zeiss, St. Gallen, Switzerland). Photographs were taken with Fujichrome Provia 400 reversal film (ISO 400; Fuji Film, Tokyo). Only faint background fluorescence was observed in specimens that underwent control staining.

The left eyes were fixed with formalin and embedded in paraffin. Sections 4  $\mu$ m thick were cut from each eye with a sliding microtome (Histoslide 2000; Leica), and stained with hematoxylin-eosin. Photographs were taken with Fujichrome Provia 100 reversal film (ISO 100; Fuji Film).

#### Results

## Type I Collagen

In the normal cornea, immunoreactivity to type I collagen was observed uniformly in the corneal stroma (Figure 1a). At 1 and 3 days after ablation, the intensity of fluorescence to type I collagen increased on the ablated surface, but no changes were observed in the other stromal layers (Figures 1b, 1c). By 7 days after ablation, the intensity of fluorescence to type I collagen had increased slightly in the shallow layers of the stroma, but neither the middle nor the deep layer had changed (Figure 1d). At 4 and 8 weeks, strong band-shaped immunoreactivity to type I collagen was observed only in the shallow layers (Figures 1e, 1f). Twenty-four weeks after ablation, the localization pattern of type I collagen was similar to that observed in the normal cornea, but strong fluorescence to type I collagen was still observed in the shallow layers (Figure 1g).

#### Fibronectin

In the normal cornea, immunoreactivity to fibronectin was observed along collagen fibrils in the corneal stroma and in Descement's membrane (Figure 2a). One day after ablation, strong immunoreactivity to fibronectin was observed on the ablated surface (Figure 2b). At 3 days, the corneal epithelium was completely resurfaced, but strong immunoreactivity to fibronectin was observed on the ablated surface (Figure 2c). At 7 days and 4 weeks, strong immunofluorescence to fibronectin was observed only



**Figure 1.** Chronological changes in the localization of type I collagen after excimer ablation. In normal cornea, immunoreactivity to type I collagen is uniformly observed in corneal stroma. After ablation, intensity of fluorescence to type I collagen was increased slightly in the shallow layer of the stroma, but no obvious changes were observed elsewhere. (a) Normal cornea; (b) 1 day, (c) 3 days, (d) 7 days, (e) 4 weeks, (f) 8 weeks, and (g) 24 weeks after ablation. Bar =  $200 \mu m$ .

in the shallow layers of the stroma (Figures 2d, 2e). At 8 and 24 weeks, strong immunoreactivity to fibronectin was still observed in the shallow layers, but the localization pattern of fibronectin in the other layers of the stroma was similar to that in the normal cornea (Figures 2f, 2g).

## Laminin

In the normal cornea, immunoreactivity to laminin was localized in the corneal epithelial basement membrane and Descement's membrane. It was also observed as thin lines within the stroma (Figure 3a). One day after ablation, immunofluorescence to laminin had disappeared from the corneal epithelial basement membrane, and no changes were observed



**Figure 2.** Chronological changes in the localization of fibronectin after ablation. In normal cornea, immunoreactivity to fibronectin is observed in the corneal stroma and in Descement's membrane. After ablation, strong immunoreactivity to fibronectin is observed on the ablated surface. Subsequently, strong immunofluorescence to fibronectin is observed in the stroma. (a) Normal cornea, (b) 1 day, (c) 3 days, (d) 7 days, (e) 4 weeks, (f) 8 weeks, and (g) 24 weeks after ablation. Bar =  $200 \,\mu$ m.

in the corneal stroma (Figure 3b). At 3 days, fragmented fluorescence-positivity to laminin was observed at the interface between the resurfaced corneal epithelium and the stroma, and the specks and the lines of fluorescence in response to laminin on the ablated stroma had increased slightly (Figures 3c, 3d). At 7 days, immunoreactivity to laminin was seen as well-defined and continuous lines in the reconstructed epithelial basement membrane. In addition, a strong band-shaped immunofluorescence was observed in the shallow layers of the stroma (Figures 3e, 3f). At 4, 8, 16, and 24 weeks, we also observed a thick continuous immunofluorescence in the reconstructed epithelial basement membrane and a bandshaped fluorescence in the shallow layer of the stroma (Figures 3g–3j).

# Type IV Collagen

In the normal cornea, type IV collagen was localized in the corneal epithelial basement membrane and Descement's membrane. Specks of fluorescence in response to type IV collagen were also observed in the corneal stroma (Figure 4a). One day after ablation, the immunoreactivity to type IV collagen in the epithelial basement membrane had disappeared, but no changes were observed in the corneal stroma (Figure 4b). At 3 days, weak discontinuous fluorescence to type IV collagen was observed in the periphery of the interface between the resurfaced corneal epithelium and the stroma. The specks of fluorescence to type IV collagen on the ablated stroma had increased slightly (Figures 4c, 4d). At 7 days and 4 weeks, immunoreactivity to type IV collagen was visualized as welldefined and continuous lines in the reconstructed epithelial basement membrane. In addition, a strong, layered immunopositivity in response to type IV collagen was observed in the shallow layers, and the specks of fluorescence in the middle and deep layers of the stroma had increased slightly (Figures 4e, 4f). At 8, 16, and 24 weeks, a thick continuous fluorescence in response to laminin remained in the reconstructed epithelial basement membrane and a layered fluorescence was present in the shallow layers of the stroma in response to type IV collagen (Figures 4g–i).



Figure 3. Chronological changes in the localization of laminin after ablation. In normal cornea, immunoreactivity to laminin is localized in the epithelial basement membrane and in Descement's membrane. After ablation, fluorescence to laminin in epithelial basement membrane is not present but fragmented fluorescence to laminin is observed at the reconstructed basement membrane. Subsequently, strong band-shaped immunofluorescence to laminin is observed in shallow layers of the stroma. (a) Normal cornea; (b) 1 day; (c) 3 days peripheral region (ablated region to right of arrow); (d) 3 days, central region; (e) 7 days, peripheral region; (f) 7 days, central region; (g) 4 weeks; (h) 8 weeks; (i) 16 weeks; and (j) 24 weeks after ablation. Bar =  $200 \,\mu$ m.



**Figure 4.** Chronological changes in localization of type IV collagen after ablation in the epithelial basement membrane and in Descement's membrane. After ablation, immunoreactivity to type IV collagen in epithelial basement membrane disappeared, and then weak discontinuous fluorescence to type IV collagen was observed in the reconstructed basement membrane. Subsequently, strong, layered fluorescence to type IV collagen was observed in shallow layer. (**a**) Normal cornea; (**b**) 1 day; (**c**) 3 days, peripheral region (ablated region to right of arrow); (**d**) 3 days, central region; (**e**) 7 days; (**f**) 4 weeks; (**g**) 8 weeks; (**h**) 16 weeks; and (**i**) 24 weeks after ablation. Bar = 200  $\mu$ m.

#### Hematoxylin-Eosin Staining

One day after ablation, the presence of keratocytes in the shallow layers of the corneal stroma had decreased (Figure 5b) in comparison with their presence in the normal cornea (Figure 5a). At 3 days, the corneal epithelial defect was completely repaired, and globular-shaped cellular components, which are thought to be activated keratocytes, had migrated into the ablated stroma (Figure 5c). At 7 days, a large number of keratocytes had migrated into the ablated stroma, and these cells were focused in the shallow layers of the stroma (Figure 5d). Four weeks after ablation, the number of keratocytes had decreased, and their shape had returned to that of normal spindle-shaped cells along collagen fibrils. However, keratocytes in the shallow layer were still globular (Figure 5e). At 8, 16, and 24 weeks, the number of keratocytes had gradually decreased to the number found in the normal cornea, although globular keratocytes were still observed in the subepithelial region (Figures 5f-5h).

## Discussion

In the present study, we used immunofluorescence techniques to investigate the chronological changes in the localization of extracellular matrix proteins, viz., type I collagen, fibronectin, laminin, and type IV collagen, after excimer laser ablation. Although no significant changes in the localization of type I collagen were observed during the experimental period, localization of fibronectin, laminin, and type IV collagen changed dramatically in the ablated area, particularly in the shallow layers of the corneal stroma. The changes in the localization of these extracellular matrix proteins were very similar to the changes in the population of keratocytes after excimer laser ablation. Therefore, these results suggest that the keratocytes that had migrated into the ablated area might be actively synthesizing and secreting fibronectin, laminin, and type IV collagen.

Type I collagen is a fibrillar protein and is a primary component of the corneal stroma.<sup>13</sup> In the present study, the intensity of fluorescence to type I collagen increased slightly in the shallow layers of the corneal stroma from 7 days to 24 weeks after ablation. This increased fluorescence might result from collagen newly synthesized by the activated keratocytes. Anderson et al<sup>18</sup> reported that type I procollagen, a precursor of type I collagen, and prolyl 4-hydroxylase, a collagen-synthesizing enzyme, stained subepithelial keratocytes in the ablated regions 3 weeks after ablation. Furthermore, the



Figure 5. Chronological changes in hematoxylin-eosin staining after ablation. After ablation, the number of keratocytes in the shallow layers of the corneal stroma decreased, and globular-shaped cellular components, which are thought to be activated keratocytes, have migrated into this area. Subsequently, their cell shape returned to normal. However, keratocytes in the shallow layer retained the globular shape. (a) Normal cornea, (b) 1 day, (c) 3 days, (d) 7 days, (e) 4 weeks, (f) 8 weeks, (g) 16 weeks, and (h) 24 weeks after ablation. Bar =  $200 \,\mu m$ .

immunostaining for type I collagen in the middle and deep layers of the stroma did not change after ablation suggesting that these areas might not be affected by laser ablation.

Fibronectin is an adhesive protein that serves as a temporary matrix for the attachment and migration of epithelial cells in wound healing.<sup>19</sup> One day after ablation, fibronectin appeared on the bare surface of the stroma. This supports the concept that fibronectin serves as a temporary matrix after both corneal epithelial debridement and stromal incision.<sup>14–16</sup> At 3 and 7 days, the intensity of fluorescence in reaction to fibronectin had increased in all layers of the stroma. These results are in good agreement with those reported by Hanna et al,<sup>20</sup> and support their suggestion that fibronectin might be synthesized and

secreted by activated keratocytes in the stroma. Furthermore, deposition of fibronectin in the shallow layers of the stroma was observed 24 weeks after ablation, suggesting that wound healing might be sustained over a long period. Indeed, the deposition of fibronectin in the shallow layers of the stroma has been observed 12 months to 18 months after ablation.<sup>18,21</sup>

Laminin and type IV collagen are glycoproteins and are major components of the basement membrane.12,22,23 After ablation, fragments of laminin and then type IV collagen reappeared at the interface of the resurfaced corneal epithelium and the stroma. Subsequently, both basement membrane components were observed as a continuous line as reconstruction of the basement membrane progressed. However, the fluorescence in response to these components was strong and thick from 4 to 24 weeks after ablation, suggesting that complete repair of the basement membrane might be a lengthy process. The same results were obtained when changes in the localization of type VII collagen, which include the anchoring fibrils of the basement membrane, were observed after excimer laser ablation in monkeys and in humans.<sup>18,24</sup> In addition, at 3 days, specks of fluorescence in response to type IV collagen and band-shaped fluorescence in response to laminin were observed in the shallow layers of the stroma. In these areas, the immunofluorescent components might have been synthesized and secreted by keratocytes that had migrated because the fluorescence was similar to that in the keratocyte populations observed after staining with hematoxylin-eosin. Hanna et al<sup>20</sup> reported that type IV collagen, laminin, fibronectin, and proteoglycans were deposited in the shallow layers of the stroma 6 days after ablation.

Using immunohistochemical techniques, many studies have investigated changes in the localization of various types of extracellular matrix proteins after excimer laser ablation.<sup>18,20,21,24,25</sup> As far as we know, however, this is the first report to investigate the chronological changes in the localization of various types of extracellular matrix proteins over a 24-week period after excimer laser ablation. In the present study, we demonstrated similar chronological changes among extracellular matrix proteins. These results suggest that various extracellular matrix proteins might act on the cooperative and/or interactive mechanisms of corneal wound healing. Furthermore, excimer laser ablation unexpectedly influenced the keratocytes over wide areas and induced sustained wound-healing reactions, such as the excess production of extracellular matrix proteins by the activated keratocytes. The excess reactions by keratocytes might cause the corneal haze and refractive regression that occurs after excimer laser ablation. Therefore, careful clinical use of excimer laser ablation is needed to understand the chronological changes, redistribution of extracellular matrix proteins, and sustained wound-healing responses by keratocytes.

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