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LABORATORY INVESTIGATIONS

HSP47 Expression in Cornea After Excimer Laser Photoablation

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Purpose: The expression of heat shock protein 47 (HSP47) was observed histologically to investigate the spatial and chronological effects of excimer laser photoablation. HSP47 expression after radial keratotomy (RK) was also investigated and compared with the effects after excimer laser photoablation.

Method: Twenty-eight male rabbits were used. The rabbits were divided with two groups and treated with either excimer laser photoablation or four radial incisions to simulate corneal refractive surgery. The chronological and spatial changes in the expression of HSP47 were observed immunohistochemically.

Results: In eyes that underwent excimer laser photoablation, HSP47 was detected in the basal layer of the epithelial cells and in the superficial stromal layer 3 days after surgery. After 5 and 7 days, HSP47 expression extended to the deep layer of the stroma and to the endothelial cells. After 14 days, HSP47 was detected only in the deep layer of the stroma and in the endothelial cells. After 28 days, HSP47 expression was reduced. In eyes that underwent RK, HSP47 was detected in the basal layer of the epithelial cells and in the stroma surrounding the wound 1 day after surgery. After 3 and 7 days, HSP47 expression did not expand further. After 28 days, HSP47 expression diminished.

Conclusions: Excimer laser photoablation affects the whole layer of the cornea, and may be caused by the shock wave that occurs as a result of photoablation. In addition, interaction among the keratocytes may propagate the stress-induced response to the whole layer of the cornea. With RK, the wound is smaller and deeper. HSP47 expression occurs earlier, but is limited to the area surrounding the wound. **Jpn J Ophthalmol 2002;46:123–129** © 2002 Japanese Ophthalmological Society

Key Words: Cornea, excimer laser, heat shock protein 47, phototherapeutic keratectomy, radial keratotomy.

Introduction

Excimer laser photoablation is useful treatment for myopia and is widely used to correct refractive error; however, the treatment can negatively affect corneal tissue. For example, corneal haze is a complication of corneal refractive surgery, and results in the loss of corneal transparency, which can significantly affect visual function by causing loss of visual acuity, decreased contrast sensitivity, astigmatism, and glare.[1–3] Corneal transparency depends on collagen fibrils forming a regular lattice with even cross-sectional diameter and spacing. When the normal collagen fibril arrangement is altered, corneal haze results. Numerous studies have correlated clinical haze after excimer laser photoablation with increased cellular infiltration, vacuole formation, increased ground substance, and collagen type III deposition in the area of the haze.[4–12] Therefore, haze prevention may be achieved by modifying the corneal wound-healing response to decrease hypercellularity and the possible deposition of abnormal extracellular matrix after excimer laser photoablation. One additional adverse effect of excimer laser photoablation is the possibility of damage to

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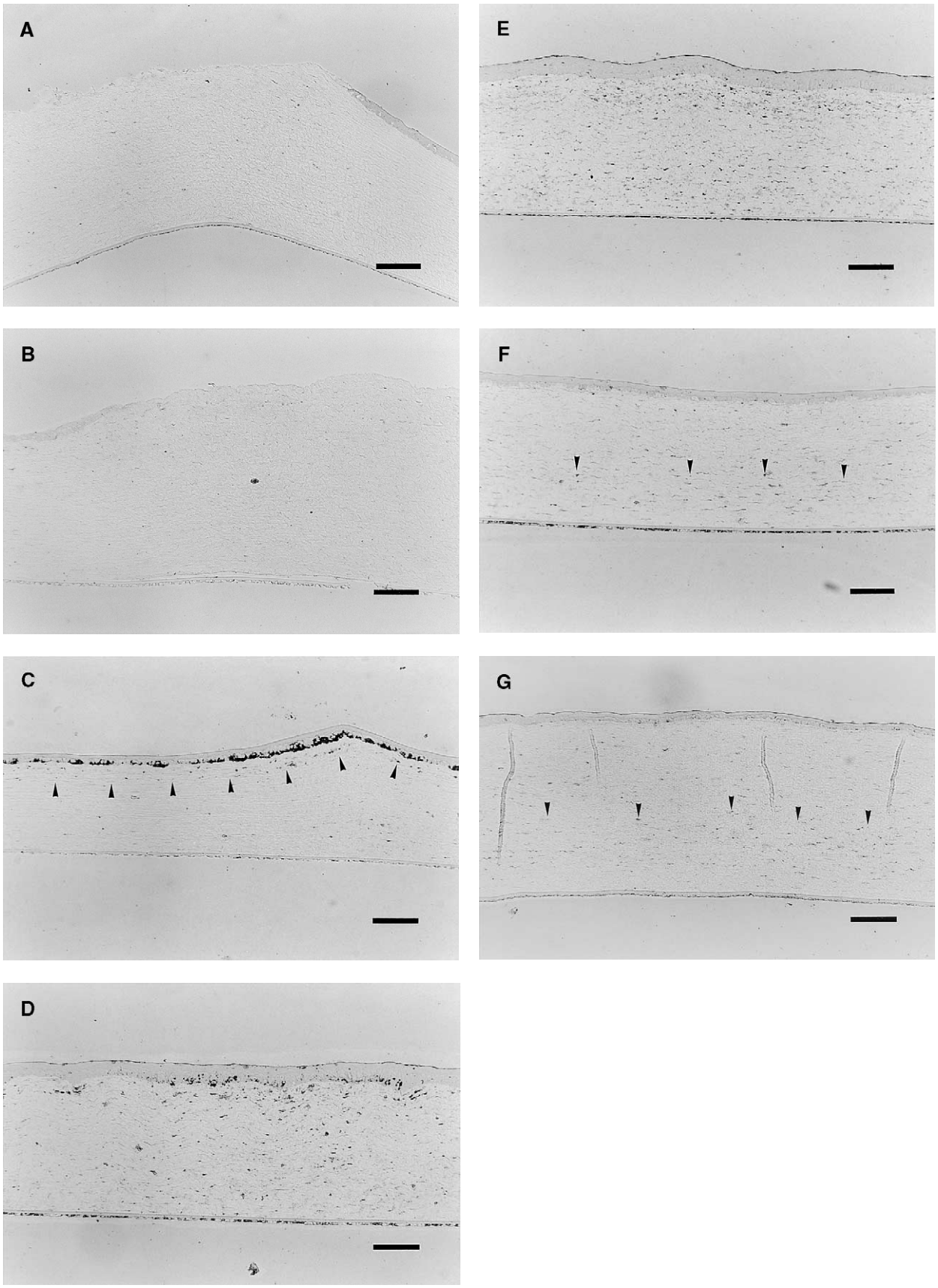

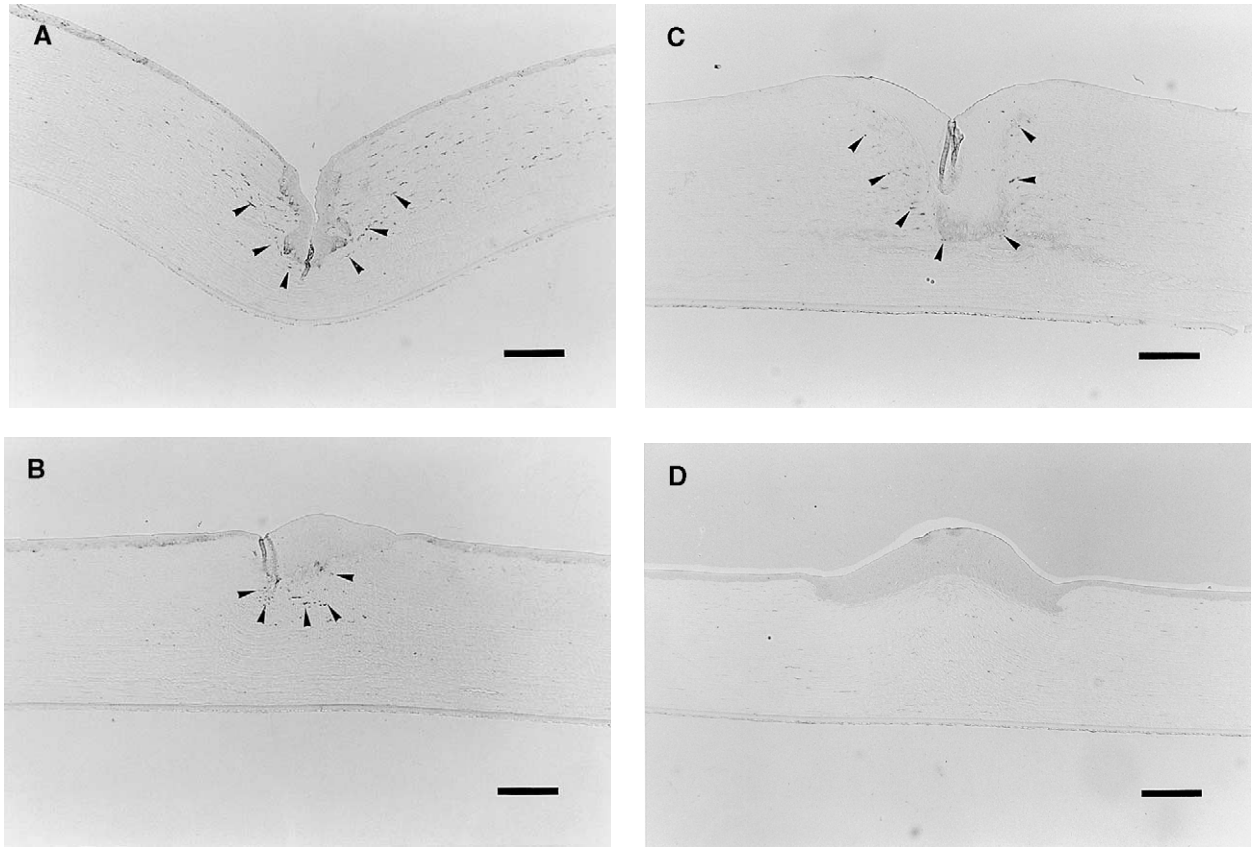

Figure 2. Immunohistochemical localization of heat shock protein 47 (HSP47) in the radial keratotomy (RK) group. Bars = 100 μm. (**A**) One day after RK. The epithelial cells have extended to the wound. HSP47 is detected in the basal layer of the epithelial cells and in the stroma surrounding the wound (arrowheads). (**B**) Three days after RK. The wound is filled by epithelial cells, and an epithelial plug is formed. HSP47 is detected only in the basal layer of the epithelial cells and in the stroma surrounding the wound (arrowheads). (**C**) Seven days after RK. HSP47 is detected only in the basal layer of the epithelial cells and in the stroma surrounding the wound (arrowheads). (**D**) Twenty-eight days after RK. HSP47 expression is reduced, especially at the basal layer of the epithelial cells.

the endothelial cells. To prevent adverse effects, it is necessary to investigate in detail the process of excimer laser photoablation.

Heat shock protein 47 (HSP47) is a stress protein, which resides in the endoplasmic reticulum, and is postulated to function as a collagen-specific molecular chaperone. HSP47 is induced in response to stress applied to cells.[13,14]

To investigate the spatial and chronological effects of excimer laser photoablation, the expression of HSP47 after photoablation of the rabbit cornea was observed histologically. At the same time, the ex-

Figure 1. Immunohistochemical localization of heat shock protein 47 (HSP47) in the phototherapeutic keratectomy (PTK) group of rabbit eyes. Bars = 100 μm. (**A**) Day of PTK. The stroma of the cornea is exposed, and the ablated area is not covered by epithelial cells. HSP47 expression is not detected. (**B**) One day after PTK. The ablated area is not covered by the epithelial cells, and the stroma of the cornea is exposed. HSP47 expression is not detected. (**C**) Three days after PTK. Ablated area is covered by the epithelial cells. HSP47 is detected in the basal layer of the epithelial cells and the superficial stromal layer (arrowheads). (**D**) Five days after PTK. The thickness of the epithelial cell layer in the ablated area is increased. HSP47 expression extends from the basal layer of the epithelium to the endothelial-side layer (deep layer) of the stroma and to the corneal endothelial cells. (**E**) Seven days after PTK. HSP47 expression extends from the basal layer of the epithelium to the endothelial-side layer (deep layer) of the stroma and to the corneal endothelial cells, as in (**D**). (**F**) Fourteen days after PTK. The thickness of the epithelial cell layer is decreased. HSP47 is detected only in the deep layer of the stroma and the corneal endothelial cells (arrowheads). (**G**) Twenty-eight days after PTK. HSP47 expression is reduced significantly (arrowheads).

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pression of HSP47 after radial keratotomy (RK) was investigated and compared with that after excimer laser photoablation.

Materials and Methods

Treatment of Animals

Twenty-eight male rabbits weighing 2.0 to 2.5 kg, were used. The use of the rabbits conformed to the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research.

Methods

The rabbits were anesthetized systemically with a venous injection of pentobarbital sodium, and divided into two groups. In the phototherapeutic keratectomy (PTK) group, the rabbit corneas were ablated (6.0 mm in diameter, 100 μm in depth and 30 Hz in frequency) using a PTK mode excimer laser (EC-5000®; NIDEK, Gamagori) in both eyes of each rabbit. At that time, the epithelium of the cornea was not removed. In the radial keratotomy (RK) group, a central clear zone, 3.0 mm in diameter, was marked on the corneal epithelium with a 3-mm optical zone marker. A diamond knife was set to 300 μm. Four radial incisions were made in both eyes of each rabbit; each extended from the border of the central clear zone and terminated 1 mm inside the limbus. Two rabbits from each group were sacrificed 0, 1, 3, 5, 7, 14, or 28 days after surgery by an overdose venous injection of pentobarbital sodium. Thereafter, the rabbit eyeballs were enucleated. In each group, 3 of the 4 eyeballs were used; their corneas were fixed 4 hours in 4% neutral buffered formalin, embedded in paraffin, and sectioned at 6 μm thickness. The remaining eyes (1 eye in each group) were used for electromicroscopic study (not shown).

Immunohistochemistry

Expression of HSP47 was detected using the primary antibody to HSP47, mouse anti-HSP47 monoclonal antibody, M16.10A1 (StressGen Biotechnologies, Victoria, BC, Canada), and a detection kit (DAKO LSAB™ Kit; DAKO, Carpenteria, CA, USA). The antibody was diluted 1:100 with phosphate-buffered saline (PBS; pH 7.4, 0.05 mol/L) containing 1% bovine serum. The sections were treated with 3% hydrogen peroxide in PBS for 20 minutes to block endogenous peroxidase activity, then rinsed in 0.02 mol/L Tris HCl buffer. This was followed by incubation with normal goat serum (DAKO) for 60 minutes at room temperature to avoid nonspecific binding of the antibodies. The sections were then incubated with the primary antibody overnight at 4°C. They were then incubated with biotinylated goat an-

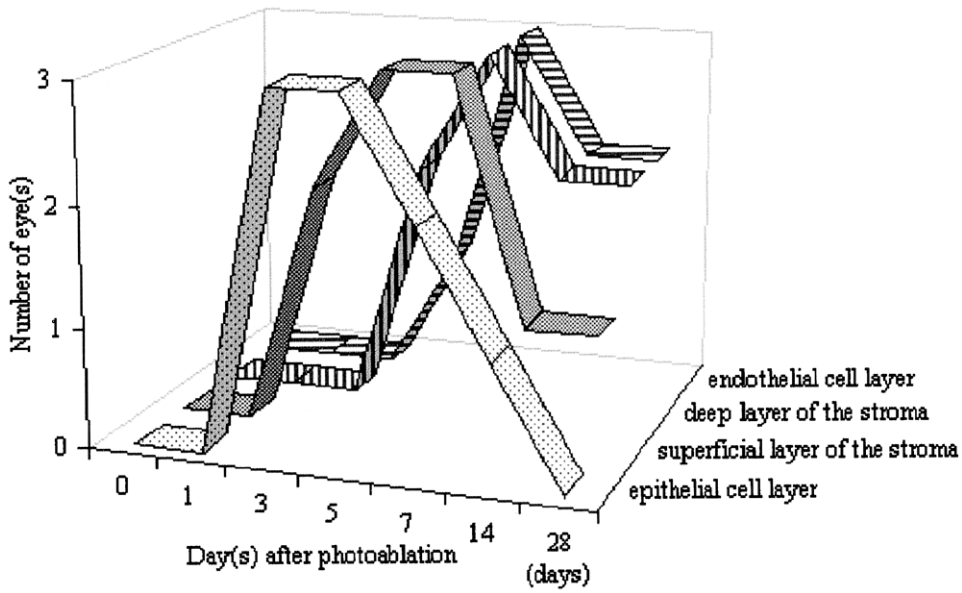

Figure 3. Time sequence of heat shock protein (HSP47) expression in corneal layers. HSP47 expression is detected first in the epithelial cells and the superficial corneal stroma and, thereafter, in the deep layer of the stroma and the endothelial cells. HSP47 expression decreased in the same sequence, from the epithelial cells and the superficial stroma, to the deep stroma and endothelial cell layer.

tibody to mouse immunoglobulin for 2 hours at room temperature and then rinsed in 0.02 mol/L Tris HCl buffer. Subsequently, the sections were incubated with peroxidase-labeled streptavidin (DAKO) for 2 hours at room temperature. Localization of peroxidase-labeled streptavidin was visualized with 3% 3-amino-9-ethylcarbazole in N,N-dimethylformamide (DAKO). As a negative control, samples were treated with nonimmune serum in place of the primary antibody. Spatial change in the expression of HSP47 was observed chronologically using an optical microscope.

Results

Effects of PTK

In the PTK group, on the day of the ablation, the stroma of the cornea was exposed, and the ablated area was not covered by epithelial cells. HSP47 expression was not detected (Figure 1A). One day after the ablation, the ablated area was not covered with epithelial cells, and the stroma of the cornea was exposed. HSP47 expression was not detected (Figure 1B). After 3 days, the ablated area was covered with epithelial cells. HSP47 was detected in the basal layer of the epithelial cells and in the superficial stromal layer. HSP47 expression was detected in the ablated area and the surrounding area (Figure 1C). After 5 (Figure 1D) and 7 (Figure 1E) days, the thickness of the epithelial cell layer in the ablated area had increased. HSP47 expression extended from the basal layer of the epithelium to the endothelial-side layer (deep layer) of the stroma and the corneal endothelial cells. After 14 days, the thickness of the epithelial cell layer had decreased. HSP47 was detected only in the deep layer of the stroma and the corneal endothelial cells (Figure 1F). After 28 days, HSP47 expression was reduced significantly (Figure 1G). In summary, the expression of HSP47 was shown first in the epithelial cells and the superficial corneal stroma, and thereafter, in the deep layer of the stroma and the endothelial cells. In addition, HSP47 expression extended to the nonablated area (Figures 3, 4A). In the same order as the appearance of HSP47, the expression of HSP47 decreased from the epithelial cells and the superficial stroma (Figures 3, 4B).

Effects of RK

In the RK group, 1 day after RK (Figure 2A), epithelial cells extended to the wound. HSP47 was detected in the basal layer of the epithelial cells and in the stroma surrounding the wound. Three (Figure 2B) and 7 (Figure 2C) days after RK, the wound was filled with epithelial cells and an epithelial plug was formed. HSP47 was detected only in the basal layer of the epithelial cells and in the stroma surrounding the wound. Twenty-eight days after RK, HSP47 expression was reduced, especially in the basal layer of the epithelial cells (Figure 2D). In summary, the expression of HSP47 appeared earlier in the RK group than in the PTK group, but only in the area surrounding the wound during our observations (Figure 5).

Discussion

All animal species are known to respond to adverse changes in their environment, such as chemical toxins, heat stress or virus infection, by increasing the levels of group of proteins referred to as heat shock or stress proteins.[15–18] It has been suggested that these proteins provide protection against the stress-induced formation of abnormal protein-protein interactions, and that they may facilitate correct folding of those proteins.[19–22] On a functional level, these effects may facilitate the restoration of metabolic pathways that have been disturbed by the stress event. So-called constitutive forms of most of these proteins are also present within unstressed cells and appear to be involved in a number of normal metabolic pathways essential to the basic functions of the cell. In particular, HSP47 is a stress protein, which resides in the endoplasmic reticulum, and is postulated to function as a collagen-specific molecular chaperone.[13,14]

In the present study, the expression of HSP47 was induced in the PTK group by excimer laser photoab-

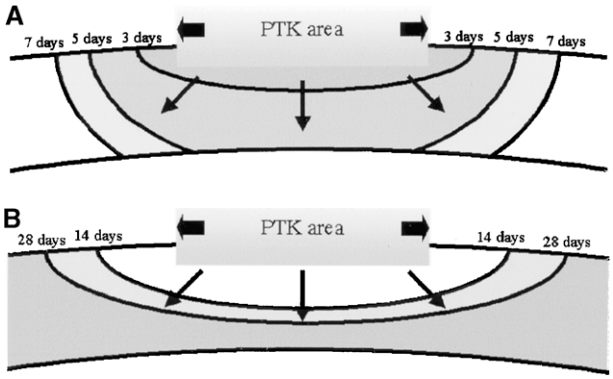

Figure 4. Spatial change of heat shock protein 47 (HSP47) expression in the phototherapeutic keratectomy group. (**A**) HSP47 expression is observed first in the epithelial cells and in the superficial corneal stroma and, thereafter, in the deep layer of the stroma and the endothelial cells. In addition, HSP47 expression extends to the nonablated area. (**B**) HSP47 expression decreases from the epithelial cells and the superficial stroma.

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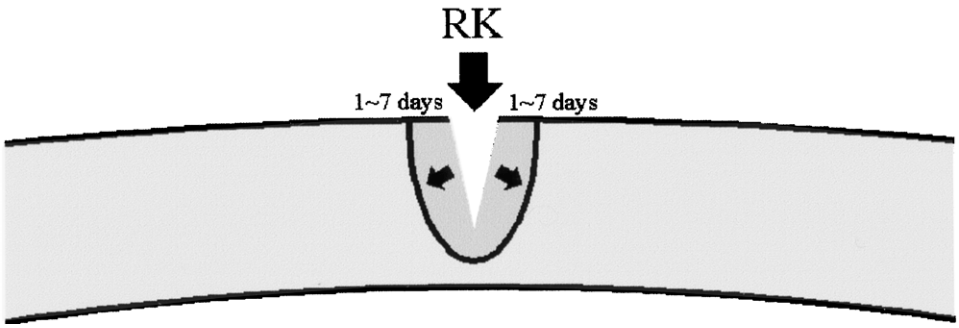

Figure 5. Spatial change of heat shock protein 47 (HSP47) expression in the radial keratotomy group. HSP47 expression is detected earlier than in the phototherapeutic keratectomy group, but observed only in the area surrounding the wound.

lation first in the epithelial cells and the superficial corneal stroma and thereafter, in the deep layer of the stroma and the endothelial cells. In addition, HSP47 expression extended to the nonablated area (Figures 3, 4A). In the same order as the appearance of HSP47, the expression of HSP47 decreased first from the epithelial cells and the superficial stroma (Figures 3, 4B).

In previous studies, histological change after excimer laser photoablation was reported only in the epithelial cells and superficial corneal stroma.[8–11] However, in this study, HSP47 was expressed in the whole layer of the cornea, which suggests that excimer laser photoablation affects the whole corneal layer. In other words, excimer laser photoablation affects even the deep layer of the stroma and endothelial cell layer, in which histological change was not shown. HSP47 functions as a collagen-specific molecular chaperone, but a previous study reported that collagen synthesis after photoablation was shown only in the superficial layer of the cornea.[23] Thus there is the possibility that HSP47 expression in the deep layer of the stroma and the endothelial cells is a stress-induced response, and not a function of the molecular chaperone. Thermal rising[24–27] or the photochemical reaction[27] to an excimer laser may induce the expression of HSP47. These effects were considered to be limited to the superficial layer of the cornea, but there is the possibility that the induction of HSP47 expression was caused by the shock wave that occurred from the photoablation.[28–31] The interaction among the keratocytes may spread the stress-induced response to the whole layer of the cornea. Photoablation was performed on a large area of the corneal stroma, resulting in a large acellular area; thus migration of keratocytes from the surrounding area was delayed and the expression of HSP47 appeared in the large affected area of the PTK group and continued longer than in the RK group. Because of this, the photoablation may have induced a stress response even in the endothelial cells.

In the RK group, the expression of HSP47 appeared earlier than in the PTK group, and occurred only in the area surrounding the wound (Figure 5). With RK, the wound is deep and narrow and V-shaped, and inflammatory cells can infiltrate the corneal stroma easily. Because of this, the inflammation after surgery is stronger and HSP47 appears earlier than with photoablation. In addition, the wound area is smaller than with photoablation and the sort of shock wave that occurs with PTK did not result. Therefore the expression of HSP47 occurred only in the area surrounding the wound and not in the deep layer of the stroma and the endothelial cells.

It is possible that HSP47 functions in the remodeling of collagen fibers in wound healing after refractive surgery. Therefore, in the clinical application of excimer laser photoablation, it is necessary to investigate the side effects not only in the corneal stroma, but also in the corneal endothelial cells.

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