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Immunohistochemical Study of Localization of Extracellular Matrix after Holmium YAG Laser Irradiation in Rat Cornea

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Purpose: To better understand the corneal responses to holmium YAG (Ho:YAG) laser irradiation, we used immunofluorescent microscopy to examine changes in the localization of the extracellular matrix components, which play important roles in the maintenance of corneal morphology and functions.

Methods: Rats were irradiated with a Ho:YAG laser. On days 1, 3, and 7 after irradiation, the eyes were enucleated and frozen. Cryosections were made with a cryostat and were stained with antibodies against type I collagen, fibronectin, type IV collagen, or laminin for immunohistochemical study.

Results: One day after Ho:YAG laser irradiation, contraction of the stromal collagen fibrils was observed. Keratocytes could not be observed at the irradiated stromal region on day 1 after irradiation. One week later, however, keratocytes returned to the irradiated area. Although the stromal collagen fibrils had contracted, they were stained by an antibody against type I collagen. Dense fluorescence for fibronectin was observed at the margin of the stromal acellular zone. Both laminin and type IV collagen were observed at the basement membrane under the corneal epithelium, regardless of whether or not the corneas had been irradiated.

Conclusion: These results suggest that Ho:YAG laser irradiation might be useful for the collagen contraction of stroma, without causing serious damage to the corneal epithelium and the basement membrane. **Jpn J Ophthalmol 2000;44:482–488** © 2000 Japanese Ophthalmological Society

Key Words: Cornea, extracellular matrix, holmium YAG laser, wound healing.

Introduction

Recently, refractive surgery has been used in an attempt to correct refractive errors in the transparent cornea. Radial keratotomy (RK), in which corneal tension is changed by incision, and photorefractive keratectomy (PRK), in which the corneal shape is trimmed by an excimer laser, are widely performed. The numerous reports of the clinical outcomes of these types of surgery indicate that they are efficacious but have limitations.[1–3]

For many years, thermokeratoplasty—using thermal coagulation to shrink stromal collagen—has been used to try to change the shape of the cornea.[4,5] Initially, this surgery, employing a thermal probe, was used to treat keratoconus.[6,7] Later it was used for hyperopic correction.[8] Thermokeratoplasty did not become popular because of the complications resulting from the surgery, such as refractive regression, corneal astigmatism, and delayed epithelial wound healing.[9,10] Since the late 1980s, keratoplasty has been performed using lasers instead of a thermal probe to correct hyperopia. Recently, thermal energy from holmium YAG (Ho:YAG) laser has been used to correct hyperopia, and clinical studies of this surgical method have been performed in the United States.[11–13]

In refractive surgery, it is extremely important to be able to predict postsurgical correction. Postsurgical correction is thought to depend on the biological

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response of the cornea to surgical invasion and the corneal wound-healing response after refractive surgery. Therefore, to obtain a good surgical outcome, it is essential to understand the biological response of the cornea to each individual surgical technique. Of particular importance are the extracellular matrix components in the cornea, which not only support the connective tissue, but also regulate cellular functions such as cellular movement, proliferation, and differentiation. Extracellular matrix components play important roles in the maintenance of normal corneal structure and of the biological responses to surgical invasion during the corneal wound healing process.[14,15] In the present study, to better understand corneal wound healing responses to Ho:YAG laser irradiation, we used an immunofluorescence technique to investigate the histological changes and localization of extracellular matrix components in irradiated rat corneas.

Materials and Methods

Ho:YAG Laser Irradiation in Rat Cornea

Thirteen male Wistar Kyoto rats (Seiwa Animal Research, Fukuoka), weighing 300–400 g, were used. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg, Nembutal®, Dainippon Pharmaceutical, Osaka). In 10 rats, both eyes were irradiated at 6–9 spots/cornea using Ho:YAG laser (Sunrise Technologies, Fremont, CA, USA). The irradiation conditions were 2.1 μm wavelength, 250 milliseconds, 5 Hz PRF, 10 pulse/spot, 9 J/cm^2. The 3 rats used as controls were not treated. After Ho:YAG laser irradiation, no medical treatment, such as antibiotic eyedrops were given, but no serious corneal disorders, such as infection and ulceration, were observed.

Care and treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histological and Immunohistochemical Examinations

Rats were euthanized by intraperitoneal injection of sodium pentobarbital at 1 day (3 rats), 3 days (4 rats), or 1 week (3 rats) after Ho:YAG laser irradiation. The eyes were enucleated, and the right eyes were immediately embedded in OCT compound (Miles, Elkhart, IN, USA) and frozen in acetone dry ice. Sections 6–8 μm thick were cut from each eye with a microtome cryostat (HM 505N; Zeiss, Oberkochen, Germany); each section was mounted on a glass slide. The specimens were then incubated with 1% bovine serum albumin fraction-V (BSA; Nacalai tesque, Kyoto) in phosphate-buffered saline (PBS) for 1 hour at room temperature to block nonspecific binding. 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 goat type I collagen diluted 1:300; rabbit anti-bovine type IV collagen diluted 1:300; rabbit anti-human fibronectin diluted 1:2,000; or rabbit anti-mouse laminin diluted 1:1,000 with 1% BSA in PBS. For control staining, rabbit normal serum (Organon Teknika, Aurora, OH, USA) at 1:300 with 1% BSA 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 (Organon) diluted 1:500 with 1% BSA in PBS was applied as a secondary antibody; the specimens were incubated for 1 hour at room temperature in a moist chamber. They were again rinsed with PBS four times for 5 minutes each rinse, and were mounted in a 1:2 glycerin:PBS solution. Sections were observed with an epifluorescence microscope (Axioskop 50; Zeiss), and photographed 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 cetylpyridinium chloride-formalin and embedded in paraffin. Sections 4-μm thick were cut from each eye with a sliding microtome (Histoslide 2000; Leica, St. Gallen, Switzerland) and stained with hematoxilin-eosin. Sections were observed with a light microscope (Axioskop 50), and photographed with Fujichrome Provia 100 reversal film (ISO 100; Fuji Film).

Results

Histological Examination (Hematoxilin-Eosin Staining)

One day after Ho:YAG laser irradiation, some differences were observed in the corneal epithelia of the 3 eyes enucleated. After the irradiation, edema and bulla formations in the intercellular spaces of the epithelium and the hyperplastic epithelium were observed in the irradiated area of one of the eyes. However, in the other 2 eyes, no abnormalities were observed in the irradiated area of the cornea, and the structure was similar to that of a normal rat cornea (Figure 1A). In the corneal stroma, the space between the collagen lamella had increased, and the regular continuous layer structure of the collagen fibrils had disappeared in all 3 eyes. These observations suggest that thermal coagulation had contracted the corneal stroma in all layers. In addition,

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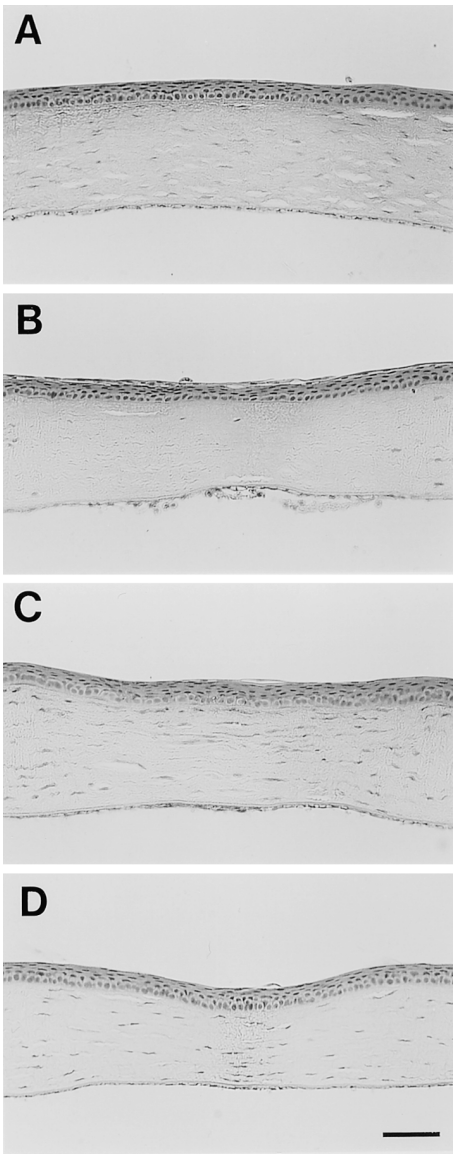

Figure 1. Histological changes in hematoxylin-eosin stained specimens of rat cornea after holmium:YAG irradiation. Normal cornea (**A**). At 1 day after irradiation (**B**), no corneal disorders were observed at center of irradiated area. Stromal collagen had contracted in all layers. Keratocytes had disappeared from irradiated area, and acellular zone had formed. Three days after irradiation (**C**), contraction of stroma was observed, and keratocytes had migrated to acellular zone. One week after irradiation (**D**), stroma had contracted, and many keratocytes had migrated to irradiated area. Bar = 100 μm.

in 2 of the 3 eyes, keratocytes had disappeared from the irradiated area, and an acellular zone was observed (Figure 1B). In the third eye, a few keratocytes were observed at the irradiated area. The retrocorneal membrane could be observed in all 3 eyes.

At 3 days after surgery, in 1 of the 4 eyes enucleated, bulla formations in the intercellular spaces of the epithelium and the hyperplastic epithelium were observed in the irradiated area, as they had been at 1 day after surgery. However, no abnormalities were observed in the other 3 eyes. In all irradiated eyes, the stroma had contracted at the irradiated area, and many keratocytes were observed at the acellular zone at 1 day after surgery (Figure 1C). The retrocorneal membrane could be observed in 2 of the 4 eyes.

After 1 week, in all the irradiated eyes, the stroma had contracted, and many keratocytes were found at the irradiated area (Figure 1D). The retrocorneal membrane could be observed in 1 of the 3 eyes enucleated.

Immunohistochemical Examination

We used immunofluorescence techniques to investigate the chronological changes in the localization of various extracellular matrix proteins after irradiation.

In the cornea of normal rats, the fluorescence specific to type I collagen was found in all the layers of the stroma (Figure 2A). After Ho:YAG laser irradiation, we observed disruption of collagen fibrils in the stroma, and the fluorescence specific to type I collagen was found in all the layers of the stroma. Although staining was somewhat heavy at the contracted stroma, no obvious changes were observed in the localization of type I collagen up to 1 week after irradiation (Figures 2B–D).

The fluorescence specific to fibronectin in the normal rat cornea was found in the stroma, epithelial basement membrane and Descemet's membrane (Figure 3A). One day after irradiation, immunoreactivity to fibronectin had diminished in the stroma, and an especially small amount of fluorescence was observed at the irradiated area (Figure 3B). Three days after irradiation, immunoreactivity to fibronectin was found to correspond to the keratocytes at the acellular zone (Figure 3C). At 1 week, localization of fibronectin corresponded to keratocytes at the irradiated area (Figure 3D). The fluorescence specific to fibronectin was also found in the retrocorneal membrane.

The fluorescence of basement membrane components—type IV collagen (Figure 4) and laminin (Figure 5)—was found in the epithelial basement membrane and Descemet's membrane in the normal rat cornea (Figures 4A, 5A), at 1 day (Figures 4B, 5B), at 3 days (Figures 4C, 5C) and at 1 week (Figures 4D, 5D) after irradiation. No changes in the localization of type IV collagen and laminin were observed even if the cornea had been irradiated. The fluorescence specific to type IV collagen and laminin was also found in the retrocorneal membrane.

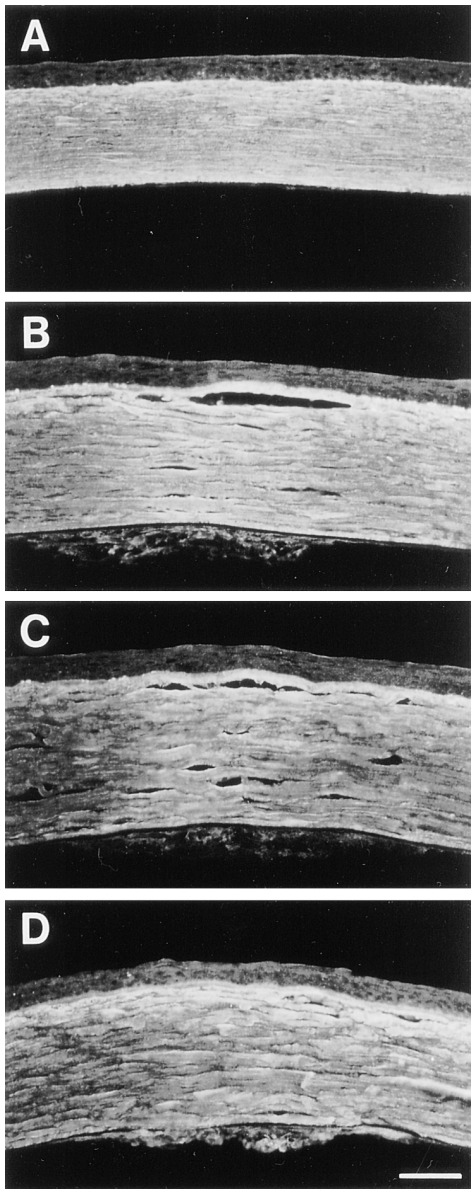

Figure 2. Localization of type I collagen after holmium:YAG irradiation. In normal cornea, type I collagen was localized in stroma in all layers (**A**). After irradiation, collagen fibrils were disrupted, but immunoreactivity to type I collagen was observed in stroma in all layers. One day (**B**), 3 days (**C**), and 1 week (**D**) after irradiation, we observed no obvious changes from normal cornea in localization of type I collagen. Bar = 100 μm.

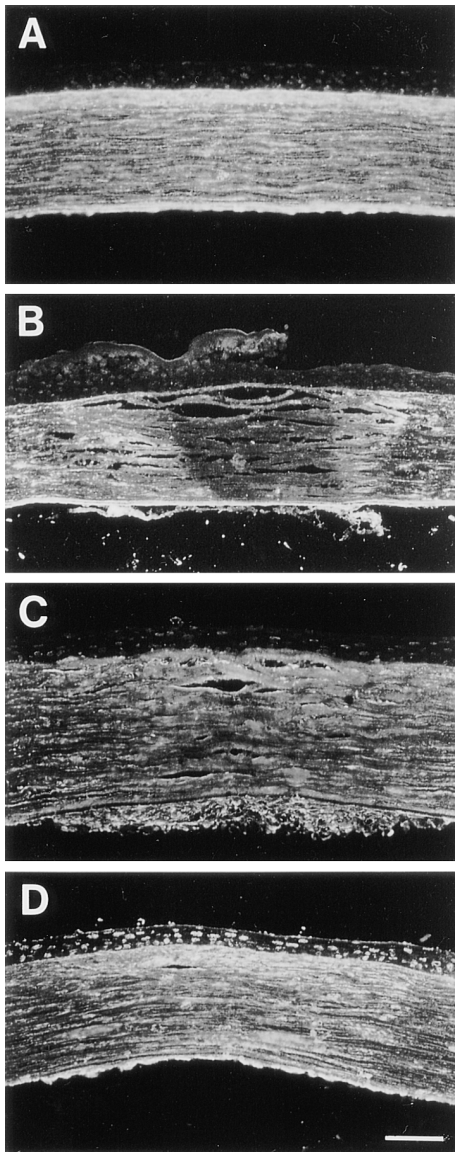

Figure 3. Localization of fibronectin after holmium:YAG irradiation. In normal cornea, immunoreactivity to fibronectin was observed in corneal stroma, epithelial basement membrane, and Descemet's membrane (**A**). One day after irradiation (**B**), immunoreactivity to fibronectin disappeared at acellular zone in stroma. At 3 days (**C**) and at 1 week (**D**) after irradiation, localization of fibronectin corresponded to migration of keratocytes from surrounding area to acellular zone. Bar = 100 μm.

Discussion

In considering the postsurgical outcome of refractive surgery by Ho:YAG laser irradiation, important problems include the condition of the stromal collagen in relation to corneal shape and postsurgical complications, such as delayed wound healing. In the present study, we investigated the histological and chronological changes in the localization of the main component of the corneal stroma, type I collagen; an important factor for wound healing, fibronectin; and basement membrane components related to maintaining a normal structure and healing wounds, type IV collagen and laminin.

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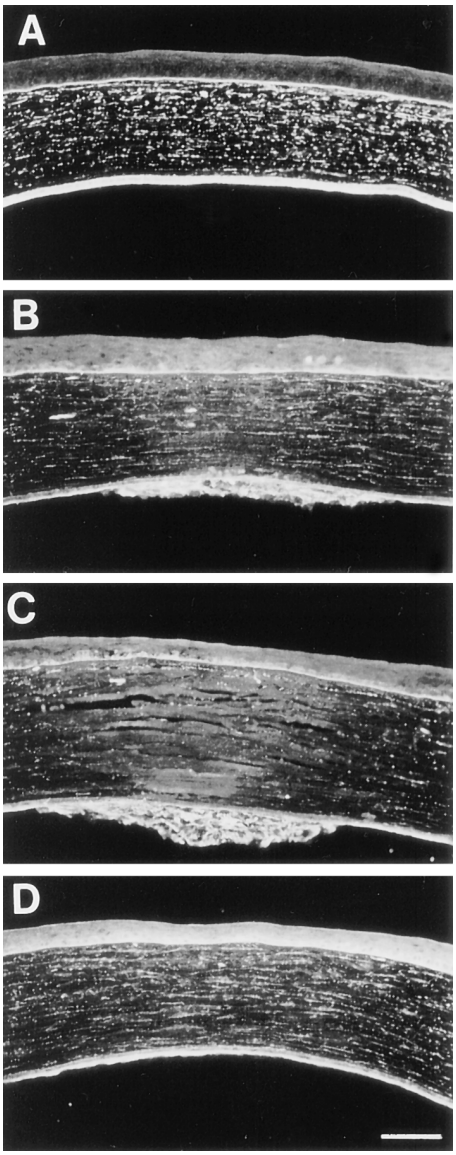

Figure 4. Localization of type IV collagen after holmium:YAG irradiation. Fluorescence specific to type IV collagen was observed at epithelial basement membrane, and Descemet's membrane in normal cornea (**A**), 1 day (**B**), 3 days (**C**), and 1 week (**D**) after irradiation. Immunoreactivity to type IV collagen could be observed at retrocorneal membrane. Bar = 100 μm.

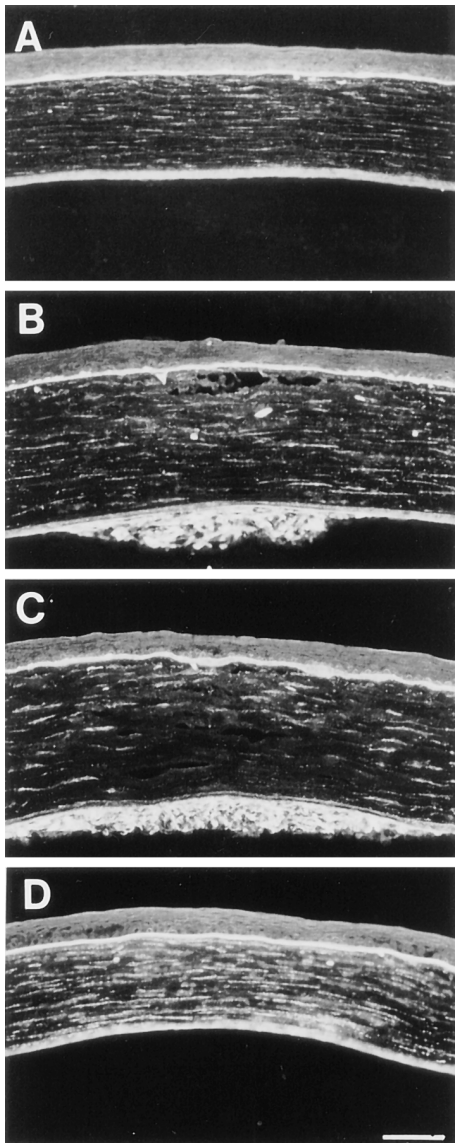

Figure 5. Localization of laminin after holmium:YAG irradiation. Fluorescence specific to laminin was observed at epithelial basement membrane and Descement's membrane in normal cornea (**A**), 1 day (**B**), 3 days (**C**), and 1 week (**D**) after irradiation. Immunoreactivity to laminin could be observed at retrocorneal membrane. Bar = 100 μm.

It is reported that collagen fibrils in stroma contract by one third after treatment with constant heat. Thermokeratoplasty changes the shape of the cornea by heating the stromal collagen fibrils so that they contract.[6,16,17] In the present study, we histologically confirmed that heat from a Ho:YAG laser contracts the stroma in rat corneas. These results are in good agreement with histological changes reported previously in human and rabbits eyes.[18–21] In addition, although type I collagen, the main component of corneal stroma had spread between the lamellar structures, no corneal abnormalities, such as ulceration, were observed.

Immediately after corneal injury, keratocytes disappear from the area adjacent to the injury. Later, keratocytes start to migrate and accumulate into the acelluar zone. These keratocytes are round in shape and metabolically active. These activated kerato-

cytes play an important role in the healing process after injury.[15,22,23] In the present study, one day after Ho:YAG laser irradiation, keratocytes disappeared from the irradiated area, and an acellular zone was formed in the stroma. Subsequently, many keratocytes were observed at the irradiated area. These results suggest that activated keratocytes gradually migrate into the acellular zone, and the wound healing process actively progresses at the stroma. We believe that these observations indicate the beginning of stromal remodeling.

It has been reported that keratocytes disappear around the injury site as a result of apoptosis and that an acellular zone develops as an immediate response to various kinds of corneal injuries.[24–26] In the present study, we are not certain whether Ho:YAG laser irradiation or simple heat damage induced keratocyte apoptosis and formation of an acellular zone. However, because keratocyte apoptosis is induced only by corneal epithelial stimuli, both mechanisms may be involved in the induction of keratocyte apoptosis.

Regarding the localization changes in fibronectin after irradiation, the immunoreactivity of fibronectin temporarily disappeared from the irradiated stroma, but strong fluorescent fibronectin was observed in the area surrounding the acellular zone 3 days after irradiation. These results suggest that fibronectin synthesis is stimulated by the migration of keratocytes to the acellular zone. In addition, these results were in agreement with our previous work, which examined the localization of fibronectin after heat coagulation with a thermal tip in rabbit cornea.[27] Similar changes in localization of fibronectin were observed after cornea stromal incision.[14,28] Therefore, we believe that fibronectin synthesis by activated keratocytes is a biological response to stromal injury.

However, irradiation did not affect the localization of the basement membrane components, type IV collagen and laminin, and no obvious changes were observed when compared with the normal rat cornea. As we used Ho:YAG laser equipment with a slit-lamp delivery system in this study, the heat energy from the irradiation focused on the corneal stroma but not on the corneal epithelium and the epithelial basement membrane. This may be why there was little damage to the basement membrane in this study. However, Koch[21] reported the disappearance of the basement membrane component, type IV collagen; the hemidesmosome component, integrin β4; and the anchoring fibril component, type VII collagen, immediately after Ho:YAG laser irradiation in rabbits. We did not observe corneal epithelial abrasion caused by our Ho:YAG laser irradiation. However, Koch[21] observed corneal epithelial abrasion and reported that it took 3 months to heal, suggesting that the damage by Ho:YAG laser irradiation is similar to that caused by corneal abrasion, corneal incision, and excimer laser irradiation. Therefore, some irradiation conditions may induce damage to the corneal epithelium and the epithelial basement membrane, and it may take a long time for the corneal structure to return to normal.

In the present study, we observed the retrocorneal membrane in corneal endothelium in over half the rats that underwent Ho:YAG laser irradiation. Koch[21] and Moreira et al[18] also observed the retrocorneal membrane in the corneal endothelium after irradiation in rabbits. The reason for this phenomenon is thought to be excess irradiation energy directed at the experimental animals. Because the corneal thickness in experimental animals (rat, about 150–200 μm; rabbit, about 350 μm) is thinner than that in the human eye, irradiation can damage the corneal endothelium in animals. In fact, when we observed the rat corneas by slit-lamp examination immediately after irradiation, we found that a large rectangular area of the cornea was opaque throughout all the corneal layers,. We did not observe the wedge-shaped opaque area reported in humans and large animals (bovine and pig),[20,29,30] suggesting that the heat from the irradiation had spread beyond the corneal endothelium. We could not irradiate all eight points of the cornea simultaneously because the rat cornea is very small, so we focused on each point individually in this study. One reason for damage to the corneal endothelium may be the difficulty of focusing on an exact point on the surface of the rat cornea, because the equipment is designed to irradiate eight points simultaneously. The damage to the corneal endothelium by Ho:YAG laser irradiation may have been caused by the limitations of the optical properties of the equipment. Therefore, the effects of irradiation on the human eye cannot be determined from the results of these rat and rabbit experiments.

As we reported in this paper, heat energy from Ho:YAG laser irradiation is effective in contracting the collagen of the stroma without causing serious damage to the corneal stroma, such as sterile necrosis and ulceration, which may be caused by other methods of heat coagulation. This result suggests that this surgery is safe as reported in clinical studies.[13,32–34] In addition, damage to the corneal epithelium and the basement membrane is minimal. When refractive sur-

gery is performed using Ho:YAG laser irradiation, transient corneal epithelial disorders such as epithelial edema do occur. Epithelial wound healing is fast, however, because the epithelial basement membrane is intact. This could mean that delayed epithelial wound healing and persistent epithelial defects do not occur. However, further studies are needed to determine the relationship between long-term histological changes and refractive regression.

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