After corneal excimer laser refractive surgery, keratocytes proliferate and produce extracellular matrixes that may result in corneal haze and regression of laser effect [1,2]. Corticosteroid eye drops are commonly used to prevent such complications after refractive surgery. However, corticosteroid-related complications such as glaucoma or cataracts prompted us to look for alternatives to control keratocyte activity.

Endothelin-1 (ET1) is a potent vasoconstrictor [3,4]. Endothelin-like immunoreactivity is found not only in vessels but also in ocular tissues [5,6]. In addition, ET1 can promote rabbit corneal epithelial wound healing [7,8]. Since the epithelial defect is the major source of ocular discomfort and pain after PRK [9,10], by giving ET1 eye drops, we may reduce the duration of pain in patients who receive PRK treatment through the promotion of epithelial healing rate. We found that ET1 has an inhibitory effect on porcine corneal keratocytes [11]. With regard to the above-mentioned
keratocyte proliferation and related extracellular matrix deposition, it would probably also be beneficial to PRK patients if ET1 inhibited the adverse effect of keratocytes in corneal stromal wound healing [1,2]. In this study, we investigated the effects of ET1 on rabbit corneas, including the rate of epithelialization, haze and extracellular matrixes after PRK.

**Materials and Methods**

**Animals**

All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eighteen New Zealand white rabbits, weighing between 2 kg and 2.5 kg, were used in the study. The rabbits were divided into groups according to the time of sacrifice: six rabbits in the 4-week group, six in the 12-week group, and six in the 24-week group. All rabbits were anesthetized before surgery with intramuscular injections of 30 mg/kg ketamine hydrochloride (Parke, Davis & Co., Detroit, MI, USA) and 5 mg/kg xylazine (Miles Inc., Shawnee Mission, KS, USA), combined with topical anesthesia of proparacaine hydrochloride 0.5% (Alcon, Rijksweg, Puurs, Belgium).

**Excimer laser and medications**

We removed the corneal epithelium with excimer laser (Schwind Keratom MultiScan Excimerlaser; Schwind eye-tech-solutions, Kleinostheim, Germany) in PTK mode with an ablation depth of 60 μm [12]. The diameter of the ablation zone was 7 mm. Following PTK, PRK (−8 D and 5 mm in diameter) was performed on both eyes. Immediately after PRK, gentamicin sulfate 0.3% eye drops (Shionogi, Japan) were applied. In addition, ET1 10−7 M eye drops (Sigma Chemicals, St Louis, MO, USA) were added to the right eyes, and phosphate-buffered salt solution (PBS) to the left eyes. After the operation, gentamicin sulfate 0.3% eye drops and ET1 10−7 M eye drops were applied five times a day for 7 days to the right eyes, and gentamicin sulfate and PBS to the left (which served as controls).

**Corneal epithelial wound healing rate, corneal haze and corneal thickness study**

Rabbit eyes were examined every 12 hours until corneal epithelialization was complete. To observe the epithelial defect, we used fluorescent staining. The longest horizontal (Lh) and longest vertical (Lv) lengths were measured, and the area of corneal epithelial defect was approximately calculated as Lh × Lv [13]. We calculated the average values of the results of each time point, and the difference between the two eyes was analyzed by paired t test. We observed corneal haze at 2, 4, 8, 12, 18 and 24 weeks after PRK. Corneal haze was defined as the following: Grade 1, haze not interfering with visibility of iris details; grade 2, mild obscuration of iris and lens; grade 3, moderate obscuration of iris and lens; and grade 4, completely opaque stroma in the area of ablation [14]. The result of corneal haze was evaluated by signed rank test. Central corneal ultrasound pachymetry was performed at weeks 4, 12 and 24. The results were analyzed by paired t test and signed rank test.

**Immunohistochemistry study of corneal extracellular matrixes**

Corneas were excised after PRK at scheduled time points: week 4, week 12 and week 24. Rabbits were anesthetized with ketamine 30 mg/kg and xylazine 5 mg/kg, and then euthanized by an intracardiac injection of an overdose of ketamine. The corneas were immediately excised and embedded in OCT compound (Optimal Cutting Temperature, Tissue-Tek OCT compound; SAKURA Co., Japan). The immunohistochemistry procedures are briefly described as follows: after being embedded in OCT compound in a chamber with a temperature of –40°C for 2 hours, the corneas were sectioned into 8-μm slices using Cryostat (Bright OTF Cryostat, Huntingdon, England). The samples were dipped in 0°C acetone for 1 minute. We added 10% normal goat serum (Zymed, San Francisco, CA, USA) to the samples, placed them in room temperature for 30 minutes, then washed them with PBS. The following primary antibodies (with concentrations shown in parentheses) were added: anti-collagen type 3 (1:4,000), anti-collagen type 4 (1:500), anti-collagen type 7 (1:1,000), anti-chondroitin sulfate (1:200) (Sigma Chemicals), and antibody to fibronectin (not diluted) (Zymed, San Francisco, CA, USA). They were incubated in a 37°C moist chamber for 1 hour and then washed with PBS. A secondary antibody, fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse IgG conjugate (1:80), was added, and the samples were placed in a 37°C moist chamber for another hour, and then washed again with PBS. The slides were sealed with glycerin (glycerin:PBS=1:1), and observed.
by fluorescent microscope (BX51TRF, exposure control unit PM-20, BH2-RFL-T3; Olympus Optical Co. Ltd., Tokyo, Japan).

**RESULTS**

Three of the rabbits died unexpectedly before the sacrificing dates and were thus excluded from the study. Two of them were sent for zootomy to the Veterinary Hospital, Department of Veterinary Medicine, National Pingtung University of Science and Technology. Only middle-sized pulmonary arterial wall hypertrophies were found in both rabbits, which is a nonspecific change in rabbits. There was no systemic evidence in the liver, kidney or brain to suggest that there was any toxic effect from ET1 (personal communication with Dr Chang, T.C., D.V.M. & M.S., Chairman, Section of Veterinary Pathology, Veterinary Hospital, National Pingtung University of Science and Technology). In addition, no significant contagion was found in the rabbits.

**Corneal epithelial wound healing rate, corneal haze and corneal thickness study**

The average size of the corneal epithelial defect was significantly smaller in ET1-treated eyes than in PBS-treated eyes at 48 and 60 hours (paired $t$ test; Figure 1). All of the corneal epithelial defects were healed in 4 days. The most severe haze did not exceed grade 2 (Table), and there was no significant difference in corneal haze between eyes treated with ET1 and those treated with PBS (signed rank test; Table). Central corneal thickness was similar in ET1-treated eyes and in control eyes on ultrasound pachymetry ($p > 0.05$, paired $t$ test and signed rank test).

**Immunohistochemistry of corneal extracellular matrixes**

In PBS-treated eyes, anti-collagen type 3 fluorescence could be found in the subepithelial and superficial stroma at 7 days. The fluorescence was most prominent at 12 weeks, and remained at 24 weeks. Type 3 collagen fluorescence was most prominent in week 4 in ET1-treated eyes, and fluorescence intensity looked similar at 4, 12 and 24 weeks. In ET1-treated eyes at day 7, when compared with PBS-treated eyes, fluorescence of type 3 collagen was more prominent in the subepithelial and superficial stroma. However, at week 24, there were no obvious differences between ET1- and PBS-treated eyes with regard to the fluorescence of type 3 collagen.

Anti-collagen type 4 fluorescence was most prominent at day 7 and week 4 in the subepithelial level, and it gradually decreased at week 12 and week 24 in PBS-treated eyes. In ET1-treated eyes, type 4 collagen fluorescence could be found at 7 days, and was most

**Table. Corneal haze after photorefractive keratotomy*†**

<table>
<thead>
<tr>
<th>Grade of haze</th>
<th>ET1</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>4</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

*Data presented as number of rabbits; †no significant difference between ET1- and PBS-treated eyes (signed rank test). ET1 = endothelin-1; PBS = phosphate-buffered salt solution.
prominent at 4 weeks. It was not obvious at 12 and 24 weeks. There was no obvious difference in type 4 collagen fluorescence between ET1 and control groups.

In PBS-treated eyes, anti-collagen type 7 fluorescence appeared in the subepithelial level at day 7. It was most prominent at week 4, and gradually decreased at weeks 12 and 24. Type 7 collagen was found in ET1-treated eyes at 7 days, was most prominent at 4 weeks, and gradually decreased at 12 and 24 weeks. There was no obvious difference between ET1- and PBS-treated eyes with regard to type 7 collagen fluorescence.

Anti-chondroitin sulfate fluorescence was most prominent in the subepithelial and superficial stroma at day 7 in the control group. The fluorescence decreased at week 4, and was not obvious at weeks 12 and 24 (Figure 2A). Chondroitin sulfate was most prominent at day 7. The fluorescence decreased at week 4, and was almost not visible at weeks 12 and 24 in the ET1 group (Figure 2B). ET1-treated eyes showed a granular fluorescent pattern in superficial stroma, while PBS-treated eyes showed a band fluorescent pattern in superficial stroma. At weeks 4, 12 and 24, the anti-chondroitin sulfate fluorescence was of low strength and showed a similar pattern in ET1- and PBS-treated eyes.

Anti-fibronectin fluorescence was noted in the subepithelial level at day 7 in PBS-treated eyes. It was most prominent at week 4 in the subepithelial and superficial stroma. However, it was not obvious at weeks 12 and 24 (Figure 3A). In ET1-treated eyes, anti-fibronectin fluorescence was present at 7 days, and was most prominent at 4 weeks and 12 weeks. It decreased at 24 weeks (Figure 3B). The fluorescence

**Figure 2.** Immunohistochemistry study of chondroitin sulfate. Bar = 50 μm. (A) Anti-chondroitin sulfate fluorescence was most prominent in the subepithelial and superficial stroma at day 7, decreased at week 4, and was not obvious at weeks 12 and 24 in the control group. (B) Chondroitin sulfate was most prominent at day 7, decreased at week 4, and was almost not visible at weeks 12 and 24 in the endothelin-1-treated group.
was about equal at 4 weeks in ET1- and PBS-treated eyes. However, in ET1-treated eyes, anti-fibronectin fluorescence lasted at least until week 24 (Figure 3).

**DISCUSSION**

Both corneal epithelium and stroma are involved in wound healing after excimer laser treatment. It seems that the epithelial defect produced by PRK is related to ocular discomfort and pain [9,10]. Clinically, bandage contact lenses, topical nonsteroidal anti-inflammatory drug (NSAID) eye drops or topical steroid eye drops can be used to relieve the pain [15]. Takagi et al reported that ET1 promoted the proliferation of corneal epithelial cells in vitro [7] and increased the healing rate of corneal epithelial wounds in vivo [8]. Although our treatment modality was different from theirs, the present study confirmed the effect of ET1. In addition, from this perspective, ET1 may be helpful in decreasing pain after PRK.

Corneal wound healing responses are mainly in the stroma [1,16]. After trauma, keratocytes in the wound bed start to migrate and proliferate [2,16]. They produce irregularly arrayed collagen and scar tissue, resulting in corneal haze, which decreases the vision of patients after operation. In addition, the deposition of new tissue is associated with corneal stroma re-thickening, which is related to myopic shift [1,2,17]. Although ET1 has an inhibitory effect on keratocytes in vitro [11], we found that ET1, $10^{-7}$M five times a day for 7 days, could not inhibit central corneal re-thickening in rabbits.

Immunohistochemistry studies of corneal extracellular matrixes after PRK without any medication modulations in monkeys [18,19] and rats [20] have
been reported. Collagen type 3 presents transiently during the corneal wound healing process and only in the superficial stroma \[18–20\]. Our study is in agreement with the above-mentioned reports that corneal wound healing can last as long as 6 months and type 3 collagen is mainly in the superficial stroma during the wound healing process \[18–20\]. Types 4 and 7 collagen are the major components of basement membrane and anchoring fibrils, respectively \[19,21\]. It is reasonable that, in our study, they were present in the subepithelial level, although in different species, the results of immunohistochemistry study of collagen types 4 and 7 in the present study and in previous reports are similar \[18–21\]. Increased anti-chondroitin sulfate fluorescence at day 7 and week 4, compared to week 12 and week 24 were noted in both ET1- and PBS-treated eyes. The transient increase in the fluorescence of anti-chondroitin sulfate during wound healing and the presence of it in the superficial stroma imply that it may be involved in the early stages of corneal wound healing \[22\]. However, further investigation is required to clarify its role in corneal wound healing.

Fibronectin is involved in cellular migration and wound healing \[18\]. It is also present during corneal wound healing \[22–24\]. The presence of fibronectin in the corneal superficial stromal level could last as long as 4 weeks after PRK \[20\]. It was proposed that the fibronectin might be derived from stromal fibroblasts for later stages of wound healing \[23\]. Our results confirmed the above-mentioned theories. In addition, we found that ET1 could increase and prolong fibronectin deposition in the superficial stromal levels. ET1 has been found to have the effect of increasing fibronectin expression in other tissues \[25,26\]. If ET1 promotes the migration of corneal epithelial cells via increasing fibronectin, it requires further study.

One issue we would like to discuss is the duration of treatment. We performed a preliminary supplementary study. Two New Zealand white rabbits received the same experimental setting described above, but instead of for 1 week, ET1 was applied for 4 weeks. We found that after 4 weeks of treatment, the corneal thicknesses in the ET1-treated eyes of the two rabbits were 87.0% and 95.6%, respectively, of the thicknesses before PRK treatment. And in the PBS-treated eyes of the two rabbits, they were 94.9% and 109.4%, respectively. Although the sample size is too small to make any conclusion, these preliminary results might indicate that ET1 has the effect of inhibiting corneal re-thickening if an appropriate treatment duration, such as 1 month, is given.

ET1 can cause vascular remodeling and vascular hypertrophy \[27–29\]. Although unexpected death occurred in three of our rabbits, and hypertrophy of middle-sized pulmonary arterial walls was found in two, it is a nonspecific change in rabbits. In addition, no other evidence was found, in the brain, liver or kidney, to suggest that there was any systemic toxic effect from ET1. However, a thorough systemic survey of ET1 in an animal study is probably needed.

In summary, ET1 10^{-7} M eye drops five times a day for 1 week can promote corneal epithelial wound healing, but it has no effect on corneal haze and corneal thickness. There was an obvious difference in fibronectin between ET1- and PBS-treated eyes, which implies that ET1 promotes corneal epithelial wound healing via increasing the amount of fibronectin.

ACKNOWLEDGMENTS

This study was supported by a research grant from the National Science Council of Taiwan (NSC 89-2314-B-037-146).

REFERENCES


在接受過雷射角膜切除術的兔子，使用內皮素-1可以增加纖維連結蛋白在角膜的表現並促進其角膜上皮傷口的癒合

賴昱宏 1  王惠珠 1,2  林昌平 3  洪秀貞 4  章順仁 5

1高雄醫學大學附設醫院  眼科
2高雄醫學大學  醫學系  眼科  4藥理學系  5公共衛生學科
3彰化基督教醫院  眼科

研究內皮素-1 (endothelin-1、ET1) 在接受過雷射角膜切除術 (photorefractive keratectomy、PRK) 之後的兔子角膜傷口癒合的影響。一共有十八隻紐西蘭白兔接受 PRK，其右眼點以 ET1 而左眼點以磷酸緩衝鹽溶液 (phosphate-buffered salt solution、PBS)。我們紀錄角膜上皮傷口大小、角膜混濁、與角膜厚度。我們利用組織免疫化學法 (immunohistochemistry) 來研究角膜的細胞外基質 (extracellular matrixes) 包括第三、四、七型膠原蛋白 (collagen)、軟骨素 (chondroitin sulfate)、與纖維連結蛋白 (fibronecin)。在雷射過後十二週與十四週，在點以 ET1 的眼睛仍然可以發現有纖維連結蛋白，但是對照組的則沒有。ET1 會加速兔子角膜上皮傷口的癒合。點以 ET1 的眼睛與對照組在角膜混濁、角膜厚度、與其他的細胞外基質則沒有明顯的差異。ET1 可以增加纖維連結蛋白在角膜基質的堆積而且可以促進角膜上皮傷口的癒合。ET1 促使纖維連結蛋白在角膜的增加也許可以解釋其增進上皮傷口癒合的可能機制。

關鍵詞：角膜傷口癒合，內皮素-1，細胞外基質，纖維連結蛋白，雷射角膜切除術

(高雄醫誌 2008;24:254-61)