Corneal Endothelial Healing Rate and the Effect of Topical Retinoic Acid

Mamoru Matsuda, John L. Ubels, and Henry F. Edelhauser

These studies were undertaken to evaluate wound healing rates of the corneal endothelium in vivo. After insertion of a 26-gauge needle into the anterior chamber of the rabbit eye through the limbus, a 5-0 nylon monofilament was introduced through the needle, and endothelial wounds were made by scratching the cells with the filament. The wounds were photographed with a wide-field specular microscope at various intervals. Montages of the wounds were made, and the areas of the wounds were determined by planimetry. Wound closure occurred rapidly in a linear manner during the first 6 hr after wounding, after which the rate of cell migration decreased. Healing rates (μm²/hr) during the first 6 hr were calculated by linear regression analysis. There was a direct linear correlation between the healing rate and initial wound area. The slope of this line for nine normal (untreated) corneas was 0.093 hr⁻¹. Nine corneas were treated with 0.1% retinoic acid in petrolatum ointment, while eight control corneas received vehicle alone. The slope of healing rate versus initial wound area for treated corneas (0.11 hr⁻¹) was significantly greater than control (0.097 hr⁻¹). This was interpreted as a stimulation of corneal endothelial migration during healing by retinoic acid. As a result of this study, a method for analysis of corneal endothelial healing rate has been developed which can be used for comparison of healing rates among treatments when initial wound area cannot be standardized. Invest Ophthalmol Vis Sci 27:1193–1198, 1986

While wound healing rates of the corneal epithelium have been calculated, the endothelial healing rate has not been evaluated in vivo. This is due mainly to difficulties in making reproducible, standardized endothelial wounds without damaging Descemet's membrane. It is also difficult to make wounds which can be conveniently observed at frequent intervals using a wide-field specular microscope.

Calculation of healing rates has provided a quantitative method for evaluating the effect of various substances on the corneal epithelial healing process in vivo. In this study, therefore, we attempted to develop a method for calculating the healing rate of the rabbit corneal endothelium, and we also tested the model by evaluating the effect of topical retinoic acid on the endothelial healing process. Topical retinoic acid was chosen for several reasons: first, it has been shown that 3H-retinol injected into the anterior chamber of the eye is taken up by the corneal epithelial and endothelial cells, especially those migrating to cover a wound; second, topical retinoic acid has been reported to be effective in promoting the healing of corneal epithelial wounds; and third, topically applied retinoic acid can penetrate the cornea and can presumably be taken up by endothelial cells.

Materials and Methods

Experimental Animals

New Zealand White rabbits of both sexes weighing 2-2.5 kg were used in this study. The investigations described adhered to the ARVO Resolution on the Use of Animals in Research. Each cornea was examined by a slit-lamp biomicroscope and a wide-field specular microscope (Keeler-Konan) before the experiment, and no pathologic findings were noted. All animals were anesthetized with an intramuscular injection of ketamine HCl (30 mg/kg) and xylazine (5 mg/kg) and...
received two drops of proparacaine topically prior to wounding and at each specular microscopic examination.

Endothelial Wounding Procedure

The wounding procedure was performed under an operating microscope. After insertion of a 26-gauge needle into the anterior chamber of the eye through the limbus, a 5-0 nylon monofilament was introduced through the needle, and endothelial wounds were made by gently scratching cells with the filament. Great care was taken to avoid collapsing the anterior chamber and disrupting Descemet's membrane. Both corneas in a total of 18 rabbits were wounded.

The wounds were photographed with the wide-field specular microscope at 0, 3, 6, 12, and 24 hr and 2, 3, and 5 days after wounding. Only the wounds in which the endothelial monolayer was completely reestablished were included in the study. Small wounds which healed by 12 hr after wounding (three corneas) and wounds which failed to heal (seven corneas), possibly due to damage involving Descemet's membrane, were excluded. The endothelial photographs were enlarged 140 times and montages of the wounds were made. The wound perimeter was traced onto paper, and the areas...
of the wounds were determined by computerized planimetry, as described previously.\textsuperscript{1,3,8} The tracing of the wound perimeter was performed on two separate occasions. The wound area was determined from each tracing and the average was calculated. The relative deviation (absolute difference in wound areas as a percentage of the original measurement) was less than 5% in any case.

Groups of Animals

The normal healing rate of the endothelium was evaluated using nine wounded corneas of six rabbits. No drug other than proparacaine was applied to these corneas during the observation period.

In a separate experiment, nine corneas of six rabbits were treated topically with 50\textsuperscript{\%} of petrolatum ointment containing 0.1\% all-trans retinoic acid (Sigma, St. Louis, MO) twice a day pre- and postoperatively, while eight corneas of six rabbits (controls) were treated with the ointment vehicle alone using the same protocol.

Results

Normal Endothelial Healing Rate

Although it was difficult to make a standardized wound, all of the wounds were roughly oval in shape (Fig. 1). Sequential specular photomicrographs of the healing process of the wound (IV) in a normal cornea are shown in Figure 1, and changes in the area of this wound are shown in Figure 2. The initial wound area was 84.0 \times 10^{3} \text{ \mu m}^{2} and decreased in a linear manner during the first 6 hr after wounding (Fig. 2) by migration and elongation of adjacent cells to cover the wound (Fig. 1A–C). Subsequently, the rate of wound closure decreased considerably (Figs. 1D, 2) and the wound was completely covered by 24 hr after wounding (Fig. 1E). The initial coverage of the wound was established by many large, irregular cells, some of which appeared to be black (Fig. 1E–F). The number of dark cells decreased thereafter (Fig. 1G), and the complete endothelial monolayer was reestablished by 5 days after wounding, although there were considerable variations in cell sizes and shapes and some black cells still remained (Fig. 1H).

The initial wound area for the nine wounds in the normal group ranged from 33–235 \times 10^{3} \text{ \mu m}^{2} (Table 1), the maximum wound representing less than 0.3% of the endothelial area. This wound area is on the order of that which might occur during intraocular surgery. Each of these wounds showed a similar healing process to the examples shown in Figures 1 and 2, and all of the wounds healed by 2 days after wounding. Because wound closure appeared to occur in a linear manner during the first 6 hr (Fig. 2), healing rates (\text{\mu m}^{2}/hr) during this period were calculated by linear regression analysis (Table 1). There was a direct correlation between the healing rate and initial wound area (r = 0.989, P \leq 0.05). A plot of initial wound areas versus wound healing rates fits a linear model with a slope of

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Area = 10^{3} \text{ \mu m}^{2}; rate = 10^{3} \text{ \mu m}^{2}/hr; r = correlation coefficient.

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**Fig. 2.** Changes in the area of the wounds I, IV (shown in Fig. 1), and VII. Note linearity for the first 6 hr of healing.

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Initial Wound Area (10^3 um^2)

Fig. 3. Corneal endothelial healing rate plotted versus initial wound area using the data shown in Table 1. Slopes and y-intercepts are shown in Table 2. V = vehicle, RA = retinoic acid.

0.093 ± 0.0017 hr⁻¹ and a y-intercept of 2.54 ± 0.22 × 10³ μm² (Fig. 3, Table 2).

Effect of Topical Retinoic Acid

The initial wound areas ranged from 41-263 × 10³ μm² in the group treated with topical retinoic acid (nine corneas) and from 45-210 × 10³ μm² in the control group (eight corneas) treated with vehicle alone (Table 1). The range and distribution of initial wound sizes was similar for all three groups of rabbits. In each of these wounds, wound closure occurred in a manner similar to the normal healing process. The healing rates (μm²/hr) during the first 6 hr were also calculated by linear regression analysis (Table 1) and again there was a positive correlation between the healing rate and initial wound area in the treated group (r = 0.995, P < 0.05) and the control group (r = 0.995). These results indicated that direct comparison of the mean healing rates (μm²/hr) between the groups was not possible.

Plots of initial wound area versus healing rate fit a linear model (Fig. 3). The slope of this line for the control group, 0.097 ± 0.0015 hr⁻¹, did not differ significantly from the normal group (t-test, P > 0.05). However, the slope of the line for the retinoic acid treated group, 0.11 ± 0.0015 hr⁻¹, was significantly different from that of the control group. The y-intercepts of the treated and control groups, 3.42 ± 0.22 × 10³ μm² and 1.65 ± 0.21 × 10³ μm², respectively, were also significantly different (Table 2).

Table 2. Slopes and y-intercepts for plots of initial wound area versus healing rates

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<th>y-intercepts (10³ μm²/hr)</th>
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<td>2.54 ± 0.22</td>
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<tr>
<td>Control</td>
<td>0.097 ± 0.0015</td>
<td>1.65 ± 0.21</td>
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<td>Treated</td>
<td>0.110 ± 0.0015*</td>
<td>3.42 ± 0.22*</td>
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Mean ± SE.

* Significantly different than control (P ≤ 0.05) t-test.

Discussion

In order to evaluate the healing rate of the corneal endothelium in vivo, the following points are of great importance. First, endothelial wounds without damage to Descemet’s membrane are essential because, when injuries involve Descemet’s membrane, the stromal cells participate in the healing process and these wounds take much longer to heal than those where the Descemet’s membrane is not involved. ¹¹ Second, the healing of the wounds must be followed and photographed by specular microscopy at frequent time intervals. This is not possible if the wounding method results in corneal edema due to extensive epithelial and endothelial damage. For these reasons, preliminary experiments demonstrated that previously reported methods of endothelial wounding, either transcorneal freezing¹²,¹³ or wounding with small sharp needles,¹¹ prevent calculation of the endothelial healing rate and therefore were not suitable methods for the present study.

In a previous study,⁵ we made endothelial wounds by scratching cells with a 4-0 nylon monofilament introduced into the anterior chamber through a 25-gauge needle. Less than 50% of these wounds healed completely. This low success rate was due mainly to difficulties in maintaining the anterior chamber during the procedure and to the low flexibility of the nylon filament. In this study, therefore, we modified the procedure by using 26-gauge needles and 5-0 nylon filaments. Despite these modifications, approximately 20% of the wounds did not heal, possibly due to damage to Descemet’s membrane, and these wounds were eliminated from the study. It remained very difficult to make a standardized wound.

Our measurements of wound area show that wound closure in the rabbit corneal endothelium occurred in an essentially linear manner during the first 6 hr and that the rates decreased thereafter (Fig. 2). Since no photographs were taken between 0 and 3 hr, no information is available concerning a possible lag time before the beginning of cell migration. However, given the extent of healing which occurred by 3 hr, it is as-
Decreases with time as contact inhibition occurs.5

The dependency of wound healing rate on initial wound area in the corneal endothelium, when the rate is expressed as μm²/hr, is in agreement with our previous findings for the corneal epithelium in which healing rate expressed as mm²/hr is also dependent on initial wound size.14 This means that, when wound size in a group of animals varies, a treatment effect, such as that of the retinoic acid used in the present study, cannot be evaluated by comparing mean healing rates for the treatment and control groups. For the corneal epithelium, this problem can be avoided by standardizing wound shape and size, or, as in our previous study,14 by calculating the linear rate of cell migration. For the corneal endothelium, wound size and shape cannot be standardized in our model, so that calculation of a linear rate of cell movement becomes a very complex problem. Alternatively, we have normalized the endothelial healing data using the linear relationship between initial wound area and healing rate during the first 6 hr expressed as μm²/hr. Statistical comparison of these lines then allows evaluation of a treatment effect.

Several points must be made concerning this method of analysis. First, the units, hr⁻¹, of the slopes of the regression lines in Figure 3 have no physiologic significance in that they do not directly describe the healing process. Each plot illustrates the linear relationship between initial wound area (μm²) and healing rate (μm²/hr), the slope being a rate constant which can be used for statistical comparison. Second, expression of the healing rate as μm²/hr cannot be directly equated with the rate of cell migration, but rather is the result of cell migration. Cells migrate toward the center of the wound at a rate of distance/unit time (μm/hr). The whole population of cells migrating from the perimeter of the wound covers a corresponding area/unit time (μm²/hr). Using the methods of the present study, this rate can conveniently be measured. Assuming that the rate of cell migration is independent of initial wound area, as we have shown in our previous study,14 wounds of equal size should have approximately equal healing rates in μm²/hr, while larger and smaller wounds will heal at faster and slower rates, respectively. This is illustrated by our data for normal and control (vehicle-treated) corneas shown in Table 1 and Figure 3. Third, since the relationship between initial wound area and healing rate (μm²/hr) for the first 6 hr is essentially linear, any treatment which increases or decreases the rate of cell migration (μm/hr) will change the slope of the regression line when data for a range of initial wound sizes are plotted (Table 1, Fig. 3). The slope changes because a change in the linear rate of cell migration will have a greater effect on the area covered per unit time for a large wound than for a small wound, due to the large wound perimeter and the use of a quantity squared as a factor in the equation. Statistical comparison of the slopes of the regression lines allows determination of a treatment effect. Fourth, extrapolation of the regression line to determine the y-intercept (Table 2) does not imply that a healing rate can be determined for a wound of zero area. Our data provide no information concerning the healing characteristics of wounds smaller than about 30 × 10³ μm². However, a significant difference in the y-intercepts between treatment groups indicates that the regression line has shifted in a positive or negative direction. This provides evidence, in addition to a change in slope, that for any initial wound size over the range studied, the endothelial healing rate has changed as a result of a given treatment.

For our data, the slopes do not differ for the normal and control groups (vehicle-treated), indicating no effect of the ointment vehicle itself on healing (Fig. 3, Table 2). The slope and y-intercept of the line for the retinoic acid-treated group are significantly different from control indicating that, for any initial wound size, over the range studied, the endothelial healing rate of a retinoic acid-treated cornea is greater than the healing rate of a control wound of equal size (Fig. 3, Table 2). This implies that the cell migration rate (μm/hr) of the treated endothelia increased. It is concluded that, under the conditions of this study, 0.1% retinoic acid ointment applied topically to the cornea promoted healing of corneal endothelium in the rabbit during the first 6 hr after wounding. The majority of the wounded area is covered during this rapid healing phase. Therefore, the retinoic acid may be beneficial in promoting the reestablishment of the barrier function of the endothelium via a more rapid restoration of a continuous monolayer of cells. This hypothesis is supported by recent data from our laboratory which show that endothelial permeability to inulin and dextran decreases as the endothelial monolayer is reestablished following wounding.15

The mechanism by which retinoic acid promotes endothelial migration is unknown, but it may be related to biosynthesis of cell surface glycoproteins. It has been shown that retinoic acid can stimulate glycoprotein synthesis in the corneal epithelium16,17 and attachment of epidermal cells in culture.18 Glycoproteins synthesized under the influence of retinoic acid may play a role in facilitating the endothelial healing by providing an adhesive matrix for cell migration.19,20

As a result of this study, a quantitative method has been developed for analysis of corneal endothelial...
healing rates and for in vivo evaluation of the effects of various drugs on cell migration during healing. Our data also suggest that topical retinoic acid can be effective in promoting endothelial migration. Cellular migration is known to be a main mechanism for coverage of defects in human corneal endothelium. In this study and in previous studies, retinoic acid does not appear to be toxic to the cornea when applied topically in low, controlled doses. Topical retinoic acid may prove to be effective in enhancing endothelial healing following ocular trauma and intraocular surgery.

Key words: corneal endothelium, endothelial wound healing, cell migration, retinoic acid

References