Corneal Re-epithelialization in Galactosemic Rats

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Abnormalities in corneal epithelial healing in diabetic patients have been described recently. Defects in corneal re-epithelialization in diabetic rats have been reported, and it was found that treatment with aldose reductase (AR) inhibitors effectively prevented these defects. Experiments using galactosemic rats to study further the role of AR in these defects, since AR is known to be the common factor involved in sugar cataractogeneses, are reported herein. Similar defects in corneal re-epithelialization in galactosemic rats as in diabetic rats were found. The delay in re-epithelialization was documented by computer planimetry. Light microscopy showed marked corneal stroma edema and wider intercellular spaces in the epithelium after complete re-epithelialization, while scanning electron microscopy revealed fewer filopodia projecting from the leading margin during the active migration stage. These defects were prevented by treating galactosemic rats with the aldose reductase inhibitor, Pfizer's Sorbinil. These suggest that AR plays a role in the defects in corneal re-epithelialization observed in diabetes. Invest Ophthalmol Vis Sci 24:563-569, 1983

Corneal epithelial healing abnormalities in diabetic patients have been found to occur with vitrectomy.1-3 We have previously observed a delay in the corneal re-epithelialization of diabetic rats compared to normal rats, with the corneas of the healed diabetic rats appearing cloudy compared to the transparent healed corneas of normal rats.4 Treatment of these diabetic rats with a variety of aldose reductase (AR) inhibitors appeared to prevent both the observed delay in corneal wound healing and the cloudy appearance of the healed corneas.

The potential involvement of AR in this phenomenon was explored, with the hypothesis that osmotic imbalance from the AR initiated accumulation of sorbitol may play a role in corneal epithelial abnormalities similar to that in sugar cataract formation. In the lens AR converts aldose sugars such as glucose and galactose to polyols that accumulate in the lens fibers leading to osmotic swelling, cell rupture and subsequent cataract formation. This cataract formation can be prevented or delayed through inhibition of AR.5,6 If the AR initiated accumulation of sugar alcohols is involved in the corneal re-epithelialization defect diabetic rats, then a similar defect should be observed in galactosemic rats.

Here we report the results of corneal scraping experiments in rats fed a 50% galactose diet that showed delayed corneal re-epithelialization after complete denudation compared to normal rats. This delay, which was quantitated by computer planimetry, could be prevented upon oral treatment with the AR inhibitor Sorbinil (S-6-fluoro-spiro (chroman-4-4'-imidazolidin-2',5'-dione).

Materials and Methods

Sprague Dawley albino rats weighing 50 g were fed a 50% galactose rat chow diet or a 50% galactose diet to which the AR inhibitor Sorbinil (Pfizer Central Research, Groton, CT) was mixed to give a dose of 60 mg/kg body wt/day (425 mg/kg chow). Control rats were fed regular laboratory rat chow. A total of 180 rats (360 eyes) were used, 60 rats (120 eyes) for each group.

After three weeks of the diet, the corneal epithelium of the rats was scraped carefully off from limbus to limbus under a Carl Zeiss dissecting microscope with a Beaver Blade 67.

Corneal re-epithelialization was followed by corneal staining with 1% fluorescein that could be observed with a UV lamp (Ultraviolet Products, Inc., San Gabriel, CA, model UVS-11 mineralight lamp) and photographed after 0, 17, 24, 48, 72, and 96 hr periods with a Kowa Fundus Camera (RC-2, Keeler Co., PA) equipped with Polaroid 107 film (ASA 3000) and a strobe flash equipped with a blue gelatin filter.

Corneal samples were taken at 0, 10, 18, 24, 30, 48, and 72 hrs for light microscopy and scanning electron microscopy (SEM). For light microscopy, the eyes were fixed overnight in 4% glutaraldehyde, 0.15 M phosphate buffer pH 7.2. Soon after the start of fixation, 10-15 min, a slit was made at the limbus...
with a razor blade. After an overnight fixation period, the eyes were transferred to 10% buffered formalin for further fixation for several days. These were then dehydrated for 2 hrs each with a series of ethyl alcohol solutions (50%, 70%, 80%, 90%, and 95%). Finally they were treated with three changes of plastic embedding mixture Solution A and catalyst (JB-4 methacrylate embedding kit, Polysciences, Inc., Philadelphia, PA), the first change overnight, followed by two additional changes at 2-hr intervals. A final embedding mixture (consisting of 20 parts Solution A and catalyst and 1 part of Solution B, at a ratio of 20:1) was poured into plastic molds. The well-infiltrated whole eyes were then dropped into molds, positioned, and the medium allowed to harden. Within 30-40 min, the medium had polymerized sufficiently. An oily residue formed on the surface was washed off with soapy water. The molds were then hardened overnight, and the block was then cut on a Sorvall JB4-A microtome. Sections of 1-2.5 inches in thickness were cut using a 1/2" thick glass knife and mounted on glass slides, stained with hematoxylin-eosin. Samples were also processed by American Histolabs (Rockville, MD).

Eyes for scanning EM studies were fixed in 4% glutaraldehyde in phosphate buffer, pH 7.2 for at least 2 hrs. They were dissected under a Carl Zeiss dissecting microscope, and the removed corneas were divided into four quadrants using a razor blade. They were then postfixed in 1% osmium tetroxide for 90 min, on ice, in a shaker. The samples were dehydrated with a series of ethyl alcohol solutions (25%, 50%, 75%, 85%, and 90% in 95% and 100%) and critically point dried using liquid carbon dioxide under a pressure of 1600 lbs/in² at 50° C Samdri PV1-3 critical point dryer (Tousimis Corp., Rockville, MD) mounted on metallic specimen holders (Cambridge sample studs) and coated with gold palladium vapor under vacuum using a Samsputter (Tousimis Corp.). Specimens were examined with a JSM-U3 Scanning EM using accelerated voltage of 8KV.

The rate of re-epithelialization was quantitated by computer planimetry as follows: using a Tetronix 4010 terminal equipped with a 4953 graphics tablet and 4923 recorder, outlines of the limbus and healed areas from polaroid photographs were traced over the graphics tablet using the NIH DIGEST digitizing program in an offline recorded mode. Raw recorded data from the tablet were then entered onto the NIH DEC-

Figs. 1A-C. A, normal Rat Cornea after reepithelialization. Cornea is clear and transparent. B, galactosemic rat cornea after reepithelialization. Cornea is edematous and hazy so that iris vessels are not clearly seen. C, Sorbinil-treated galactosemic rat cornea after reepithelialization. Cornea is clear and transparent as in normal controls.
10 computer system using the DIGTAPE program and these raw data were converted into a series of curves containing x,y coordinate points using the DIGIT procedure. The curves were entered into MLAB mathematical modeling program, and areas enclosed by the curves were numerically integrated. From these curves healing rates for control rats, galactose untreated, and galactose-treated rats were expressed as a percent of the area healed. These were analyzed using the NIH Prophet computer system's regression and statistical procedures. Healing curves were fit to the general allosteric equation 
\[ y = 100 \left( \frac{B_1B_2x(1 + B_3x)^{B_5-1} + (1 + x)(B_5-1)}{(B_3(1 + B_2x)^{B_5} + (1 + x)^{B_5})} \right) \]
where \( B_1, B_2, \) and \( B_3 \) are constants.

Results

Following epithelial scraping, an initial lag period of 10–15 hrs was observed before the conjunctival epithelium began to migrate covering the denuded cornea. In the second phase (15–48 hrs) the control and Sorbinil-treated galactosemic rat corneas appeared to heal more rapidly than the untreated galactose rats.

Normal and Sorbinil-treated galactosemic rat corneas appeared to be completely re-epithelialized by 54 hr, while the untreated galactosemic cornea healed by 60 to 72 hrs. The healed corneas of the normal and Sorbinil-treated galactosemic rats were clear in appearance while the healed corneas of the untreated galactosemic rats appeared edematous and hazy (Fig. 1).

In order to quantitate the observed corneal healing rate, computer planimetry techniques using available NIH digitizing software were employed. This basically consisted of generating a series of curves (x,y coordinates) representing the outlines of corneal limbus and healed areas entered into the computer by tracing polaroid photographs over a graphics tablet. Once entered into the computer, the areas bound by these curves could be determined by integration. The feasibility of this method was initially investigated by partially denuding normal rat corneas using no 2, 3, or 4 trephines to delineate 2, 3, and 4 mm areas in the corneal surface. Photographs of these denuded corneas were then taken by holding the rats at an approximate 90°, 45°, or 30° angle to the lens of a Kowa Fundus Camera. Analysis of the areas, expressed as % area denuded, as shown in Table 1, indicates that this method yielded reproducible results with little error. Moreover, the photographic angle appeared to have no significant effect in the calculated % area denuded—an important point since eye movements of the rats in subsequent experiments could not be readily controlled.

### Table 1. Reproducibility of computer planimetry: rat corneas were treated with a trephine and areas enclosed were denuded

<table>
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<tr>
<th>Trephine number</th>
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<th>Total mean</th>
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\* n = 4-6.

Using this method the rate of corneal healing expressed as % area healed at 0, 17, 24, 48, 72, and 96 hrs was obtained for normal, Sorbinil-treated, and untreated galactosemic rats. From the means of each group, s-shaped healing curves were generated by fitting the observed data to the general allosteric equation of Monod, Wyman, and Changeux.6

As shown in Figure 2, similar healing curves were generated for the normal and Sorbinil-treated galactosemic rats, while a similar healing curve displaced to the right was generated for the untreated galactosemic rats. The similar shape of all three curves indicate similar healing rates in all three groups while
rightward displacement of the untreated galactosemic healing curves indicates an initial delay in the onset of healing. Statistical analysis at 17, 24, and 48 hrs indicated significantly less area healed in the galactosemic rats compared to either the normal or Sorbinil-treated rats (P < .05). No significant differences in the % area healed between the normal and Sorbinil-treated rats could be observed.

Differences between the untreated corneas of galactosemic rats and either the treated or normal rats could also be microscopically detected. Scanning electron microscopy (SEM), especially at 15–24 hrs after scraping, revealed numerous rufflings and pseudopodia formation at the healing margins of the normal or Sorbinil-treated corneas (Fig. 3A). The corneas of the untreated galactosemic rat, however, had cells with little ruffling and pseudopodia formation, and in some instances the cells appeared flat (Fig. 3B). No difference in the surfaces of completely healed corneas of either the normal, treated, or untreated galactosemic rats could be distinguished by SEM.

Light microscopy of the corneas of untreated galactosemic rats showed the presence of leukocytes in both the corneal surface and stroma 15–24 hrs after initial scraping. Moreover, upon healing, light microscopy revealed these corneas to be more edematous than the control or Sorbinil-treated rats. The corneal stroma of these edematous corneas appeared to be thicker with the epithelium containing wider intercellular spaces (Fig. 4).
Discussion

When the corneas of galactosemic rats are denuded by limbus to limbus scraping, a significant delay in the rate of re-epithelialization compared to normal rat corneas is observed. Moreover, the healed corneas of these galactosemic rats appear edematous and hazy compared to normal rats. Oral treatment of the galactosemic rats with the AR inhibitor Sorbinil prevents both the observed healing delay and cloudy appearance of the galactosemic corneas. These results are similar to those previously observed in diabetic rats.

Healing rate curves were constructed through regression analysis of the % area healed at various time points obtained through computer planimetry. These resulted in three similar s-shaped curves of similar healing rates with a rightward displacement of the untreated galactosemic curve indicating an initial delay in the onset of healing (Fig. 2). Significantly less ($P < 0.05$) healing was observed at 17–24 and 48 hrs in the galactosemic vs either normal or Sorbinil-treated rats.

Scanning electron microscopy of the healing margin of normal and Sorbinil-treated galactosemic rats at onset of healing (15–24 hrs after scraping) revealed cells characterized with numerous rufflings and pseudopodia as described by Buck et al. On the other hand, the healing margin in the untreated galactosemic rat corneas had cell with little or no ruffling and pseudopodia before healing. Light microscopy of the
healed corneas revealed that untreated galactosemic corneas were more edematous than either the Sorbinil-treated or normal corneas with thicker stromas and wider intercellular spaces in the epithelium.

The fact that epithelial abnormalities can be demonstrated in galactosemic as well as diabetic rats and that these abnormalities can be corrected with several different aldose reductase inhibitors strongly suggest that aldose reductase is involved in the diabetic epithelial defect of these animals. Moreover, this delay in corneal re-epithelialization in diabetic rats appears dependent on the blood glucose levels with levels of 600–700 mg/dl necessary for significant delays. This may explain in part the failure to observe these effects in diabetic rabbits.

The findings, that delay in re-epithelialization can be shown in both diabetic and galactosemic rats and that various AR inhibitors can correct the defect, suggest that AR participates in the development of the diabetic corneal abnormalities.

Key words: galactosemic corneas, re-epithelialization, aldose reductase, inhibitors, computer planimetry
Figs. 4A-B. Light microscopy of re-epithelialized galactosemic rat corneas. A, the corneal stroma in untreated rats appear thicker and the epithelium contains wider intercellular spaces. B, the corneas of Sorbinil-treated rats appear similar to the normal controls (plastic methacrylate embedding, Toluidine blue, X360).

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References