Diabetes mellitus and the rabbit corneal epithelium

Judith Friend, Timothy C. Kiorpes, and Richard A. Thoft

Diabetes mellitus has been shown to be a factor in the development of corneal epithelial abnormalities in stressed human eyes, but the biochemical basis for this is not known. To see if sorbitol pathway activation might be involved, ocular surface epithelial healing rates and metabolites of the glycolytic and sorbitol pathways were measured in alloxan-diabetic rabbits. As in humans, corneal epithelial healing rates were not decreased in the diabetic rabbits, suggesting that the rabbit may be an appropriate model for human disease. Increased levels of glucose, glycogen, and sorbitol were found in the diabetic corneal epithelium compared with normal. However, the sorbitol accumulation only mounted to 1.0 mOsm/L of tissue water, which implies that osmotic damage secondary to corneal epithelial cell sorbitol accumulation might not be a significant factor in corneal epithelial abnormalities of diabetes.

Key words: diabetes, sorbitol pathway, conjunctiva, cornea, rabbit, ocular surface epithelium, epithelial healing rates

The existence of corneal epithelial abnormalities in patients with diabetes mellitus has been reported several times in the past.1-5 Persistent and recurrent epithelial defects, vascularization, and superficial punctate staining have all been observed in diabetic patients, particularly after ophthalmic surgical procedures. In diabetic animals, on the other hand, few corneal abnormalities have been observed. Decreased epithelial healing rates in diabetic and galactosemic rats compared with those in normal animals have been reported,6,7 but a study in humans showed corneal epithelial healing rates to be normal.8 This discrepancy between the human and rat re-epithelialization in diabetes in part prompted this investigation of diabetic rabbit epithelium.

The mechanism responsible for corneal epithelial changes in diabetes may be similar to that postulated for other tissues. In lens and nerve, for example, it has been shown that activation of the sorbitol pathway may be involved in the tissue damage seen in diabetes. In the sorbitol pathway, glucose is metabolized to sorbitol by the enzyme aldose reductase. This is followed by conversion of the sorbitol to fructose with polyol dehydrogenase. This pathway may not be very active at normal tissue levels of glucose, but when excess glucose is present in the tissues, saturating the glycolytic pathway enzymes (e.g., hexokinase), the sorbitol pathway may produce significant amounts of sorbitol. Because cell walls are impermeable to sorbitol, the molecule accumulates intracellularly, causing an osmotic imbalance that may lead to cell swelling and disruption. The role of the sorbitol pathway...
Previous investigations have shown that the sorbitol pathway is present in rabbit and rat corneal epithelium and that there is an elevation of sorbitol in diabetic human corneal epithelium as compared to normals. In addition, aldose reductase inhibitors (which decrease sorbitol production) restore epithelial healing to normal in diabetic and galactosemic rats. It seems possible therefore that the human corneal epithelial abnormalities in diabetics might be related to activation of the sorbitol pathway.

The purpose of the work to be reported here is (1) to measure corneal epithelial healing rates in diabetic rabbits, (2) to assess the role, if any, of metabolites of the sorbitol pathway in corneal epithelial disease in diabetic rabbits, and (3) to determine if enough similarities to human diabetic corneal epithelial observations exist to make the rabbit a good experimental model for the human disease.

**Methods**

**Animal preparation.** Forty-seven rabbits, weighing 2 to 3 kg, were made hyperglycemic with alloxan. After intramuscular chlorpromazine hydrochloride–ketamine hydrochloride anesthesia, intravenous alloxan was injected into the peripheral ear vein. The initial dose was 30 mg/kg, followed 1 week later by 110 mg/kg if the blood sugar of the rabbit was less than 200 mg/dl. Blood glucose levels were monitored weekly. Thirty-three (70%) of the rabbits had blood sugar levels of 200 mg/dl or more for 2 successive weeks and were used for experiments. Of the remainder, 11 did not become hyperglycemic and three died.

**Tissue preparation.** The corneal epithelium was scraped off with a No. 15 blade, frozen on the blade in liquid nitrogen, lyophilized, and weighed. Glycogen, or glucose, fructose, and sorbitol, were measured in single epithelial samples as described below. Animals were left 1 hr without food before samples were taken.

**Hydration.** In some cases the epithelium was weighed before as well as after lyophilization. The wet and dry weights were used to calculate epithelial hydration (H = mg H2O/mg dry weight).

**Metabolite analysis.** The amount of glycogen was measured by the hexokinase method after extraction of the glycogen in absolute alcohol and hydrolysis in 2N sulfuric acid for 90 min. Glycogen is expressed as micromoles of glucose per gram dry weight of tissue.

Glucose, fructose, and sorbitol were all measured in single epithelial samples by gas-liquid chromatography. Samples were extracted in water, deproteinized with chloroform:methanol, then dried. Dried samples were derivatized for 90 min with 200 \( \mu l \) of a trimethylsilyl reagent with pyridine (Sigma Sil A), followed by redrying and resuspension in hexane to a final volume of 25 \( \mu l \). Injection volume was 5 to 10 \( \mu l \). The gas chromatography was performed on an FID Hewlett-Packard 5830 with a 6 foot SE-30 column as described previously.

Inositol (10 nmol) was added to each sample as an internal standard. Levels of glucose, fructose, and sorbitol in the tissue are expressed as amol per gram dry weight of tissue.

Blood glucose was measured in whole blood samples collected from rabbits that had been 1 hr with no food. The hexokinase reaction after deproteinization in perchloric acid and neutralization was used to measure the glucose.

**Functional analyses**

**Epithelial healing rates.** These were determined by planimetry of standardized successive photographs of the epithelial defects at various times after scraping. Defects were stained with Richardson's stain (1% methylene blue: 1% Azure II, 1:1). In some cases, only an 8 mm diameter area of central corneal epithelium was removed, in which case the healing rate measured was that of the corneal epithelium. In other cases, the entire cornea was scraped limbus to limbus, in which case the healing rate measured was that of conjunctival epithelium. Twenty-one diabetic and 12 normal eyes were scraped centrally, and 10 diabetic and eight normal eyes were scraped limbus to limbus.

**Appearance.** The twenty-one diabetic and 12 normal rabbit eyes after central scraping, and the 12 diabetic rabbit eyes with corneas that had not been touched, were followed by slit-lamp and

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**Table I. Blood glucose levels (mg/dl) in normal and diabetic rabbits**

<table>
<thead>
<tr>
<th></th>
<th>Normal 84 ± 3 (11)</th>
<th>Diabetic 291 ± 19 (27)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>p</strong></td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Values are averages ± S.E.M.; number of determinations in parentheses.
Table II. Corneal epithelial metabolites (μmol/gm dry weight) in normal and diabetic rabbits

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Normal</th>
<th>Diabetic</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>217 ± 8 (11)</td>
<td>337 ± 17 (12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose</td>
<td>9 ± 1 (8)</td>
<td>53 ± 7 (9)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.7 ± 0.4 (3)</td>
<td>3.8 ± 0.1 (9)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.3 ± 0.2 (3)</td>
<td>4.2 ± 0.9 (8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hydration</td>
<td>3.2 ± 0.3 (12)</td>
<td>3.1 ± 0.1 (18)</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are averages ± S.E.M.; number of determinations in parentheses.

Table III. Epithelial healing rates (mm²/hr) in normal and diabetic rabbits

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normal</th>
<th>Diabetic</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central defect (8 mm diameter):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.89 ± 0.05 (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.13 ± 0.06 (21)</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total defect:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.76 ± 0.11 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.93 ± 0.11 (10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are averages ± S.E.M.; number of determinations in parentheses.

Results

Blood glucose levels in the diabetic animals immediately before the corneas were used for biochemical analyses or for studies of epithelial healing rates averaged 291 mg/dl, which is about 3½ times the normal level (Table I).

Epithelial metabolites. As shown in Table II, glycogen levels were 1½ times normal, glucose was 5.8 times normal, sorbitol at least 5 times normal, and fructose 14 times normal. The epithelial hydration, on the other hand, was normal.

Functional analyses

Healing rates (Table III). If a central defect was made and the wound was covered by epithelium of corneal origin, the epithelial healing rates of the diabetic eyes were significantly faster than those of the normal eyes (p < 0.01). If, on the other hand, the corneas were being covered with epithelium of conjunctival origin, the normal and diabetic healing rates were not significantly different (Table III).

Appearance. Neither the diabetic nor the normal eyes showed any signs of defect formation, vascularization, or superficial punctate keratopathy in the month they were followed, whether or not the central corneal epithelium had been scraped.

Discussion

Metabolic changes of the rabbit corneal epithelium have been demonstrated in this rela-

Glasslight examination for 1 month to see if epithelial abnormalities such as defects, punctate staining with fluorescein, or vascularization occurred.

However, as discussed above, it has been suggested that accumulations of sorbitol pathway products, especially of sorbitol, might cause osmotic cellular damage resulting in corneal epithelial complications in diabetes. To evaluate this possibility, the total osmotic change expected from sorbitol and fructose accumulation in the diabetic rabbit corneal epithelium (7 μmol/gm dry weight of tissue, Table II) was calculated and found to be approximately 2.3 mOsm/L of tissue water. In other diabetic tissues, the osmotic contribution by these molecules has been found to be considerably higher. In diabetic rat lenses, for example, where polyol increases have been shown to be osmotically significant, sorbitol levels of 30 mOsm/L of tissue water have been found. The osmotic effect in rabbit corneal epithelium is therefore much more pronounced.
Table IV. Epithelial healing in diabetes

<table>
<thead>
<tr>
<th>Animal</th>
<th>Source of epithelium</th>
<th>Healing rate vs. normal</th>
<th>Blood glucose (mg/dl)</th>
<th>Epithelial sorbitol*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>Conjunctiva</td>
<td>Depressed</td>
<td>500 to 600</td>
<td></td>
</tr>
<tr>
<td>Rabbits</td>
<td>Conjunctiva</td>
<td>Same</td>
<td>200 to 300</td>
<td></td>
</tr>
<tr>
<td>Rabbits</td>
<td>Cornea</td>
<td>Increased</td>
<td>200 to 300</td>
<td>1.2</td>
</tr>
<tr>
<td>Humans</td>
<td>Cornea</td>
<td>Same*</td>
<td>150 to 200</td>
<td>ca0.3</td>
</tr>
</tbody>
</table>

*Expressed as mOsm/L of tissue water.

Table IV. Epithelial healing in diabetes

less and would seem not to be high enough to cause a significant osmotic imbalance. The possibility still exists that the small increase in sorbitol might be significant if it were confined to only some of the cells or if it interfered directly with cell function, perhaps by altering pyridine nucleotide ratios.

We were unable to detect any functional abnormalities in these diabetic rabbit eyes in terms of increased corneal epithelial breakdown or vascularization. This is in direct contrast to the situation in humans, where it has been shown that stressed eyes in long-term controlled diabetics show an increased frequency of epithelial abnormalities.1-5 In diabetic rats, as in humans, functional abnormalities of the surface have been noted. Stressed diabetic rat corneas, healing after total scraping, were cloudier than were normal corneas after scraping,7 which may have been related to increased stromal or epithelial edema.

One indicator of function that has been measured in diabetes is the rate of healing of the ocular surface epithelium (Table III). We found the normal corneal epithelial healing rate was 0.89 mm²/hr, which is comparable to previously reported values.21, 23, 24 In the diabetic animals, however, we found an increased corneal epithelial healing rate. Measurement of healing rates of conjunctiva over cornea is subject to great variation,24 but in the present study, conjunctiva in diabetic rabbits heals at approximately the same rates as in normal rabbits. Because increased levels of glucose and glycogen are found in diabetic corneal epithelium and because those compounds are important in epithelial metabolism and healing,25 an increased healing rate might not be unexpected. This finding of normal to increased corneal epithelial healing rates in rabbits conflicts with the reports of decreased rates in diabetic rats.6, 7 It agrees, however, with the observation that diabetic humans have normal epithelial healing rates.8

What, then, is the possible role of the sorbitol pathway in diabetic changes in these three species (Table IV)? In diabetic rats, not only is the corneal epithelial healing rate decreased, but it can also be returned to normal by treatment with topical aldose reductase inhibitors (drugs that prevent the accumulation of sorbitol). This implies a significant role of the sorbitol pathway in surface abnormalities in diabetic rats.6, 7, 14 To date, however, little accumulation of sorbitol pathway products in diabetic rat corneal epithelium has been demonstrated. In humans and rabbits, on the other hand, the epithelial healing rates are normal to high, with only the humans showing abnormalities in the quality and persistence of the epithelium. Neither the rabbit nor the human shows epithelial levels of sorbitol and fructose that might be expected to have an osmotic effect. These observations are summarized in Table IV.

There are at least three possible explanations for these differences. First, there may be species differences. It is possible, for example, that the enzymes of sorbitol pathway in the rat corneal epithelium are significantly more active than in that of the humans and rabbits. Although this might account for the low accumulation of sorbitol in the rabbits and humans (Table II),5, 15 Graham et al.9 (personal communication) also found little sorbitol accumulation in diabetic rat cornea, suggesting that the sorbitol pathway is not particularly active in that tissue either.

*Prof. Charles R. Graham, Loyola College, Baltimore, Md., Chairman, Baltimore Eye Bank.
What may be of greater importance, however, is the difference in the blood glucose in the three species. It must be noted that the rats were significantly more hyperglycemic than the humans or rabbits. The rats only had about 290 mg/dl whereas the rat levels were 500 to 600 mg/dl (Table IV). The humans were well-controlled patients with blood sugar levels in the 150 to 200 mg/dl range. Whether the difference in healing rates only reflects the substantial difference in blood sugar or a real metabolic difference among the species has not been resolved.

Another complicating factor is the acute nature of the diabetes in rats and rabbits, contrasted to the ongoing, albeit controlled, diabetes of many years' duration in humans. Despite its acute nature, however, the rabbit model more closely mimics the controlled human diabetic situation. Information derived from the rabbit experiments, coupled with the clinical observations in humans, provides little evidence that the accumulation of sorbitol plays an important role in human diabetic corneal epithelial complications.

We thank Jin H. Kinoshita, Ph.D., for helpful discussions and Benigno Pecezon, Ph.D., for the use of the Hewlett-Packard 5830A gas chromatograph.

REFERENCES