Reduction of Basement Membrane Thickening in Diabetic Cat Retina by Sulindac

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Capillary basement membrane thickening is one of the earliest histologic lesions in diabetic retinopathy. Its pathogenesis is not understood; however, recent evidence suggests that aldose reductase may play a role. In this study, a new animal model, the diabetic cat, was used to determine whether retinal capillary basement membrane thickening occurred early in the course of hyperglycemia, and if so, whether it could be inhibited by sulindac, an aldose reductase inhibitor. Retinal capillary basement membrane thickness was significantly increased in diabetic cats compared to normal cats (114 ± 15 vs 72 ± 12 nm, mean ± SEM). Basement membrane thickness was significantly less in sulindac-treated diabetic cats (93 ± 9 nm) compared to the untreated diabetic cats (114 ± 15 nm). In addition, quantitation of endothelial cells, pericytes, and contacts between endothelial cells and pericytes in electron micrographs revealed that they were not reduced in number in untreated diabetic cats compared to normal or sulindac-treated diabetic animals. Invest Ophthalmol Vis Sci 31:457-463, 1990

Although diabetic retinopathy is one of the leading causes of blindness in the world, its pathogenesis is little understood. Animal models are useful because they can provide tissue for study at all stages of the disease and can be used for therapeutic trials involving drugs or procedures that are potentially hazardous to patients. At the present time, however, animal models for the study of diabetic retinopathy are scarce. Therefore, we developed a short and technically simple procedure for rendering cats diabetic by partial pancreatectomy alone or combined with local injection of alloxan into the artery supplying the remaining bit of pancreas.¹ We chose cats for this (and other studies) because their eyes are large enough for surgical and experimental manipulations and have been used extensively for studying retinal physiology. In addition, cats are less expensive to maintain than are dogs or monkeys and live longer than rodents, allowing us to follow the progress of the disease for many years. Lastly, diabetic cats do not develop cataracts, such that continuous visualization of the fundus is possible.²

Retinal capillary basement membrane thickening is one of the earliest histologic lesions in diabetic retinopathy and is considered the fundamental structural lesion of the small blood vessels and the ultrastructural hallmark of diabetic retinopathy.³ Basement membrane thickening in the retinal microvasculature was first described with the light microscope by Ashton⁴ and by Friedenwald 5±6 40 years ago. Later, numerous electron microscopic studies demonstrated that the lamina densa of the retinal capillary basement membrane is increased in thickness in diabetic humans,⁷ dogs,⁸±10 rats,¹¹±¹³ and galactosemic dogs¹⁴ and rats.¹⁵±¹⁷ Recent evidence suggests that aldose reductase may play a role in basement membrane thickening.¹⁵±¹⁸ The purpose of this study was to determine if basement membrane thickening occurs in the retinal capillaries of diabetic cats, and if so, whether it could be prevented by oral administration of the aldose reductase inhibitor sulindac. Sulindac was selected because it is a widely used antirheumatic drug with established clinical safety in humans.

Materials and Methods

Animals

Sixteen adult cats weighing 4.5–5.5 kg were used in these studies. Thirteen were rendered diabetic by partial pancreatectomy by the simple surgical procedure developed in our laboratory.¹ All of the diabetic cats were kept in poor metabolic control by a single daily injection of protamine zinc insulin I (PZI insulin (Eli Lilly, Indianapolis, IN) in the morning. Blood glucose was measured at least twice a month in the morning before the insulin injection. The insulin
dose for each cat was adjusted as needed to keep the morning blood glucose level between 300 and 400 mg/dl. There was no significant difference between the blood glucose levels of the sulindac-treated diabetic cats and the untreated diabetic cats (386 ± 23 vs 340 ± 32 mg/dl respectively; mean ± SEM). We attempted to measure glycosylated hemoglobin by standard laboratory methods, but spurious results were obtained, probably because there are two major hemoglobins in variable amounts in each erythrocyte in cat blood.\textsuperscript{19} All of the animals were allowed dry cat food ad lib, and were given canned cat food at the time of the insulin injections. A powdered pancreatic enzyme supplement was added to the canned food fed to the diabetic cats.\textsuperscript{1}

Four of the diabetic cats were given sulindac orally in a single daily dose of 10 mg/kg body weight as soon as hyperglycemia was established (within 1–2 weeks after pancreatectomy). One sulindac-treated cat was euthanized at each of the following intervals after the beginning of treatment: 3, 5, 8, and 10 months. Six untreated diabetic cats were euthanized after 3 (2 cats), 5 (2 cats), 8 (1 cat), or 10 (1 cat) months’ duration of hyperglycemia. The cats were first anesthetized with ketamine hydrochloride (22 mg/kg body weight) and acepromazine (1 mg/kg body weight) IM, and then killed by intracardiac injection of an overdose of sodium pentobarbital. All experiments with these animals were carried out according to the ARVO Resolution on the Use of Animals in Research.

Tissue Processing

Both eyes of each cat were enucleated quickly, cut at the equator, and immersed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. On the 2nd day, the eyes were carefully dissected to provide 8–10 strips of tissue from the central and midperipheral retina. These strips were postfixed in osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in low-viscosity embedding medium for electron microscopy. Thick sections were cut for light microscopy and were used to select areas for thin sectioning. Ultrathin sections of retina were cut on an ultramicrotome and stained with lead citrate and uranyl acetate.

**Electron Microscopy**

Forty retinal capillaries from each cat (20 from the superficial capillary net and 20 from the deep capillary net) were photographed at 10,000× using a JEOL (Peabody, MA) 1200EX transmission electron microscope.

**Computerized Image Analysis**

A LeMont (State College, PA) OASYS video-input image analyzer was used for image analysis. Electron micrograph negatives were placed on a fluorescent light box, and a well focused image was obtained on the video monitor using the video camera. The magnification of the image was calibrated with a micrometer. The image was transmitted to the imaging board, which converted the analog input signal to a digital array. The array was stored in a high-speed random access memory (RAM) buffer which permitted computer access to the stored image. The image array was 512 × 480 pixels, and 256 different gray levels could be distinguished for each pixel by the

<table>
<thead>
<tr>
<th>Cats</th>
<th>Duration of diabetes (months)</th>
<th>Baseline membrane thickness (nm) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Both capillary nets</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>59 ± 7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>72 ± 2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>84 ± 9</td>
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<tr>
<td></td>
<td>3</td>
<td>72 ± 12</td>
</tr>
<tr>
<td>Untreated diabetic</td>
<td>3</td>
<td>94 ± 26</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>119 ± 25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>104 ± 19</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>109 ± 17</td>
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<tr>
<td></td>
<td>10</td>
<td>121 ± 24</td>
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<tr>
<td></td>
<td></td>
<td>138 ± 21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>114 ± 15</td>
</tr>
<tr>
<td>Treated diabetic</td>
<td>3</td>
<td>101 ± 16</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>99 ± 13</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>88 ± 17</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>83 ± 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93 ± 9</td>
</tr>
</tbody>
</table>

Last row of data in each group is the mean ± SEM for that group.
The magnification of the digital image was calibrated with a micrometer. A digitizer tablet integrated in the analyzer was used to trace first the inner and outer borders of the basement membrane of each capillary. The space between the borders was filled with a set gray level value that did not appear elsewhere in the image. This level was then pseudocolored (blue) for display. Data collected from the image analyzer consisted of the percent area of the screen occupied by the basement membrane borders and the percent area of the screen occupied by the space between the borders.

Calculations

Percent-area data from the image analyzer were manually entered into a spreadsheet and database program (Knowledgeman; Micro Data Base Systems, Lafayette, IN) on an IBM PC/XT computer (Boca Raton, FL). The final magnification of the image was first determined and a value for the length of the pixel was calculated.

Basement membrane length (L) was calculated by the following formula:

$$ L = \frac{(\text{total screen area} \times \text{percent area of line} \times 0.01)}{2} $$

Basement membrane thickness (W) was calculated by the following formula:

$$ W = \frac{\text{basement membrane area}}{\text{basement membrane length}} $$

Electron microscopy and image analysis were done by one masked investigator. Statistical analysis was done with the student t-test.

Reproducibility of the Method

The reproducibility of the method of image analysis was tested first by analyzing ten retinal capillaries on 3 separate days. Then each of another ten capillaries were analyzed three times consecutively on 1 day. Basement membrane measurements were compared
by calculating the relative coefficient of variation (CV). The mean CV of basement membrane measurements that were repeated on successive days was 4.8%, and that of measurements done on the same day was 2.6%.

Quantitation of Retinal Capillary Cells and Endothelial Cell Contacts with Pericytes

The number of endothelial cells, of pericytes (or pericyte processes), and of contacts between endothelial cells and pericytes was counted on at least 33 electron microscopic negatives from each cat retina. Statistical analysis was done with the student t-test.

Results

Basement Membrane Measurements

There was a significant increase in retinal capillary basement membrane thickness in untreated diabetic cats as compared to normals (114 ± 15 nm vs 72 ± 12 nm, mean ± SEM; P < 0.04) (Table 1). An increase was observed at all time periods studied in both the superficial and deep capillary nets of diabetic animals (Table 1).

A significant decrease in retinal capillary basement membrane thickness was evident in sulindac-treated diabetic cats as compared to untreated diabetics (93 ± 9 nm vs 114 ± 15 nm; P < 0.04) (Table 1).

There was no significant difference in basement membrane thickness between the superficial and the deep capillary nets of the retina in normal or diabetic cats (Table 1).

Electron Microscopy

The majority of thickened basement membranes in diabetic cats examined by electron microscopy showed homogeneous thickening without glycogen accumulation, vacuolization, inclusions, or lamination of the basement membrane (Figs. 1, 2). Loss of endothelial cells or pericytes could not be demonstrated by quantitative analysis from the electron mi-
Table 2. Comparison of cell numbers and contacts between retinal capillary endothelial cells and pericytes

<table>
<thead>
<tr>
<th>Cats</th>
<th>No. of eyes (N)</th>
<th>No. of endothelial cells</th>
<th>No. of pericytes</th>
<th>No. of endothelial cell-pericycle contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>1.01 ± 0.01</td>
<td>3.38 ± 0.62</td>
<td>3.04 ± 0.05</td>
</tr>
<tr>
<td>Untreated diabetic</td>
<td>6</td>
<td>1.02 ± 0.05</td>
<td>4.23 ± 0.78</td>
<td>2.63 ± 0.50</td>
</tr>
<tr>
<td>Sulindac-treated</td>
<td>4</td>
<td>1.01 ± 0.01</td>
<td>3.67 ± 0.31</td>
<td>2.07 ± 0.55</td>
</tr>
</tbody>
</table>

All numbers are mean ± SEM. There are no statistically significant differences between groups at the 95% confidence level.

crographs. There was no difference in the number of cells or the number of contacts between endothelial cells and pericytes in diabetic cats compared to normal cats (Table 2). In sulindac-treated diabetic animals, the retinal capillary basement membranes were visibly thinner than in the untreated diabetic cats (Figs. 3, 4), but there was no difference in the number of endothelial cells, pericytes, or contacts between endothelial cells and pericytes, in treated and untreated cats (Table 2).

Discussion

Significant thickening of the retinal capillary basement membrane was present in diabetic cats as early as 3 months after the onset of hyperglycemia. A linear increase in basement membrane thickness over time (5-10 months) was demonstrated in the four untreated diabetic animals (Table 1). Retinal capillary basement membranes progressively increase in width with increasing duration of galactosemia in rats.15,16,18
and hyperglycemia in spontaneously diabetic BB-rats, and with aging in normal rats. In the latter study, the basement membrane was also thicker in the superficial capillary net. We found no significant difference between basement membrane thickness in the two retinal capillary nets in normal or diabetic cats.

The pathogenesis of capillary basement membrane thickening in diabetes remains unexplained. Possible mechanisms include nonenzymatic glycosylation of basement membrane collagen, enzymatic glycosylation of hydroxylysine residues, impaired proteoglycan synthesis with a compensatory increase in synthesis of other basement membrane components, or increased aldose reductase activity.

We showed that an aldose reductase inhibitor, sulindac, caused a significant decrease in retinal capillary basement membrane thickness in treated as compared to untreated diabetic cats, but not a decrease to normal levels. The effect of sulindac was greater after 8 and 10 months of treatment compared to the effect at earlier time points.

Sulindac was selected for use in this study for several reasons. First, it is a widely used antirheumatic drug (Clinoril), and its clinical safety is established.

Second, its potency as an aldose reductase inhibitor has been demonstrated. For example, it significantly decreases sorbitol formation in lens and nerve tissue incubated in high glucose media. Third, it has been shown to have a significant beneficial effect on the breakdown of the blood-retinal barrier in patients with IDDM and minimal or no retinopathy (as assessed by vitreous fluorophotometry after 6 months of oral treatment). The dosage in that study was approximately 5.7 mg/kg body weight per day (assuming a body weight of 70 kg) given in two 200-mg doses per day. Based on that study, we administered 10 mg/kg body weight per day to our cats in one dose per day. Higher or more frequent doses may have a greater effect. To our knowledge, other metabolic or physiologic effects of sulindac in cats have not been reported, but further studies are planned in our laboratories.

Because sulindac has other effects (e.g., antiinflammatory) besides aldose reductase inhibition, the possibility remains that the decrease in basement membrane thickening was by means of some other mechanism; however, prevention of retinal capillary basement membrane thickening in diabetic and galactosemic rats by several other aldose reductase in-
thickening of retinal capillary basement membranes was prevented by the concomitant daily administration of the aldose reductase inhibitor sorbinil$^{15,24}$ and by the structurally unrelated aldose reductase inhibitor tolrestat.$^{18,24}$ In another study, the aldose reductase inhibitor dl-spiro-(2-fluorofluoren-9'4'-imidazoline)-2's-dione (AI 1567) prevented retinal capillary basement membrane thickening in streptozotocin-diabetic rats.$^{17}$

A subjective observation in our study was that basement membrane thickening separates the pericytes from the endothelial cells and decreased the number of contacts between the two cell types; however, quantitative analysis of the contacts did not support this observation. Robison and Nagata have reported decreased cell contacts in galactosemic rats.$^{27}$ Orlidge and D'Amore have shown that pericytes inhibit endothelial proliferation in a coculture system when cellular contact is allowed, but not when the two cell types are prevented from making physical contact.$^{28}$ It was surprising to us that the number of cells and cell contacts was maintained even though the pericyte processes appear to be pushed away from the endothelial cells by the thickened basement membrane in diabetic cats (Fig. 2); however, Tilton et al also reported that capillary basement membrane thickening preceded any evidence of pericyte degeneration in diabetic rats that had been hyperglycemic for 9 months.$^{13}$

**Key words:** aldose reductase, basement membrane, cat, diabetic retinopathy, sulindac

**References**