chemical results of cultured monkey mural cells, provides evidence for the attractive hypothesis that the aldose reductase initiated accumulation of sorbitol may be involved in the selective degeneration of human mural cells in non-proliferative diabetic retinopathy.

Key words: aldose reductase, diabetes, retinopathy, mural cell, pericytes, microangiopathy, immunohistochemistry

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References

Galactose-induced Retinal Capillary Basement Membrane Thickening: Prevention by Sorbinil

Robert N. Frank, Richard J. Keirn, Alexander Kennedy, and Karin W. Frank

Normotensive and spontaneously hypertensive rats fed a 30% galactose diet until 15–21 months of age developed significant (P < 0.05) retinal capillary basement membrane thickening, compared with animals fed a standard test diet. Animals on the high galactose diet containing 250 mg/kg of the aldose reductase inhibitor, Sorbinil, did not develop basement membrane thickening. No cytologic abnormalities of pericytes or endothelial cells were noted, and pericyte:endothelial cell nuclear ratios did not differ in the various experimental groups. This model reproduces a characteristic lesion of diabetes mellitus in non-diabetic animals, and should facilitate study of the biochemical mechanisms of basement membrane thickening. Invest Ophthalmol Vis Sci 24:1519–1524, 1983

Thickness of capillary basement membranes is characteristic of diabetes mellitus.12 Its pathogenesis, however, remains unexplained. We have been attempting to develop a model of diabetic retinopathy in the laboratory rat by a number of methods, including the production of galactosemia by long-term feeding of a diet enriched in galactose, and the induction of diabetes or galactosemia in spontaneously hypertensive (SHR) rats. In the course of these experiments, we discovered that the prolonged (15–21 months) feeding of a high galactose diet produced significant thickening of retinal capillary basement membranes in both normotensive and hypertensive rats. The basement membranes not only were thickened, but also frequently demonstrated focal abnormalities that have been described previously in human subjects with diabetic retinopathy. Simultaneous administration of the aldose reductase inhibitor, Sorbinil (d-6-fluorospiro[chroman-4,4'-imidazoline]-2',5'-dione; CP-45,634, Pfizer), prevented the abnormality. This animal model should be useful in the study of the mechanism of capillary basement membrane thickening, and may have relevance to the pathogenesis of one of the most common vascular lesions of diabetes mellitus.

Materials and Methods. Normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) female rats were entered into the study at 6 weeks of age. Control animals were fed the standard Ralston Purina® test diet, in pellet form. Other rats were placed on a diet in which a portion of the carbohydrate (30% of the total weight of the diet) was replaced by galactose,
or the high galactose diet to which 250 mg/kg diet of Sorbinil (kindly provided by Dr. Michael J. Peterson, Pfizer Central Research, Groton, CT) had been added. Although the animals consumed different amounts of diet at different ages and weights, we did not measure food consumption, nor did we vary the concentration of the drug in the diet. Since a rough average daily food consumption for a rat is approximately 15 g/day, each animal in the galactose-plus-Sorbinil groups received about 3 mg of drug/day.

Animals received water and food ad libitum. They were weighed weekly, and systolic blood pressures were measured periodically by a tail cuff sphygmomanometer (Narco Biosystems, Houston, TX).

Rats were sacrificed after 15–21 months on the diet by etherization, followed by exsanguination by cardiac puncture. One eye was fixed in formalin for light microscopy histologic study and trypsin-digest preparations of the retinal vessels, while the anterior segment of the other eye was removed, the posterior segment was bisected through the optic nerve, and one-half was fixed in 3% glutaraldehyde buffered in 0.1 M cacodylate and the other half of the eye was processed for eventual use in other studies. Glutaraldehyde-fixed retinas were washed in buffer and secondarily fixed in 1% OsO4 in cacodylate buffer for 1 hour, washed again in buffer and distilled water, stained en bloc in 2% aqueous uranyl acetate, dehydrated, and embedded in Epon. Thin sections (cut to a silver interference color) were collected on grids and stained with uranyl acetate and lead citrate before viewing. Sections were prepared from the posterior retinas, within 2 mm of the optic nerve, and capillaries for measurement were taken from the inner nuclear and inner plexiform layers. At least 10 capillaries per eye, taken in equal numbers from at least two tissue blocks, were photographed at a standard microscope magnification of ×7,500 (checked daily by photographing calibration grids), and enlarged to a final magnification of ×18,000 when the negatives were printed. Prints were labeled with code numbers prior to measurement by an observer, who therefore was unaware of the animal from which they were taken. Measurements, to the nearest 0.1 mm, were made of the basement membrane surrounding the endothelial cell layer, and were taken perpendicular to the plane of the membrane. Basement membrane thickness was measured either by the “mean basement membrane thickness” method of Siperstein, in which the mean of 20 measurements is taken, where 20 equidistant radii originating from the center of the lumen intersect the endothelial basement membrane, or by the “minimum basement membrane thickness” method of Williamson, in which the two minimum measurements for each capillary are averaged, provided they are separated by at least 1 cm of circumference on the enlarged photomicrograph.

We modified these methods, first, by including for measurement basement membrane segments located between endothelial cells and pericyte somata or processes (Fig. 1A). This was done because, in many retinal capillaries, pericytes occupy a very large portion of the circumference of the vessel, thus leaving only a small region that could be measured if the methods for measurement were used as described originally. Second, we averaged measurements from individual vessels directly to derive the values presented in Table 1, rather than taking their logarithms, as was done by Williamson et al. This was done because histograms of measurements of individual vessels from each experimental group showed approximately normal distributions, with kurtosis between 3 and 4 and skewness between 0 and 0.5. Williamson’s data from populations of human subjects were highly skewed, necessitating the logarithmic transformation.

We did not measure basement membrane thickness in areas where focal lesions (fibrillar collagen deposits, vacuolization) were located.

Pericyte and endothelial cell nuclei were counted from the electron micrographs. We found trypsin-digest preparations of the rat retinal vascular tree difficult to evaluate, since unequivocal morphologic differences between pericyte and endothelial cell nuclei, such as are found in human retinal capillaries, are not seen.

**Results.** Basement membrane measurements are presented in Table 1. In both normotensive and hypertensive rats, basement membrane thickness was significantly greater in galactose-fed animals than in animals of comparable ages that received the control diet ($P < 0.05$, unpaired $t$-test). Animals that received galactose and Sorbinil had basement membrane thickness no greater than in the control group ($P = 0.5$) and significantly ($P < 0.05$) thinner than in rats fed the high galactose diet alone. Photomicrographs of representative capillaries are shown in Figure 1. We observed several vessels in the galactose-fed groups that demonstrated vacuolization or deposition of fibrillar collagen within the basement membranes (Figs. 1C, D), similar to those described in diabetic humans.

Counts of pericyte and endothelial cell nuclei (Table 1) demonstrated a pericyte:endothelial cell ratio of 1:3, with no statistically significant variation among the different experimental groups either by contingency table analysis, or by comparing all possible pairs of groups by the continuity-corrected chi$^2$ test. Serum glucose levels, measured just prior to sacrifice...
Fig. 1. Electron micrographs of representative retinal capillaries. (A) Capillary from a control (WKY) animal age 15 months. Thin arrows in this, and other photomicrographs, indicate the endothelial basement membrane. Open arrows indicate portions of basement membrane enveloping pericyte processes (P), which were not measured in this study. EN indicates an endothelial cell nucleus. The calibration bar applies to all parts of this figure, which are printed at the same magnification. (B) Uniform basement membrane thickening is evident in this capillary from a hypertensive rat after 15 months on the high-galactose diet. (C) A portion of a capillary from a 15-month, galactose-fed WKY rat showing extensive, focal basement membrane thickening and degeneration. Fibrillar collagen (C) is present within the basement membrane. RBC indicates an erythrocyte within the capillary lumen. (D) Portion of a capillary from a 21-month, galactose-fed WKY rat showing diffuse basement membrane thickening and “Swiss cheese” vacuolization (heavy arrows).
Table 1. Basement membrane thickness (BMT) and nuclear counts in electron micrographs of rat retinal capillaries

<table>
<thead>
<tr>
<th></th>
<th>ABMT (A) ± SD</th>
<th>MBMT (A) ± SD</th>
<th>PN</th>
<th>EN</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY Control (7)</td>
<td>1,604 ± 312</td>
<td>1,016 ± 172</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>WKY + 30% gal. (7)</td>
<td>2,033 ± 376</td>
<td>1,223 ± 181</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td>SHR Control (7)</td>
<td>1,594 ± 352</td>
<td>969 ± 202</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>SHR + 30% gal. (6,9)</td>
<td>1,594 ± 361</td>
<td>982 ± 195</td>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td>SHR + 30% gal. + Sorb. (8)</td>
<td>2,307 ± 623</td>
<td>1,362 ± 325</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>1,543 ± 435</td>
<td>973 ± 252</td>
<td>11</td>
<td>36</td>
</tr>
</tbody>
</table>

ABMT = Average (mean) BMT; MBMT = Minimum BMT; PN = Pericyte nuclei; EN = Endothelial nuclei. Numbers of animals in each group are given in parentheses after the description of the group in the first column. Further subdivision of each group by age is given in the caption of Figure 2.

by a glucose oxidase method (Glucostat, Worthington Chemical Co., Freehold, NJ) were 142 ± 28 mg/dl in control and 143 ± 32 mg/dl (mean ± SEM) in galactose-fed animals. There were no significant differences in any of the six subgroups shown in Table 1. Serum galactose levels, measured by a galactose oxidase procedure,7 were 65 ± 20 mg/dl in galactose-fed animals and 3 ± 1 mg/dl in controls, also with no significant differences in the various subgroups. Galactose-fed animals gained weight steadily, but at a lower rate than those on the control diet. Sorbinil did not affect the weight gain. Mean weights (±1 SEM) for the normotensive control group were 220 ± 11 g after 10 weeks in the study, 298 ± 3 g after 40 weeks, and 331 ± 4 g after 70 weeks. Comparable figures for the normotensive, galactosemic animals were: 209 ± 4 g (10 weeks); 257 ± 5 g (40 weeks); and 285 ± 28 g (70 weeks); for normotensive, galactosemic, Sorbiniltreated rats, 210 ± 4 g (10 weeks); 243 ± 4 g (40 weeks); and 282 ± 11 g (70 weeks). For the hypertensive animals, the trends over time and the intergroup relationships were similar, although SHR rats in each group were 30-40 g lighter at comparable ages than were the WKY animals. Although only a single measurement of serum glucose and galactose was carried out in each animal and we did not determine diurnal variations in these hexose levels, we believe that the intergroup comparisons are valid, since these were highly inbred animals maintained under identical environmental conditions. In particular, Sorbinil did not appear to alter the serum galactose levels, nor the dietary intake, since weight gain was virtually identical in the galactose and galactose-plus-Sorbinil groups. All of the galactose-fed animals that did not receive Sorbinil had developed grossly evident cataracts at the time of sacrifice; most had cataracts after four months on the diet. No cataracts were present in control animals. Two galactose-plus-Sorbinil-fed normotensive animals also had cataracts, but none were evident in the galactose-and-Sorbinil-fed hypertensive rats. Mean systolic blood pressure, measured within one month of sacrifice, was 85 ± 10 mmHg in the WKY animals and 150 ± 20 mmHg in the SHR rats. Galactose or Sorbinil feeding did not alter the blood pressure.

We measured basement membrane thickness in retinal capillaries of groups of four normotensive rats at ages 6, 9, and 12 months, and included data from these animals along with those of the 16.5- and 22.5-month-old normotensive controls (maintained on the experimental regimen for 15 and 21 months) in Figure 2. Consistent with the studies of Kilo et al in humans,8
and of Bloodworth, et al in rats and dogs, we find a steady increase in retinal capillary basement membrane thickness with age in our WKY control animals. Although the galactose-fed animals are of two different ages, their capillary basement membranes are substantially thicker than those of comparable controls (Fig. 2). For this reason, we felt it appropriate to average the galactose-fed animals are of two different ages, their capillary basement membranes are substantially thicker than those of comparable controls (Fig. 2). For this reason, we felt it appropriate to average the basement membrane data from the 15- to 21-month age groups in Table 1.

**Discussion.** These results demonstrate that prolonged feeding of galactose to rats, with resultant substantial elevation of serum galactose levels, produces retinal capillary basement membrane thickening markedly similar to that observed in diabetes mellitus. Although our method of measuring basement membrane thickness is somewhat at variance with technique used previously, we believe that this does not affect our conclusions. Measurement of basement membrane segments between endothelial cells and pericytes may cause the absolute values of the thicknesses to vary from those that would have been obtained using the methods of Siperstein or of Williamson, but should not affect the intergroup differences. Of particular importance is the fact that all measurements were made by an observer who was masked as to the identity of the capillaries being evaluated.

The calculation of pericyte-to-endothelial cell ratios using electron micrographs might be affected by consistent differences in length or volume, between pericyte and endothelial nuclei, as well as by their number. However, any bias introduced by this method should affect all of the experimental groups equally, and will not alter intergroup differences. Although we counted only relatively small numbers of nuclei in each experimental group, we believe our conclusion that the ratio of pericytes to endothelial cells in the retinal vessels is unaffected by a high galactose diet or by Sorbinil remains valid. The statistical method we used for the comparison is an adaptation of the \( \chi^2 \) test for cases when the sample size is relatively small. Although we did not use trypsin-digest preparations for nuclear counts because the identity of many of the capillary nuclei in rat retinal preparations was equivocal, we can state with certainty that pericyte "ghosts," indicative of pericyte dropout (and hence, a likely alteration in the nuclear ratio) were never observed in these preparations in any of the groups.*

Our results do not prove that the mechanism of galactose-induced capillary basement membrane thickening is identical to that which occurs in diabetes, but there are several reasons for believing this to be likely. Glucose and galactose enter several common metabolic pathways that are relevant to diabetes, including the "sorbitol pathway" and enzymatic bonding to basement membrane collagens, and nonenzymatic condensation with proteins. That an aldose reductase inhibitor, which prevents or retards certain other abnormalities of diabetes, also prevents galactose-induced capillary basement membrane thickening is of great interest, although its biochemical explanation is not yet apparent, since sugar alcohols have not been shown to play any role in basement membrane synthesis or degradation. Using this animal model, however, it should be possible to approach this problem experimentally.

**Key words:** capillary basement membrane thickening, aldose reductase, galactose, diabetic retinopathy, intramural pericytes, endothelial cells, morphometry

From the Kresge Eye Institute, Wayne State University School of Medicine, Detroit, Michigan. Supported by grants EY-01857 and EY-02566 from the National Eye Institute. Submitted for publication April 7, 1983. Reprint requests: Robert N. Frank, M.D., Kresge Eye Institute of Wayne State University School of Medicine, 3994 John R Street, Detroit, MI 48201.

**References**


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* A few calculations using the formula for the continuity-corrected \( \chi^2 \) value will show how much the pericyte-to-endothelial ratio must change before significant differences in the experimental groups appear. We compare here the cell ratios between the WKY control and WKY + galactose groups, and between the SHR control and SHR + galactose groups (Table 1). For the sake of brevity, we consider the extent to which the pericyte count must decrease (and the endothelial cell count must correspondingly increase) in the galactose-fed groups to produce a significant difference. These calculations permit an estimate of the validity of our conclusion that galactose feeding produces no significant pericyte dropout. A significant difference is first seen if the pericyte count for the WKY + galactose group decreases from nine to two nuclei, with an increase in endothelial nuclei from 30 to 37 (continuity-corrected \( \chi^2 = 4.9, P = 0.027 \)). Note that, if the conventional \( \chi^2 \) test were used, significance occurs if the pericyte count is decreased from nine to three, with the endothelial cell count raised from 30 to 36 (\( \chi^2 = 4.7, P = 0.031 \)). However, the continuity-corrected \( \chi^2 \) for this cell ratio is only 3.4, (\( P = 0.066 \)). For the SHR animals, reduction in the pericyte count in the galactose-fed group to only one nucleus, with the endothelial cell count raised to 32, yields continuity-corrected \( \chi^2 = 3.56, P = 0.06 \), while the conventional \( \chi^2 \) value is 5.12, \( P = 0.024 \).
Flicker Threshold and Pattern VEP Latency in Ocular Hypertension and Glaucoma

Adam Atkin, Ivan Bodis-Wollner, Steven M. Podos, Murray Wolkstein, Lee Mylin, and Susan Nitzberg

Latency of the pattern visual-evoked potential (PVEP) was measured in 24 ocular hypertensive (OHT) patients, eight open-angle glaucoma (OAG) patients, and 37 control subjects. The PVEP stimulus was a 2.3 cycle/degree sinusoidal grating, counterphase-modulated at 1 Hz. Field size was 9 degrees and mean luminance 1.7 log ft-lamberts. For 22 of the 32 patients, a psychophysical measure of dynamic contrast sensitivity at 8 Hz (DRC) was obtained with a 4 degrees diameter stimulus, by determining the mean value for the contrast sensitivities to a homogeneous flickering field and to a 1.2 cycle/degree counterphase-flickering grating. Patient DRC values were compared with previously published control data from 21 subjects. Mean PVEP latencies of both the OHT and the OAG patients were greater than normal (P < 0.001), with the OAG value larger than the OHT value (P < 0.001). Mean DRCs were lower than normal (P < 0.002) for both patient groups, with the OAG value lower than the OHT value (P < 0.025). DRC correlated with PVEP latency for these patients (r = −0.66, P < 0.001). Invest Ophthalmol Vis Sci 24:1524–1528, 1983

Open-angle glaucoma (OAG) patients, as well as some ocular hypertensive (OHT) patients with normal visual fields, have shown abnormalities in a measure of the contrast required to detect flicker and flickering patterns at 8 Hz ("dynamic response coefficient" or DRC). Further, optic nerve damage in glaucomatous eyes has been reported prior to definite field loss; and increased latencies of visually evoked potentials (VEPs) have been reported in glaucoma patients and some OHT patients. Discerning such early abnormalities may eventually permit the systematic detection of glaucomatous damage prior to the development of frank scotomata.

Materials and Methods. This is a retrospective study: patient and control data (Table 1) were selected from test results obtained previously, either for diagnostic purposes or to set diagnostic standards of normality, and also from control data of a previous study.

Thirty-two patients with open angles and intraocular pressures (IOPs) above 21 mmHg in at least one eye were compared with two control groups. About half the patients were using antiglaucomatous medication; however, no eye receiving pilocarpine was included. The patients were subdivided into an ocular hypertension (OHT) group of 24 patients with no evidence of visual field defects, and an open-angle glaucoma (OAG) group of eight patients who did show typical glaucomatous visual field defects. One OHT patient had pigmentary dispersion syndrome, and one had exfoliation syndrome. The OAG group comprised three patients diagnosed as primary open-angle glaucoma, one patient with pigmentary glaucoma, one with Sturge-Weber, and two with exfoliation syndrome. The OAG and OHT groups did not differ with respect to mean values of IOP, or of known duration of IOP elevation. However, compared with the OHT patients,