

A Role for the Polyol Pathway in the Early Neuroretinal Apoptosis and Glial Changes Induced by Diabetes in the Rat

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We tested the hypothesis that the apoptosis of inner retina neurons and increased expression of glial fibrillary acidic protein (GFAP) observed in the rat after a short duration of diabetes are mediated by polyol pathway activity. Rats with 10 weeks of streptozotocin-induced diabetes and GHb levels of $16 \pm 2\%$ (mean \pm SD) showed increased retinal levels of sorbitol and fructose, attenuation of GFAP immunostaining in astrocytes, appearance of prominent GFAP expression in Müller glial cells, and a fourfold increase in the number of apoptotic neurons when compared with nondiabetic rats. The cells undergoing apoptosis were immunoreactive for aldose reductase. Sorbinil, an inhibitor of aldose reductase, prevented all abnormalities. Intensive insulin treatment also prevented most abnormalities, despite reducing GHb only to $12 \pm 1\%$. Diabetic mice, known to have much lower aldose reductase activity in other tissues when compared with rats, did not accumulate sorbitol and fructose in the retina and were protected from neuronal apoptosis and GFAP changes in the presence of GHb levels of $14 \pm 2\%$. This work documents discrete cellular consequences of polyol pathway activity in the retina, and it suggests that activation of the pathway and "retinal neuropathy" require severe hyperglycemia and/or high activity of aldose reductase. These findings have implications for how to evaluate the role of the polyol pathway in diabetic retinopathy. *Diabetes* 52:506–511, 2003

The clinically important lesions that are caused by diabetes in the retina are those of blood vessels, but evidence is also mounting that neural and glial cells of the retina are affected early in both human and experimental diabetes (1). The most prominent neuronal abnormality is apoptosis of cells whose size and location are consistent with ganglion cells (2). The glial changes described to date relate to the pattern and level of expression of glial fibrillary acidic protein (GFAP). This intermediate filament protein is expressed in the normal retina mostly by astrocytes (the glia confined to the innermost region of the retina) and minimally by

Müller cells (the glia that share functions with astrocytes in the inner retina but span the whole thickness of the retina with their radial processes) (3). In diabetes, Müller cells acquire prominent GFAP immunoreactivity throughout the extension of their processes (4,5), whereas astrocytes progressively lose GFAP immunoreactivity (6) and may also decrease in number (7); the levels of GFAP measured in the whole retina are increased (4,5).

The retinal neuroglial abnormalities are observed before the characteristic lesions of diabetic microangiopathy; they occur predominantly in the inner retina, where the capillaries also reside; and they involve cells that are topographically and functionally connected with vessels. Understanding their mechanisms and consequences may thus prove instrumental to an accurate reconstruction of the pathogenesis of the vascular lesions. In this study, we tested the hypothesis that neuronal apoptosis and altered expression of GFAP in the diabetic retina are mediated by increased activity of the polyol pathway. The hypothesis was formulated because the retina is known to accumulate sorbitol in experimental diabetes (8), and the retinal cells best documented to contain immunoreactivity for aldose reductase are ganglion and Müller cells (9–12).

RESEARCH DESIGN AND METHODS

Experimental animals. Experiments were performed in rats and mice. Sprague-Dawley male rats (~230 g body wt) were randomly assigned to one of the following groups: control, diabetic, diabetic on intensive insulin treatment, or diabetic treated with sorbinil. Diabetes was induced with streptozotocin (57.5 mg/kg body wt) dissolved in citrate buffer, pH 4.5, and injected via the tail vein. Development of diabetes (blood glucose >250 mg/dl) was verified 1 week after streptozotocin injection. Body weight was recorded three times a week in the diabetic rats, and 2–4 units of NPH insulin were administered subcutaneously as needed to prevent weight loss without preventing hyperglycemia. The rats assigned to intensive insulin treatment received 6–8 units s.c. NPH insulin daily; those treated with sorbinil (Pfizer) received the drug at 65 mg · kg body wt⁻¹ · day⁻¹ mixed with the powdered diet. The rats were killed after 10 weeks (2.5 months) of diabetes. C57BL/6J male mice were rendered diabetic with multiple streptozotocin injections (50 mg/kg body wt) administered intraperitoneally on 5 consecutive days (13). The diabetic mice received 0.1–0.3 units s.c. NPH insulin twice a week as needed to prevent weight loss. Mice were killed after 10 or 24 weeks of diabetes together with age- and sex-matched controls. Blood was obtained by cardiac puncture for the measurement of GHb (Glyc-Affin GHb assay; PerkinElmer, Norton, OH). From each animal, one eye was fixed in 10% buffered formalin at room temperature for the preparation of retinal whole-mounts or sections, and the contralateral retina was isolated under the dissecting microscope and either homogenized for protein studies (14) or weighed and extracted in 6% ice-cold perchloric acid to measure sorbitol and fructose content. The measurements of retinal sorbitol and fructose were performed by the laboratory of Dr. Peter Oates at Pfizer using fluorometric enzyme assays (15,16).

Immunohistochemistry. Immunohistochemistry was performed on whole-mounted retinas prepared and processed as described by Barber et al. (2) with

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GFAP, glial fibrillary acidic protein; TUNEL, terminal transferase dUTP nick-end labeling.

TABLE 1
Weight, GHb, and retinal sorbitol and fructose levels in the four groups of rats studied

	<i>n</i>	Weight (g)	GHb (%)	Retinal sorbitol (nmol/g wet wt)*	Retinal fructose (nmol/g wet wt)*
Control	20	525 ± 45	4.6 ± 0.9	65 ± 7	33 ± 15
Diabetes	17	295 ± 77†	16.5 ± 1.7†	177 ± 76†	232 ± 101†
Diabetes with intensive insulin	13	435 ± 44†‡	11.7 ± 1.4†‡	86 ± 35‡	43 ± 49‡
Diabetes with sorbinil	19	268 ± 35†	16.2 ± 2.4†	45 ± 10‡	36 ± 36‡

Data are means ± SD. *Measurements were performed in four rats from each group; †*P* < 0.005 vs. control; ‡*P* < 0.005 vs. diabetic rats.

minor modifications. Eyes fixed in formalin for 20 min were washed in PBS, and the retina was isolated and fixed again in formalin for 10 min, mounted vitread side up on silane-coated slides, and stored at -20°C. The whole mounts were dehydrated through graded alcohols, defatted in xylene overnight at 4°C, then rehydrated, permeabilized with 0.3% Triton X-100 for 15 min, and treated with proteinase K (20 µg/ml) for 30 min at room temperature. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min at room temperature. The preparations were blocked with 2% BSA in PBS and incubated for 2 h at room temperature, with the primary antibodies diluted in blocking buffer containing 0.3% Triton X-100. The primary antibodies were rabbit anti-cow GFAP (1:3,000; Dako, Carpinteria, CA), mouse anti-rat vimentin (1:40; Sigma, St. Louis, MO), and rabbit anti-aldose reductase (1:1,000) (17). All antibodies were tested in immunoblots of rat retinal proteins and documented to react with a unique band of the expected molecular size. After PBS washes, the preparations were reacted with the appropriate secondary antibodies conjugated with Cy3. Negative controls were obtained by substituting the primary antibodies with an equivalent concentration of the appropriate nonimmune IgG. The preparations were observed under the fluorescence microscope, and multiple fields were photographed.

Terminal transferase dUTP nick-end labeling. The whole-mounted retinas used for immunohistochemistry were washed extensively in PBS and tested with the terminal transferase dUTP nick-end labeling (TUNEL) reaction to detect apoptosis (in situ cell death detection kit; Roche, Mannheim, Germany). In the specimens used to quantitate apoptosis, the fluorescent TUNEL signal was converted to peroxidase signal (TUNEL POD; Roche), and 3-amino-9 ethylcarbazole (Vector, Burlingame, CA) was used to visualize the reaction. Positive controls were whole-mounted retinas treated with DNase-I to fragment DNA and retinas obtained from rats subjected to unilateral optic nerve crush 6 days before sacrifice to induce ganglion cell apoptosis. Negative controls were whole-mounted retinas treated with DNase I but incubated in the absence of terminal transferase. The number of TUNEL⁺ nuclei was counted in the whole retina.

Immunoblotting. The retinas were homogenized in ice-cold lysis buffer (14), and the proteins were resolved by electrophoresis on 12% SDS-PAGE and electroblotted. The blots were probed with the GFAP antibodies (1:40,000), and immunoreactive bands were detected and quantitated as previously described (14). The results are expressed as densitometric units per microgram of protein.

Statistical analysis. The data are summarized with the mean ± SD. The results of the rat studies were analyzed by ANOVA, followed by Fisher's multiple comparison test; the results of the mouse studies were analyzed with the unpaired *t* test.

RESULTS

Sorbinil prevents GFAP changes in the retina of diabetic rats. Intensive insulin treatment partially normalized the weight gain and GHb values in the diabetic rats, whereas sorbinil had no effect on these parameters. However, both intensive insulin and sorbinil prevented sorbitol and fructose accumulation in the retinas of diabetic rats (Table 1). The normal rat retina showed GFAP immunostaining in a delicate stellar-reticular pattern characteristic of astrocytes (Fig. 1A). In the retina of diabetic rats, this orderly pattern was replaced by discrete coarse processes and patches of variable shapes (Fig. 1B). The switch was attributable to new positivity for GFAP acquired by the Müller cells because the pattern was similar to that exhibited in control retinas by vimentin (Fig. 1C). Vimentin, like GFAP, is an intermediate filament protein;

however, unlike GFAP, it is normally expressed by Müller cells (18). Intensive insulin treatment largely, albeit not completely, prevented the GFAP pattern observed in the diabetic rats (not shown). Sorbinil afforded complete prevention (Fig. 1D). In accordance with the fact that Müller cells constitute up to 90% of the nonneuronal cells in the retina, their acquisition of GFAP expression in the diabetic rats was accompanied by an increase in retinal GFAP levels (Fig. 1E). In the diabetic rats treated with intensive insulin or sorbinil, the GFAP levels were not different from control values (*P* = 0.5), although the

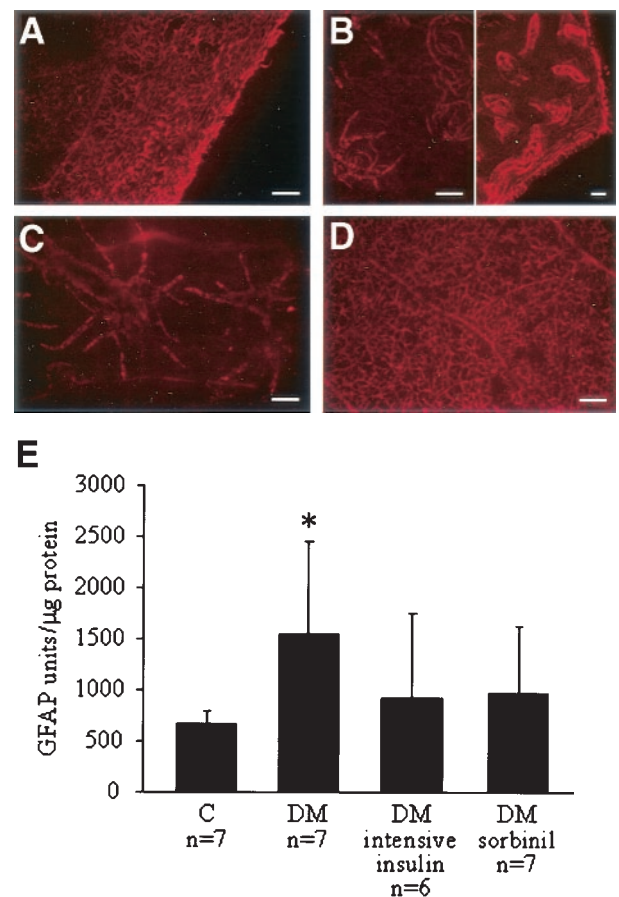


FIG. 1. Sorbinil prevents GFAP changes in the retina of diabetic rats. GFAP immunofluorescence in the retina of a control rat (A) shows the delicate reticular pattern characteristic of astrocytes, which is replaced in the retina of a diabetic rat (B) by discrete coarse processes, shown at two different magnifications. The coarse processes are similar to those seen in the retina of a control rat (C) immunostained for vimentin to identify Müller cells. D: Sorbinil prevented the GFAP changes induced by diabetes. The bar represents 100 µm. E: GFAP levels in retinal lysates as measured by immunoblots. Bars represent the mean ± SD of the values obtained in the indicated number of rats. C, control; DM, diabetes. **P* = 0.02 vs. control.

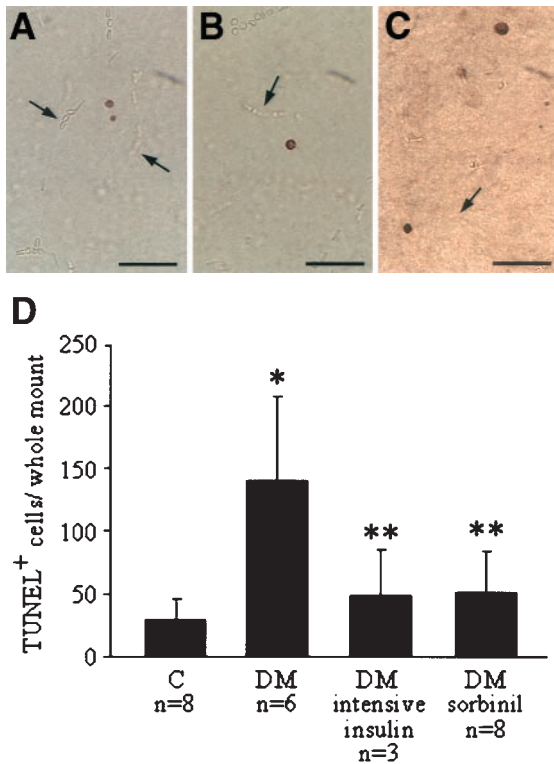


FIG. 2. Sorbinil prevents neural apoptosis in the retina of diabetic rats. Brown TUNEL⁺ nuclei (A and B) in the retina of diabetic rats are not associated with capillaries (arrows) and have size and morphology similar to the TUNEL⁺ ganglion cell nuclei (C) in the retina of a rat subjected to optic nerve crush. The bar represents 25 μ m. D: Number of TUNEL⁺ nuclei counted in the whole-mounted retinas. Bars represent the mean \pm SD of the counts in the indicated number of rats (one retina each). C, control; DM, diabetes. * $P < 0.0001$ vs. control; ** $P < 0.005$ vs. diabetes.

difference versus the diabetic values did not achieve statistical significance ($P = 0.1$).

Sorbinil prevents neural apoptosis in the retina of diabetic rats. In the whole-mounted retinas, different planes of focus permitted the observation of at least two layers of the capillary network. The TUNEL⁺ nuclei observed in both the diabetic and control rat retinas were located in the planes bracketed by capillaries, but they were not associated with the capillary wall (Fig. 2A and B). Both the location and the morphology of the TUNEL⁺ nuclei in the retina of diabetic rats were similar to those observed in the retina of a rat subjected to unilateral optic nerve crush 6 days before it was killed to serve as positive control for the identification of apoptotic ganglion cells (Fig. 2C). TUNEL⁺ nuclei were much more frequent in the retina of diabetic than control rats, and both intensive insulin and sorbinil treatment prevented the excess apoptosis caused by diabetes (Fig. 2D).

We sought to determine whether apoptotic nuclei belonged to cells endowed with aldose reductase. In a whole-mounted retina of a diabetic rat (Fig. 3), the cytoplasm of most cells in the superficial layers stained for aldose reductase, with nuclei of various size appearing as negative images. Also the vessels appeared as negative images, their profile being marked by aldose reductase immunoreactivity, possibly attributable to glial cell processes. The TUNEL⁺ nuclei observed in this retina were of

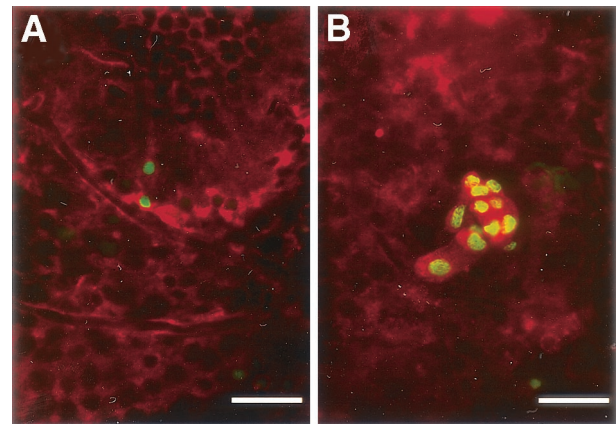


FIG. 3. Apoptotic neurons in the inner retina of diabetic rats are immunoreactive for aldose reductase. The superficial layers of neurons (A) show diffuse aldose reductase immunofluorescence (red), with the nuclei as well as the vessels appearing as negative images; the TUNEL⁺ nuclei show green fluorescence (A and B). Bar represents 25 μ m.

large size and belonged to cells positive for aldose reductase (Fig. 3), likely ganglion cells.

GFAP and apoptosis in the retina of diabetic mice. In the lens, mice exhibit only 10% of the aldose reductase activity present in the rat, and even under conditions of sustained hyperglycemia, they do not accumulate sorbitol or fructose or develop cataracts (19). We investigated whether the retina in diabetic mice was also protected from accumulation of sorbitol and fructose and from the development of neuroglial abnormalities. Mice with a diabetes duration matched to that of the rats in this study (10 weeks), as well as mice with 24 weeks' duration of diabetes, showed GHb levels significantly higher than those of age-matched controls and similar to those of diabetic rats but no increased sorbitol content in their retina (Table 2). Fructose was undetectable in all samples. The pattern of GFAP staining typical of astrocytes appeared undisturbed in the retina of diabetic mice irrespective of the duration of diabetes, and there was no indication of Müller cell reactivity (Fig. 4B and C). The prevalence of TUNEL⁺ nuclei was much lower in the mouse than in the rat retina (compare Figs. 4D and 2D), and although there was a tendency toward a greater number of TUNEL⁺ nuclei in the older mice, the numbers recorded in the 10- and 24-week study were not statistically different. The diabetic mice did not show any increase in the prevalence of apoptotic neural cells over their age-matched controls.

TABLE 2
GHb and retinal sorbitol levels in mice with 10- or 24-week duration of diabetes

	GHb (%)	Retinal sorbitol (nmol/retina)*
Control	4.3 \pm 0.3	0.5 \pm 0.1
Diabetes duration 10 weeks	13.9 \pm 1.4 [†]	0.6 \pm 0.2
Control	4.4 \pm 0.7	0.2 \pm 0.1
Diabetes duration 24 weeks	14.2 \pm 2.2 [†]	0.3 \pm 0.1

Data are means \pm SD. Each group contained 5–6 mice. *Reported in nanomoles per retina because the wet weight recorded for the mouse retinas in two consecutive experiments was not consistent; [†] $P < 0.005$ vs. respective age-matched control.

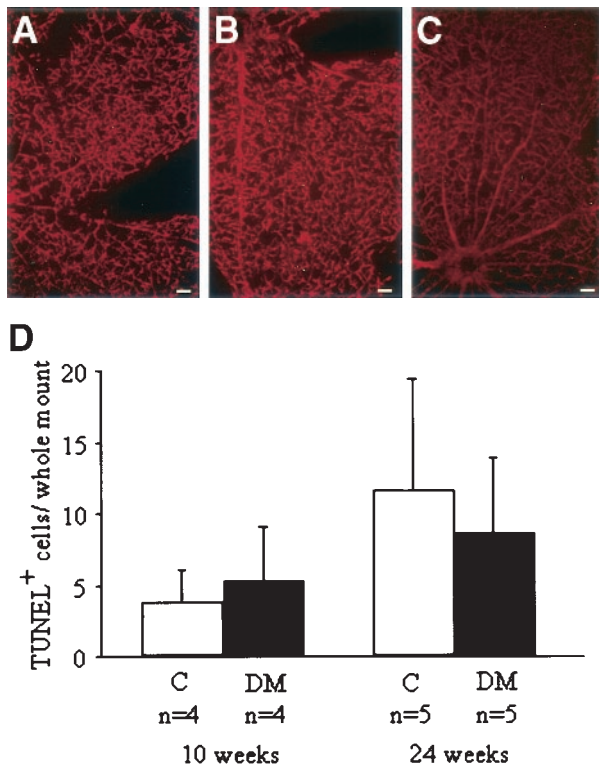


FIG. 4. GFAP and TUNEL in the retina of diabetic mice. GFAP immunofluorescence in the retina of a control mouse (A) shows the characteristic astrocyte pattern, which is not altered by diabetes of 10-week (B) or 24-week (C) duration. The bar represents 100 μm . D: Number of TUNEL⁺ nuclei counted in the whole-mounted retinas. Bars represent the means \pm SD of the counts in the indicated number of mice (one retina each). C, control; DM, diabetes.

DISCUSSION

This work documents that activation of the polyol pathway accounts for neuronal apoptosis and glial changes induced by diabetes in the retina, and it suggests that these events occur when hyperglycemia is severe. It also adds the retina to the tissues in which the polyol pathway is more active and more consequential in the diabetic rat than in the diabetic mouse, a finding that may prove useful in probing the relationship between neuroglial and vascular abnormalities in the development of diabetic retinopathy.

The polyol pathway has been studied in relation to all complications of diabetes, but most extensively and successfully in relation to peripheral neuropathy. Aldose reductase is present in Schwann cells (20), the glia of peripheral nerves; sorbitol accumulates in the sciatic nerve to a degree that can have osmotic, in addition to metabolic, consequences (21); and inhibitors of aldose reductase prevent or reduce the slowing of nerve conduction velocity and loss of small myelinated nerve fibers caused by diabetes (22). It is now apparent that the polyol pathway also plays a role in the "retinal neuropathy" caused by diabetes. Among the mechanisms proposed for the pathogenicity of the pathway in peripheral nerves (20,23), osmotic stress is unlikely to be involved in the neuroretinal abnormalities because the increase in retinal sorbitol levels was far from attaining the required micromolar range (21). Carbonyl stress, oxidative stress, and pseudohypoxia can all affect cellular homeostasis, and at

least a portion of the neuroretinal changes is likely to represent the stereotypical response to stress characteristic for the affected cell types. Ganglion cells die by apoptosis in ocular hypertension; in normal pressure glaucoma, a disease in which blood flow abnormalities may cause ischemia (24); in retinas damaged by light (25); and in several genetic diseases in which ubiquitous mitochondrial defects are manifested with a remarkably selective loss of ganglion cells, whereas other energy-dependent retinal cell types, such as photoreceptors, are unaffected (26). GFAP upregulation is a stereotypical response of glial cells to injury or neural damage (27), but this cannot be the only explanation for what occurs in the diabetic retina, where Müller cells acquire, but astrocytes decrease, their GFAP immunoreactivity. The decrease is not caused by massive astrocyte demise because the typical astrocyte pattern of GFAP immunoreactivity is restored in diabetic rats by short-term insulin administration (6). The mechanisms for the discordant effects of diabetes on the GFAP content of Müller cells and astrocytes may need to be sought in the regulation of GFAP expression. GFAP mRNA levels are increased in the diabetic rat retina (C.G. and M.L., unpublished observations), indicating that gene transcription may be one of the sites at which polyol pathway activity induces GFAP expression in Müller cells. The elements that regulate GFAP transcription are not located in the same region of the gene in Müller cells and astrocytes (28), and this may make such elements susceptible to additional and, in the case of diabetes, divergent modulation. It is currently unknown whether astrocytes have aldose reductase. If they do, polyol pathway activity appears to reduce or silence their constitutive GFAP expression. If they do not, the GFAP downregulation may occur in response to the events taking place in Müller and ganglion cells. Either case would represent a new paradigm for GFAP regulation because in the context of other retinal pathologies, both Müller cells and astrocytes upregulate GFAP expression (28).

We treated a group of diabetic rats with intensive insulin to verify that neuronal apoptosis and glial changes were in fact a consequence of diabetes rather than of streptozotocin toxicity. A noteworthy finding in these experiments was that insulin treatment prevented to a large extent retinal neuroglial abnormalities and the accumulation of sorbitol and fructose, despite only partial amelioration of hyperglycemia. This suggests that in the retina, the polyol pathway is activated and pathogenic only beyond a glycaemic threshold. If the observations are confirmed, the concept could help reconcile reports that are in apparent disagreement. On the one hand, sorbinil has been found not to prevent the development of diabetic retinopathy in dogs (29) or lessen its progression in patients (30). On the other hand, the frequency of an allelic variant of aldose reductase that confers increased expression of the gene (measured in white blood cells) is found to be significantly greater in diabetic patients with retinopathy than in those without retinopathy (31). Because retinal sorbitol levels in diabetic dogs were only minimally increased (in keeping with GHB elevated to only 8%) (29), one may speculate that the modest hyperglycemia did not lead to a degree of polyol pathway activation that would burden cells, and therefore there was little room for sorbinil to show a

beneficial effect. Along this line of reasoning, inhibition of the polyol pathway would yield maximal returns when its activity is high either as a consequence of severe hyperglycemia or because of elevated levels of expression of aldose reductase.

Two sets of data will help us determine whether aldose reductase inhibitors can be expected to be beneficial in diabetic retinopathy. The first data set is more conclusive information on whether retinal vascular cells contain aldose reductase and are affected by polyol pathway activity. Aldose reductase immunoreactivity was not detected in human (12), rat (9), or dog (11) retinal vessels, but it was reported in the retinal pericytes and endothelial cells of the BB rat (10) and in cultured human pericytes (32). The second data set is information showing whether the retinal neuroglial abnormalities that we have found mediated by the polyol pathway play a role in the development of diabetic retinopathy. In the diabetic rat, neuronal apoptosis was sufficient to account for a 14% reduction in the thickness of the inner retina after 7.5 months of diabetes (2), a change that could have an impact on visual processing. In diabetic patients, however, there is no direct clinical counterpart to loss of ganglion cells or other inner retinal cells. Neuroglial abnormalities could impact on the manifestations and course of the retinal microangiopathy of diabetes. Vascular endothelial growth factor, the powerful permeability factor that is produced in the retina by neurons and glia (33,34), has been found increased in the diabetic rat retina (34), and the increase was prevented by aldose reductase inhibition (35). Dysfunctional glial cells could further contribute to the increased permeability of retinal vessels if they alter their contribution to the formation of tight junctions (36,37). Material attributable to GFAP-positive Müller cells is present in the former lumen of ghost vessels in human diabetic retinopathy, raising the possibility of a glial role in vascular occlusion (38,39). Neurons in distress may produce molecules that change the microenvironment of capillary cells, and cumulative neuronal loss may send involuntional signals to vessels, contributing to the obliterative phase of diabetic retinal microangiopathy. These possibilities need to be addressed experimentally. The diabetic mouse can become useful in this context because it could be induced to activate the polyol pathway in the retinal neuroglial cells of interest, and it could be examined for the impact of the phenotype on the course and manifestations of retinal microangiopathy.

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