

Müller Cell Changes in Human Diabetic Retinopathy

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Vascular cells may not be the only cells affected by diabetes in the retina. In particular, abnormalities of the b-wave of the electroretinogram in diabetic patients with absent or minimal microangiopathy have pointed to possible dysfunction of Müller cells, the principal glia of the retina. In this study, we sought evidence for diabetes-induced Müller cell abnormalities by testing the expression of three proteins (Bcl-2, glutamine synthetase [GS], and glial fibrillar acidic protein [GFAP]) that are solely or predominantly expressed in Müller cells and show a reproducible pattern of changes in the context of retinal injuries or degenerations. Retinas obtained postmortem from a total of 14 donors aged 65 ± 6 years with 10 ± 4 years of diabetes and histological evidence of microangiopathy and 18 age-matched nondiabetic donors were examined by immunohistochemistry and immunoblotting. The typical Müller cell pattern of Bcl-2 and GS immunostaining was similar for both intensity and distribution in the nondiabetic and diabetic retinas, as were the levels of the two proteins. In contrast, GFAP staining, largely confined to the most proximal retina in the nondiabetic donors, was in most diabetic retinas present along the entire length of the Müller cell processes, throughout the outer retina. Accordingly, the level of GFAP was increased in the diabetic retinas (161 ± 106 densitometric units/ μg protein vs. 55 ± 45 in the nondiabetic retinas, $P = 0.03$). These data provide evidence for selective biosynthetic changes of Müller glial cells in diabetes. Because Müller cells produce factors capable of modulating blood flow, vascular permeability, and cell survival, and their processes surround all blood vessels in the retina, a possible role of these cells in the pathogenesis of retinal microangiopathy deserves to be investigated. *Diabetes* 47:445–449, 1998

Some studies have suggested that retinal microangiopathy is just a facet of a more global retinal dysfunction occurring in diabetes. Abnormalities in the electroretinogram have been reported to precede vasculopathy (1) or accompany early vascular lesions (2), and the abnormalities in the b-wave of the elec-

troretinogram have pointed to a possible involvement of Müller cells, the site of generation of the b-wave. Several characteristics of Müller cells—which are the principal glial cells of the retina (3)—make them a possible target of diabetic hyperglycemia and, if so, a possible cause for dysfunction of both neural and vascular cells in the retina. Müller cells have GLUT1 (4) and an elevated rate of glycolysis, the products of which fuel mitochondria oxidative metabolism in photoreceptors (5). Through their processes, the cells span the depth of the retina and surround neuronal cell bodies, axons, and vessels (3,6) and subserve the critical housekeeping roles of removing from the extracellular space potassium and neurotransmitters released during neural activity (3). In particular, being the only cells in the retina endowed with the enzyme glutamine synthetase (GS) (7,8), they transform the glutamate taken up via high-affinity carriers into glutamine, which is then returned to the neural cells for glutamate resynthesis (5).

Müller cells, akin to brain astrocytes (9), synthesize factors that induce the formation of tight junctions and thus confer barrier properties to the retinal vessels (10). In addition, they synthesize or store a number of growth factors (11) that may have trophic or regulatory functions for various cell types in the retina. These characteristics make an assessment of Müller cell function in diabetes relevant to two well-known features of diabetic microangiopathy—vascular leakage and capillary obliteration. We have recently demonstrated the occurrence of microvascular cell apoptosis in human and experimental diabetic retinopathy (12), and one of the mechanisms leading to apoptosis is loss of survival signals provided by neighboring cells (13).

Müller cell changes have mostly been studied in the context of retinal insults and inherited or age-related retinal degenerations. Changes that recur in various combinations across the diverse pathologies are increased expression in Müller cells of the anti-apoptotic molecule Bcl-2 and of the intermediate filament protein glial fibrillar acidic protein (GFAP) and decreased expression of GS (14–16). To ascertain whether diabetes affects Müller cell characteristics, the expression of the three proteins was compared in the retina of patients with nonproliferative diabetic retinopathy and age-matched nondiabetic individuals.

RESEARCH DESIGN AND METHODS

Eye donors and specimens. Human eyes were obtained from certified eye banks through the National Disease Research Interchange. Criteria for inclusion in the study were age <75 years, absence of retinal or hematological diseases or uremia, absent administration of chemotherapy or life-support measures, diabetes duration of <15 years to address mostly background retinopathy (17), and the fewest possible chronic pathologies other than diabetes. We studied eyes from a total of 14 diabetic and 18 nondiabetic individuals. In seven of the diabetic donors

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BSA, bovine serum albumin; GFAP, glial fibrillar acidic protein; GS, glutamine synthetase; HRP, horseradish peroxidase; Ig, immunoglobulin; PBS, phosphate-buffered saline; TBST, Tris-buffered saline with Tween.

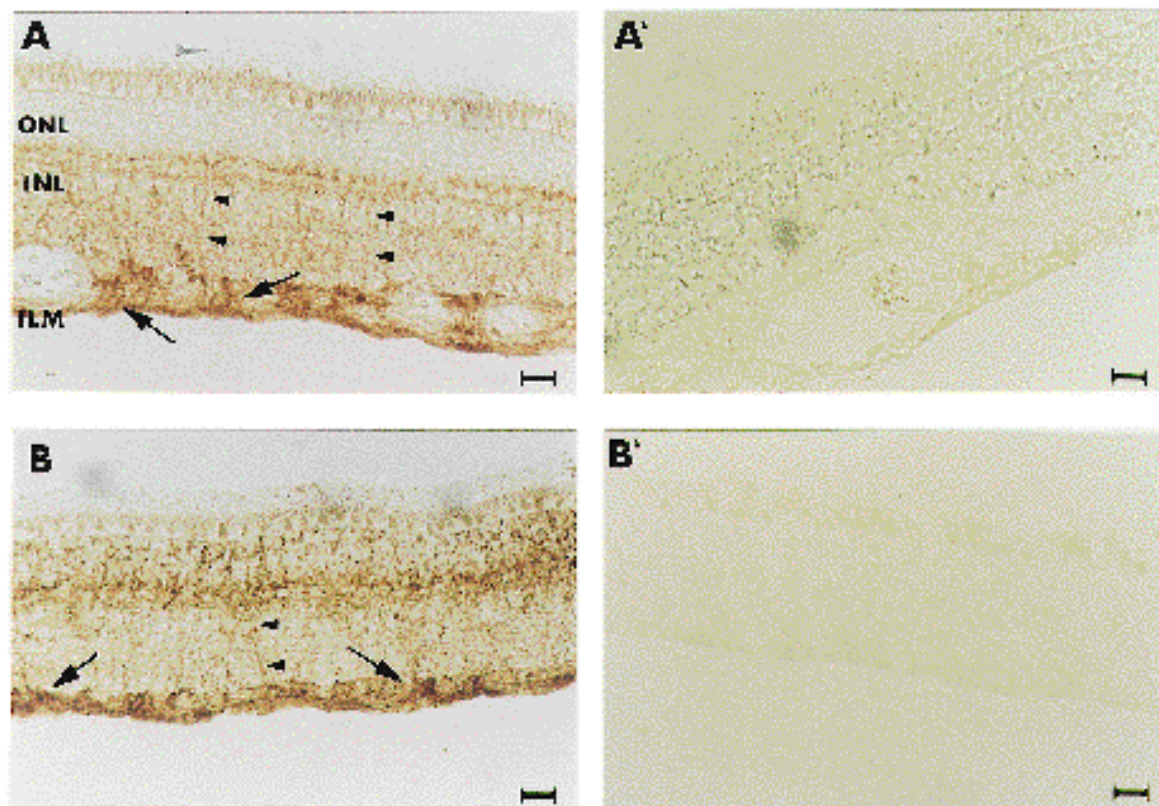


FIG. 1. Bcl-2 (**A**) and GS (**B**) immunohistochemistry in the retina of a 56-year-old nondiabetic male donor. Peroxidase reaction product for both proteins is prominent in the inner retina, along the inner limiting membrane (ILM), in Müller cell endfeet (arrows), and Müller cell radial fibers (arrowheads) extending through the inner nuclear layer (INL). The outer nuclear layer (ONL), while negative for Bcl-2, shows GS reaction product in the Müller cell arborizations surrounding the nuclei of the photoreceptors. The vessels are negative. **A'**: Negative control for Bcl-2 prepared as described in METHODS and photographed in phase microscopy to obtain a visible image. **B'**: Negative control for GS obtained as described in METHODS. Bars represent 25 μ m.

(age 64 ± 6 years, duration of diabetes 9 ± 3 years) and seven of the nondiabetic donors (age 64 ± 7 years), one eye was processed for immunohistochemical studies on retinal sections and the other for extraction of total retinal proteins for Western analysis. In the remainder diabetic (age 67 ± 7 years, duration of diabetes 10 ± 4 years) and nondiabetic (age 67 ± 5 years) donors, one eye was processed for retinal immunohistochemistry and the other for retinal trypsin digestion (18) to study the microvasculature. For the diabetic and nondiabetic eyes, the time elapsed from death to enucleation was 3.5 ± 1 and 3.1 ± 1 h, respectively; time to fixation 11.2 ± 4 and 11.6 ± 3 h; and time to processing of the fresh samples destined to protein extraction 33 ± 3 and 30 ± 7 h.

The eyes destined for morphological studies were fixed in 10% buffered formalin by the eye banks. Those eyes to be used for retinal immunohistochemistry were fixed in formalin for 48 h, and after three washes in rinse buffer (0.1 mol/l sodium phosphate, 0.15 mmol/l calcium chloride, 5% sucrose), the eyes were immersed in 30% sucrose in rinse buffer overnight at 4°C for cryopreservation. The following day, the retinas were isolated, cut into quadrants, and embedded in optimum cutting temperature (OCT) compound (Miles, Elkhart, IN) on dry ice. Radial sections of 6 μ m thickness were cut in a cryostat and set onto Silane-coated slides (Polysciences, Warrington, PA), air dried, and stored at -20°C . The eyes destined for retinal protein extraction were kept at 4°C by the eye banks and shipped to the laboratory on ice. The retina was immediately isolated, detached from the pigmented epithelium, and transferred to 1 ml of homogenization buffer (30 mmol/l Tris-HCl, 10 mmol/l EGTA, 5 mmol/l EDTA, 1% Triton X-100, 250 mmol/l sucrose, and 1 mmol/l phenylmethylsulfonyl fluoride, pH 7.5). The homogenate was sonicated three times for 2 s and centrifuged at $16,000g$ for 15 min at 4°C . The supernatant was collected and stored at -80°C .

Immunohistochemistry. The retinal sections were rehydrated in phosphate-buffered saline (PBS) for 20 min, blocked with 2% bovine serum albumin (BSA) in PBS for 10 min, and incubated overnight in a moist chamber with the primary antibody diluted in PBS containing 2% BSA and 0.5% Triton X-100. Incubation was at room temperature for the Bcl-2 antibody and at 4°C for the other antibodies. The monoclonal mouse anti-human Bcl-2 antibody (clone Bcl-2/100; Pharmingen,

San Diego, CA) was used at 5 $\mu\text{g/ml}$; the monoclonal mouse anti-chicken GS antibody in ascites fluid (gift of Dr. P. Linsler) at a dilution of 1:20; and the polyclonal rabbit anti-cow GFAP antibody (Dako, Carpinteria, CA) at 0.8 $\mu\text{g/ml}$. After three PBS washes, the specimens were incubated for 1 h at room temperature with a biotinylated goat anti-mouse immunoglobulin (Ig) G (Vector Laboratories, Burlingame, CA), and endogenous peroxidase was inactivated by a 30-min incubation in 3% H_2O_2 in glass distilled water. The specimens were then treated with the avidin-biotin-peroxidase complex, and the peroxidase reaction product was developed with diaminobenzidine for 10 min. No counterstaining was performed. Negative controls were obtained by substituting the primary antibody with an equivalent concentration of nonimmune mouse IgG, fraction for Bcl-2 and rabbit IgG for GFAP and by omitting the primary antibody for GS because its concentration in the ascites fluid was not measured.

Immunoblotting. The protein concentration of each retinal lysate was determined with the Bradford method (19) using BSA as the standard. The lysates were diluted 1:3 in Laemmli Sample Buffer (Bio-Rad, Hercules, CA), supplemented with 100 mmol/l dithiothreitol, and boiled for 5 min. Scalar amounts of total protein from each retina were electrophoresed on 12% SDS-polyacrylamide gels as described by Laemmli; each gel always included samples from an equal number of diabetic and nondiabetic retinas. Proteins were transferred onto nitrocellulose membranes by electroblotting. The membranes were blocked overnight at 4°C in 5% dried milk in Tris-buffered saline with Tween (TBST) buffer (10 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, and 0.05% Tween-20) and then incubated with the primary antibody diluted in TBST (1 $\mu\text{g/ml}$ for the Bcl-2 antibody, 1:50 for the GS antibody, and 0.1 $\mu\text{g/ml}$ for the GFAP antibody) for 1 h at room temperature. After three washes with TBST, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 30 min at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, IL). Densitometric analysis of the autoradiographs was performed with the model 330A computing densitometer (Molecular Dynamics, Sunnyvale, CA). The values recorded for the increasing amounts of sample proteins were averaged to yield

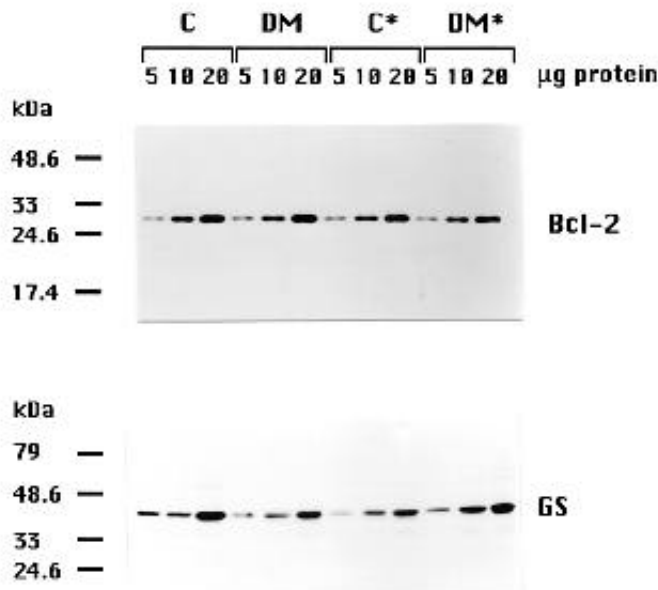


FIG. 2. Immunoblot analysis of retinal Bcl-2 and GS. The indicated amounts of retinal proteins from two nondiabetic (control) donors (C, age 67 years; C*, age 72 years) and two diabetic donors (DM, age 68 years, diabetes duration 5 years; DM*, age 68 years, diabetes duration 11 years) were electrophoresed on 12% SDS-polyacrylamide gels and immunoblotted with anti-Bcl-2 and anti-GS antibodies. The intensity of the signals is linearly related to input protein and similar in control and diabetic retinas. Molecular weight markers are indicated on the left.

the Bcl-2, GS, or GFAP concentration in each sample (densitometric units per microgram total protein).

Statistical analysis. The data are summarized with the mean \pm 1 SD. Statistical analysis was performed with the unpaired Student's *t* test.

RESULTS

The topography of Bcl-2 immunoreactivity in the adult human retina indicates expression of the anti-apoptotic molecule mostly, if not solely, in Müller cells (Fig. 1A). Staining was prominent in the most proximal retina located to Müller cell endfeet and extended in the radial fibers of the Müller cells to the outer plexiform layer. The outer retina was remarkably negative, as were the walls of the large blood vessels in the inner retina. Immunohistochemistry performed on trypsin digests of both nondiabetic and diabetic subjects indicated that retinal capillaries also fail to stain for Bcl-2 (data not shown).

All but one of the diabetic subjects in whom vascular histopathology was examined had characteristic lesions of retinal microangiopathy. The trypsin digests, each corresponding to one-sixth of the retina, showed microaneurysms, pericyte ghosts, and acellular vessels. Among the nondiabetic subjects, one showed a microaneurysm and another occasional acellular capillaries. The distribution and intensity of Bcl-2 immunoreactivity was not altered in the diabetic retinas, even when the trypsin digest of the contralateral retina showed signs of microangiopathy.

The pattern of GS immunoreactivity (Fig. 1B) was similar to that of Bcl-2 in the inner retina, with intense staining of the Müller cell endfeet anchored to the internal limiting membrane and surrounding the blood vessels, and of the radial processes. GS reaction product was, however, also prominent

in the outer retina, in accordance with the functional role of the enzyme in the transamidation of glutamate released by photoreceptors. The pattern and intensity of GS immunoreactivity was not altered in the diabetic retinas.

In Western immunoblots (Fig. 2), the lysates from both nondiabetic and diabetic retinas showed a unique band of the expected molecular mass of 26 kDa with the Bcl-2 antibodies and a unique band of 42 kDa with the GS antibodies. Bcl-2 content (in densitometric units per microgram protein) was similar in nondiabetic (69 ± 10) and diabetic (75 ± 15) retinas. Also, the GS content (in densitometric units per microgram protein) did not differ in the nondiabetic (66 ± 16) and diabetic (54 ± 12) retinas. To ensure that the small sample size had not precluded the identification of differences, GS content was tested in the retinal lysates of four additional diabetic and four nondiabetic subjects with characteristics (age 66 ± 3 years for the diabetic and 65 ± 4 years for the control subjects, duration of diabetes 11 ± 5 years) similar to those of the original cohort. In the total group of 11 diabetic patients, the retinal GS content was 64 ± 18 densitometric units/ μ g protein, identical to that (63 ± 15) of the control subjects. The mean interassay coefficient of variation (samples from four retinas tested in two different assays) was 10%, indicating satisfactory reproducibility of the quantitation procedure.

GFAP immunoreactivity (Fig. 3) in the retinal sections of nondiabetic donors was confined to the proximal retina, concentrated in the nerve fiber layer, Müller cell endfeet, and around blood vessels. In most diabetic retinas, however, the GFAP-positive filaments within the Müller cell processes appeared greatly thickened in the inner retina and extended the length of the entire Müller cell throughout the outer retina. In immunoblots, GFAP antibodies detected a major band of the expected molecular mass of 50 kDa (Fig. 4), and the intensity of the signal was increased in the diabetic retinas (161 ± 106 densitometric units/ μ g protein vs. 55 ± 45 in the nondiabetic retinas, $P = 0.03$) (Fig. 4).

DISCUSSION

Because the clinically important manifestations of the effects of diabetes on the retina derive from the microangiopathy, we and others have addressed to date the pathogenesis of diabetic retinopathy focusing on retinal vessels and/or vascular cells. The observations presented here of selective abnormalities of Müller glial cells in the retina of diabetic patients provide not only evidence for diabetes-induced dysfunction of an additional cell type but also the indication that a thorough understanding of diabetic retinopathy will require an integrated view of the interactions between vascular and nonvascular cells in the retina.

The finding that Bcl-2 in the adult human retina appears confined to the Müller cells confirms observations made in the rat retina (15). During both human (20) and murine (15,21) embryonic development, Bcl-2 is expressed also in the retinal ganglion cells and their fibers, but it disappears rapidly from these locations in postnatal life. Bcl-2 is instead present in neurons of the adult human cerebral cortex (22), and it is therefore surprising that retinal neurons, which are also long-lived postmitotic elements, should not be endowed with this powerful anti-apoptotic defense. Within the paradigm that long-lived cells are among the cells that preferentially express Bcl-2 (22), it was also somewhat surprising not to detect Bcl-2 in retinal vascular cells, which are known to

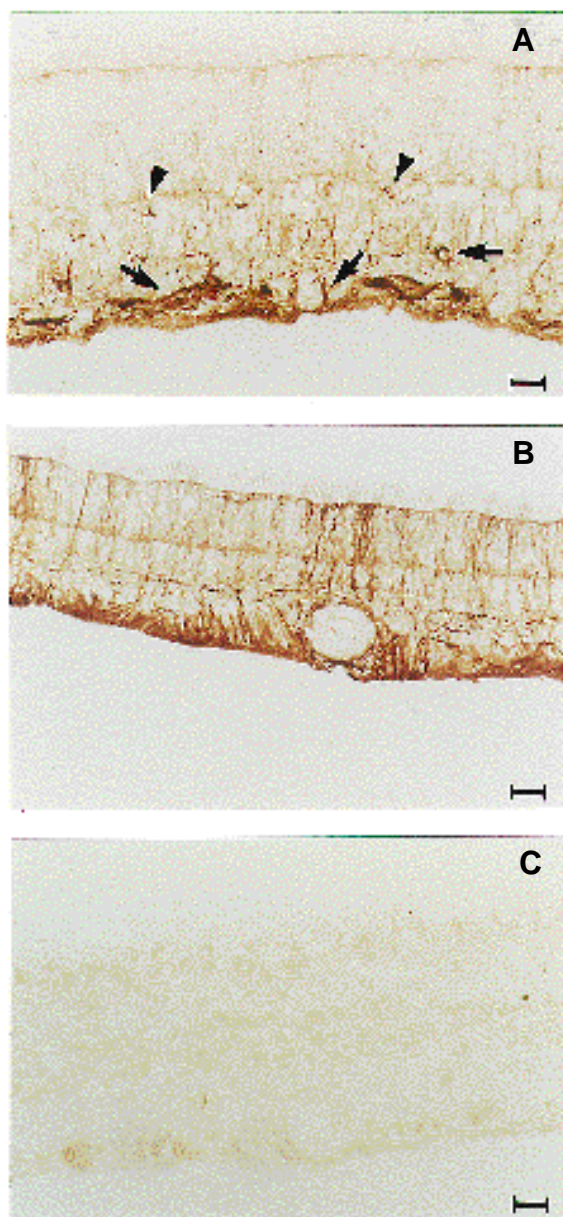


FIG. 3. GFAP immunohistochemistry in the retina of a nondiabetic female donor aged 72 years (*A*) and a diabetic female donor aged 74 years with a 12-year history of diabetes (*B*). In the nondiabetic retina, peroxidase reaction product is evident along the inner limiting membrane, around vessels sectioned radially and tangentially (arrows), and in thin radial fibers reaching the inner nuclear layer. The GFAP-positive processes around the inner network of vessels belong to both astrocytes and Müller cells; those around the outer network (arrowheads) belong to Müller cells because astrocytes are restricted to the innermost retina. In the diabetic retina, the Müller cell endfeet show abundant GFAP immunoreactivity, and the radial processes stain intensely throughout both the inner and outer retina. *C*: Negative control, obtained as described in METHODS. Bars represent 25 μ m.

have a very low turnover (23). On the other hand, both endothelial and smooth muscle cells in other vascular beds have been reported to be negative for Bcl-2 (24,25).

Changes in Müller cell biosynthetic activities may be informative of specific pathological processes occurring in the

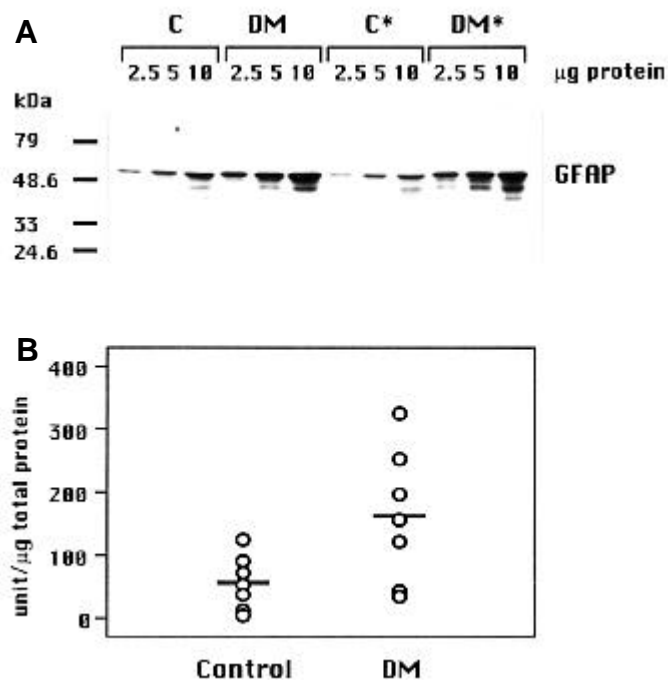


FIG. 4. Immunoblot analysis of retinal GFAP. *A*: Donors tested and methods are those described in the legend to Fig. 2; the GFAP bands are more intense in the diabetic retinas. *B*: Retinal GFAP in the seven nondiabetic (control) and seven diabetic (DM) donors tested. The bars represent the group means.

retina. Upregulation of Bcl-2 expression in retinal Müller cells occurs after optic nerve transection (15), light damage (14), and in the retina of rats with inherited retinal dystrophy (16). An event common to these pathologies is the degeneration of neuronal elements, and the Müller cell upregulation of Bcl-2 may thus signal the disruption of glial-neural interactions. Similarly, the decreased expression of GS reported in retinal light damage (14) and retinal dystrophy (16) is likely to reflect the loss of photoreceptors, the major population of glutamate-releasing neurons in the retina. The absence of changes in the levels of Bcl-2 or GS in the diabetic retinas could thus be interpreted as consistent with the notion that human diabetic retinopathy does not have clinically prominent features of neuronal damage.

It should be noted, however, that in vitro glutamine synthesis by the retinas of rats with 9 months of streptozotocin-induced diabetes and Zucker diabetic rats was found to be reduced when compared with synthesis by control retinas (26). The discrepancy with our observations may only be an apparent one if the enzymatic activity of the protein is affected by diabetes more than its steady-state level, or if the greater severity of diabetes in the experimental animals leads to more profound changes in Müller cell properties, eventually consequent to alterations in the neural retina. Alternatively, the possibility that in our studies with human specimens accurate recovery of GS—the only soluble protein among the three examined—might have been compromised by events occurring in the postmortem period could be entertained. Such a possibility is, however, unlikely in view of the maintained discrete topographical localization of the immunoreactivity to Müller cells and the concordant information

obtained with immunohistochemistry and immunoblotting of total retinal lysates. Moreover, postmortem events should have similarly affected the diabetic and nondiabetic retinas.

The mechanism(s) and significance of the increased GFAP content in the retinas of diabetic donors are for the moment unknown. GFAP is present in normal Müller cells of several species (8), and its expression is upregulated by a variety of retinal insults (14,16,27) and also as an early event in age-related retinal degenerations (28). Increased GFAP expression may reflect glial cells replication, as it occurs in retinal detachment or upon administration of basic fibroblast growth factor (27,29), but in most circumstances reflects a state of glial hypertrophy, since astrocytes and Müller cells are generally mitotically quiescent. The finding that in the retinas of diabetic donors only the abundance of a cytoskeletal protein was increased points to the occurrence of glial cell hypertrophy, since an increase in Müller cell number would also be expected to result in a larger retinal content of Bcl-2 and GS. Whether the increased GFAP observed in the Müller cells of diabetic donors represents a primary response to the metabolic perturbation (perhaps induced by increased rates of Müller cell glycolysis) or a stereotypical reaction to other diabetes-induced processes in the retina, including vascular abnormalities, should now be investigated. Increased GFAP immunoreactivity (26) as well as ultrastructural changes (30) have also been observed in the Müller cells of rats with long-term experimental diabetes, and time-course studies should thus be possible in these models.

The investigation could profitably be extended to other biosynthetic properties of Müller cells that bear on the well-known abnormalities of retinal vessels in diabetes. Müller cells express nitric oxide synthase (3) and endothelin (31) and may thus contribute to blood flow regulation. They produce molecules that confer barrier properties to vascular endothelium (10) and produce or store growth factors active on vascular cells (11). If functionally relevant Müller cell abnormalities prove to be early events in the development of diabetic retinopathy, preventive strategies may need to enlarge or even shift their target.

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