Glial Reactivity, an Early Feature of Diabetic Retinopathy

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PURPOSE. To characterize early structural gliotic reactions in retinal Müller cells, astrocytes, and microglia in experimentally induced diabetes.

METHODS. Rats were rendered diabetic by streptozotocin injection and killed after 2, 4, 12, or 20 weeks. Cell densities were determined in flatmounted retinas and transverse semithin sections. Expression of glial fibrillary acidic protein (GFAP) was localized on frozen sections or flatmounts by immunofluorescence and confocal microscopy, and GFAP content was evaluated by Western blot analysis. Microglial cells were visualized by binding of isolectin B4 or staining with antibodies to phosphotyrosine residues. The integrity of the blood–retinal barrier was assessed by intravenous injection of Evans blue.

RESULTS. The density of Müller cells and microglia was significantly increased at 4 weeks of diabetes compared with nondiabetic controls. GFAP expression in Müller cells was not detected at 4 weeks but was prominent at 12 weeks. The number of astrocytes was significantly reduced at 4 weeks in the peripapillary and far peripheral retina. Shape changes of microglial cells indicated functional activation. Leakage of the blood–retinal barrier was observed at 2 weeks of hyperglycemia, the earliest time point investigated.

CONCLUSIONS. The leakage of the blood–retinal barrier before glial reactivity suggests that glia are early targets of vascular hyperpermeability. The individual glial cell types react differentially to the diabetic state. Müller cells undergo hyperplasia preceding GFAP expression, and microglial cells are activated, whereas astrocytes regress. This glial behavior may contribute decisively to the onset and development of neuropathy in the diabetic retina. (Invest Ophthalmol Vis Sci. 2000;41:1971–1980)

Breakdown of the blood–retinal barrier and occurrence of microaneurysms are generally considered to be clinical hallmarks of diabetic retinopathy (DR). However, a growing body of evidence documents that functional impairment arises well before the earliest clinical manifestations of vasculopathy become discernible. Sensitive psychophysical examination methods, such as contrast sensitivity, color vision, and focal ERG, clearly show that these parameters are altered early on as a potential consequence of hyperglycemia, not only in diabetic individuals but also in glucose-intolerant, nondiabetic and even in glucose-tolerant, obese individuals. However, no sign of vasculopathy could be detected by the commonly used clinical observation methods, indicating that neuronal impairment occurs much earlier in the pathogenesis of DR than originally assumed. This raises the question of the extent of involvement of the cellular elements of the neural parenchyma, neurons, and glia in the onset of DR.

The particular spatial arrangement of retinal macroglial cells (astrocytes and Müller cells) that are intercalated between vasculature and neurons points to their important role in the uptake of glucose from the circulation, its metabolism, and transfer of energy to neurons. Recently, these crucial functions have been recognized to be interdependent. Uptake of glucose and glycolysis in glial cells are closely linked to the release of glutamate from neurons and its uptake by glia, a process that is coupled with Na\(^+\)–K\(^+\)–adenosine triphosphatase (ATPase) activity.

In view of their intricate metabolic interdependence, dysregulation of a number of cell functions in both glial and neuronal cells can be anticipated under hyperglycemic conditions in which high glucose concentrations are found in the neural parenchyma because of increased permeability of the blood–retinal barrier. It is likely that the limiting factor in glutamate and glucose uptake by glial cells is Na\(^+\)K\(^+\)–ATPase, the activity of which decreases very rapidly in the hyperglycemic tissue. Impairment of the glial sodium pump has been related to increased glutamate release from injured neurons as well as to enhanced oxidative stress.

Reactive gliosis is a general response to injury and inflammation in the adult brain. It is characterized by upregulation of various kinds of molecules, the best known of which is glial fibrillary acidic protein (GFAP). Less frequently, glial hyperplasia is also observed. In Müller cells of the retina, de novo expression of GFAP is indicative of any kind of impairment of the retina, whether induced by glaucoma, retinal detachment, light damage, mechanical lesioning, or, notably, experimental and spontaneous human DR. In contrast, retinal astrocytes may not only acquire gliotic features but may decrease in number in
situations in which vessel damage with increased permeability of the blood-retinal barrier or a massive loss of neurons occurs.

Similar to macroglia, microglial cells are highly dynamic and capable of assuming different morphologies and functions in response to changes in their local chemical and cellular environment. Microglial activation is rapid and often precedes that of macroglia. This does not necessarily imply, however, that activated microglial cells induce macroglial reaction. Rather, the micro- and macroglial functions are interrelated in vivo, and both cell types may mutually induce their reactivity by the release of soluble factors. Moreover, the acquisition of glial features enables both macro- and microglia to participate in the initiation of an immune response.

Given the role of glial cells as communicators between vessels and neurons, understanding their behavior in diabetes may provide the necessary clues to interrelate diabetic vasculopathy and neuropathy. The present study was undertaken to record, in the diabetic rat model, glial alterations at an early time point of hyperglycemia, during the first 4 weeks after injection of streptozotocin (STZ). Local vascular leakage and reactivity of all three glial cell types, manifesting as hyperplasia of Müller cells, a decrease in cell density of astrocytes, and activation of microglial cells, are the earliest structural changes observed, long before overexpression of GFAP occurs.

**MATERIALS AND METHODS**

**Animals, Induction of Diabetes, and Anesthesia**

Experiments were performed on 63 albino rats of the Wistar-Kyoto (WKY) strain at 2, 4, 12, and 20 weeks of diabetes. The ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Swiss Law on Animal Protection were followed in all respects. Housing of the rats was standard, with food and water supplied ad libitum. The fluorescent light source (500 lux), cycled to 14 hours on and 10 hours off, provided an average intensity of 90 lux in the cages. Experimental and control animals were from the same breed, housed under identical conditions, and killed the same day.

Streptozotocin (STZ, 60 mg/kg body weight; Sigma, Buchs, Switzerland) was injected in one dose into the tail vein. At the time of injection, the body weight of each experimental group varied between 160 g and 175 g, but average weights were identical for the STZ-injected and the control animals of the same experiment. At 20 weeks of diabetes, the animals were 26 weeks old.

Blood glucose levels were determined with a glucose analyzer (Beckman Instruments, Zürich, Switzerland), once a week in 2- and 4-week diabetic animals or once every 4 weeks in 20-week diabetic animals. Final measurements were recorded at the end of the experiments, before animals were killed.

Anesthesia was induced by 2% isoflurane in an air stream and maintained with an intraperitoneal injection of Pentothal (Abbott, Cham, Switzerland). The animals were killed by intracardiac injection of 1 M KCl before enucleation of the eyes.

**Tissue Preparation**

**Semithin Sections for Cell Nuclei Counting.** Enucleated eyes were opened at the ora serrata and immersed in fixative solution I (1.5% glutaraldehyde, 1% freshly prepared formaldehyde, 2 mg/ml tannic acid, and 100 mM phosphate buffer [pH 7.4]) for 15 to 30 minutes (room temperature). The posterior segment was cut from the anterior, fixed in fixative II (2.5% glutaraldehyde in phosphate buffer [pH 7.0], and tannic acid) for a further 4 hours (room temperature), and cut into pieces before extensive washing in 100 mM cacodylate buffer (pH 7.4, 4°C). After osmification (30 minutes in 0.5% osmium tetroxide, 50 mM cacodylate buffer, and 0.8% ferrocyanide), the tissue blocks were stained in uranyl acetate, dehydrated through a graded series of ethanol, and embedded in Epon.

**Visualization of Vessel Leakage by Evans Blue**

Increased vascular permeability was assessed after 2 weeks of induction of hyperglycemia by injection of Evans blue, which binds to plasma albumin. Under deep anesthesia, rats weighing approximately 200 g were kept on a warming plate (37°C, 20 minutes) before injection, through the femoral vein, of 200 μl of 2% (wt/vol) Evans blue (Sigma) dissolved in sterile physiological solution. The animals were returned to the warming plate for 20 minutes before death. Retinas were rapidly isolated in 10% formaldehyde, flattened, and immediately viewed and photographed under fluorescent light (excitation filter 546 nm, barrier filter 590 nm).

**Staining Procedures**

**Indirect Immunoperoxidase and Immunofluorescence Staining.** Müller cells were visualized in Bouin-fixed paraffin sections with either polyclonal rabbit antiserum to CRALBP (kind gift of Dean Bok, Jules Stein Eye Institute, University of California, Los Angeles, CA) or S-100 protein (Dako Diagnostics, Zug, Switzerland), both diluted 1:100 in Tris-buffer (pH 8.0), and 150 mM NaCl. Before staining, deparaffinized and rehydrated sections were treated with 0.5% H2O2 in TBS (30 minutes) to block endogenous peroxidase and washed in TBS before incubation in the first antibody (45 minutes, 37°C). Immunoreactivity for CRALBP was revealed by a biotin-coupled secondary goat anti-rabbit antibody and subsequent incubation in a biotin-streptavidin-peroxidase complex (Dako, dilution according to the manufacturer) and for S-100 protein by donkey anti-rabbit F(ab)2 fragments coupled to peroxidase (dilution 1:100; Jackson Immunoresearch, Milan Analytica, La Roche, Switzerland). All incubation mixtures and intermediate washing steps for S-100 protein (but not for CRALBP) contained 1% Tween 20. After short fixation in 1.5% formaldehyde, the sections were rinsed in 50 mM Tris-HCl (pH 7.6) and reacted with diamino-
benzidine (DAB, 2 mg/ml) in Tris buffer containing 100 mM imidazole and 0.4% nickel chloride.

Microglial cells were visualized in retinal flatmounts with polyclonal rabbit antibodies to phosphotyrosine residues (dilution 1:100; Transduction Laboratories, Maechler, Basel, Switzerland) after blocking endogenous peroxidase and preincubation in TBS containing 0.5% Triton X-100 and 1 mg/ml bovine serum albumin. Secondary antibodies were peroxidase-coupled F(ab)2 fragments directed against rabbit IgGs (Jackson ImmunoResearch). The tissue was incubated (4°C) for 4 days with the first antibody and for 3 days with the second. Peroxidase was revealed by the DAB reaction, as indicated.

**GFAP Staining.** Retinal flatmounts stored in glycerol buffer were directly embedded in Tissue Tek (Miles, Bayer, Zürich, Switzerland) and cryosectioned. Sections were fixed in methanol and acetone (7 and 5 minutes, respectively) at −20°C, air dried, and rehydrated in TBS-0.05% Triton X-100. Monoclonal antibody to GFAP (clone GA-5, Boehringer–Mannheim, Rotkreuz, Switzerland) was used at a dilution of 1:5. Sections were incubated for 45 minutes at 37°C, washed in TBS-X 100, and further incubated (30 minutes, 37°C) in donkey anti-mouse IgGs coupled to Texas red (dilution 1:200; Jackson ImmunoResearch). After washing, the sections were rinsed in ethanol and mounted in Mowiol (Plüss-Staufer, Offingen, Switzerland).

In flatmounted retina, astrocytes were visualized by incubation in the monoclonal antibody to GFAP (2.5 days, 4°C) and donkey anti-mouse IgGs coupled to Texas red (1.5 days, 4°C). Incubation in the first antibody was made in the presence of 1% Triton X-100 and 1 mg/ml bovine serum albumin. Retinas were mounted in a mixture (3:7) of 100 mM Tris-HCl (pH 9.5) and glycerol containing 50 mg/ml n-propyl gallate.

**Lectin Staining.** For cell counting, microglial cells were visualized by isolectin B4 of *Griffonia simplicifolia* coupled to peroxidase (dilution 1:50; Sigma). Blocking of endogenous peroxidase as well as reaction with DAB were as has been described.

**Confocal Microscopy**

Formaldehyde-fixed whole retinas were preincubated in TBS containing 0.5% Triton X-100 and 0.5 mg/ml bovine serum albumin and incubated (4 days, 4°C) in a cocktail of monoclonal antibody to α-smooth muscle actin (supernatant diluted 1:10; kind gift of Giulio Gabbiani, Department of Pathology, University of Geneva, Switzerland) and polyclonal rabbit antiserum to GFAP (dilution 1:40; Bio-Science Products, Emmenbrücke, Switzerland). Secondary antibodies were donkey anti-mouse and anti-rabbit IgGs coupled to Texas red and fluorescein (Jackson Immunoresearch), respectively. The double-stained tissue was viewed, vitreous side toward the objective, in an inverted microscope (LSM 410; Carl Zeiss, Oberkochen, Germany) equipped with two lasers at wavelengths of 488 and 543 nm for the simultaneous excitation of fluorescein and Texas red, respectively. Optical sections were viewed using a ×40 oil objective at an interval of 0.5 μm. Extended-focus images of the complete data set were performed and processed by computer (Imaris software; Bitplane, Zürich, Switzerland) program with an algorithm for simulated fluorescent process to induce shadowing.

**Western Blot Analysis of GFAP**

Isolated retinas were rinsed in 100 mM PIPES-EGTA-MgCl2 buffer, drained, and solubilized by boiling in sodium dodecyl sulfate (SDS) sample buffer (pH 8.9). Protein concentration was determined using the BCA Protein Assay (Pierce, Socrinch, Lausanne, Switzerland). Total proteins (25 μg per slot) were electrophoresed on a 5% to 15% SDS-polyacrylamide gradient gel and blotted onto nitrocellulose.49 After they were blocked in TBS containing 0.05% Tween 20 and 5% fat-free milk powder, blots were incubated in a mixture of monoclonal anti-GFAP antibody (clone GA-5, dilution 1:3000; Sigma) and a rabbit anti-actin50 antisem (dilution 1:2000), washed, and further incubated in a mixture of secondary antibodies (donkey anti-mouse and -rabbit F(ab)2 coupled to peroxidase, dilution 1:4000; Jackson Immunoresearch). Blots were reacted with a chemiluminescence reaction product for bioluminescence (ECL; Amersham, Zürich, Switzerland).

**Cell Counting and Data Analysis**

**Müller Cells.** On semithin transverse sections, Müller cell nuclei in the inner nuclear layer, readily recognizable by their characteristic angular shape and homogeneous nucleoplasm (Fig. 1), were counted as a means to evaluate Müller cell

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932906/ on 06/26/2017)
number. Tissue sections were viewed with a microscope (Axioskop, Zeiss) using a ×63 oil objective lens. Images were captured with a video camera (ProgRes 3008; Kontron, Zürich, Switzerland), digitized on a computer (Macintosh; Apple Computer, Cupertino, CA), and projected onto a 20-in. screen. Nuclei were counted directly on the projected image corresponding to a stretch of a retinal section 135 μm in length. We evaluated the right eye retinas of seven animals at 2 and 20 weeks of diabetes, and of five animals at 4 weeks of diabetes. The same number of animals from the same breed served as control subjects. From each retina, four tissue blocks (two each from the peripheral and central retina) were chosen, and three images of each tissue block (i.e., 12 images per retina) were recorded.

Astrocytes. Cells were counted in retinal wholemounts of six 4-week diabetic animals and six age-matched control animals. Photomicrographs were taken on black-and-white film (HP5; Ilford Imaging, Marly, Switzerland) from GFAP-stained, fluorescent wholemounts using a ×20 objective lens. Nega-
tives were printed at a magnification of ×165. The number of astrocytes contained in a square area corresponding to 0.09 mm² of retinal tissue was determined. These areas were selected in a manner that avoided large blood vessels. Five to nine such areas could be counted for each retina, depending on the region. Peripapillary areas were termed central; those in the midperipheral retina, mid; and those in the far periphery, peripheral.

Microglial Cells. Cell counts were performed on flat-
mounted, lectin-stained retinas using an ocular reticulum of 1 mm² and a ×40 objective lens. Five areas per retina were randomly chosen, and the microglial cell number contained in the assigned square unit was determined. Seven to nine retinas per experimental group were analyzed.

Statistics
All counts were recorded in a double-blind manner. Data reported are means ± SD. For statistical evaluation, analysis of variance (ANOVA) and the Bonferroni test were used. P < 0.05 was considered significant.

RESULTS

Diabetic State

Body Weight. STZ-treated animals lost weight during the first 2 weeks after injection, began to put on weight, and, at 4 weeks of diabetes, weighed 86% of the weight of control rats (Table 1). At 20 weeks of diabetes, the animals had markedly lost weight again to reach a value similar to that at the time of injection.

Basal Glycemia. STZ-treated animals showed a fourfold increase in blood glucose levels at 2 and 4 weeks of diabetes. At 20 weeks, blood glucose was more than fivefold compared with that of the age-matched control animals (Table 1).

Permeability of Retinal Vessels. To consider the possibility that an increase in permeability of retinal vessels precedes glial modifications, we assessed the integrity of the blood–retinal barrier by intravascular injection of Evans blue,47 2 weeks after induction of hyperglycemia. Focal leakage of the dye from capillaries and larger vessels was noted in the retinal flatmounts (not shown), corroborating findings obtained by different techniques on compromised blood–retinal barrier early in diabetes.52,55

Müller Cells

Identification. To identify Müller glia at the light microscopic level, we stained Bouin-fixed transverse retinal sections with antibody to CRALBP and S-100 protein. As shown in Figure 1, immunoreactivity for both CRALBP (Fig. 1a) and S-100 (Fig. 1b) was confined to Müller cell structures, particularly prominent in the end feet at the vitreoretinal interface. Staining also outlined the characteristic angular nuclei in the inner nuclear layer. These distinct nuclear profiles were used as criterion to determine Müller cell density in semithin, meth-
ylene blue–stained sections (Fig. 1c).

Cell Density. To observe the dynamics of the Müller cell population, proliferation studies using bromodeoxyuridine (BrdU) incorporation and its immunocytochemical visualization on cryosections were attempted. This method turned out to be unsuitable for observing cell dynamics over several weeks, because the longest incorporation period without risk of toxicity to the organism is less than 2 days.54 In our experiments an incorporation period of 2 days yielded only a few labeled cells, too small a number for statistical evaluation. We therefore determined the number of Müller cells by counting, in semithin sections, the number of their nuclei at 2, 4, and 20 weeks after STZ injection (Fig. 2). During the 20-week exper-
imental period, control rats showed a steady decrease in Müller cell nuclei, possibly due to light damage in retinas of the albino phenotype. In STZ-injected animals, at 2 weeks of hypoglycemia, Müller cell number was similar to that in age-matched control animals. At 4 weeks, however, it was significantly higher (P < 0.017), and, at 20 weeks, it was 1.6 times the control value (P < 0.0001).

Because the statistical ANOVA (not shown) did not reveal heterogeneity among the individual measurements (n = 12) of a given tissue sample, the observed hyperplasia in the diabetic animals appeared to be evenly distributed throughout the whole retina.

Table 1. Body Weight and Blood Glucose Levels in Diabetic (STZ) and Age-Matched Control (C) Rats

<table>
<thead>
<tr>
<th>Weeks of Diabetes</th>
<th>n</th>
<th>Body Weight</th>
<th>Blood Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>STZ</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>6</td>
<td>225 ± 15</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>234 ± 19</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7</td>
<td>429 ± 21</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7</td>
<td>7.0 ± 1.2</td>
</tr>
</tbody>
</table>

Data for body weight are in mean grams ± SD and for blood glucose are in mean millimolar ± SD. n, number of animals.
Astrocytes

In the normal rat retina, the distribution of astrocytes is essentially uniform (55, 56) (Fig. 3), but, as was determined in GFAP-stained flatmounts, the cell number in the different retinal regions was heterogeneous (Fig. 3; Table 2) being higher in the peripapillary and midperipheral retina than in the far periphery. In 4-week diabetic retinas, astrocyte density was significantly lower in the peripapillary region ($P < 0.03$) and in the far periphery ($P < 0.01$), whereas in the midperiphery no difference between diabetic and control animals was noted.

GFAP Expression

Expression of GFAP in 4-week diabetic retinas was monitored by both immunofluorescence microscopy and immunoblot analysis. As in age-matched control retinas (Fig. 4a), staining in the diabetic animals (Fig. 4b) was confined to the innermost retinal layer, where astrocytes are located. Müller cell processes were not stained or were inconsistently stained in both the diabetic and the control group. Faint GFAP staining of Müller cells seemed not to be correlated with the hyperglycemic state of the animals.

Expression of GFAP at 4 weeks after injection was further evaluated by Western blot analysis (Fig. 4c). Immunoblots of retinal extracts of eight control and eight diabetic animals each showed high variability of the GFAP content in both groups. The densitometrically measured ratio between the actin (inter-

TABLE 2. Density of Astrocytes as Determined by GFAP Staining in Flatmounted Retinas of Age-Matched Control and Diabetic Animals at 4 Weeks of Diabetes

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>Diabetic</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>43.61 ± 1.13</td>
<td>40.52 ± 0.76</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Mid</td>
<td>43.28 ± 0.94</td>
<td>43.98 ± 0.83</td>
<td>0.58</td>
</tr>
<tr>
<td>Peripheral</td>
<td>36.80 ± 0.98</td>
<td>33.82 ± 0.61</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Cell counts were made on photographic prints from the peripapillary (central), midperipheral (mid) and far peripheral (peripheral) retina. Areas chosen were devoid of larger vessels. $n$, number of areas counted corresponding to 0.09 mm$^2$ of tissue. Data are means ± SD.
nal standard) and GFAP bands revealed no difference between diabetic and control retinas (not shown).

Taken together, the results show that no evident de novo expression of GFAP in Müller cells nor overexpression in astrocytes took place at an early stage (4 weeks) of DR. However, in long-standing diabetes, Müller cells indeed expressed GFAP, as shown by confocal imaging of retinal flatmounts (Fig. 5). Double-staining for GFAP and α-smooth muscle actin of a 12-week diabetic retina (Fig. 5a) and a normal age-matched (Fig. 5b) retina shows the spatial arrangement at the vitreoretinal interface of GFAP-containing glial elements and blood vessels. In the control retina (Figs. 5b, 5d), GFAP staining was restricted to astrocytes, whereas in the diabetic retina (Figs. 5a, 5c), both astrocytes and Müller cell end feet were stained. The tortuous profiles of the end feet indicating hypertrophy of GFAP-containing intermediate filaments were especially well seen in images from which the data set representing the vasculature was subtracted (Fig. 5c). By contrast, when compared with the normal retina (Fig. 5d), astrocytic profiles, notably the processes investing axonal bundles, were scanty in diabetic tissue (Fig. 5c), and the starlike cell bodies were irregularly distributed.

Microglia

Morphology. In the normal retina, isolectin B4 outlined fine arborizations of the microglial cells (Figs. 6a, 6b). Staining of these cells in the diabetic retina was enhanced at 4 weeks after STZ injection (Figs. 6c through 6f). Close to the vitreoretinal interface, parenchymal microglial cells embraced ganglion cell bodies (Figs. 6c through 6f) or contacted axonal bundles (not shown), and paravascular cells were apposed to blood vessels (Fig. 6c). Enlarged cytoplasmic processes with a granular content suggested synthetic or phagocytic activity (Figs. 6d, 6e, 6f).

In retinas at 20 weeks of diabetes, staining with isolectin B4 (Fig. 6g) and for phosphotyrosine residues (Figs. 6h, 6i) revealed microglial cells of a stout morphology with pronounced nodular enlargements along their cellular processes. The cells were frequently associated with blood vessels (Fig. 6g). Moreover, a strong tendency of microglia to invade outer retinal layers was noted (not shown).

Cell Density. The number of isolectin B4-stained microglial cells was recorded in flatmounted retinas at 2, 4, and 20 weeks of diabetes (Fig. 7). The average cell number was still similar in 2-week diabetic and control animals, became significantly higher in the 4-week diabetic group \( (P < 0.0001) \), and was nearly double in the 20-week group \( (P < 0.0001) \).

DISCUSSION

In an attempt to identify possible links between vasculopathy and neuropathy in short-term experimental diabetes, we investigated glial behavior in the retina of hyperglycemic rats, paying particular attention to the first 4 weeks after STZ injection. Because the integrity of the blood-retinal barrier is impaired even at mildly elevated blood glucose concentrations,57 an increase in vessel permeability is expected to take place rather early in diabetes. Indeed, results in two studies and in the present one have shown that retinal vessels become leaky within the first 2 weeks after induction of hyperglycemia,52,53 and, 4 weeks after injection, leukocytes adhere to the endothelium.58 By this time, all three glial cell types—Müller cells, astrocytes, and microglia—exhibit quantitative and structural alterations. In the current study, microglial cells displayed a phenotype characteristic of activation, reflected by an increase in cell number and changes in cellular shape. Müller cells and astrocytes showed opposite reactions. Müller cells increased in cell density, whereas astrocytes decreased in number and continued to decline over the next few months.

Because of their close topological association with blood vessels, glial cells are predestinated targets of vascular alter-
monocytes produce, under high-glucose conditions, tumor necrosis factor (TNF)-α and interleukin -6. We thus suspect that the observed glial reactivity was a direct consequence of leakage of glucose and such inflammatory agents from the vasculature into the neural parenchyma.

Surprisingly, in the diabetic retina, hyperplasia of Müller cells precedes GFAP overexpression, which is generally considered to be the key feature of gliosis. Many types of insults to the retina lead to a rapid upregulation of GFAP in Müller cells rather than to hyperplasia. However, proliferation of brain astrocytes has been observed in response to neural trauma. Hyperplasia and GFAP expression may thus not necessarily be induced by the same mechanisms. Because our statistical ANOVA indicated that Müller cell numbers were homogeneous within a given tissue sample, hyperplasia seems not to be regionally restricted. This points to free diffusion of the mitogenic signal throughout the retinal parenchyma, once the permeability of the blood-retinal barrier is increased. Among the large diversity of glial mitogens, both high glucose concentrations and TNF-α seem likely to promote glial cell proliferation in vivo, because they are effective mitogens in vitro.

Whereas Müller cells increased during the first month of hyperglycemia, astrocytes of the central and peripheral retina diminished in number. Although the two glial cell types share important functions, this contrasting reaction early in diabetes points to the existence of fundamental differences in their activation pathways and metabolism. Similarly, sodium-bicarbonate cotransporters in the rat retina differ in astrocytes and Müller cells and, in the cat retina, particulate glycogen is stocked primarily in Müller cells but not in astrocytes, suggesting differences in their handling of glucose. Because astrocytes preferentially contact ganglion cell bodies, their axons, and larger vessels, it is possible that they are influenced by this environment in quite a different manner than Müller cells, which extend across the entire neural retina.

Figure 5. Expression of GFAP in retinal flatmounts of a 12-week diabetic rat (a, c) and an age-matched control (b, d). Double immunofluorescence to visualize GFAP (glia, revealed by fluorescein isothiocyanate [FITC]) and α-smooth muscle actin (medial layer of blood vessels [V], revealed by Texas red). Analysis by confocal microscopy and simulated fluorescent image treatment to introduce shading. (a, b) Combined data set of both channels. (c, d) Data set of the FITC channel only to display the overall distribution of GFAP-stained structures. (∆) Ghost of the vessel. (c) Note the abundance in the diabetic retina of tortuous filament bundles (arrowheads) in Müller cells and the scarcity of astrocytic filaments (A) associated with ganglion cell axons (arrows). (d) GFAP staining in the control retina revealed astrocytes only. Bar, 50 μm.

Signals for microglial activation may be of various origins and natures. In the neural parenchyma they may emanate from both activated macroglial cells and damaged neurons.

During early diabetes, microglial cells are closely apposed to ganglion cell bodies and axons. This association could favor an initial microglial activation by compromised neurons, with release of free fatty acids being a likely inducer. During long-standing diabetes, however, microglial cells are frequently
found in outer retinal layers and in association with blood vessels. This behavior may be triggered by an increasingly damaged blood–retinal barrier.\textsuperscript{75,78,79}

In conclusion, all three cell types of retinal glia exhibit strikingly rapid reactivity to hyperglycemia that is preceded by increased vessel permeability. We propose that leakage from the vascular bed not only leads to increased glucose levels within the neural parenchyma capable of inducing glial reactivity by itself but sets free a number of blood-derived factors that are potential additional triggers. In this view, initial development of diabetic neuropathy relies, to a large extent, on altered glial behavior. This, in turn, disturbs the crucial functional interdependence of glia and neurons and thereby aggravates glucose-induced neuronal damage. Analysis of neuronal suffering and death during early DR is in progress.

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