Remodeling of Retinal Capillaries in the Diabetic Hypertensive Rat

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PURPOSE. To document the effect of sustained systemic hypertension on the integrity and ultrastructural morphology of retinal capillaries in diabetic and nondiabetic rats.

METHODS. Normotensive (strain Wistar–Kyoto; WKY) and genetically hypertensive (spontaneously hypertensive; SHR) rats were rendered diabetic by intravenous streptozotocin injection. At 20 weeks of diabetes, endothelial cells, pericytes, and extracellular matrix were evaluated by ultrastructural morphometry. Serum albumin was localized by immunofluorescence microscopy.

RESULTS. The endothelial cell layer was markedly thinner in the diabetic normotensive animals. The number of intercellular junctions was reduced in both the nondiabetic and diabetic hypertensive group but less so in the diabetic normotensive group. No significant endothelial cell loss was noted in either of the experimental groups, whereas the number of pericytes and the number of their cytoplasmic processes were reduced in diabetic and hypertensive animals. Significant thickening of the basement membrane and increased permeability to serum albumin were observed in diabetic and hypertensive rats and were strongly enhanced in the combined diseases.

CONCLUSIONS. Endothelial thinning and shape changes from an elaborate to a simpler form as well as rounding up of the pericytes and loosening of their vascular sheaths indicate remodeling of the vascular wall during chronic diabetes and sustained hypertension, before a characteristic vasculopathy becomes manifest. The combination of diabetes and hypertension enhances these features, as well as basement membrane thickening and breakdown of the blood–retinal barrier. (Invest Ophthalmol Vis Sci. 1999;40:2405–2410)

Essential hypertension, prevailing in persons with diabetes, increases the risk of nephropathy and morbidity and death of cardiovascular disease. It is also one of the most important risk factors for diabetic retinopathy, influencing both its development and severity, and thereby plays a major role in visual loss in diabetic patients.

Because both hypertension and diabetes have an impact on the vascular wall, they may act in an additive or synergistic manner. Indeed, thickening of the basement membrane, degeneration of pericytes, and rarefaction of vessels are features common to both diseases, whereas constriction of the capillaries in the hypertensive state or their dilation in the diabetic state are divergent traits. The mechanism through which hypertension worsens diabetic retinopathy is still not fully understood. Diabetic patients in whom a controlled rise in systemic blood pressure is performed, show impairment of retinal vascular autoregulation and hyperperfusion. This situation may lead to an increase in vascular wall shear stress and, eventually, to lasting endothelial damage. Moreover, in spontaneously hypertensive diabetic rats (SHR strain), the deposition of advanced glycation end products (AGEs) in arterioles and the occurrence of acellular retinal capillaries and microthromboses are strongly enhanced but can be corrected by aminoguanidine treatment that prevents the formation of AGEs. These observations suggest that increased nonenzymatic glycation due to hyperglycemia accelerates the development of a hypertensive vasculopathy.

In this study, we documented the effect of diabetes on the structure of retinal capillaries in SHR and normotensive (Wistar–Kyoto; WKY) rats. The model of the SHR rat shares several features with essential hypertension in humans and is thus well suited for studying the combined diseases of hypertension and diabetes. Morphologic changes were assessed by ultrastructural morphometry at 20 weeks of diabetes, a time point when acellular vessels are not observed yet. In addition, we evaluated, by immunofluorescence microscopy, the extent of vascular permeability to serum albumin.

MATERIALS AND METHODS

Animals

Male rats of the WKY and SHR strains (Iffa Credo, Lyon, France) weighing 240 g, were rendered diabetic by a single intravenous injection of 60 mg/kg body weight streptozotocin (Sigma, Buchs, Switzerland) at 7 weeks of age.

The animals were kept on a 14-hour-on–10-hour-off light cycle and fed a normal diet with water ad libitum. At monthly intervals, body weight, blood glucose levels after an overnight fast, and basal glucose levels were determined (glucose analyzer; Beckman Instruments, Geneva, Switzerland; Table 1).

Under the same experimental conditions, systolic blood pres-
sure was measured by indirect tail-cuff plethysmography in 12-week-old animals and at the end of the study (i.e., at 27 weeks) before the animals were killed.

All animal care and handling were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with the Swiss Law on Animal Protection. Anesthesia was induced with a gas mixture containing 2% isoflurane and 35% oxygen and maintained with an intraperitoneal injection of thiopental (Pentothal; Abbott, Cham, Switzerland). The animals were killed by an intracardiac injection of 1 M KCl and the eyes enucleated immediately.

### Tissue Processing

The morphometric study was performed on seven animals in each of the following four experimental groups: WKY-N (normotensive nondiabetic), WKY-D (normotensive diabetic), SHR-N (hypertensive nondiabetic), and SHR-D (hypertensive diabetic). Eyes were enucleated and fixed by immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.2% tannic acid. The eyes were immersed in cacodylate buffer and postfixed with 0.5% osmium tetroxide in the presence of 0.8% potassium ferrocyanide in 50 mM cacodylate buffer. After block staining in 2% uranyl acetate in 50% ethanol, tissue pieces were washed overnight in several changes of cacodylate buffer and postfixed with 0.5% osmium tetroxide in the presence of 0.8% potassium ferrocyanide in 50 mM cacodylate buffer. After block staining in 2% uranyl acetate in 50% ethanol, the tissue was dehydrated through a graded series of ethanol and embedded in Epon. Electron micrographs of thin sections were taken with a video camera at a magnification of 14,776 and transferred to a computer (AcerPower Pentium 133; Hewlett Packard, Geneva, Switzerland). Images were acquired by means of a frame grabber board (Matrox PCI; Matrox Electronic Systems Ltd, Dorval, Quebec, Canada).

### Computer-Assisted Morphometric Measurements and Statistical Analysis

Because structural differences exist among the capillaries of the individual layers of the retina,26 we evaluated only the capillaries of the outer plexiform layer, which harbors half the capillary bed. Electron micrographs of 10 to 12 randomly selected capillaries were taken from four different tissue blocks corresponding to up to 84 capillaries per experimental group. Only cross-sectioned capillaries were considered. A software program (KS 300, Kontron, Zurich, Switzerland) was used for morphometric analysis.

On each capillary, the following primary measurements were taken (Fig. 1A): 1) inner basement membrane length (IBML, segment surrounding the endothelial cells) and outer basement membrane length (OBML, segment surrounding both pericytes and endothelial cells). Correspondingly, the area circumscribed by the IBML was the one occupied by the capillary lumen plus the endothelial cell cytoplasm, and the area circumscribed by the OBML was the total area of the vessel including both endothelial cell and pericyte; 2) the area of the capillary lumen; 3) the width of the basement membrane, using the two-point minimum method27 that avoids an overestimation of its thickness.28 The measurements were obtained exclusively in segments that were lined by perivascular glial cells; 4) pericyte area and pericyte length (PL); 5) number of endothelial cell nuclei and pseudopod pairs at intercellular junctions per cross-section through the capillary; and 6) number of pericyte nuclei and processes per cross-section.

From these primary measurements, other parameters were calculated: the endothelial cytoplasmic area, measured as the total endothelial cell area delineated by the IBML minus the luminal and nuclear areas, and the portion of the capillary circumference (IBML) covered by pericyte cytoplasmic processes (PL), expressed as the percentage of total IBML (PL/IBML).

The inner and outer basement membrane areas calculated from the values of IBML and OBML were used to evaluate the caliber of the capillaries.14 The measurements of the endothelial cytoplasmic area and of the number of pseudopod pairs (derived from the number of intercellular junctions), taken together with the number of endothelial nuclei, were used as the parameter of endothelial cell shape. Pericyte loss was evaluated using the number of pericyte nuclei and processes and cell adhesion by the proportion of the IBML covered by pericytes. Pericyte hyperplasia was evaluated by the number of pericyte nuclei, pericytic processes, and cytoplasmic area.

All measurements were performed in a masked manner by investigators without prior knowledge of the capillary origin. All parameters are shown as mean ± SD. The significance of differences between groups was tested by analysis of variance and Student–Newman–Keuls test.

### Assessment of Vascular Permeability

Retinas were snap frozen and embedded (Tissue-Tek, Miles, Elkhart, IN). Five cryostat sections of each retina were immunofluorescently stained with a rabbit antiserum to rat serum to reveal the presence of the extracellular matrix protein, fibronectin, and the50

### Table 1. Physical and Metabolic Parameters of Normotensive Nondiabetic (WKY-N) and Diabetic (WKY-D), as well as Spontaneously Hypertensive Nondiabetic (SHR-N) and Diabetic (SHR-D) Rats at 27 Weeks of Age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY-N</th>
<th>WKY-D</th>
<th>SHR-N</th>
<th>SHR-D</th>
<th>P</th>
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<tbody>
<tr>
<td>Number of animals</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>429 ± 21</td>
<td>165 ± 11</td>
<td>397 ± 29</td>
<td>175 ± 15</td>
<td>0.001†‡§</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>135 ± 13</td>
<td>145 ± 8</td>
<td>195 ± 5</td>
<td>188 ± 16</td>
<td>0.001†‡§</td>
</tr>
<tr>
<td>Fasting blood glucose (mM/l)</td>
<td>5.1 ± 0.4</td>
<td>18.9 ± 4</td>
<td>3.9 ± 0.8</td>
<td>20.0 ± 2</td>
<td>0.001†‡§</td>
</tr>
<tr>
<td>Basal blood glucose (mM/l)</td>
<td>7.0 ± 1.2</td>
<td>38.0 ± 3</td>
<td>6.2 ± 1.5</td>
<td>40.7 ± 3</td>
<td>0.001†‡§</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Statistical comparison assessed by analysis of variance and Student–Newman–Keuls test.

† WKY-N compared with WKY-D.
‡ WKY-N compared with SHR-D.
§ SHR-N compared with SHR-D.
|| WKY-N compared with SHR-N.
¶ WKY-D compared with SHR-D.
albumin (dilution 1:50; Anawa, Wangen, Switzerland) and then in goat anti-rabbit IgS coupled to Texas red (dilution 1:200; Jackson Immunoresearch–Milan Analytica; La Roche, Switzerland). The specimens were examined in a masked manner for the presence of extravasated serum albumin. The sections were semiquantitatively graded for intensity of extravascular fluorescence using the following subjective grading scale: no staining, −; moderate staining, +; intense staining, ++.

RESULTS

Weight and Blood Glucose

At 27 weeks of age, WKY-N and SHR-N animals did not differ significantly in their body weight or in their blood glucose levels (Table 1). Both the WKY-D and the SHR-D groups had a significantly lower body weight and higher blood glucose levels (fasting and basal) than the WKY-N, and SHR-N animals. The mean systolic blood pressure was significantly higher in all SHR animals, whether diabetic or not. No increase in systolic blood pressure was induced by the diabetic state in normotensive or hypertensive animals.

Morphometric Measurements of Retinal Capillaries

Caliber. No significant differences were noted between the four experimental groups in the caliber of the capillaries measured (Table 2).

Basement Membrane. The SHR-N, SHR-D groups and the WKY-D group showed a significant increase in basement membrane thickness compared with WKY-N rats (Table 2). The increase was most pronounced in the SHR-D group and significantly higher than in both, the WKY-D and SHR-N groups, suggesting that both diseases act in an additive or even synergistic manner. Thickening was focal and confined to segments lining the perivasular glia limitans (Figs. 1B, 1C, 1D). This local disturbance was reflected in the increased SD of both diabetic groups (Table 2).

Endothelial Cells. In the WKY-D group, a significant decrease of the cytoplasmic area and a concomitant significant increase in the lumen area (Table 2) reflect a marked thinning of the endothelial cell layer. A similar, though less pronounced tendency was observed in the SHR strain. The number of endothelial cells, estimated by the number of nuclei per cross-section, was similar in all four experimental
Diabetic (SHR-D) Rats, Nondiabetic (WKY-N) and Diabetic (WKY-D) Rats, and Spontaneously Hypertensive Nondiabetic (SHR-N) and SHR-D animals indicating a marked diminution of IBML covered by the pericytes was decreased in WKY-D, statistically significant, in the SHR-N group. The percentage of IBML covered by the pericytes was decreased in WKY-D, statistically significant, in the SHR-N group. An earlier estimation of the endothelial cell number was based on the number of cytoplasmic pseudopod pairs that are regularly associated with intercellular junctions (cf. Fig. 1; ref. 29). Because their number may also vary with the complexity of cell shape (sections through rectangular or roundish cells display less pseudopod pairs than cells of a more elaborate form), we took the significant decrease in the number of pseudopod pairs in the SHR-N and SHR-D groups as an indication of a less intricate cell shape rather than as a sign of loss of cells.

**Pericytes.** The number of pericytes, as reflected by the number per cross-section, was significantly decreased in the WKY-D and SHR-D groups compared with the nondiabetic groups. This cell loss was particularly significant in the SHR-D animals and was also noted, although not statistically significant, in the SHR-N group. The percentage of IBML covered by the pericytes was decreased in WKY-D, SHR-N, and SHR-D animals indicating a marked diminution of cell-wrapping around the outer vessel surface associated with both the diabetic and hypertensive diseases. This feature was accompanied by a significant decrease in the number of pericyte processes. It is noteworthy, however, that the overall cytoplasmic area remained unchanged, except in the SHR-N animals, which showed a higher value. Besides cell loss and a decreased number of cytoplasmic processes, the results reflect hypertrophic modification and a change in the pericyte shape, from flattened to roundish, as exemplified in Figures 1B, 1C, and 1D.

### Immunohistochemical Assessment of Blood–Retinal Barrier Permeability

In WKY-N retinas, fluorescent staining for serum albumin was strictly confined to the vessel lumen, resulting in a relatively weak yet distinct outlining of the vascular profiles (Fig. 2A). In the SHR-N tissue (Fig. 2B), staining was enhanced and the definition of the vascular profile blurred because of leakage of serum albumin into the perivascular tissue. In SHR-D retinas (Figs. 2C, 2D), extravascular staining was observed in a fraction only of the vascular profiles (C), whereas other profiles, even within the same tissue section, showed no sign of leakage (D). A semiquantitative evaluation of serum albumin extravasation revealed an increase in vascular permeability in both WKY-D and SHR-N retinas (Table 3). This increase was most marked in SHR-D animals, and all the examined tissue sections displayed serum albumin extravasation.

### Table 2. Ultrastructural Morphometry of Capillaries in the Outer Plexiform Layer of the Retina in Normotensive Nondiabetic (WKYN) and Diabetic (WKY-D) Rats, and Spontaneously Hypertensive Nondiabetic (SHR-N) and Diabetic (SHR-D) Rats

<table>
<thead>
<tr>
<th></th>
<th>WKY-N</th>
<th>WKY-D</th>
<th>SHR-N</th>
<th>SHR-D</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Number of animals</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
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<tr>
<td>Caliber of retinal capillaries</td>
<td></td>
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<tr>
<td>Inner basement membrane area (μm²)</td>
<td>36.5 ± 5</td>
<td>41.2 ± 3</td>
<td>36.7 ± 4</td>
<td>35.4 ± 3</td>
<td>&lt;0.05,† &lt;0.01,‡ &lt;0.005,‡ &lt;0.03,§ &lt;0.01‖</td>
</tr>
<tr>
<td>Outer basement membrane area (μm²)</td>
<td>50.2 ± 8</td>
<td>55.9 ± 5</td>
<td>52.1 ± 5</td>
<td>50.7 ± 5</td>
<td></td>
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<tr>
<td>Basement membrane thickness (nm)</td>
<td>111.8 ± 14</td>
<td>132.8 ± 19</td>
<td>131.8 ± 8</td>
<td>150.3 ± 20</td>
<td></td>
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<tr>
<td>Endothelial cells</td>
<td></td>
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<tr>
<td>Endothelial cytoplasmic area (μm²)</td>
<td>15.6 ± 2</td>
<td>11.5 ± 3</td>
<td>12.5 ± 4</td>
<td>13 ± 3</td>
<td>&lt;0.02*</td>
</tr>
<tr>
<td>Lumen area (μm²)</td>
<td>9.5 ± 4</td>
<td>16.1 ± 1.7</td>
<td>13.2 ± 2</td>
<td>13.1 ± 3</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Endothelial pseudopod pairs/cross-section</td>
<td>0.47 ± 0.2</td>
<td>0.30 ± 0.1</td>
<td>0.25 ± 0.1</td>
<td>0.15 ± 0.05</td>
<td>&lt;0.05,† &lt;0.002,‡ &lt;0.01‖</td>
</tr>
<tr>
<td>Endothelial nuclei/cross-section</td>
<td>0.78 ± 0.1</td>
<td>0.79 ± 0.2</td>
<td>0.63 ± 0.2</td>
<td>0.69 ± 0.2</td>
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<tr>
<td>Pericytes</td>
<td></td>
<td></td>
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<tr>
<td>Pericyte length (μm)</td>
<td>9 ± 3</td>
<td>5.3 ± 0.5</td>
<td>6.4 ± 1</td>
<td>5.4 ± 0.9</td>
<td>&lt;0.003*, &lt;0.04,† &lt;0.008‡</td>
</tr>
<tr>
<td>IBML covered by pericytes (%)</td>
<td>35.8 ± 12</td>
<td>19.4 ± 1</td>
<td>24.8 ± 4</td>
<td>21.4 ± 4</td>
<td>&lt;0.001*, &lt;0.04,† &lt;0.003‡</td>
</tr>
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<td>Pericyte processes/cross-section</td>
<td>2.3 ± 0.7</td>
<td>1.7 ± 0.2</td>
<td>1.5 ± 0.5</td>
<td>1.9 ± 0.3</td>
<td>&lt;0.02*, &lt;0.03‡</td>
</tr>
<tr>
<td>Pericyte cytoplasmic area (μm²)</td>
<td>6.7 ± 3</td>
<td>6.8 ± 1</td>
<td>8.3 ± 3</td>
<td>6.6 ± 3</td>
<td></td>
</tr>
<tr>
<td>Pericyte nuclei/cross-section</td>
<td>0.37 ± 0.1</td>
<td>0.24 ± 0.06</td>
<td>0.29 ± 0.1</td>
<td>0.18 ± 0.1</td>
<td>&lt;0.04,‡ &lt;0.02‡</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. Statistical comparison assessed by analysis of variance and Student–Newman–Keuls test.

* WKY-N compared with WKY-D.
† WKY-N compared with SHR-N.
‡ WKY-N compared with SHR-D.
§ WKY-D compared with SHR-D.
‖ SHR-N compared with SHR-D.
DISCUSSION

In the present study, we showed that chronic diabetes in normotensive (WKY-D) and sustained systemic hypertension in nondiabetic (SHR-N) rats induced microvascular remodeling characterized by a thickened extracellular matrix (basement membrane) along the glia limitans, loss of pericytes, shape changes in both pericytes and endothelial cells, and an increased permeability to serum albumin. The combination of both diseases markedly enhanced most of these features.

Our results confirm previous reports showing that both diabetes and hypertension, regardless of their dissimilar triggering mechanisms, lead to hyperpermeability of the blood–retinal barrier. Leakage is dramatically enhanced in the superimposed diseases, as was also noted in previous studies, although it maintains its focal character typical of the chronic diabetic state. Uncontrolled passage of glucose and serum factors through a compromised blood–retinal barrier are likely to stimulate abluminal cells to overproduce extracellular matrix components. The resultant thickening of the basement membrane and deposition of unusual collagen polymers in turn could lead to qualitative and quantitative changes in the extracellular molecular sieve properties. The most likely candidate cells to upregulate the synthesis of extracellular matrix components are, besides endothelium and pericytes, the glial cells, specifically the Müller cells which sheathe capillaries of the outer plexiform layer.

The conspicuous tendency of pericytes toward reduction of the number of processes, toward becoming round, and toward decreasing contact with the underlying matrix indicates impaired cell adhesion. Although our results do not provide an explanation regarding the underlying mechanisms, they point out that the decrease in vessel sheathing is an early sign of pericyte loss resulting not only from cell death but also from detachment. In diabetic and hypertensive arteries of the human retina, we in fact observed single smooth muscle cells detaching from the vascular wall.

By contrast, we could not document any significant loss of endothelial cells at 20 weeks of diabetes or hypertension. The endothelial layer is significantly thinner in the diabetic rat, however, and, to a lesser extent, in the hypertensive rat, as deduced from a decrease in the endothelial surface area and a concomitant increase in the capillary lumen. In addition, our numeric evaluation of endothelial pseudopod pairs as a measure of endothelial junctions indicates that endothelial cells in both diabetic and hypertensive animals displayed a less elaborated, simpler shape than in the normotensive nondiabetic control animals. Superposition of both diseases renders this feature most conspicuous. Acquisition of a simpler cell morphology may well represent a transitory stage in the progression of endothelial disease before cell loss, acellular capillaries, and microaneurysms are noted.

In our study, the vascular caliber did not differ significantly in the four experimental groups, although we noted a slight tendency of diabetic capillaries to be enlarged. Because the capillary diameter in the outer plexiform layer is likely to be regulated by the pericytes, this would imply that the regulatory activity of the remaining pericytes in capillaries of diabetic rats is sufficient to maintain a vascular tone within normal range. Moreover, in none of the experimental groups studied did we note vasoconstriction as was previously reported in

TABLE 3.

| Number of Animals With Evidence of Extravasation of Serum Albumin as an Indication of Blood–Retinal Barrier Breakdown and Semiquantitative Evaluation of the Severity of the Leakage |
|-----------------|-----------------|-----------------|-----------------|
| Number of animals | WKY-N | WKY-D | SHR-N | SHR-D |
| Blood-retinal barrier breakdown | 0/7 | 5/7 | 3/7 | 7/7 |
| Severity of extravasation | – | + | + | ++ |
older (44 weeks) hypertensive rats. This feature may manifest itself only in an advanced state.

In conclusion, the observed alterations in the vascular wall during an intermediate stage of diabetes and/or hypertension showed, besides thickening of the basement membrane and hyperpermeability of the blood–retinal barrier, changes in endothelial and pericyte morphology and weakening of the pericyte sheath. These features document a loss of vascular integrity, which may well be the origin of massive vessel leakage and vascular ischemia. They occurred indiscriminately in diabetic and hypertensive animals. However, the enhancement of the pathologic traits in animals with both diseases underscores the importance of a tight control of hypertension in diabetic patients.

Acknowledgments
The authors thank Sabina Scarpino, Alain Conti, and Nicole Gilodi for skilful technical assistance.

References