Increased Prevalence of Microthromboses in Retinal Capillaries of Diabetic Individuals

Daria Boeri, Michele Maiello, and Mara Lorenzi

In diabetic retinopathy, capillary nonperfusion and eventual obliteration can lead to retinal ischemia and sight-threatening neovascularization. The occurrence of retinal microthrombosis in human diabetes has long been suspected and occasionally observed but never systematically demonstrated. We used trypsin digestion to isolate the intact vascular network from retinas obtained postmortem from nine diabetic donors (age 64 ± 11 years, duration of diabetes 6 ± 4 years; mean ± SD) and eight age-matched nondiabetic donors. Topographically matched sectors (each one-sixth of retina) of diabetic and nondiabetic retinas were tested sequentially with antibodies to fibrin cross-linking factor XIII and platelet glycoprotein (GP)-IIIa to identify fibrin-platelet thrombi. In some trypsin digests, we also examined vascular cell apoptosis. The retina from a nondiabetic donor, 24 years of age, who had died of trauma, was used to exclude confounding influences caused by the postmortem period. When compared with those of nondiabetic donors, the retinas of diabetic donors showed double the number of capillary segments with colocalized immunostaining for factor XIII and GP IIIa (P = 0.02). The total area of the positive segments was fourfold greater in the diabetic than in the nondiabetic donors (P = 0.02) and correlated with the duration of diabetes (r = 0.71, P < 0.05). Large thrombi were six times more frequent in the diabetic donors (P = 0.03), and there was a significant topographical association of microthrombosis with apoptotic cells in both diabetic and nondiabetic vessels (P = 0.0001). Hence, diabetes of short duration was found to be associated with a greater than normal number and size of platelet-fibrin thrombi in the retinal capillaries. These thrombi can contribute to capillary obliteration and retinal ischemia and may be a practical target for early drug intervention. *Diabetes* 50:1432–1439, 2001

RESEARCH DESIGN AND METHODS

Eye donors and specimens. The human eyes from anonymous donors were provided by certified eye banks through the National Disease Research Interchange. The donors were selected based on age <80 years, diabetes duration ≤15 years, and fewest possible chronic pathologies other than diabetes. Criteria for exclusion were retinal or hematological diseases, uremia, and use of chemotherapy or life support measures. The characteristics of the donors and specimens are reported in Table 1. The eyes of a 24-year-old male donor who had no medical history and had died of trauma in a motor vehicle accident were used as negative controls to identify vascular events attributable to the postmortem period. All eyes were fixed in 10% buffered formalin by the eye banks as soon as possible after death.

To isolate the vascular network from the neural retina, each retina was cut in six sectors, and each sector was separately digested with crude trypsin according to the method of Cogan et al. (10), with modifications previously reported (11). The trypsin digests were mounted on Silane-coated slides, air-dried, and stored at −20°C.

Immunohistochemistry. It was necessary to compare topographically matched sectors of diabetic and nondiabetic retinas in view of reports that the distribution of acellular capillaries is not uniform throughout the retina of experimentally diabetic animals (12). Because not all sectors were available from all retinas, on account of usage in concurrent studies, the diabetic and nondiabetic specimens to be compared were matched as follows (Table 1): superior temporal, three and three; central temporal, four and two; central nasal, one and one; inferior nasal, one and one; and superior nasal, one and zero diabetic and nondiabetic specimens, respectively.
TABLE 1
Characteristics of the donors and specimens studied and number of retinal capillaries positive for thrombi

<table>
<thead>
<tr>
<th>Donors (no.)</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Diabetes duration (years)</th>
<th>Diabetes therapy</th>
<th>Cause of death</th>
<th>Other pathologies/medications</th>
<th>Time to exudate clearance (h)</th>
<th>Time to capillary fixation (h)</th>
<th>Retina sector</th>
<th>Number of positive capillaries</th>
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<tr>
<td>Diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>77</td>
<td>F</td>
<td>5</td>
<td>Insulin</td>
<td>COPD</td>
<td>CHF</td>
<td>3.5</td>
<td>12</td>
<td>Central nasal</td>
<td>60</td>
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<tr>
<td>2</td>
<td>75</td>
<td>M</td>
<td>3</td>
<td>Insulin</td>
<td>MI</td>
<td>CAD</td>
<td>2.2</td>
<td>9</td>
<td>Central temporal</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>M</td>
<td>5</td>
<td>Oral agents</td>
<td>CVA</td>
<td>CAD, on Coumadin</td>
<td>2.0</td>
<td>13</td>
<td>Central temporal</td>
<td>101</td>
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<tr>
<td>4</td>
<td>45</td>
<td>M</td>
<td>7</td>
<td>Unknown</td>
<td>Cardiomyopathy</td>
<td>A-fib, on Coumadin</td>
<td>2.5</td>
<td>6</td>
<td>Central temporal</td>
<td>53</td>
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<td>5</td>
<td>57</td>
<td>M</td>
<td>10</td>
<td>Insulin</td>
<td>CPA</td>
<td>CAD, on aspirin</td>
<td>5.0</td>
<td>7</td>
<td>Superior temporal</td>
<td>87</td>
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<tr>
<td>6</td>
<td>76</td>
<td>M</td>
<td>5</td>
<td>Insulin</td>
<td>MI</td>
<td>None reported</td>
<td>2.5</td>
<td>13</td>
<td>Inferior nasal</td>
<td>82</td>
</tr>
<tr>
<td>7</td>
<td>57</td>
<td>M</td>
<td>2</td>
<td>Diet</td>
<td>CPA</td>
<td>Arrhythmias</td>
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<td>11</td>
<td>Superior temporal</td>
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<td>8</td>
<td>66</td>
<td>F</td>
<td>15</td>
<td>Insulin</td>
<td>Trauma</td>
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<td>9</td>
<td>56</td>
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<tr>
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<td>73</td>
<td>F</td>
<td></td>
<td>Ruptured AA</td>
<td>None reported</td>
<td></td>
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<td>MI</td>
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<td>4.5</td>
<td>12</td>
<td>Superior temporal</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>F</td>
<td></td>
<td>CHF</td>
<td>COPD</td>
<td></td>
<td>2.5</td>
<td>14</td>
<td>Superior temporal</td>
<td>61</td>
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<tr>
<td>5</td>
<td>63</td>
<td>M</td>
<td></td>
<td>MI</td>
<td>CHF, on Coumadin</td>
<td></td>
<td>5.5</td>
<td>16</td>
<td>Superior temporal/</td>
<td>22</td>
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<tr>
<td>6</td>
<td>67</td>
<td>M</td>
<td></td>
<td>MI</td>
<td>CAD, on cholestyramine; given heparin</td>
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<td>14</td>
<td>Inferior nasal</td>
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<tr>
<td>7</td>
<td>64</td>
<td>M</td>
<td></td>
<td>MI</td>
<td>CAD, HTN</td>
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<td>5.5</td>
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<td>Superior nasal</td>
<td>38</td>
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<tr>
<td>8</td>
<td>68</td>
<td>F</td>
<td></td>
<td>MI</td>
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<td>12</td>
<td>Inferior nasal</td>
<td>4</td>
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AA, aortic aneurysm; CAD, coronary artery disease; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; CPA, cardiopulmonary arrest; CVA, cerebrovascular accident; HTN, hypertension; MI, myocardial infarction. *Counts were performed on trypsin digests, each representing one-sixth of the retina. The results for nondiabetic subject no. 5 are the average of two experiments performed on two different trypsin digests (superior temporal and superinferior sectors, respectively).

The trypsin digests were tested sequentially with antibodies to the A-chains of factor XIII and to platelet glycoprotein (GP)-IIa. Factor XIII is a transglutaminase that catalyzes the covalent cross-linking of fibrin monomers to form a stable complex during clotting. Both platelet and plasma factor XIII contain A-chains, and immunodetection of factor XIII A-subunit has been found most sensitive in revealing capillary thrombi (13). However, the A-subunit has sequence homology with tissue transglutaminases. Hence, to ensure specific identification of luminal thrombi, the trypsin digests were also immunostained for factor XIIIA, the sensitivie in revealing capillary thrombi (14).

In situ cell death. The retinal trypsin digests of two diabetic and two nondiabetic donors were tested for microvascular cell apoptosis with the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) method using the In Situ Cell Death Detection Kit by Boehringer Mannheim Biochemicals (Indianapolis, IN), as previously described (11). The negative control received only the label solution without the terminal transferase. In these specimens, the TUNEL assay was performed first, and factor XIIIA was detected with secondary antibodies conjugated to Cy3 (red fluorescence) to facilitate discrimination from the green fluorescent signal of TUNEL-positive nuclei.

Analysis. Each trypsin digest was surveyed systematically (11) under the microscope to identify the outcome under study (TUNEL-positive nuclei, factor XIIIA immunostaining, and GP IIaA immunostaining). The observers were blind to the identity of the donors. At each sequential step, the areas of interest were located in the specimen by recording the coordinates and were photographed. Pictures were stored for image analysis, which was performed using the National Institutes of Health Image Program (http://rsb.info.nih.gov/nih-image). To ensure maximal specificity, the number of capillary segments positive for markers of microthrombosis was counted based on GP IIaA immunostaining. The positive capillary areas were computed independently for factor XIIIA and for GP IIaA, using only the areas in which immunostaining for the two antigens colocalized. Hence, the factor XIIIA–positive areas entering the analysis were only those also positive for GP IIaA. To test the association of TUNEL-positive (apoptotic) vascular cells with microthrombosis, the area occupied by the capillary network in the trypsin digest was measured and transformed into a sum of segmental units. These were computed by dividing the total capillary area in each trypsin digest by the average size of a thrombus (calculated, in turn, from the sum of the areas positive for both factor XIIIA and GP IIaA divided by the number of such areas).
The TUNEL-positive images could then be attributed to capillary units positive for thrombosis or negative for thrombosis.

Data are summarized as the mean ± SD. Statistical analysis was performed with the unpaired *t* and the Mann-Whitney rank-sum tests with similar results. The *χ*² test statistic was used to analyze the frequency of association of TUNEL-positive vascular cells with microthrombosis.

**RESULTS**

The diabetic and nondiabetic donors were of similar age (64 ± 11 and 63 ± 9 years, respectively), and for almost all donors, the causes of death as well as the pathologies other than diabetes were cardiovascular (Table 1).
The known duration of diabetes was 6 ± 4 years, which in epidemiological studies of patients with late-onset diabetes is associated with a 40–50% prevalence of retinopathy, almost exclusively nonproliferative (16). In the diabetic and nondiabetic groups, the time elapsed from death to enucleation of the eyes was 3 ± 1 and 4 ± 1 h respectively, and the time to fixation was 13 ± 6 and 11 ± 4 h. There was no evidence of neovascularization in the retinas examined.

The images in Fig. 1 are representative of those observed in the retinal capillaries of both nondiabetic and diabetic donors. Factor XIIIA immunostaining showed a faint diffuse or an occasionally patchy background fluorescence, most likely attributable to vascular wall transglutaminases. Staining of the capillary walls was often more marked along the edges, yielding a rail-like appearance (Fig. 1A). Such areas were negative for GPIIIa. In contrast, in other capillary segments, intense and homogeneous factor XIIIA fluorescence identified discrete and compact intraluminal deposits corresponding to clumps of platelets staining for GPIIIa. Such areas, in which immunostaining for the two antigens colocalized, were counted as thrombi. Factor XIIIA staining was often more homogeneous than GPIIIa staining of the same area (Fig. 1A and C), suggesting detection not only of platelets, but also of plasma factor XIII trapped in the fibrin that enmeshed the platelet aggregates (17). The more homogeneous factor XIIIA immunostaining was reflected in the image analysis in values numerically larger than those computed for GPIIIa immunostaining of the same area.

In the 24-year-old donor who had died of trauma, the one-sixth of retina examined showed only four positive capillaries, and the immunostained areas were extremely small. The total area of factor XIIIA positivity was 810 μm², and that of GPIIIa positivity was 416 μm². The number and extension of retinal capillary segments showing dual staining for factor XIIIA and for GPIIIa were greater in the two study groups, which consisted of older donors; and all measurements yielded significantly larger values in the diabetic retinas (Fig. 2). In the diabetic group (n = 9), the number of positive capillary segments was 60 ± 29 per one-sixth of retina (vs. 32 ± 18 in the nondiabetic group, n = 8, P = 0.02); the total area of factor XIIIA–positive segments was 29,793 ± 14,089 μm² (vs. 7,544 ± 8,558; P = 0.01), and the total area of GPIIIa-positive segments was 17,061 ± 13,884 μm² (vs. 4,231 ± 3,600; P = 0.02). In individual donors, the total area of factor XIIIA–positive capillary segments correlated with the duration of diabetes (r = 0.71, P < 0.05). The correlation between the total number of positive capillaries and duration of diabetes (r = 0.47) was not significant in the small group studied. None of the measurements correlated with age in the diabetic and nondiabetic donors; if the 24-year-old donor was included in the nondiabetic group, r was 0.49. The findings were consistent within the same retina. In two different retinal sectors of a nondiabetic donor, each representing one-sixth of retina, the number of positive capillary segments was 21 and 24, respectively (Table 1).

Two of the nondiabetic donors were known to have received heparin at the time of the myocardial infarction that was the cause of death (Table 1). In one of these donors, there were only 7 retinal capillary segments positive for factor XIIIA and for GPIIbIIIa, whereas there were 26 in the other, a value close to the group mean. In one nondiabetic and two diabetic donors who were known to have been on Coumadin treatment, the number of positive capillary segments was 22, 53, and 101, respectively, values within the range of the respective group. Aspirin treatment was reported for only one subject (diabetic donor no. 5 in Table 1), who also had a number of positive capillary segments within the group range. In the absence of laboratory data on the efficacy of anticoagulation and without knowledge of the duration, dose, and compliance with aspirin treatment, it is not possible to draw conclusions on the effects of the drugs on retinal microthrombosis.

Studies of experimental retinal microthrombosis have shown that the duration of occlusion increases with the length of the vascular segment involved (18). We thus compared in nondiabetic and diabetic trypsin digests the prevalence of capillaries showing areas of factor XIIIA immunoreactivity in excess of 1,000 μm², equivalent to four times the average size of the thrombus measured in nondiabetic vessels. Such large thrombi were rare within the group of nondiabetic donors (1.2 ± 1.6 per one-sixth of retina, with a total extension of 3,056 ± 2,704 μm²); and in the diabetic donors, both their number (7.6 ± 5.3 per one-sixth of retina) and total extension (13,640 ± 9,757 μm²) (2) were severalfold greater (P < 0.03 for both measurements). The microvascular network of a diabetic donor was reconstructed from individual microscopic fields (Fig. 3) to provide a perspective for the areas

**FIG. 2.** Quantitative analysis of platelet-fibrin thrombi in the retina of diabetic (DM) and nondiabetic control (C) donors. A: Capillary segments showing GPIIIa immunoreactivity were counted throughout each trypsin digest, representing one-sixth of retina. B: The total capillary area occupied by factor XIIIA immunostaining (left) and by GPIIIa immunostaining (right) were computed in each trypsin digest with the aid of the National Institutes of Health Image program. Only the areas positive for both antigens entered the analysis; the larger values for factor XIIIA versus GPIIIa immunostaining reflect its more homogeneous appearance, as shown in Fig. 1. The bars represent the mean ± SD.
occupied by these larger thrombi in relationship to the total capillary area and to show their features and topography. Of the 11 capillary segments in which factor XIIIA immunoreactivity was in excess of 1,000 \( \mu \text{m}^2 \) (four times the average area of the thrombus measured in nondiabetic vessels), 5 were located in the posterior polar region, a preferential site of ischemic events in diabetic retinopathy (19).

A cellular abnormality we have previously noted in the retinal capillaries of diabetic subjects, which may relate to microthrombosis in numerous ways, is accelerated apoptosis of vascular cells (11). The topographical relationship of apoptotic (TUNEL-positive) cells and thrombi was examined in two diabetic and two nondiabetic donors. Capillary thrombi were much more numerous than TUNEL-positive cells, but the latter were often detected adjacent to or within microthrombi (Fig. 4). The observed associations of TUNEL-positive nuclei and microthrombi were 12 versus the 0.03 that was expected in diabetic vessels, and 1 vs. 0.01 expected in nondiabetic vessels (\( P = 0.0001 \)), indicating that microvascular cell apoptosis and microthrombosis occur in contiguity not by chance alone.

**DISCUSSION**

In retinas obtained postmortem from type 2 diabetic individuals, we observed more numerous and larger microthrombi than in retinas of age-matched nondiabetic individuals. We found a correlation between the total area of capillary thrombi and the known duration of diabetes, which must, however, be interpreted with caution, in view of the limited sample examined and the known difficulty in precisely dating the onset of type 2 diabetes. We also observed a greater than expected frequency of association of microthrombi with apoptotic vascular cells.

A caveat to be addressed outright is that the study was performed on postmortem samples. Human retinas for
systematic laboratory studies are accessible only through the eye donor programs but could be expected to be informative specimens, because there are differences between intravital thrombi and thrombi formed postmortem. In accordance with the evidence that platelets do not become activated postmortem (20), white thrombi consisting of compact aggregation of platelets and firm fibrin can only be formed during intravital clotting. Thrombi formed postmortem predominantly contain erythrocytes, a variable number of leukocytes, a few platelets, and a loose fibrin network (21). The reagents used in our study were directed at platelets and fibrin and would not have detected thrombi with other components. Moreover, the reagents detected only platelet aggregates, not platelets suspended in blood, and colocalized the platelet aggregates with cross-linked fibrin. Direct experimental confirmation that the processing of the tissue and the postmortem period could not in themselves generate the appearance of microthrombosis was sought by studying the eyes of a young donor who died of trauma and had no medical history. In the retina of this individual, only a minuscule number and extension of thrombi were recorded. The larger number of microthrombi found in the retinal vessels of the study groups could not in themselves generate the appearance of microthrombosis was sought by studying the eyes of a young donor who died of trauma and had no medical history. The reagents used in our study were directed at platelets and fibrin and would not have detected thrombi with other components. Moreover, the reagents detected only platelet aggregates, not platelets suspended in blood, and colocalized the platelet aggregates with cross-linked fibrin. Direct experimental confirmation that the processing of the tissue and the postmortem period could not in themselves generate the appearance of microthrombosis was sought by studying the eyes of a young donor who died of trauma and had no medical history. In the retina of this individual, only a minuscule number and extension of thrombi were recorded. The larger number of microthrombi found in the retinal vessels of the study groups could not in themselves generate the appearance of microthrombosis was sought by studying the eyes of a young donor who died of trauma and had no medical history. The retinal trypsin digest shown in Fig. 3 was tested sequentially with the TUNEL assay (A), antibodies to factor XIIIA (B), and antibodies to GPIIIa (C) and stained with periodic acid Schiff–hematoxylin (D). The two TUNEL-positive nuclei in A correspond, respectively, to the round eccentric nucleus of a pericyte (arrowhead) and to the elongated nucleus of an endothelial cell (arrow), recognizable in C and D. The intervening capillary segment shows immunostaining for factor XIIIA and for GPIIIa, which identifies a microthrombus. The surrounding capillaries show some degree of pericyte loss but visible endothelial cell nuclei and can be presumed to be patent. The scale bar represents 50 μm.

Many of the systemic abnormalities that make both type 1 and type 2 diabetic patients more prone to large vessel disease are prothrombotic: hyperreactive platelets (22), decreased vascular prostacyclin production (22), endothelial dysfunction resulting in increased circulating levels of von Willebrand factor (22) and leukocyte adhesion molecules (23,24), hypercoagulability (25), and decreased fibrinolysis (25). These may also contribute to microvascular disease. Increased levels of intercellular adhesion molecule-1 (26) and of plasminogen activator inhibitor-1 mRNA (27), as well as decreased levels of tissue plasminogen activator (28), are specifically found in retinal vessels of diabetic compared with nondiabetic individuals. Intercellular adhesion molecule-1 can facilitate the leukostasis described in diabetic retinal vessels (6,7) and potentially leads to occlusions and endothelial injury or death. The combined reciprocal changes of plasminogen activator and its inhibitor can compromise fibrinolysis, thus permitting extension of thrombi and lessening recanalization. Additionally, the accelerated apoptosis occurring in diabetic retinal capillaries (11) can provide a trigger for microthrombosis insofar as apoptotic endothelial cells become procoagulant (29) and generate a proinflammatory environment (30). Indeed, in this study, we observed a significant association of vascular cell apoptosis and thrombosis within the same capillary segment. The relationship of endothelial activation and apoptosis with microthrombosis is likely to be a circular one. On the one hand, endothelial activation and apoptosis are prothrombotic, and on the other hand, ischemia consequent of microthrombosis can lead to vascular cell death and/or enhance endothelial leukocyte–adhesive interactions (31). An added contribution of systemic hypertension to the increased prevalence of microthromboses observed in the diabetic donors cannot be excluded but was not apparent in our small cohort. Hypertension was explicitly reported in two of the diabetic and one of the nondiabetic donors, and the number and extension of retinal microthrombi in these subjects were in the mid-range or lower range of the distribution for the respective group.

The nondiabetic individuals examined in this study also...
showed retinal microthromboses. The occurrence of these microthromboses may explain the extensive capillary obliteration found in the peripheral (32) and central (33) retina of older individuals but will not result in the retinopathy typical of diabetes. Thus, one wonders whether and how the microthromboses observed in the diabetic donors in our study relate to the development of retinal microangiopathy. The results of trials with antiplatelet agents suggest that they may be related, and this and other studies propose possible mechanisms. In humans, aspirin administration had no beneficial effect in advanced nonproliferative diabetic retinopathy (34) but significantly slowed the progression of early retinopathy (35), akin to ticlopidine (36), and in dogs completely prevented the development of acellular capillaries when begun at the time of induction of diabetes (37). In their aggregate, these findings suggest that platelet-mediated events are important relatively early in the process of capillary obliteration in diabetes. Microthromboses may be especially damaging to diabetic vessels for at least three reasons. As shown in this study, they appear to be more frequent and extensive and/or less susceptible to recanalization, heightening the risk of ischemia. In turn, ischemia reperfusion appears to cause an exaggerated inflammatory response in diabetic microvessels (31). Lastly, vascular endothelium already challenged by hyperglycemia (38) is likely to be less tolerant of added stress and to enter an accelerated cycle of death (11) and replication (39). More extensive microthrombosis accompanied by an exaggerated inflammatory response and accelerated death and turnover of the endothelium can be mechanisms for irreversible architectural changes in the microvessels.

It is becoming apparent that at least three processes can contribute to retinal capillary occlusion and obliteration in diabetes: proinflammatory changes, microthrombosis, and apoptosis. These processes have been documented in both human (11,26, and this study) and experimental (4–7,11) diabetes. Their sequence has not been specifically investigated, but the studies in experimental animals indicate that the proinflammatory changes and leukostasis are the earliest detectable events (observed after 1 week of diabetes) (7) and that increased frequency of microthrombosis and apoptosis becomes detectable midway between the onset of diabetes and the appearance of characteristic histological changes of the capillaries (8–10 months) (4,5,11). However, the reduced retinal blood flow observed in type 1 diabetic patients still free of retinopathy (40) and the beneficial effect of short-term aspirin in improving retinal blood flow in these patients (41), together with the diminishing effect of aspirin as retinopathy progresses (34), suggests that microthrombosis may be occurring earlier than it is detected. It is conceivable that the earliest capillary occlusions caused by platelet-fibrin thrombi are transient and escape detection but leave behind a substantial degree of capillary damage.

The results of this study and of the aspirin trials point to a mechanism for the causation of retinal ischemia in diabetes that could be approached with clinically available drugs. The American Diabetes Association (42) has recently issued recommendations for the use of aspirin in diabetic patients, targeted to both secondary and primary prevention of large vessel disease. When implemented early, such strategy may extend its benefits to retinopathy. Ultimately, sustained effects of antiplatelet therapy in preventing diabetic retinopathy will depend on whether the therapy targets a primary mechanism of capillary damage and whether the abnormalities that it may not prevent remain free of clinical consequences. The possibility that doses of aspirin achieving anti-inflammatory effects in addition to antiplatelet effects may be a superior strategy also needs investigation.

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REFERENCES