Early Complement Activation and Decreased Levels of Glycosylphosphatidylinositol-Anchored Complement Inhibitors in Human and Experimental Diabetic Retinopathy

Jing Zhang, Chiara Gerhardinger, and Mara Lorenzi

Diabetic retinal microangiopathy is characterized by increased permeability, leukostasis, microthrombosis, and apoptosis of capillary cells, all of which could be caused or compounded by activation of complement. In this study, we observed deposition of C5b-9, the terminal product of complement activation, in the wall of retinal vessels of human eye donors with 9 ± 3 years of type 2 diabetes, but not in the vessels of age-matched non-diabetic donors. C5b-9 often colocalized with von Willebrand factor in luminal endothelium. C1q and C4, the complement components unique to the classical pathway, were not detected in the diabetic retinas, suggesting that C5b-9 was generated via the alternative pathway, the spontaneous activation of which is regulated by complement inhibitors. The diabetic donors showed a prominent reduction in the retinal levels of CD55 and CD59, the two complement inhibitors linked to the plasma membrane by glycosylphosphatidylinositol anchors, but not in the levels of transmembrane CD46. Similar complement activation in retinal vessels and selective reduction in the levels of retinal CD55 and CD59 were observed in rats with a 10-week duration of streptozotocin-induced diabetes. Thus, diabetes causes defective regulation of complement inhibitors and complement activation that precede most other manifestations of diabetic retinal microangiopathy. These are novel clues for probing how diabetes affects and damages vascular cells. *Diabetes* 51:3499–3504, 2002

The metabolic abnormalities of diabetes cause damage to blood vessels throughout the body. Damage to the retinal capillaries is manifested with abnormal permeability that can lead to macular edema and with occlusion and obliteration that can engender retinal ischemia and unregulated angiogenesis. Underlying processes documented to date are microthrombosis (1), leukostasis (2), and accelerated apoptosis of vascular cells (3). Whether these are the only processes operative in causing retinal vascular damage in diabetes and how they are triggered are the objects of ongoing investigation.

Activation of the complement cascade can both compound and initiate thrombosis, leukostasis, and apoptosis. On the one hand, microthrombi and leukostasis can cause ischemia-reperfusion, which activates complement via the classical pathway (4), and apoptotic endothelial cells can trigger the alternative pathway (5). On the other hand, complement activation is procoagulant, proinflammatory, and proapoptotic (6), and primary activation of complement on autologous cells can occur because of insufficient activity of plasma and/or cell surface inhibitors. Spontaneous cleavage of plasma C3 generates C3b, which attaches covalently to the endothelial cell surface through its reactive thioester group (5). Additional activation steps are normally prevented by complement inhibitors (7). Among these are the cell surface membrane co-factor protein (MCP; CD46) and decay accelerating factor (DAF; CD55), which act to restrict the activity of the C3/C5 convertase enzymes, and protectin (CD59), which inhibits the final step in the assembly of the membrane-attack complex (MAC), the terminal and potentially cytotoxic product of complement activation. Targeted mutation of the gene encoding Crry, the rodent complement inhibitor that exhibits both MCP- and DAF-like activity, causes embryonic lethality because of complement activation at the fetomaternal interface (8). Inherited deficiency of CD59 in humans causes paroxysmal nocturnal hemoglobinuria (9), and experimental neutralization of CD59 activity in rats augments complement-mediated glomerular damage (7).

Glomerular structures (10) and endoneurial microvessels (11) of patients with diabetes show signs of complement activation. Decreased availability or effectiveness of complement inhibitors in diabetes is suggested by the findings that high glucose in vitro selectively decreases on the endothelial cell surface the expression of CD55 and CD59 (12), the two inhibitors that are glycosylphosphatidylinositol (GPI)-anchored membrane proteins (7), and that CD59 undergoes nonenzymatic glycation that hinders its complement-inhibitory function (13). In this work, we investigated whether complement activation is a feature of human nonproliferative diabetic retinopathy and is associated with changes in inhibitory molecules. We extended the study to an animal model of...
nonproliferative diabetic retinopathy to test the universal-
ity and timing of abnormalities.

RESEARCH DESIGN AND METHODS

Eye donors and specimens. Human postmortem eyes were obtained from
Eye donors and specimens.

Groups

<table>
<thead>
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<th>Immunochemical studies</th>
<th>Age (years)</th>
<th>Sex (M/F)</th>
<th>Diabetes duration (years)</th>
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<th>Eyes: time to processing (h)*</th>
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Data are means ± 1 SD. *The time elapsed from death to retina processing was longer for the eyes used for protein isolation (fresh samples) than for the eyes used in the morphological studies because the latter were fixed by the eye banks before shipment.

TABLE 1
Characteristics of eye donors and specimens

Animals and specimens. Six-week-old male Sprague-Dawley rats (Taconic
Animals and specimens. Rats (Taconic Farms, Germantown, NY) were assigned randomly to a diabetic and a control group. Diabetes was induced by intravenous administration of streptozotocin (55 mg/kg body wt, dissolved in citrate buffer pH 4.5). The care of the diabetic rats and insulin treatment to prevent weight loss were described previously (14). The rats were killed after 10 weeks of diabetes. A total of nine diabetic and nine control rats were studied; from each rat, one retina was embedded in OCT to prepare frozen sections, and the other was homogenized in lysis buffer for isolation of protein.

Immunohistochemistry. Sections (10 μm) and trypsin digests from the
Immunohistochemistry. Sections (10 μm) and trypsin digests from the formalin-fixed human retinas were rehydrated and blocked as described (15) and incubated overnight at 4°C with the primary antibodies diluted in PBS containing 2% BSA and 0.3% Triton X-100. The reactions were visualized by peroxidase immunohistochemistry or immunofluorescence (15,16). The primary antibodies were mouse monoclonal antibodies (mAb) aE11 anti-human C5b-9 (0.5 μg/ml; Dako), Capringtona, CA), which react with a neoepitope on poly C9 exposed upon formation of MAC; IA4 anti-human smooth muscle
Immunohistochemistry. Actin with mouse mAb AC-15 (1:200,000; Dako); E4.3 anti-human CD46 (1 μg/ml; Santa Cruz Biotechnol, Santa Cruz, CA); MEM-43 anti-human CD59 (1 μg/ml; Serotec, Oxford, U.K.); and rabbit polyclonal antibodies to human C3d (1:1,000), Clq (1:1,000), C4c (1:1,000), and von Willebrand factor (vWF; 1:7,000), all from Dako. The rat retinal sections were fixed in acetone and studied with mAb 2A1 anti-rat C5b-9 (1:150; provided by W.G. Couser). Negative controls were obtained by substituting the primary antibodies with an equivalent concentration of nonimmune isotypic mouse IgG or rabbit IgG as appropriate.

Immunoblotting and immunoprecipitation. For isolation of protein, the
Immunoblotting and immunoprecipitation. Fresh retinas were homogenized in ice-cold lysis buffer containing phospha-
Itase and protease inhibitors as described (16). The homogenate was sonicated three times for 2 s and centrifuged at 16,000g for 15 min at 4°C, and the supernatant was collected and stored in aliquots at −80°C. Protein concentration was determined with the Bradford method using BSA as standard (protein assay kit; Bio-Rad, Hercules, CA). Retinal proteins were resolved by SDS-PAGE and immunoblotted as described (14). For the study of CD46, CD55, and CD59, SDS-PAGE was under nonreducing conditions. In the human retinas, CD46 was detected with rabbit polyclonal antibody H-294 (1:1,000; Santa Cruz), CD55 with mAb BRIC 216 (1 μg/ml; Serotec), and CD59 with both mAb MEM-43 (1 μg/ml) and goat polyclonal Ab N-20 (1:1,000; Santa Cruz). Neuron-specific enolase was detected with mAb BBS/NCV/H414 (1 μg/ml; Dako). In the rat retinas, CD46, CD55, and CD59 were detected with rabbit polyclonal antibody H-294 (1:1,000), H319 (1:200), and R-79 (1:1,000), respectively (all from Santa Cruz), and β-actin with mouse mAb AC-15 (1:200,000; Sigma, St. Louis, MO).

To determine whether the antibodies chosen could detect CD59 quantita-
To determine whether the antibodies chosen could detect CD59 quantitatively in diabetic samples irrespective of glycation, we tested the antibodies against red blood cells (RBCs) CD59 was digested in vitro. Normal human RBCs were incubated in standard storage medium containing 55 mmol/l glucose or in storage medium containing 150 mmol/l glucose for 40 h at 30°C, following a protocol previously described (17). Glycation of hemoglobin was measured with the Gly-Affin GHb Assay (Perkin Elmer Wallac, Nortn, OH). Equal amounts (200 μg) of solubilized protein from the high- and control-glucose RBC were incubated with mouse mAb BRA-106 anti-human CD59 (20 μg/ml; Ancell, Bayport, MN) at 4°C overnight. The immunocomplexes were precipitated with the addition of protein-G Sepharose (Sigma) for 2 h, and the resulting immunoprecipitates were analyzed by Western blotting (14). CD59 glycation, known to lead mostly to formation of Amadori product (13), was measured with a polyclonal antibody (provided by N. Taniguchi) that reacts with ε-(1-deoxyhexitoyl)-lysine, the reduced form of the Amadori product (18). Hence, the blots were treated with NaBH4 (50 μmol/l in PBS) for 4 h at room temperature, followed by a 15-min wash in PBS acidified to pH 5.0 with acetic acid to stop the reaction. After additional PBS washes, the membrane was blocked and reacted with the anti–hexitol-lysine antibody (1:1,000). The amount of immunoprecipitated CD59 was measured in companion blots probed with the anti-CD59 mAb MEM-43 or polyclonal antibody N-20. Similar immunoprecipitation experiments were performed with lysates of individual retinas of diabetic and nondiabetic donors to assess the level of glycation of retinal CD59 in human diabetes.

Statistical analysis. Data are summarized as the mean ± SD. Statistical analysis was performed with the unpaired t test.

RESULTS

MAC deposition in human diabetic retinal vessels.
RESULTS MAC deposition in human diabetic retinal vessels. Radial retinal sections from 12 of the 15 diabetic donors studied showed C5b-9 immunostaining. The staining localized at small and mid-size vessels (Fig. 1), which were mostly negative for smooth muscle actin. In contrast, no staining or only occasional punctiform staining was observed in vessels of nondiabetic donors. Also, the retinal capillaries, not readily visualized in sections and therefore studied in trypsin digest preparations, showed MAC immunostaining in the diabetic but not nondiabetic donors (Fig. 1). In diabetic retinal vessels observed in confocal microscopy, MAC was seen on the endothelial surface, often colocalized with vWF (Fig. 2). Occasionally, MAC was also detected in the middle and outer layers of the wall of large vessels, concentric to the lumen and possibly reflecting accumulation over time in basement membranes (Fig. 2).

MAC colocalizes with C3 but not with complement components of the classical pathway. In the retinal sections of diabetic donors, MAC showed perfect colocalization with C3, the complement component on which the
classical and alternative pathways of activation converge. In confocal microscopy, colocalization was captured on the surface of vascular endothelium (data not shown). In the same retinas, C1q and C4, the two complement components unique to the classical pathway, were either not detected at all or detected occasionally in vascular lumens reflecting their presence in blood (Fig. 3). This pattern is consistent with complement activation occurring in diabetic retinal vessels via the alternative pathway.

Selective decrease in GPI-anchored complement inhibitors in diabetic retinal vessels. Because activation of the alternative pathway of complement is critically modulated by inhibitors, we compared the levels of inhibitors in the retina of diabetic and nondiabetic donors and investigated their topography in the human retina. In SDS-PAGE and Western blot (Fig. 4A), CD55 was detected as a unique band of 70- to 80-kDa apparent molecular mass; its levels were reduced by 48% in the diabetic retinas when compared with the control retinas (P = 0.001). CD59 migrated as a broad band at 20–25 kDa, reflecting a mixture of glycoforms (19), and its levels measured using either Ab MEM-43 or N-20 were decreased as well in the retinas of diabetic donors (P = 0.01). CD46 showed in the retina the two isoforms (65 and 55 kDa) reported in other organs and accounted for by alternative splicing, leading to different degrees of glycosylation (20). An additional band migrating at 48 kDa possibly represents intracellular precursors (20). In contrast to CD55 and CD59, which are anchored to the plasma membrane by GPI linkage, CD46 is.

FIG. 1. MAC in retinal vessels of diabetic donors. A–D: In retinal sections immunostained for C5b-9, the brown product of the peroxidase reaction is present in the vessels of two diabetic donors (A and C; arrows) but not in vessels of nondiabetic donors (B and D). E and F: Retinal trypsin digests show C5b-9 immunofluorescence in the capillaries of a diabetic (E) but not in those of a nondiabetic (F) donor. Bar = 40 μm.

FIG. 2. MAC localization within retinal vessels of diabetic donors. In retinal sections immunostained for both C5b-9 (red fluorescence) and vWF (green fluorescence), C5b-9 (arrows) is detected on the vascular endothelium (A) and in deeper layers of the wall of a large vessel (B) and in several areas colocalizes with vWF (yellow, arrowheads). Bars = 5 μm.

FIG. 3. Absent C1q and C4 staining in the retina of diabetic donors. Consecutive retinal sections from a diabetic donor were immunostained for C5b-9 (A, red fluorescence) and C1q (B, green fluorescence); sections from a different donor were stained for C5b-9 (C) and C4 (D). Vessels are positive for C5b-9 (arrows) but not for C1q or C4. Bars = 40 μm.
a transmembrane protein, and its levels were similar in diabetic and nondiabetic retinas (the sum of the three bands was used to measure CD46 levels in each sample).

The measurement of CD59 levels took into account that this protein is a target of nonenzymatic glycation in diabetes. Exposure of human RBCs to 150 mmol/l glucose in vitro resulted in nonenzymatic glycation of hemoglobin (7.4 vs. 5.1% in control RBCs incubated in 55 mmol/l glucose) and of CD59 (Fig. 4B). However, the intensity of the CD59 band as detected by mAb MEM-43 or polyclonal antibody N-20 was similar in the immunoprecipitate from RBCs exposed to 55 or 150 mmol/l glucose, indicating that documented glycation of CD59 did not affect its reactivity with the two antibodies (Fig. 4B). Confirmation was obtained in immunoblots comparing the levels of CD59 in glycated and nonglycated RBC lysates not subjected to immunoprecipitation (data not shown). There was a suggestion that CD59 was more glycated in diabetic than in nondiabetic retinas (the ratio of the hexitol-lysine to the CD59 signal was 1.3 ± 0.8 in the diabetic and 0.7 ± 0.4 in the nondiabetic retinas), but the difference was not statistically significant.

We did not find antibodies suitable for immunohistochemical detection of CD55 in the fixed human retina, but both CD59 and CD46 could be localized exclusively to vessels (Fig. 5). Retinal vessels thus seem to be the site where the selective decrease in GPI-anchored complement inhibitors occurs in diabetes.

**DISCUSSION**

We found that retinal vessels, one of the main targets of the long-term effects of diabetes, show evidence of complement activation early in diabetes, associated with a prominent and selective decrease in the levels of GPI-anchored complement inhibitors. This indicates that complement regulation is altered in diabetes and suggests mechanisms for the pathogenesis of diabetic microangiopathy.

The presence of MAC in diabetic retinal vessels is likely to reflect local assembly, possibly on the endothelium. Confocal images captured colocalization of MAC and vWF, which, coupled with the knowledge that the C5b-9 complex becomes partially embedded within the plasma membrane of target cells (21), indicates that vascular...
endothelium is one of the sites of MAC assembly in diabetic retinal vessels. However, in larger retinal vessels of diabetic donors we also observed MAC deposits removed from the luminal endothelium. Increased permeability of retinal vessels in diabetes cannot be the only explanation for MAC deposited in the vessel wall because there was no evidence of extravasation of complement components (we tested C1q and C4), which have molecular sizes smaller than MAC. Moreover, when complement is activated by the subendothelial extracellular matrix, the reaction seems not to proceed to MAC formation (22). In the renal glomeruli of patients with diabetes, immunoelectron microscopy had shown that MAC is deposited on membranous structures, most likely cellular debris trapped between layers of existing and newly formed lamellae of basement membranes (10). Reduplication of basement membranes is a characteristic feature of diabetic vessels (23). It is thus possible that MAC forms initially on the luminal endothelium of diabetic vessels and that, over time, fragments of disrupted plasma membrane still bearing MAC become embedded within adjacent basement membranes. Such a paradigm would be consistent with the absence of MAC deposits in the wall of the larger retinal vessels of diabetic rats, which were studied after only 10 weeks of diabetes.

The observations in the rat model identify complement activation and decreased levels of inhibitors as early events in the course of diabetes, detectable before other manifestations of retinal vascular pathology. The human retinal vessels were from donors with a duration of diabetes that had set the stage also for the occurrence of vascular cell apoptosis (3) and microthrombosis (1), both of which could contribute to complement activation (4,5). However, these two processes are not detected in the diabetic rat model until after 6–8 months of diabetes (3,14,24), and this points to complement activation as an upstream event. Decreased levels of complement inhibitors seem likewise to be upstream of other known vascular pathology. Complement regulators are lost from necrotic cells and tissues (25), but the prominent decrease in CD55 and CD59 was disproportionate to the small number of apoptotic cells detected at any given time in the retinal vessels of diabetic donors (3) and preceded by several months the development of acellular capillaries in the diabetic rats (3). The decreased levels of inhibitors also cannot be readily attributed to complement activation because the latter results in upregulation of inhibitors (7). The reduced levels of CD55 and CD59 thus may be viewed as a primary effect of diabetes and one of the mechanisms for complement activation in diabetic vessels.

The selective decrease in GPI-anchored complement inhibitors suggests effects of diabetes on common regulatory steps in the synthesis or the processing of these molecules. Studies in cultured endothelial cells have shown that, similar to what we observed in diabetes, high glucose concentrations decreased cellular content of both CD55 and CD59 but not CD46 and that the levels of GPI-anchored inhibitors decreased on the cell surface but increased in the culture medium (12). Transfer to a glucose medium induced in yeast spheroplasts the activation of an endogenous GPI-specific phospholipase C (26). Collectively, these observations propose that a mechanism for the selectively reduced levels of CD55 and CD59 in diabetic retinal vessels may be sought in hyperglycemia-induced activity of phospholipases capable of cleaving GPI anchors. Hyperglycemia can additionally compromise the protective effects of CD59 (and other complement inhibitors) by causing nonenzymatic glycation in the vicinity of the active site (13). In this context, one may ask why patients who have diabetes with poor metabolic control do not manifest symptoms of paroxysmal nocturnal hemoglobinuria. Complete deficiency of CD59 is probably required for the syndrome to become apparent (9). In addition, the mechanism that leads to decreased levels of CD59 (and CD55) in diabetes may be cell- or tissue-specific and not involve RBCs. It was noteworthy in our experiments that the RBCs exposed to high glucose in vitro manifested extensive Amadori adduct formation on CD59 but did not show decreased CD59 levels.

This work proposes several unsuspected pathways whereby diabetes/hyperglycemia may alter the phenotype of vascular cells to induce the characteristic abnormalities
of retinal microangiopathy (27). C3a and C5a anaphylatoxins generated during complement activation could contribute to increased permeability and neutrophil adhesion. Even if not lytic, repeated attacks by MAC will, at a minimum, burden the endothelium with the energetic and metabolic cost involved in recovering from the attacks (28) and may lead to a state of chronic activation (6), heightening the risk of acute events such as apoptosis and microthrombosis over time. The finding that diabetes alters the synthesis or processing of GPI-anchored proteins further widens the spectrum of implications for the endothelial phenotype to include possible effects on other GPI-linked molecules and on signaling systems compartmentalized in the plasmalemmal microdomains in which GPI-anchored molecules cluster (26,29). New experiments will address these issues to clarify further the biology of diabetic vascular disease and to test whether the events surrounding complement activation and/or complement activation itself should become targets in preventative strategies.

ACKNOWLEDGMENTS
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REFERENCES