Glycation Inactivation of the Complement Regulatory Protein CD59

A Possible Role in the Pathogenesis of the Vascular Complications of Human Diabetes

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Micro- and macrovascular diseases are major causes of morbidity and mortality in the diabetic population, but the cellular and molecular mechanisms that link hyperglycemia to these complications remain incompletely understood. We proposed that in human diabetes, inhibition by glycation of the complement regulatory protein CD59 increases deposition of the membrane attack complex (MAC) of complement, contributing to the higher vascular risk. We report here *1*) the generation and characterization of an anti–glycated human CD59 (hCD59) specific antibody, *2*) the detection with this antibody of glycated hCD59 colocalized with MAC in kidneys and nerves from diabetic but not from nondiabetic subjects, and *3*) a significantly reduced activity of hCD59 in erythrocytes from diabetic subjects, a finding consistent with glycation inactivation of hCD59 in vivo. Because hCD59 acts as a specific inhibitor of MAC formation, these findings provide a molecular explanation for the increased MAC deposition reportedly found in the target organs of diabetic complications. We conclude that glycation inactivation of hCD59 that leads to increased MAC deposition may contribute to the extensive vascular pathology that complicates human diabetes. *Diabetes* 53:2653–2661, 2004

Micro- and macrovascular complications cause substantial morbidity and mortality for patients with diabetes, who are at higher risk for heart disease and stroke, blindness, kidney failure, and extremity amputations. Treatment of chronic diabetic complications consumes nearly 15% of health care dollars in the U.S. Extensive epidemiological evidence clearly demonstrates that prolonged hyperglycemia underlies chronic complications of diabetes (1,2). Hyperglycemia drives protein glycation, the nonenzymatic reaction of glucose with free α- or ε-amino groups in proteins that results first in the formation of a Schiff base or aldimine and then of the more stable ketoamine via the Amadori rearrangement. The ketoamine moiety can also undergo further rearrangements, leading to the formation of advanced glycation end products (AGEs) (3). When glycation involves its active site, the glycated protein may lose function, as reported for glycated hemoglobin or AGE basic fibroblast growth factor (4).

Consistent with a role of protein glycation in the pathogenesis of diabetic complications is the strong correlation found among hyperglycemia, protein glycation, and AGE formation. In addition to protein glycation, several mechanisms have been proposed to contribute to hyperglycemic-related pathology, as reviewed recently (5). However, the cellular and molecular mechanisms by which hyperglycemia causes vascular damage still remain incompletely understood. It is interesting that it has been noted that humans are particularly prone to develop extensive vascular disease as a consequence of diabetes (6,7). We proposed that a novel mechanism that provides for increased vascular risk in humans with diabetes could involve inactivation by glycation of the complement regulatory protein human CD59 (hCD59) (8).

The complement system consists of a group of >12 soluble proteins that interact with one another in three distinct enzymatic activation cascades known as the classical, alternative, and lectin pathways. All activation pathways converge to form the membrane attack complex (MAC). The MAC is a circular polymer of 12–18 monomers of the C9 complement protein with the capacity to insert into cell membranes and form a transmembrane pore of an effective internal radius of 5–7 nm (9). Influx of salt and water through the MAC pore induces colloidosmotic swelling and lysis of MAC-targeted cells such as gram-negative bacteria or heterologous erythrocytes. We have demonstrated that during the assembly of the MAC pore, there is a phase when the MAC pore is transient and reversible, permitting opening and closing/resealing of the plasma membrane (10–12). These transient MAC pores can generate significant changes in the membrane permeability of autologous cells without compromising their viability and

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Received for publication 20 February 2004 and accepted in revised form 6 July 2004.

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AGE, advanced glycation end product; DAF, decay-accelerating factor; hCD59, human CD59; MAC, membrane attack complex; PMP, platelet-derived microparticle; PNH, paroxysmal nocturnal hemoglobinuria; RBC, red blood cell.

TABLE 1
Clinical characteristics of 12 patients with diabetic nephropathy

Patient no.	Sex	Age (years)	Plasma creatinine (mg/dl)	Proteinuria (mg/24 h)	Pathology
1	F	66	2.5	11	Diffuse intracapillary glomerulosclerosis with interstitial fibrosis
2	F	59	2.0	13	Glomerulosclerosis with nodule formation
3	F	55	5	2.5	Advance diffuse and nodular glomerulosclerosis
4	M	77	2.9	423	Diffuse and nodular diabetic glomerulosclerosis
5	F	52	N/A	N/A	Diffuse and nodular diabetic glomerulosclerosis moderately advanced
6	F	45	N/A	N/A	Diffuse mesangial sclerosis
7	M	55	3.5	170	Diffuse intracapillary glomerulosclerosis with priminent hyaline arteriolar sclerosis
8	M	59	1.8	233	Diffuse and nodular diabetic glomerulosclerosis moderately advanced
9	F	50	N/A	N/A	Intracapillar glomerulosclerosis with interstitial fibrosis
10	M	71	2.7	>55	Advanced diffuse and nodular glomerulosclerosis
11	M	62	1.8	233	Diffuse intracapillar glomerulosclerosis
12	F	46	2.3	>300	Glomerulosclerosis with interstitial fibrosis
13	N/A	N/A	N/A	N/A	N/A

thereby mediate physiological and/or pathological responses (13). For example, complement activation and nonlytic MAC formation on endothelium activates endothelial cells (14) and induces the release into the extracellular medium of growth factors such as basic fibroblast growth factor and platelet-derived growth factor (15,16), interleukin-1 (12), monocyte chemotactic protein-1 (17), and von Willebrand factor (18). Acting auto- and paracrinically on extracellular receptors, these molecules released through the MAC pore, respectively, promote *1*) proliferation of fibroblasts and endothelial and smooth muscle cells (15); *2*) inflammation attracting monocytes and macrophages to the site of focal complement activation (19), and inducing the expression of proinflammatory adhesion molecules such as E-selectin, vascular cell adhesion molecule-1, intracellular adhesion molecule-1 (20), and P-selectin (14); and *3*) thrombosis by inducing the expression of tissue factor (17) and the exposure of binding sites for clotting factor Va (21).

To downregulate complement activity and protect "self" cells from the catastrophic effect of complement activation/amplification, >10 plasma- and membrane-bound inhibitory proteins, including CD59, have evolved to restrict complement activation at different stages of the activation pathways. CD59, a key inhibitor of MAC formation, is a glycan phosphatidylinositol-linked membrane protein that is universally expressed in cells. We have previously reported that *1*) glycation inactivates hCD59 because the protein contains a glycation site formed by amino acid residues K41 and H44 at the active domain, *2*) inactivation of hCD59 by glycation increases MAC-induced growth factor release from endothelial cells in vitro, and *3*) hCD59 is glycated in vivo because it is present in the urine of diabetic subjects (8). On the basis of these findings, we proposed that glycation inactivation of hCD59 in diabetes could increase MAC deposition in diabetic tissues and link the complement system to the pathogenesis of diabetic vascular complications. The previously reported findings of increased MAC deposition in diabetic kidneys (22), nerves (23), and retinas (24) are consistent with this hypothesis, but the mechanism that induces MAC deposition in diabetic tissues has not been investigated in humans. Here, we report an immunocytochemistry study in human diabetic and nondiabetic kidney and nerve biopsy samples showing the presence of glycated hCD59 colocalized with MAC in diabetic but not in nondiabetic tissues. We also show that erythrocytes of diabetic patients have a significantly reduced activity of hCD59 and increased sensitivity to MAC-mediated lysis. Together, these findings indicate that glycation inactivation of hCD59 may explain increased MAC deposits in diabetic tissues and contribute at the molecular level to the vascular proliferative pathology that complicates human diabetes.

RESEARCH DESIGN AND METHODS

Human samples. Renal samples were obtained from the Pathology Department of Brigham and Women's Hospital. Table 1 summarizes the renal function and pathological records of 12 of the 13 patients with diabetic nephropathy. Nerve biopsies, provided by Dr. A. Hayes (Columbia-Presbyterian, New York, NY), were the same samples reported previously (23). All samples were obtained with approval by the institutional review boards of the respective institutions.

Generation of anti–glycated hCD59 polyclonal antibody. The antigen was a 14–amino acid peptide ($hCD59_{37–50}$) synthesized by solid-phase chemistry using an ε-glucitol-lysine residue at the position corresponding to lysine 41 in the native protein. This glycated peptide was conjugated to keyhole limpet hemocyanin and injected into rabbits for immunization as described previously (25). The antibody was purified from rabbit immune serum by affinity chromatography with the antigen peptide.

Production of recombinant hCD59 and confirmation of the specificity of the anti–glycated hCD59 antibody. Following the strategy reported previously (26–28), NH_2-terminal FLAG-tagged hCD59 was generated by transfecting into CHO cells a construct that contained hCD59 with the FLAG sequence. Recombinant human FLAG-hCD59 was purified using the anti-FLAG M2 affinity GEL column (Sigma, St. Louis, MO) and then incubated with and without glucose (0.5 mol/l at 37°). Aliquots were tested at different time intervals by immunoblotting with either anti-hCD59 antibody YTH53.1 (Serotec, Raleigh, NC) or anti–glycated hCD59 antibody. The specificity of the purified antibody was tested by immunoblotting of recombinant glycated and nonglycated hCD59 and glycated albumin, all reduced by incubation with 50 mmol/l sodium borohydride (1 h at room temperature) before SDS-PAGE.

Immunohistochemistry analysis of tissue samples. Eight-micron serial cuts from paraffin-embedded tissue samples were stained with *1*) anti–ulex Europaeus agglutinin antibody (Vector Laboratories, Burlingame, CA), *2*) mouse anti-human MAC neoantigen monoclonal antibody (Quidel, San Diego, CA), *3*) anti–glycated hCD59 antibody, *4*) anti-hCD59 monoclonal antibody (Bric 229; International Blood Group Reference Laboratory, Bristol, U.K.), and

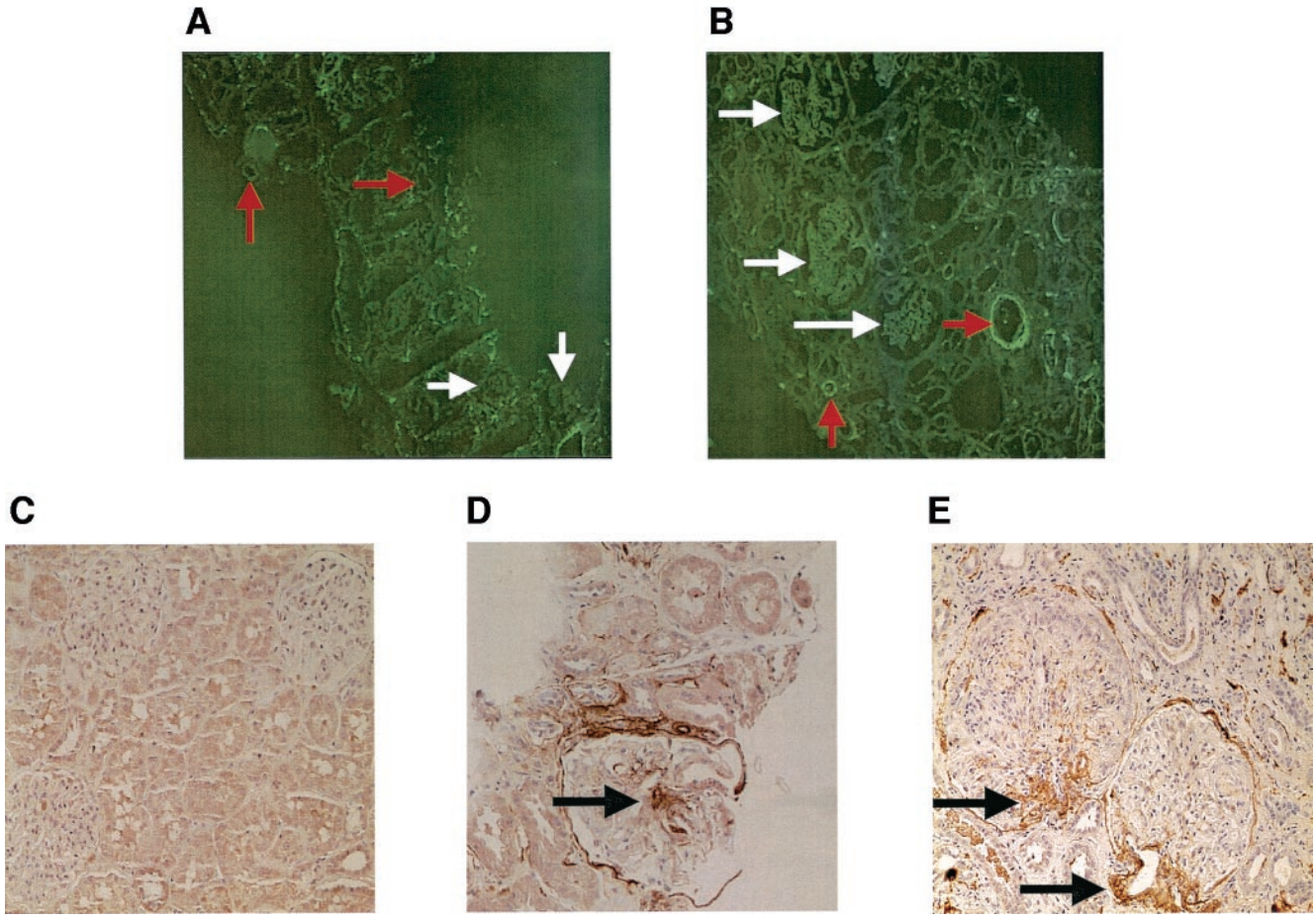

FIG. 1. MAC deposition in diabetic nephropathy. MAC deposition was found in the glomeruli and middle-size arteries of a kidney that was transplanted into a diabetic patient (*B*) but not in a biopsy sample of minimal-change nephropathy stained simultaneously (*A*). The white and red arrows, respectively, show glomeruli and blood vessels. MAC deposition in diabetic (*D* and *E*) but not in nondiabetic (*C*) nephropathy in archival samples of renal biopsies stained with anti-MAC antibodies. Arrows, MAC deposition.

4) mouse biotinylated IgG isotype (Vector Laboratories) used as negative control for MAC staining. For staining with anti–glycated hCD59 antibodies, tissue sections were exposed to 100 mmol/l sodium borohydride (60 min at room temperature) for reduction. For secondary antibodies, we used horse anti-mouse for MAC and hCD59 staining and goat anti-rabbit for glycated hCD59 (23) (negative controls for glycated hCD59 were stained with goat anti-rabbit secondary antibody only). Secondary antibodies were conjugated with horseradish peroxidase. Two independent investigators blindly scored the samples on the basis of the intensity difference between staining by each specific antibody and the respective negative control. In the kidney samples, sclerotic glomeruli were not included in the analysis.

Complement-mediated hemolytic assay and measurement of hCD59 activity. Hemolysis of human red blood cells (RBCs) was assessed by two methods: *1*) classical pathway activation by sensitization with anti-human RBC antibodies (Research Diagnostics, Flanders, NJ) and *2*) human MAC formation assay with purified human C5b-6, C7, C8, and C9, as we described previously (11). The functional activity of hCD59 was measured using a neutralizing rat monoclonal anti-hCD59 antibody (YTH 53.1) for 1 h at 37°C. Activity of hCD59 was estimated as the percentage of MAC-induced lysis in the presence minus MAC-induced lysis in the absence of the anti-hCD59 neutralizing antibody.

RESULTS

Extensive MAC deposition in a kidney transplanted into a diabetic patient to treat diabetic nephropathy. CD59 is a glycan phosphatidylinositol-anchored membrane protein that specifically inhibits formation of the MAC. hCD59 is inactivated by glycation of its K41 residue because it contains a glycation site formed by residues K41–H44, as we demonstrated in vitro (8). Glycation inactivation of hCD59 allows increased MAC formation in complement-target cells. If glycation of hCD59 as a consequence of hyperglycemia in diabetes were to limit similarly its function in vivo, then one would expect increased MAC deposition in diabetic tissues. This prediction has been confirmed by several studies that demonstrated MAC deposition in kidneys (22), nerves (23), and retinas (24) of diabetic patients with minimal or no MAC deposits in the same tissues from nondiabetic individuals. However, the mechanism underlying this reported MAC deposition in diabetic tissues is still poorly understood. Indeed, MAC deposition in diabetic tissues could be secondary to hyperglycemia or could also be due to an intrinsic higher sensitivity to complement-mediated damage and MAC deposition in the diabetic tissues. As a preliminary approach to address this complex issue, we took advantage of one unusual case of a poorly controlled diabetic patient who required a renal transplant for treatment of diabetic nephropathy. Two years later, the patient developed signs of progressive renal failure, and a kidney biopsy was obtained to rule out late rejection of the transplanted kidney. The pathology diagnosis revealed early recurrent diabetic nephropathy with no signs of late rejection. Staining of paraffin-embedded sections of this biopsy with primary anti-MAC and fluorescent secondary antibodies showed intense MAC deposition in the glomeruli and

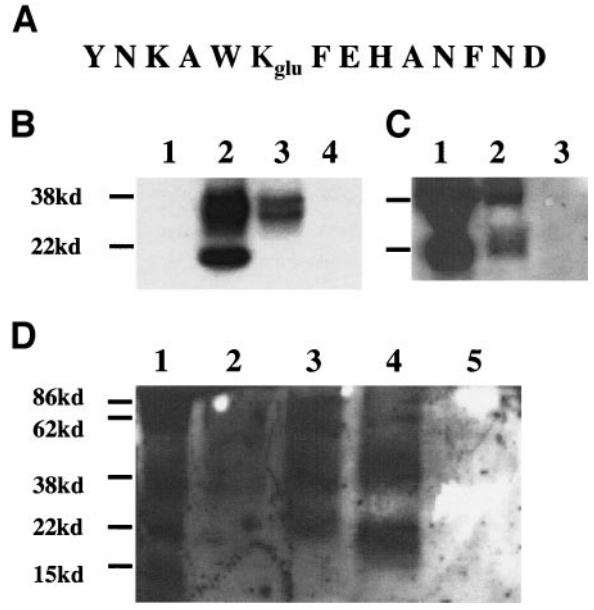

FIG. 2. Generation of anti–glycated hCD59 polyclonal antibody. *A*: Glycated hCD59 peptide was synthesized with a glycated lysine in the K41 equivalent position (Kglu). *B*: Expression of recombinant hCD59 (rhCD59) in CHO cells was detected by immunoblot analysis with anti-FLAG antibody. *Lanes 1* and *4*, no expression of hCD59; *lanes 2* and *3*, expression of hCD59. *C*: Purified rhCD59 was incubated with and without glucose (0.5 mol/l at 37°C), and aliquots were tested at different time points by immunoblotting with anti-hCD59 antibody (YTH53.1). *Lane 1*, rhCD59 (no glucose); *lane 2*, rhCD59 (glucose for 7 days); *lane 3*, rhCD59 (glucose for 21 days). *D*: Same samples as in *C* were immunoblotted with anti–glycated hCD59 antibody, and commercially available glycated albumin was used to control reactivity with other glycated proteins. *Lane 1*, molecular marker; *lane 2*, rhCD59 (no glucose); *lane 3*, rhCD59 (glucose for 7 days); *lane 4*, rhCD59 (glucose for 21 days); *lane 5*, glycated albumin.

middle-size arteries of the transplanted kidney (Fig. 1*B*). In contrast, a simultaneously stained control kidney biopsy from patients with minimal-change glomerulonephritis showed no or minimal MAC deposits (Fig. 1*A*). Also, staining kidney biopsies from diabetic subjects (5 and 8 in Table 1) and nondiabetic subjects by immunocytochemistry showed extensive MAC deposition in the diabetic (Fig. 1*D* and *E*) but not in the nondiabetic (Fig. 1*C*) samples, a finding consistent with results reported previously (22). These results confirm that MAC deposition occurs in diabetic kidneys; importantly, MAC deposition in a transplanted kidney with pathological evidence of diabetic nephropathy suggests that MAC deposits in diabetic kidneys may be secondary to chronic hyperglycemia rather than a coincidental primary disorder of the diabetic kidney. To better understand the mechanism underlying increased MAC deposition in diabetic tissues, we generated an antibody that specifically recognizes glycated hCD59 and investigated the presence of glycated hCD59 in diabetic and nondiabetic tissues.

Generation of anti–glycated hCD59 antibody. The presence of hCD59 has been documented extensively in human tissues, including kidneys and nerves (29). To generate an antibody that specifically recognizes glycated but not nonglycated hCD59 in target organs of diabetic complications, we synthesized an hCD59 peptide using a glycated lysine for the K41 equivalent residue (Fig. 2*A*). The synthetic glycated peptide was used for rabbit immunization and generation of a polyclonal antibody. To confirm the specificity of the antibody, we incubated FLAG-tagged hCD59, purified by an anti-FLAG affinity column from CHO cells (Fig. 2*B*), with and without glucose, and aliquots were tested at 7 and 21 days by immunoblot analysis with either anti-hCD59 YTH53.1 (Fig. 2*C*) or anti–glycated hCD59 (Fig. 2*D*) antibodies. The blot with anti-FLAG antibody showed multiple bands of hCD59, with one prominent band at ~20 kD and another at ~40 kD, the latter product of CD59 dimerization, as previously reported (30). The YTH53.1 anti-hCD59 antibody neutralizes its activity because it recognizes an epitope within the active site of the protein that centers around W40 (8). Glycation of hCD59 abrogates its immunorecognition by the YTH53.1 antibody, probably because the glycation motif K41-H44 is adjacent to the active site, as we previously reported (8). Consistently, the YTH53.1 anti-hCD59 antibody strongly recognized hCD59 when it was nonglycated but progressively lost recognition of the protein exposed to glucose, showing a weak band at day 7 and no recognition at day 21 (Fig. 2*C*). In contrast, anti–glycated hCD59 antibody recognized hCD59 only after exposure to glucose. The increased recognition of the glycated protein after 21 days as compared with 7 days is consistent with the rate of glycation inactivation of the purified protein in vitro that we reported previously (8). Furthermore, the anti–glycated CD59 antibody did not recognize commercially available glycated albumin (Fig. 2*D*).

MAC and glycated hCD59 colocalize in diabetic human kidneys and nerves. If glycation inactivation of hCD59 contributes to the increased MAC deposition in diabetic tissues, then one would expect to find that MAC and glycated hCD59 colocalize in tissue samples from diabetic subjects. We immunostained serial sections of kidney and sural nerve biopsy samples with anti–glycated hCD59 and anti-MAC antibodies. For detection of glycated hCD59, tissue sections were preincubated with 100 mmol/l sodium borohydride for reduction because the anti–glycated CD59 antibody recognizes the reduced form of the Amadori product. The results demonstrate the presence of glycated hCD59 in both kidneys (Fig. 3) and nerves (Fig. 4) from diabetic but not from nondiabetic patients. Staining of serial sections of the same specimens with anti-MAC antibodies showed extensive MAC deposition in diabetic kidneys and nerves but not in tissues from nondiabetic subjects. Importantly, in both diabetic kidneys and nerves, glycated hCD59 colocalized with MAC deposits (Figs. 3 and 4, Table 2). In diabetic kidneys, glycated hCD59 and MAC localize primarily in glomerular capillaries (Fig. 3), whereas in the diabetic nerves, glycated hCD59 and MAC colocalize in endoneurial microvessels (vasa nervorum), as demonstrated by staining an additional serial section of the same sample with anti–endothelium-specific ULEX-1 lectin antibodies (Fig. 4). Thus, glycated hCD59 and MAC colocalize in diabetic kidney and nerve tissues. This colocalization of glycated hCD59 and MAC found in ~60% of diabetic kidneys and nerves but not in any of the same tissues from nondiabetic subjects supports the hypothesis that glycation of hCD59 in diabetes contributes to the increased MAC deposition found in the target organs of diabetic complications.

Decreased hCD59 activity in diabetic RBCs. The hypothesis that glycated hCD59 allows for increased MAC

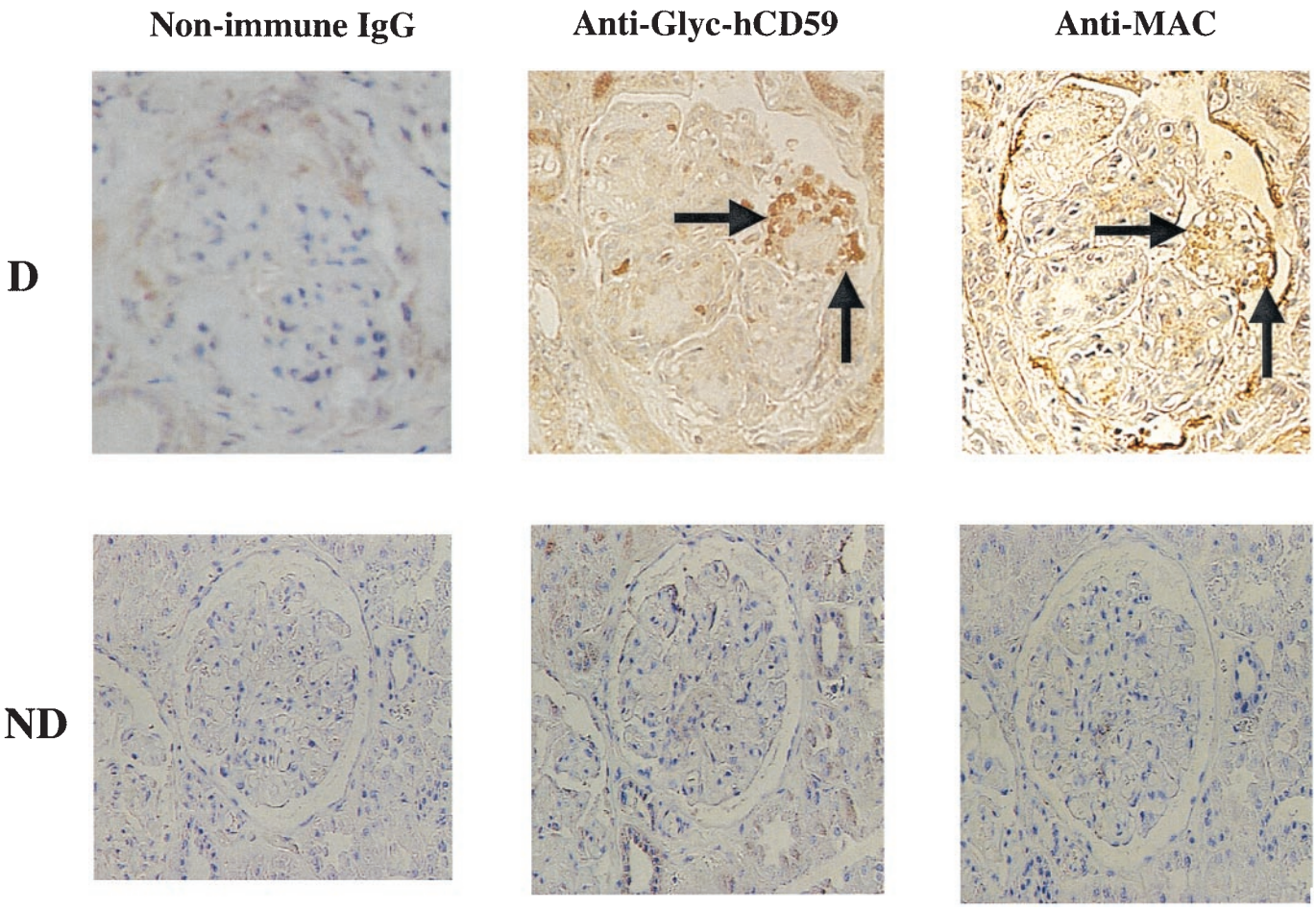

FIG. 3. Glycated hCD59 colocalizes with MAC in diabetic human kidneys. Serial sections of the same glomerulus in a diabetic (D) and nondiabetic (ND) kidney stained with anti-MAC or anti–glycated hCD59 antibodies. The arrows indicate positive staining areas for glycated CD59 and MAC in the same glomerulus. (The minor distortion in the anatomy of the glomeruli shown is due to the different planes of the serial sections and the incubation with sodium borohydride before staining with anti–glycated hCD59.) Magnification ×20.

deposition in diabetic target organs and thereby plays a role in the development of diabetic complications suggests that hCD59 is inactivated by glycation in vivo as it is in vitro. Indeed, in vitro glycation of either RBCs or human endothelial cells increases *1*) MAC deposition on the cell membranes, *2*) MAC-mediated lysis in the case of RBCs, and *3*) growth factor release in the case of endothelial cells. However, it would be very difficult to establish a comparable direct cause-effect relationship between the observed glycation of hCD59 in kidneys and nerves from diabetic subjects and the loss of its functional activity as an inhibitor of MAC formation in those tissues in vivo. As an indirect approach to establish glycation inactivation of hCD59 in diabetic subjects, we tested ex vivo the sensitivity of diabetic and nondiabetic RBCs to MAC-mediated lysis as a functional measure of hCD59 activity. If glycation were to inactivate hCD59 in diabetic subjects, then one would expect that RBCs from individuals with diabetes would have an increased sensitivity to complement-mediated lysis and decreased hCD59 protection against human MAC. To test these predictions experimentally, we exposed RBCs from diabetic (HbA_{1c} >8%) and nondiabetic (HbA_{1c} <4%) individuals to the purified terminal complement components necessary to form the MAC. The results demonstrate that diabetic RBCs have a much higher sensitivity to MAC-mediated lysis than nondiabetic controls (Fig. 5*A*). Similar results were obtained when antibody-sensitized RBCs were exposed to human serum to form the MAC via the classical pathway of complement activation (Fig. 5*B*). The increased sensitivity to MAC-mediated lysis in diabetic RBCs is consistent with decreased activity of hCD59. This interpretation was confirmed measuring hCD59 activity in RBC membranes from the same individuals using an anti-hCD59 neutralizing antibody. MAC-mediated lysis in the presence of a saturating concentration of the neutralizing antibody minus MAC-mediated lysis in the absence of the antibody represents the protective activity of hCD59 against MAC formation in the RBC membranes. Diabetic RBCs exhibited an 8–10 times lower hCD59 activity than normal nondiabetic control RBCs (Fig. 5, inset). Thus, diabetic RBCs express a highly reduced hCD59 activity and increased sensitivity to MAC-mediated lysis, a finding that provides functional evidence for glycation inactivation of hCD59 in circulating RBCs in vivo.

DISCUSSION

We have proposed that increased MAC deposition as a result of glycation inactivation of hCD59 could contribute

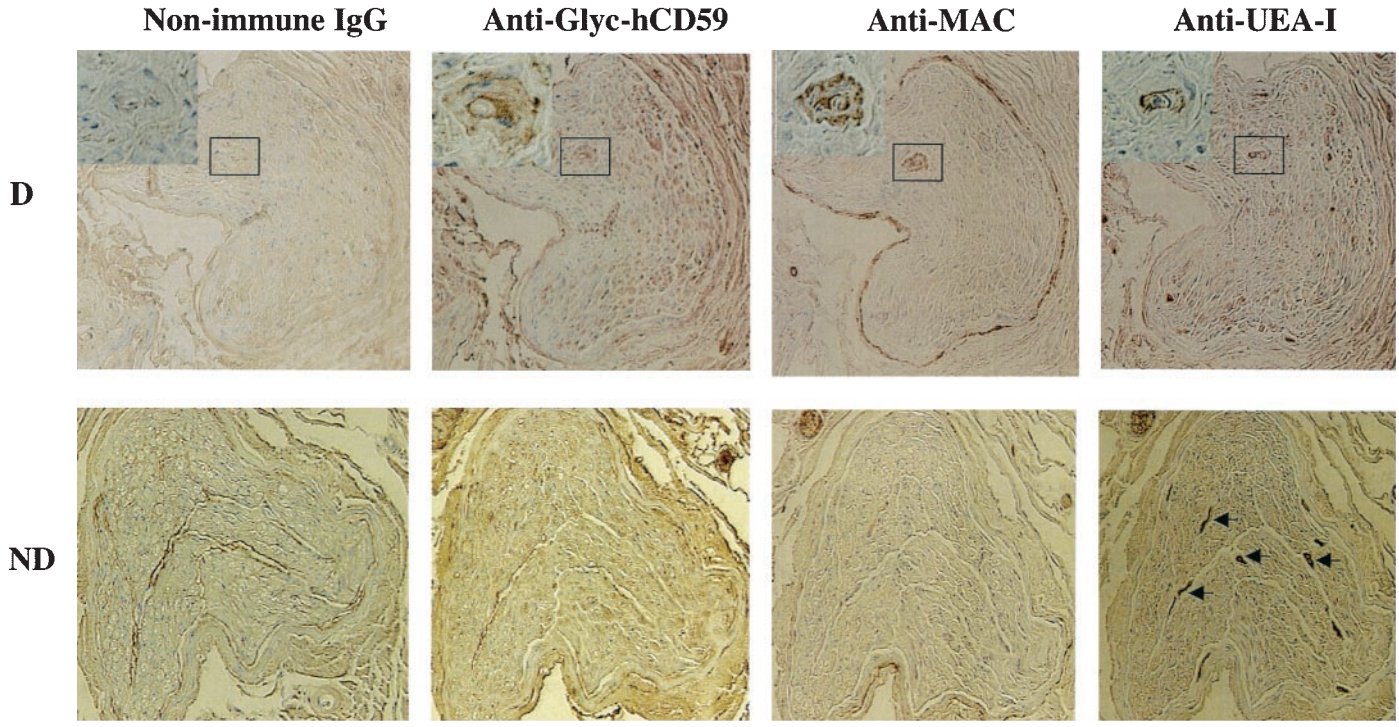

FIG. 4. Glycated hCD59 colocalizes with MAC in diabetic human nerves. Serial sections of a diabetic (D) and a nondiabetic (ND) nerve were stained with anti-MAC or anti–glycated hCD59 antibodies. Staining with anti–ulex Europaeus agglutinin-I antibody shows the endoneurial microvessels (arrows in the nondiabetic sample). All pictures were taken at ×20; the squares delineate the region amplified in the inset. Magnification ×20; ×60 in inset.

to the pathogenesis of the vascular complications of human diabetes (8). Our current studies demonstrate that *1*) glycated hCD59 is present and colocalizes with MAC in diabetic kidneys and endoneurial microvessels from diabetic but not from nondiabetic patients (Figs. 3 and 4, Table 2), *2*) there is a significant reduction of hCD59 functional activity in diabetic RBCs demonstrated by an increased sensitivity to MAC-mediated lysis detected with two well-established methods of complement-mediated lysis (Fig. 5), and *3*) there is significantly lower activity of hCD59 in RBCs from diabetic as compared with nondiabetic subjects (Fig. 5). Together, the results of this work indicate that glycation inactivates hCD59 in diabetic subjects and provide a probable molecular explanation for the reported findings of increased MAC deposition in diabetic kidneys (22), nerves (23), and retinas (24). These findings support the hypothesis that the increased complement deposition in diabetic tissues as a result of the glycation inactivation of hCD59 plays a role in the high sensitivity of humans to develop diabetic vascular complications.

TABLE 2
Immunostaining for glycated hCD59 and MAC in kidney and nerve biopsies

	Nephropathy		Neuropathy	
	Diabetic individuals	Nondiabetic individuals	Diabetic individuals	Nondiabetic individuals
n	13	17	12	14
GlycCD59 (%)	58	0	60	0
Colocalized MAC + GlycCD59 (%)	57	0	58	0

Serial sections of paraffin-embedded renal and sural nerve tissue obtained by biopsies from diabetic and nondiabetic individuals were immunostained with either anti–glycated hCD59 or anti-MAC antibody. Results are percent of positive and negative staining for each marker.

Complement activation and MAC formation on "self" cells can be highly deleterious and, therefore, are tightly restricted at different levels of the activation pathways. Three membrane proteins, decay-accelerating factor (DAF), membrane cofactor protein, and CD59, efficiently protect the human endothelium from complement attack. Thus, it seems that inactivation of a single component of this well-orchestrated protection machinery would not be sufficient to cause unrestricted MAC formation. However, several lines of evidence strongly suggest that CD59 is the most relevant of these complement regulatory proteins. First, CD59 knockout mice exhibit a phenotype comparable to the complement-mediated hemolytic anemia known in humans as paroxysmal nocturnal hemoglobinuria (PNH) (31), which is not observed in DAF knockouts (32). In humans, members of families who carry an isolated total deficiency of DAF on all circulating blood cells do not manifest signs of PNH (33). In contrast, an index patient with a global deficiency of CD59 exhibited a severe PNH phenotype noted at the unusually young age of 13 years (34). Consistent with a key role of CD59 in protecting cells from MAC-mediated attack is the increased MAC deposition observed in cell membranes after deletion (31), absence (34), or inhibition (35) of CD59. In endothelium, absence or inhibition of CD59 results in increased MAC-induced phenomena, including the release of growth factors and proinflammatory and prothrombotic cytokines that may, respectively, promote cell proliferation, inflammation, and thrombosis in the vascular wall (12). The

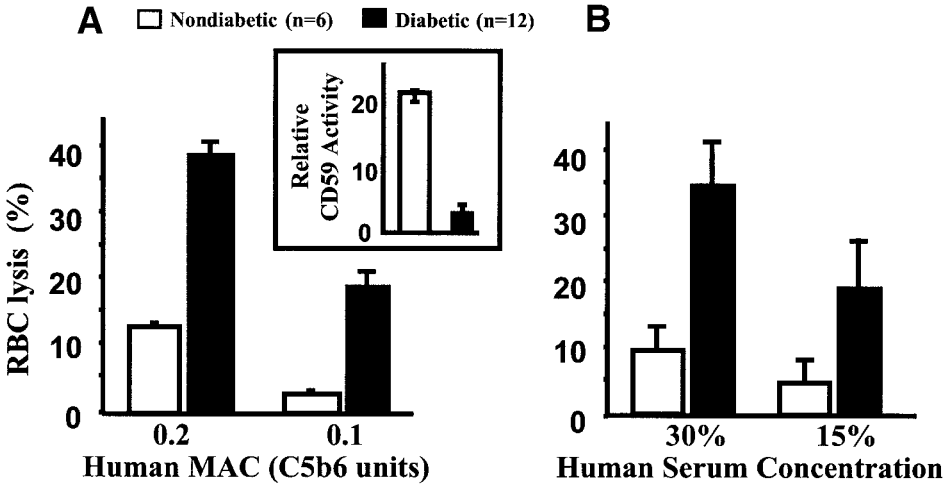

FIG. 5. Increased sensitivity to MAC-induced lysis and decreased hCD59 activity in diabetic RBCs. Diabetic RBCs (n = 12; HbA_{1c} >8%) are much more sensitive than nondiabetic RBCs (n = 6; HbA_{1c} <4%) to lysis induced by addition of purified terminal complement components to form the MAC (*A*) or by activating the classical pathway with anti-human RBC antibody and two different concentrations of human serum (15 and 30%; *B*). *Inset*: Diabetic RBCs have an 8–10 times lower CD59 activity than nondiabetic RBCs.

present finding that glycated hCD59 colocalized with MAC in glomerular capillaries of diabetic kidneys and microvessels of diabetic nerves (Figs. 3 and 4) is consistent with the hypothesis that glycation inactivates hCD59 in vivo as it does in vitro. The alternative possibility that increased MAC deposition in diabetic retinas could be due to decreased expression of CD59 has been suggested by Zhang et al. (24). We did not observe a decreased level of hCD59 expression, as detected by immunocytochemistry, in the diabetic kidneys and nerves assessed in these studies, although the possibility that in human diabetes there might be some reduction in the expression levels of hCD59 (and/or other complement regulatory proteins) that could also contribute to increased MAC deposition requires further investigation. It has also been shown that in the serum of diabetic subjects, antibodies are generated to neo-antigens in glycated and glyoxidized proteins (36). These antibodies represent an additional source of complement activation that may have an amplification effect in the context of impaired complement regulation as a result of glycation inactivation and/or reduced expression of hCD59.

Our previous in vitro experiments with purified and recombinant hCD59 as well as with human RBCs and endothelial cells demonstrated that hCD59 is inhibited by glycation of its K41 residue (8). Although a direct experimental demonstration of decreased activity of hCD59 in the endothelium or other tissues in diabetic patients would be difficult to obtain with available technologies, studies are under way in our laboratory to analyze the effect of hyperglycemia on the function of hCD59 transgenically expressed in mice. In the work reported here, we investigated ex vivo the loss of the protective effect of hCD59 in the RBCs of diabetic patients as an indirect indicator of the effect of hyperglycemia on hCD59 function. The results showed that the RBCs of poorly controlled diabetic patients (HbA_{1c} >8%) have a much higher sensitivity to MAC-mediated hemolysis. Comparable results in a small group of patients with type 1 diabetes were reported by Morgan et al. (37). Increased sensitivity to MAC-mediated lysis would be expected if glycation inhibits hCD59 function in vivo as it does in vitro. Consistently, we observed a significant decrease of hCD59 activity in the same RBCs from diabetic patients as compared with nondiabetic control subjects. This reduced activity of hCD59 could explain the increased reticulocyte count and shorter RBC lifespan, both suggestive of mild hemolytic anemia, reportedly observed in diabetic patients with high glycemic exposure (38,39). A comparable decrease of hCD59 activity in the endothelium of diabetic individuals would explain the increased MAC deposition that we and others have now demonstrated in all principal target tissues of diabetic complications (Figs. 3 and 4) (22–24). This increased MAC deposition would induce MAC-mediated release of growth factors and proinflammatory and prothrombotic cytokines, thereby contributing to the extensive vascular pathology seen in diabetic complications. The extent of MAC deposition and MAC-induced phenomena would depend, on the one hand, on the degree of functional inhibition of hCD59 and, on the other hand, on the level of complement activity of the individual. Heterogeneity in the level of complement activity among individuals has been well documented (40).

Patients with diabetes have an increased risk of thrombosis; diabetic platelets are hyperactive, and plasma from patients with diabetes contains higher levels of platelet release products, including platelet-derived microparticles (PMPs) (41). PNH platelets deficient in plasma membrane CD59 are exquisitely sensitive to MAC-induced activation and release of PMP, indicating that the deletion of this complement inhibitor from platelets contributes to the tendency toward thrombosis in PNH patients (42). Spontaneous platelet activation with increased PMPs are also found in CD59b knockout mice (31). Thus, glycation inactivation of hCD59 could contribute, at least in part, to the high incidence of thrombosis in diabetic patients.

Several pathways of intracellular signaling have been proposed as mediators of the vascular complications of diabetes, including upregulation of the polyol pathway (43), oxidative stress (44), and activation of protein kinase C-β (45). Recently, a unifying theory suggested that all of these pathways are mechanistically linked by hyperglycemia-induced generation of superoxide ions via the mitochondrial respiratory chain and activation of the transcription factor nuclear factor-κB (5). However, studies strongly suggest that the MAC also functions as a cell-signaling complex that stimulates cell proliferation, inflammation, and thrombosis by releasing growth factors and cytokines from endothelium (15,16). The MAC also *1*) activates pathways of intracellular signaling, including

Ca^{2+}-sensitive and Ca^{2+}-insensitive protein kinase C (46), the adenylyl cyclase system (47), G proteins, and mitotic signaling through Ras, Raf-1, ERK1, and phosphatidylinositol 3-kinase (48); *2*) stimulates oxygen radical generation by leukocytes (49); and *3*) upregulates the transcription factor nuclear factor-κB (50). This apparent convergence of MAC- and hyperglycemia-induced pathways strongly suggests that increased MAC-induced signaling as a consequence of glycation inactivation of hCD59 could act synergistically with other hyperglycemia-related pathways to produce the long-term vascular complications of human diabetes.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health Grants R01 DK52855, R01 DK62294, and M01 RR01032.

We are grateful to Dr. D.C. Tosteson and Dr. M.T. Tosteson for continuous support and critical comments. We are grateful to A. Shahasafaei for technical help with immunostaining and to Katie Hazard for editing the manuscript.

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