Functionally inhibitory antibody to the plasma membrane complement complement inhibitor CD59 has been used to investigate control of the terminal complement proteins at the endothelial cell surface. Antibodies against purified human erythrocyte CD59 (polyclonal anti-CD59 and monoclonal antibodies [MoAbs] 1F1 and 1F5) were found to bind specifically to monolayers of cultured human umbilical vein endothelial cells, and by Western blotting to recognize an 18- to 21-Kd endothelial protein. When bound to the endothelial monolayer, anti-CD59 (immunoglobulin G or Fab fragment) potentiated membrane pore formation induced upon C9 binding to C5b-8, and augmented the C5b-9-induced cellular responses, including stimulated secretion of von Willebrand factor and expression of catalytic surface for the prothrombinase enzyme complex. Although potentiating endothelial responses to the terminal complement proteins, anti-CD59 had no effect on the response of these cells to stimulation by histamine. Taken together, these data suggest that human endothelial cells express the CD59 cell surface inhibitor of the terminal complement proteins, which serves to protect these cells from pore-forming and cell-stimulatory effects of the C5b-9 complex. These data also suggest that the inactivation or deletion of this cell surface regulatory molecule would increase the likelihood for procoagulant changes in endothelium exposed to complement activation in plasma.

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from erythrocyte membranes (P18), preparation of rabbit antibody to the erythrocyte inhibitor (anti-P18), and preparation of Fab fragments of anti-P18 IgG have been reported previously in detail.

The full-length sequence of the 18- to 21-Kd erythrocyte inhibitor has been reported and has established its identity with the leukocyte antigen CD59. Therefore, the antibody against the erythrocyte inhibitor, previously referred to as anti-P18, will be designated anti-CD59 henceforth. Monoclonal antibodies (MoAbs) 1F1 and 1F5, reactive with CD59, were prepared by Drs Motowo Tomita (Showa University, Tokyo, Japan) and Hidechika Okada (Nagoya City University School of Medicine, Nagoya, Japan).

Radioiodination of antibodies. MoAbs 1F1 and 1F5 were radiolabeled with IODO-GEN (Pierce Chemical, Rockford, IL). Specific activities were 6,221 cpm/ng (1F1) and 4,856 cpm/ng (1F5).

Binding of radiolabeled antibodies to CD59 to cultured human endothelial cells. Except where specified, all cell experiments were performed using Hanks balanced salt solution, modified to contain 10 mmol/L HEPES and 1% bovine serum albumin (Hanks-HEPES-BSA). Cells were washed free of serum-containing medium and fixed 10 minutes with 1% paraformaldehyde in phosphate-buffered saline, pH 7.4. Cells were washed three times to remove fixative, and incubated with 125I-anti-CD59, 125I-1F1, or 125I-1F5 in the presence and absence of a 20-fold excess of the unlabeled antibody for 30 minutes at room temperature. Cells were then washed rapidly five times with 1 mL vol of Hanks-HEPES-BSA chilled to 4°C, solubilized in 4% sodium dodecyl sulfate (SDS), and radioactivity measured.

Immunoblotting of endothelial proteins with antibody to CD59. Second-passage HUVE were grown to confluence (75 cm²). After washing free of medium, cells were removed by trypsinization. The washed cells were pelleted, denatured at 100°C in 10% SDS and 20 mmol/L N-ethylmaleimide (without reduction), and subjected to polyacrylamide gel electrophoresis under nonreducing conditions. After transfer to nitrocellulose and blocking with 10% nonfat dry milk, blots were incubated overnight with either polyclonal anti-CD59 (10 µg/mL) or MoAb 1F1 (10 µg/mL) and developed with affinity-purified goat antirabbit IgG or goat antimouse IgG conjugated to alkaline phosphatase (Sigma, St Louis, MO). Purified human erythrocyte CD59 served as molecular weight standard.

Complement assembly on human endothelial cells. Confluent endothelial cells were washed three times with Hanks-HEPES-BSA. The cells were then incubated with rabbit anti-endothelial IgG (5 mg/mL) for 15 minutes at 23°C, washed once, and incubated with C8-deficient serum for 10 minutes at 37°C. The cells were then washed three times with buffer chilled to 4°C, and incubated with anti-CD59 (IgG or Fab fragments) or Hanks-HEPES-BSA only (see figure legends) at 4°C for 30 minutes. Finally, cells were washed twice and incubated at 37°C with C8 and/or C9 at the concentrations described in the figure legends. In some experiments, radiolabeled C8 or C9 was used to measure cell surface binding (see legend to Fig 6).

Quantitation of C5b-9-induced changes in membrane permeability using 3H-2-deoxy-D-glucose (3H-DG). For 24 hours before an experiment, cells were grown in culture medium (300 µL) containing 3H-DG (ICN Radiochemicals, Irvine, CA; 33 Ci/mmol/L) 4 µCi/well for 24 hours. Cells were then washed and complement complexes assembled as described above, including incubation with anti-CD59 IgG (0 to 1 mg/mL) before addition of C8 and C9. Ten minutes after addition of C8 and/or C9, supernatants were removed and pooled with two subsequent washes (100 µL). Cells were lysed with 125 µL of 2% Triton X-100 (Calbiochem, San Diego, CA), each well was washed twice, and washed pooled with lysates. Supernates and lysates were counted after addition of 4 mL of Aquasol (New England Nuclear Research Products, Boston, MA).

**RESULTS**

Expression of the CD59 antigen in HUVE. Human erythrocytes, platelets, and leukocytes have been shown to express membrane proteins that serve to restrict activation of the terminal complement proteins at the surface of these cells. In the case of platelets, neutralization of this C5b-9–regulatory function with an antibody against CD59 has been shown to potentiate complement-induced cell activation. The resistance of human endothelial cells in culture to the pore-forming and cytolytic effects of these proteins suggested the possibility that these cells also express C5b-9–regulatory proteins. To explore this possibility, we first demonstrated that 125I-anti-CD59 (IgG) bound specifically to endothelial monolayers, with saturation observed at 500 µg/mL and half-maximal binding observed at approximately 100 µg/mL (data not shown). Moreover, two MoAbs reactive with erythrocyte CD59 (1F1 and 1F5) were found to bind specifically to the endothelial plasma membrane (Table 1).

The presence of CD59 antigen in these cultured human endothelial cells was confirmed by Western blotting with both polyclonal and MoAbs against purified human erythrocyte CD59 (Fig 1). Immunohistochemical evidence for the expression of the 1F5 epitope on human vascular endothelial has been recently reported by Nose et al.18 The binding of these MoAbs at saturation suggests that human endothelial cells in culture express approximately 2 x 10⁵ molecules of CD59 antigen per cell, equivalent to 100 molecules/µm² of luminal surface. By comparison, human erythrocytes are estimated to express approximately 25,000 CD59 molecules/cell, equivalent to approximately 200 molecules/µm² membrane surface. Whether this endothelial antigen is structurally identical to the 18- to 21-Kd phosphatidylinositol-linked protein isolated from human erythrocytes remains to be determined.

**Table 1. Binding of CD59 MoAbs 1F1 and 1F5 to Endothelial Monolayers**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specific Binding* (molecules/µg/cell)</th>
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<tr>
<td>125I-1F1</td>
<td>151,000 ± 26,000</td>
</tr>
<tr>
<td>125I-1F5</td>
<td>188,000 ± 6,000</td>
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*Binding of each antibody was performed at a saturating concentration of 5 µg/mL. Nonspecific binding was measured in the presence of a 20-fold excess of each unlabeled antibody and was 15% of total (1F1) and 25% of total (1F5). Data are mean ± SD.
Effect of anti-CD59 on C5b-9 pore formation. The expression of CD59 epitopes by human endothelial cells suggested a complementary C5b-9-regulatory function of the endothelial plasma membrane. We therefore examined whether functionally inhibitory antibody to the CD59 antigen (anti-CD59) altered the assembly and activation of the complement pore on these cells. In these experiments, C5b67 complexes were first deposited on the endothelial plasma membrane, followed by incubation at 4°C with anti-CD59 (IgG or Fab fragments). C5b-9 assembly was then completed by addition of C8 and C9, with incubation at 37°C (see Experimental Procedures). The efflux of 3H-DOG from the endothelial cytoplasm was used to monitor formation of the C5b-9 pore in the plasma membrane. As illustrated by Fig 2, C5b-9 pore formation was potentiated by addition of anti-CD59, with apparent saturation of this effect at 0.5 mg/mL IgG. At the submaximal concentration of C9 used in this experiment, anti-CD59 caused a fourfold increase in the C5b-9–induced efflux of 3H-DOG. By contrast, this antibody had no effect on the efflux of 3H-DOG from C5b-8 cells (omitting C9).

Potentiation of C5b-9 induced stimulatory responses by anti-CD59. The capacity of anti-CD59 to increase the C5b-9–mediated change in plasma membrane permeability (Fig 2) suggested that this antibody might also potentiate the cell-activation responses induced on C5b-9 binding to the endothelial surface. In cells exposed to anti-CD59 (Fab fragments), C5b-9–induced prothrombinase activity was increased approximately threefold when compared with identically treated cells not exposed to antibody (Fig 3). By contrast, the prothrombinase activity measured for control cells was unaffected by incubation with anti-CD59.

In addition to inducing expression of membrane prothrombinase sites, the C5b-9 proteins have been shown to stimulate secretion of vWF multimers from endothelial storage granules. As shown in Fig 4, this secretory response to C5b-9 increased with increasing anti-CD59, and was augmented approximately fourfold at saturating concentrations of Fab (>250 μg/mL). As shown in Fig 5A, anti-CD59 (Fab) potentiated the C5b-9–induced secretory response at all
ENDOTHELIAL CELL COMPLEMENT C5b-9 INHIBITOR

Fig 3. Effect of anti-CD59 on C5b-9-induced exposure of catalytic surface for prothrombinase assembly. C5b67 complexes were deposited on endothelial monolayers and these cells then incubated at 4°C with anti-CD59 (0 or 0.35 mg/ml Fab). After washing, C8 (0.5 µg/well) and C9 (12.5 ng/well) were added, cells incubated at 4°C, and prothrombinase activity measured as described in Experimental Procedures. C5b67 denotes concentrations of C9 tested. By contrast, this antibody had no effect on histamine-induced vWF secretion at any concentration of histamine (Fig 5B). The data of Fig 5 suggest that anti-CD59 potentiates the C5b-9–induced response by directly affecting activation of the complement pore, and does not potentiate receptor-mediated endothelial secretory responses.

Effect of anti-CD59 on C5b-9 assembly. The data of Figs 2 through 5, suggest that anti-CD59 specifically inhibits a C5b-9–regulatory protein on the endothelial surface. In red blood cells, CD59 has been shown to restrict hemolysis by C5b-9 and to reduce the incorporation of C9 into membrane C5b-9, thereby decreasing the number of C9 bound to concentrations of C9 tested. By contrast, this antibody had no effect on histamine-induced vWF secretion at any concentration of histamine (Fig 5B). The data of Fig 5 suggest that anti-CD59 potentiates the C5b-9–induced response by directly affecting activation of the complement pore, and does not potentiate receptor-mediated endothelial secretory responses.

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DISCUSSION

Our data suggest that the human endothelial plasma membrane contains an 18- to 21-Kd inhibitor of the terminal complement proteins that shares both functional and antigenic properties with the 18- to 21-Kd C5b-9–inhibitor, CD59, previously detected in the human erythrocyte and leukocyte plasma membrane. In erythrocytes, this protein appears to serve a key role in restricting the cytolytic consequence of C5b-9 assembly. In addition to contributing to the normal resistance of human endothelial cells to lysis by complement, our data suggest that this membrane component serves directly to attenuate the capacity of the C5b-9 proteins to evoke thrombogenic responses from these cells, which normally express predominantly anticoagulant properties.

When C5b67-containing endothelial cells were exposed to anti-CD59, C5b-9 pore formation (as indicated by 1H-DIG release, Fig 2) was enhanced fourfold, suggesting that this antigen limits C8 and C9 binding to C5b67 sites or limits subsequent activation of the pore. Similarly, anti-CD59 enhances hemolysis of C5b67-erythrocytes exposed to C8 and C9, and enhances secretion and prothrombinase activity of C5b67-platelets exposed to C8 plus C9. The protective effect of CD59 in C5b-9–mediated hemolysis appears to be related to this protein’s capacity to limit the number of C9 that bind to C5b-8 deposited on the erythrocyte surface. Human platelets exposed to anti-CD59 show enhanced susceptibility to the stimulatory effects of the C5b-9 complex and increased binding of C9 to C5b-8, suggesting that epitopes recognized by anti-CD59 include a functional domain(s) which interfere(s) with the binding and/or activation of C9 by membrane bound C5b-8. In endothelial cells, we did not observe increased C9 binding to C5b-8 on cells exposed to anti-CD59 (Fig 6). One interpretation of these data is that in endothelial cells the cell surface complement regulatory protein(s) that are inhibited by anti-CD59 restrict a conformational change in the C5b-9 complex necessary for pore formation and cell activation, rather than reducing C9 binding to C5b-8 per se. Alternatively, we have demonstrated previously that C5b-9 induces the formation of membrane microparticles that are shed from the endothelial surface and contain the membrane-inserted C5b-9 proteins. It is likely, therefore, that our inability to detect increased binding of 125I-C9 to C5b-8 on anti-CD59–treated endothelial monolayers is related to a concomitant increase in this vesiculation of membrane-embedded C5b-9 complexes from the surface of these cells. Of note in platelets, increased membrane incorporation of C9 after exposure to anti-CD59 has been shown to be largely accounted for by C5b-9
The capacity of anti-CD59 to potentiate the C5b-9-induced vWF secretion and prothrombinase activity of endothelial cells suggests that a deletion or inactivation of the membrane protein recognized by this antibody might potentiate the procoagulant response of these cells exposed to low levels of complement activation. Alternatively, increased expression of CD59 on the plasma membrane of vascular endothelial cells, as might potentially be achieved by transfection with the human CD59 gene, might render these cells resistant to the cytolytic and cell-stimulatory effects of the terminal complement proteins. Such resistance to activated complement would be of particular benefit in protecting complexes present on microparticles that were released from the platelet plasma membrane.15

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Fig 5. Effect of anti-CD59 on histamine-stimulated vWF secretion. Endothelial monolayers were incubated with either 0 (○) or 500 μg/mL anti-CD59 Fab (△) and the dose-dependent stimulation of vWF secretion determined for C5b-9 (A) and histamine (B). (A) After incubation of C5b67 monolayers with anti-CD59, C8 (0.5 μg/well) and C9 (0 to 125 ng/well) were added, and secreted vWF quantitated after 10 minutes, 37°C. Data shown are mean ± SD, n = 3, and are representative of two experiments performed. (B) After incubation of endothelial monolayers with anti-CD59, histamine (0 to 0.1 mmol/L) was added, and secreted vWF quantitated after 10 minutes, 37°C. Data shown are mean ± SD, n = 2, and are representative of three experiments performed.

components present on microparticles that were released from the platelet plasma membrane.15

The capacity of anti-CD59 to potentiate the C5b-9-induced vWF secretion and prothrombinase activity of endothelial cells suggests that a deletion or inactivation of the membrane protein recognized by this antibody might potentiate the procoagulant response of these cells exposed to low levels of complement activation. Alternatively, increased expression of CD59 on the plasma membrane of vascular endothelial cells, as might potentially be achieved by transfection with the human CD59 gene, might render these cells resistant to the cytolytic and cell-stimulatory effects of the terminal complement proteins. Such resistance to activated complement would be of particular benefit in protecting complexes present on microparticles that were released from the platelet plasma membrane.15

Fig 6. Effect of anti-CD59 on the incorporation of C8 and C9 into membrane C5b-9. C5b67 was deposited on endothelial monolayers and the binding of 125I-C8 (A) and 125I-C9 (B) determined for endothelial monolayers treated with either 0 (△) or 500 μg/mL (▲) anti-CD59 Fab. (A) After incubation with anti-CD59, 125I-C8 was added at concentrations on abscissa and incubated 12 minutes, 37°C. After washing five times with ice-cold Hanks-HEPES-BSA, the monolayer were solubilized in 4% SDS, and radioactivity determined. Nonspecific binding was determined by omitting C8-deficient serum (○; see Experimental Procedures). (B) The binding of 125I-C9 was performed at concentrations shown on abscissa, measured in the presence of 0.5 μg C8 per well (4 μg/mL). Nonspecific binding was performed in the absence of C8 (○). Data shown are mean ± SD, n = 2, and are representative of two experiments so performed.
xenogeneic organ transplants, or in ameliorating hyperacute allograft rejection.

ACKNOWLEDGMENT

The authors gratefully acknowledge the advice and suggestions of Dr Therese Wiedmer (Oklahoma Medical Research Foundation) and the excellent technical assistance of Elizabeth A. Smith, Darlene Schwartzott, and Janet Heuser. Factors Va, Xa, and prothrombin were generous gifts from Dr Charles T. Esmon (Oklahoma Medical Research Foundation), and MoAbs IF1 and IF5 were generously provided by Drs Motowo Tomita (Showa University, Tokyo, Japan) and Hidechika Okada (Nagoya City University School of Medicine, Nagoya, Japan).

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Regulatory control of the terminal complement proteins at the surface of human endothelial cells: neutralization of a C5b-9 inhibitor by antibody to CD59

KK Hamilton, Z Ji, S Rollins, BH Stewart and PJ Sims