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Elizabeth J. Hindmarsh and Rory M. Marks

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Vascular endothelium is continuously exposed to plasma complement, which could generate a potent proinflammatory signal if activated on the vascular wall. Normal endothelium, however, expresses an anti-inflammatory phenotype, which includes resistance to complement fixation. As activated endothelium converts to a proinflammatory phenotype, we investigated the effect of cytokines on endothelial susceptibility to complement fixation. Cytokine-treated HUVEC were exposed to human serum as a source of complement, and C3 deposition was quantified. IL-1β and TNF-α in combination with IFN-γ markedly increased endothelial C3 deposition; however, immunofluorescence microscopy revealed that the endothelial cells had retracted, and that bound C3 was concentrated not on cells but in areas of exposed subendothelial extracellular matrix (ECM). Studies with cell-free ECM indicated that complement activation required only ECM exposure and was independent of cellular activation. C3 deposition on ECM was reproduced by reconstituting the alternative pathway, which generated a stable C3 convertase on ECM, but not on endothelial cells. C3b and iC3b were identified on ECM exposed to purified alternative pathway components and serum, respectively. In conditions associated with endothelial disruption, exposure of subendothelial ECM could induce complement fixation and contribute to inflammation and vascular damage. The Journal of Immunology, 1998, 160: 6128–6136.

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2 Address correspondence and reprint requests to Dr. Rory M. Marks, Department of Internal Medicine, Division of Rheumatology, MSRB1, Room 5520, University of Michigan Medical Center, Ann Arbor, MI 48109-0680. E-mail address: rmarks@umich.edu

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**Antisera**

Goat antiserum to human C3 and murine mAb to human fibronectin were obtained from Calbiochem (San Diego, CA). Goat antisera to human C5 and C9 and murine mAb to human C5b-9 neoantigen were purchased from Quidel, mAb to human collagen type IV (clone COL-94) and laminin (clone to LM-89) were obtained from Sigma, and goat antiserum to human C5 from Willebrand factor (vWF) was purchased from Atlantic Antibodies (Scarborough, ME). Normal goat serum was obtained from Life Technologies, and normal murine IgG1 was purchased from Coulter (Miami, FL). Rabbit F(αβ′)2 anti-goat IgG was obtained from Cappel (West Chester, PA). Mouse anti-goat IgG, fluorescein-conjugated rabbit anti-goat IgG, horseradish peroxidase-conjugated rabbit F(αβ′)2, anti-goat Ig, and B-phycoerythrin-conjugated donkey F(αβ′)2 anti-goat IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa 488 goat anti-mouse IgG conjugate and Alexa 568 goat anti-rabbit IgG conjugate were purchased from Molecular Probes.

**Endothelial cell culture and preparation of extracellular matrix**

HUVEC were isolated (15) and cultured on gelatin-coated tissue culture dishes (0.2% gelatin, 30 min) in medium 199 supplemented with 20% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml endothelial cell growth supplement, and 100 μg/ml heparin at 37°C in 5% CO2. Cells were confirmed as endothelial by typical cobblestone morphology at confluence and binding of Ab to vWF. HUVEC were used in experiments at the second and third passages after primary isolation. Most experiments were performed on cells that had been transferred to gelatin-coated 96-well flat-bottom plates at 104 cells/well and grown to confluence. Cells were treated with cytokines in culture medium for periods up to 72 h.

To prepare cell-free ECMs, unstimulated HUVEC were cultured for 3 to 6 days before confluence, washed with cation-free PBS, and released from the culture surface by incubation with 0.02% EDTA in cation-free PBS at 37°C, followed by gentle trituration. Removal of cells was monitored by phase contrast microscopy. ECMs were washed three times with PBS and used immediately. Fluoroimmunoassay using monospecific antisera confirmed that ECMs had incorporated endothelium-generated components of naturally occurring basement membranes: type IV collagen, fibronectin, and vWF.

**Immobilization of matrix proteins**

Ninety-six-well tissue culture plates were coated overnight at 4°C with 50 μg/well of Matrigel (1/50 dilution), laminin (25 μg/ml), type IV collagen (25 μg/ml), fibronectin (25 μg/ml), vitronectin (50 μg/ml) or gelatin (25 μg/ml). Dilutions were performed in cation-containing PBS. Coated wells were then treated for 2 h at 37°C with gelatin (0.2%) to block nonspecific protein binding sites.

**Complement activation**

HUVEC monolayers or ECM were incubated for varying periods at 37°C with 50 μl of human serum diluted in PBS, or with purified complement components diluted in PBS containing 10 mg/ml HSA. Purified complement components were used at concentrations equivalent to 33% serum (C3 (400 μg/ml), factor B (70 μg/ml), factor D (0.32 μg/ml), type IV collagen (10 μg/ml) or gelatin (25 μg/ml)). Dilutions were performed in cation-containing PBS. Coated wells were then treated for 2 h at 37°C with gelatin (0.2%) to block nonspecific protein binding sites.

**Fluorescence microscopy**

Single color. HUVEC were grown to confluence in gelatin-coated tissue culture dishes for 3 days postconfluence, with or without cytokines added during the final 36 h. For simultaneous detection of cell membranes and C3, HUVEC membranes were labeled with 5,5′-dithiobis-(2-nitrobenzoic acid) (10 μM) in PBS for 60 min at 37°C. In some studies, ECMs were then prepared by releasing the cells, as previously described. Complement fixation on HUVEC or cell-free ECM was induced by a 30-min incubation with 50% human serum, and C3 was detected by sequential incubation with goat anti-human C3 (1/500), mouse anti-IgG IgG (1/200), and the green-emitting fluorochrome conjugate Alexa 488-goat anti-mouse IgG (1/50). For simultaneous detection of C3 and matrix proteins, complement fixation was induced on cell-free ECM, and C3 was detected by sequential incubation with goat anti-human C3 (1/500), rabbit anti-IgG IgG (1/200), and the red-emitting fluorochrome conjugate Alexa 568-goat anti-rabbit IgG (1/50). Matrix proteins were detected with murine mAb to fibronectin (1/500) or collagen type IV (10 μg/ml), followed by Alexa 488-goat anti-mouse IgG (1/50). Cells/ECM were mounted in PBS, and epifluorescence was visualized using a Nikon Optiphot-2 microscope with 31003 and 31002 filter sets (Chroma Technology Corp., Brattleboro, VT). Controls showed no significant difference in Alexa 488 using the 31002 filter set or of 5,5′-dithiobis-(2-nitrobenzoic acid) (3) and Alexa 568 using the 31003 filter set.

**Analysis of C3 fragments by Western blotting**

Wells were exposed to complement and washed three times with PBS, and covalently bound C3 fragments were released by 2-h incubation at 37°C with 130 μl of 25 mM carbonate buffer (pH 11) containing 25 mM methanol and 1% SDS. Samples were diluted with 65 μl of 3X Laemmli sample buffer, proteins were resolved on the basis of m.w. by SDS-PAGE of 6% gel running under reducing conditions and electrophoresed to nitrocellulose. Membranes were blocked with 5% gelatin in PBS containing 0.2% Tween-20 (PBST) for 2 h at 37°C, then probed with goat anti-human C3 (1/10,000) in 5% gelatin/PBST for 1 h at room temperature. Blots were washed three times with PBST, incubated with horseradish peroxidase-conjugated rabbit F(αβ′)2 anti-goat Ig (1/3,000,000) for 1 h at room temperature, and washed five times with PBST. Ab binding was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL).
Measurement of soluble C5a, C5a des-Arg, and SC5b-9

Human serum (100%) was incubated alone, with cell-free ECM, or with HUVEC opsonized with rabbit antiserum for 30, 60, and 120 min at 37°C. Complement activation was stopped by placing the samples on ice with addition of 4 mM EDTA. The C5a/C5a des-Arg concentration was measured using the SC5b-9 enzyme immunoassay kit (Quidel). Background C5a levels were assessed using serum/4 mM IFN-γ with IL-1β.

Results

Complement activation on endothelial cells

Spontaneous complement activation on endothelium was assessed by incubating HUVEC monolayers with 50% human serum as a source of complement. Endothelial binding of C3 was used as an index of complement fixation and was quantified by fluoroimmunoassay (Fig. 1A). A low level of C3 deposition was consistently observed on untreated HUVEC; however, this was no greater than that associated with cell-free, gelatin-coated wells (used in all wells to promote endothelial adherence). A positive control consisting of HUVEC opsonized with complement-fixing rabbit anti-HUVEC IgG followed by exposure to 50% human serum led to considerable endothelial C3 deposition.

C3 binding was similarly assessed on HUVEC pretreated with cytokines for 72 h (Fig. 1B). Treatment with the individual cytokines IL-1β, TNF-α, and IFN-γ led to increased C3 binding compared with that by untreated HUVEC; however, the greatest increases resulted from combinations of IL-1β or TNF-α with IFN-γ. LPS had no effect on C3 binding, nor did it influence the stimulatory effects of TNF-α or IFN-γ. Increased C3 deposition due to IL-1β or TNF-α in combination with IFN-γ was readily apparent after 24-h cytokine treatment, but did not occur consistently at shorter exposure times (8 h or less; data not shown); this correlated with the onset of visible morphologic changes. Untreated monolayers consisted of closely packed polygonal cells; after exposure to TNF-α or IL-1β, cells became elongated, an effect even more pronounced in cultures treated with IFN-γ (alone or combined with other cytokines). Cells treated with IL-1β or TNF-α in combination with IFN-γ were also less densely packed, with areas of intercellular retraction. LPS-treated cells, in contrast, were only mildly elongated and remained densely packed. Similar morphologic changes following extended treatment with cytokines (1–5 days) have been described previously and were accompanied by monolayer disorganization and exposure of the substratum (17–19).

Distribution of bound C3

The distribution of C3 bound to HUVEC monolayers after exposure to human serum was examined by immunofluorescence microscopy (Fig. 2A). A low level of C3-associated fluorescence was observed on the surface of untreated HUVEC (Fig. 2A.i). No specific fluorescence was apparent on control cells exposed to normal goat serum instead of anti-human C3 (Fig. 2A.ii). The pale yellow fluorescence observed in the photomicrographs is attributable to cellular autofluorescence; in contrast, fluorescein-derived fluorescence is characteristically apple green.

HUVEC undergo considerable morphologic change after prolonged treatment with cytokines, as noted above. HUVEC pretreated for 24 h with TNF-α and IFN-γ, exposed to human serum, and assayed for C3 deposition showed the same minimal cell-associated fluorescence as untreated cells (Fig. 2A.ii). However, gaps in the monolayer were apparent where cells had retracted or detached, exposing subcellular material, and these regions were associated with an intense, homogeneous fluorescent signal. Control cells treated with human serum followed by normal goat serum instead of anti-human C3 showed no specific fluorescence (Fig. 2A.iv).

To confirm that bound C3 was associated with the substratum rather than the endothelial surface, HUVEC were exposed to human serum, and the cells were nonenzymatically released before detection of C3. After the removal of most cells, the subcellular matrix was again noted to be highly fluorescent, with the C3-associated fluorescent signal appearing as a halo around sites at which cells had previously been attached. Fluorescent regions were far more extensive on matrix derived from cytokine-treated HUVEC (Fig. 2A.v) than on that from untreated cells (Fig. 2A.v), consistent with the greater exposure of subcellular matrix after cytokine treatment; however, fluorescence intensities were similar. Identical results were obtained using HUVEC activated with IL-1β plus IFN-γ (data not shown). These observations demonstrate that the increased C3 deposition on cytokine-treated cultures was due to retraction of viable cells with resultant exposure of subcellular material, to which C3 then bound.

C3 deposition colocalizes with matrix proteins

Cell debris and intracellular components have previously been associated with complement activation (20, 21). To determine whether the C3 binding observed was occurring on the ECM or on residual cell debris, we compared sites of C3 deposition with those of residual cellular material and of matrix proteins by dual-color fluorescence microscopy (Fig. 2B). Postconfluent HUVEC, either unstimulated or treated with IL-1β and IFN-γ, were labeled with the red-emitting fluorescent plasma membrane probe 5,5′-Ph2DiIC18(3) (22) (Fig. 2B.i). ECMs were then prepared from unstimulated HUVEC using EDTA, which causes cells to progressively loosen and detach from the substratum (Fig. 2B.ii). Cell-free ECMs and cytokine-treated HUVEC monolayers were incubated with human serum to permit complement activation, followed by immunodetection of C3; sites of residual cell membrane material (red) and C3 deposition (green) were then assessed simultaneously. Comparison of Figure 2B, iii and iv (cytokine-treated HUVEC), clearly indicates that although residual membrane fragments remained in association with the exposed ECM (Fig. 2Biii), these regions did not correspond to sites of C3 fixation (Fig. 2Biv).
the patterns of fluorescence observed were entirely different. Figure 2B.iii includes a particularly prominent region of residual membranous material, yet this area was not a focus for C3 deposition. Similarly, there was no correspondence between sites of C3 deposition and residual membrane fragments on cell-free ECM (not shown). In contrast, simultaneous immunodetection of sites of C3 deposition (Fig. 2B.vi) and a representative matrix protein, fibronectin (Fig. 2B.v), revealed identical mesh-like fibrillar patterns. A similar comparison of the distribution of C3 and collagen IV yielded identical results (not shown). These data indicate that bound C3 was associated with the subendothelial matrix exposed by cellular retraction or detachment, not with residual cellular debris.

We then directly examined the ability of ECM to activate complement by assessing C3 deposition on a selection of purified matrix proteins. Tissue culture wells were coated with Matrigel, laminin, collagen type IV, fibronectin, or vitronectin and then incubated with human serum with or without 10 mM EDTA for 30
min at 37°C (Fig. 3). Gelatin-coated wells were used as a negative control. Matrigel (a soluble matrix preparation containing predominantly type IV collagen, laminin, entactin, and heparan sulfate proteoglycan) bound a high level of C3 in a cation-dependent manner, indicating dependence on complement activation. C3 deposition was also high on human vitronectin (found in plasma and extracellular matrices, including endothelium-derived ECM) (23, 24), while intermediate levels bound to human fibronectin (found in plasma and subendothelial basement membrane) (19, 25). In contrast, laminin and type IV collagen, both major components of basement membranes (26), bound no more C3 than did gelatin; adsorption of these matrix proteins to wells was confirmed by fluoroimmunoassay (data not shown).

Comparison of complement activation on HUVEC and subendothelial ECM

To more directly contrast the ability of subendothelial ECM to activate complement with that of the intact HUVEC monolayer, the time courses of C3 binding to HUVEC, cell-free ECM, and cell-free gelatin-coated wells were compared (Fig. 4). Wells were exposed to 50% human serum for times up to 130 min, and C3 binding was assessed by fluoroimmunoassay. C3 deposition occurred at a similarly low level on both intact HUVEC monolayers and gelatin. In contrast, C3 deposition on ECM progressively increased over 100 min, plateauing at a level that ranged from 2.5- to 4-fold greater than that on HUVEC in three separate experiments. C3 deposition was also dependent on serum concentration, increasing over the range from 10 to 30% serum (data not shown). The absence of binding in the presence of EDTA (Fig. 4) indicates that C3 deposition on ECM was a consequence of complement activation, rather than nonspecific adsorption from serum, or unamplified C3 binding. Data shown were obtained using serum from a single donor; ECM C3 deposition was also demonstrated using sera from all of nine different donors (data not shown), indicating that the serum-associated complement activation observed on ECM was unlikely to be due to an ECM-specific allo-Ab.

Alternative pathway activation on ECM

The occurrence of spontaneous C3 fixation on ECM suggested that complement was likely to be activated via the alternative pathway. This was directly examined by reconstituting the alternative pathway with purified components C3, factor B, factor D, and properdin and assessing the binding of C3 (Fig. 5). A level of C3 deposition comparable to that induced by serum occurred when all four components were combined in serum-equivalent concentrations and incubated with ECM. Addition of C3 together with factors B and D, but without properdin, led to C3 deposition only slightly higher than that occurring with C3 alone, indicating that properdin, which stabilizes the alternative pathway C3 convertase (27), has a major effect on alternative pathway activation on ECM. The alternative pathway C3 convertase complex C3bBbP is responsible for cleaving additional molecules of C3 to C3b, which may then also bind to the target surface; the formation and the stability of this complex comprise one of the major complement

![FIGURE 3](image-url) C3 deposition on immobilized matrix proteins. Wells were coated with Matrigel (1/30 dilution), laminin (Ln; 25 μg/ml), type IV collagen (Coll. IV; 25 μg/ml), fibronectin (Fn; 25 μg/ml), vitronectin (Vn; 50 μg/ml), or gelatin (25 μg/ml) and then blocked with gelatin (0.2%). Coated wells were incubated with 50% human serum with or without 10 mM EDTA for 30 min at 37°C, and C3 deposition was assessed by fluoroimmunoassay. Results are expressed as the mean ± SEM of triplicate samples and are corrected for nonspecific Ab binding.

![FIGURE 4](image-url) Time course of C3 deposition on subendothelial extracellular matrix and endothelial cells. ECM, untreated HUVEC, or gelatin-coated (control) wells were incubated with 50% human serum as a source of complement or with 50% human serum containing 50 mM EDTA (serum/EDTA) for 10 to 130 min at 37°C. C3 binding was assessed by fluoroimmunoassay and expressed as the mean fluorescence intensity ± SEM of triplicate samples. Results are corrected for nonspecific Ab binding.

![FIGURE 5](image-url) Alternative pathway complement activation on subendothelial ECM. ECMs were incubated for 60 min with 40 μl of 29% human serum or combinations of purified alternative pathway components at equivalent concentrations (C3 (350 μg/ml), factor B (61 μg/ml), factor D (0.29 μg/ml), and properdin (6 μg/ml)). C3 binding was assessed by fluoroimmunoassay and expressed as the mean fluorescence intensity of duplicate samples. Error bars indicate the range. Nonspecific fluorescence is indicated by Ab binding in the absence of added serum or complement proteins (Nil).
amplification and control elements. We therefore examined the ability of ECM to support the formation of a stable alternative pathway C3 convertase complex. ECM was initially incubated for 15 min with either serum or purified alternative pathway proteins to permit formation of the C3 convertase. ECMs were then washed and incubated for an additional 45 min with fresh serum or purified C3, and C3 deposition was quantified by fluoroimmunoassay (Fig. 6A). Two sequential incubations with serum resulted in a successive increase in C3 deposition; this was due to complement activation rather than nonspecific C3 adsorption, as addition of EDTA to the serum prevented most of the C3 binding. This result indicated that complement activation could be initiated and progress in the presence of the inhibitory regulators factors H and I, which are present in serum and convert C3b to the convertase-inactive form iC3b. An initial incubation with C3, factor B, factor D, and properdin, followed by a second incubation with C3 alone, elicited the highest level of C3 binding observed, far exceeding binding of C3 alone. This is consistent with formation of a stable alternative pathway C3 convertase on ECM during the initial incubation, enabling C3 cleavage and fixation to continue during the secondary incubation with C3 alone. Conversely, little additional binding occurred when C3 was added to ECM preincubated with serum (data not shown), suggesting that individual C3 convertases formed in the presence of serum regulatory factors were short lived and did not persist long enough to activate subsequently added C3. In contrast to results obtained with ECM, a similar experiment performed using intact HUVEC monolayers resulted only in low levels of C3 deposition following primary incubation with serum, C3, or alternative pathway complement proteins, with subsequent addition of serum or C3, indicating failure to maintain an effective C3 convertase (Fig. 6B).

**FIGURE 6.** Alternative pathway complement activation generates a stable C3 convertase on subendothelial ECM. ECM (A) and untreated HUVEC (B) were initially incubated for 15 min with 40 μl of 33% human serum, 33% serum containing 50 mM EDTA (serum + EDTA), C3 (400 μg/ml), or a combination of C3, factor B (70 μg/ml), factor D (0.32 μg/ml), and properdin (6.7 μg/ml; serum-equivalent concentrations). After washing, wells were either left untreated or were further incubated for 45 min with fresh serum, serum + EDTA, or C3. C3 binding was assessed by fluoroimmunoassay and expressed as the mean fluorescence intensity ± SEM of quadruplicate samples (serum) or the mean ± range of duplicate samples (purified complement components).

**FIGURE 7.** Generation of C3b and iC3b during two-stage complement activation on subendothelial ECM. ECMs and intact HUVEC monolayers were treated with serum or purified alternative pathway complement proteins as described in Figure 6. Bound C3 fragments were released as described in Materials and Methods and analyzed by SDS-PAGE and Western blotting. Each lane contains protein released from an equivalent surface area. Standards: purified C3 (lane 1), C3b (lane 2), iC3b (lane 3). ECM: C3, 15-min incubation (lane 4); C3, 15-min incubation, followed by C3, 45-min incubation (lane 5); C3, factor B, factor D, and properdin, 15-min incubation (lane 6); C3, factor B, factor D, and properdin, 15-min incubation, followed by C3, 45-min incubation (lane 7); serum, 15-min incubation, followed by serum, 45-min incubation (lane 8). HUVEC: C3, 15-min incubation (lane 9); C3, 15-min incubation, followed by C3, 45-min incubation (lane 10); C3, factor B, factor D, and properdin, 15-min incubation (lane 11); C3, factor B, factor D, and properdin, 15-min incubation, followed by C3, 45-min incubation (lane 12). C3 α- and β-chains are identified by arrows. Molecular weight markers (×10−3) are identified between gels.

**Generation of C3b and iC3b**

ECM-associated C3 convertase activity was further demonstrated by identifying the C3 fragments bound to ECM in the experiment described above and in Figure 6. C3 consists of α- and β-chains of 119,000 and 75,000 m.w., respectively. The C3 convertase cleaves a small fragment (C3a) from the α-chain, generating C3b with an α'-chain m.w. of 111,000. C3b may be processed to the convertase-inactive form iC3b by cleavage of the α'-chain by factor I and cofactors, yielding two fragments of 68,000 and 43,000 m.w. (28). To determine the form of ECM-associated C3, covalently bound C3 fragments were released from the ECM with methylamine, resolved on the basis of m.w. by SDS-PAGE, and detected by Western blotting (Fig. 7). Lanes 1 to 3 of Figure 7 illustrate the α-chain m.w. associated with standard preparations of C3, C3b, and iC3b.

On ECM incubated with C3 alone for 15 min (Fig. 7, lane 4) or for the two incubations totaling 60 min (lane 5), only uncleaved C3 was detected. Incubation with C3, factor B, factor D, and properdin for 15 min resulted in a mixture of intact C3 together with a lesser amount of C3b (Fig. 7, lane 6). A subsequent 45-min incubation with C3 also yielded a mixture of C3 and C3b (Fig. 7, lane 7), with a higher proportion of C3b than that detected in lane 3. As expected, no iC3b was formed, as factor I and cofactors were not supplied. Incubation with serum for 60 min resulted in almost complete conversion of bound C3 to iC3b (Fig. 7, lane 8).

Evaluation of bound C3 fragments recovered from an intact HUVEC monolayer revealed a much reduced level of C3 cleavage compared with that on ECM. As on ECM, C3 added alone was uncleaved by HUVEC (Fig. 7, lanes 9 and 10). Incubation of
serum incubated at 37°C for 30 to 60 min. C5a was substantially increased in serum incubated on Ab-opsonized HUVEC, particularly with longer incubation times (60–120 min); however, no significant changes occurred with ECM exposure. As C5a levels in serum exposed to ECM for 60 and 120 min were no higher than those in control serum, the slight increase seen after 30 min is unlikely to be significant. In addition, ECM exposure did not increase formation of the SC5b-9 complex in serum (data not shown). Thus, complement activation on ECM was associated with C3 fixation, but there was no detectable terminal pathway activation.

Discussion

Normal vascular endothelium does not support complement activation, consistent with its anti-inflammatory phenotype. Our initial aim was to identify inflammatory mediators able to convert endothelium to a form susceptible to complement activation, which we hypothesized could comprise part of a normal inflammatory response. Using an in vitro model, we found that cytokine-stimulated endothelium did indeed become a target for complement activation; surprisingly, this was due not to changed cell surface properties, as enhanced C3 deposition was not associated with the cell membrane, but instead to a morphologic change in the endothelial monolayer. Cytokine-induced retraction of endothelial cells exposed the substratum, which was an effective complement target. The ability of subendothelial ECM to activate complement was confirmed by demonstrating a close correspondence between the morphologic patterns of C3 and matrix proteins on exposed ECM as well as the ability of selected isolated matrix proteins to support C3 fixation. While cultured HUVEC cannot fully represent all types and functions of vascular endothelium found in vivo, these cells have been particularly valuable for investigating inflammatory mechanisms; whether some or all subendothelial basement membranes in vivo share the ability of HUVEC-derived ECM to act as a complement target remains to be determined. Support for the proposition that subendothelial matrix in vivo can activate autologous complement is provided by reports describing activation of the alternative pathway on isolated glomerular basement membrane and on type II collagen (30, 31).

Spontaneous activation of complement on ECM suggested that the alternative pathway was responsible, and this interpretation was supported by the finding that purified alternative pathway components could reproduce the complement activation observed with whole serum. However, the possibility of simultaneous Ab-independent activation of the classical pathway has not been excluded (32, 33). Alternative pathway activation is initiated by the spontaneous binding of a small number of C3b molecules to a suitable acceptor surface. The features that define target specificity for the alternative pathway are not fully defined; however, the ability to assemble and maintain the C3 convertase C3bBb, which amplifies further deposition of C3b, is a critical determinant (27). C3b binds covalently to nucleophiles, typically hydroxyl groups, on acceptor surfaces (34); in terms of their expression of suitable nucleophiles, some surfaces are intrinsically more accommodating to C3b binding, and thus supportive of complement activation, than others (35). The ability of ECM to support C3b deposition and C3 convertase formation indicates that it does present suitable nucleophilic sites. Carbohydrates, with their high hydroxyl content, are preferred targets for the alternative pathway (35); subendothelial matrix is rich in proteoglycans and glycoproteins (e.g., laminin, entactin, and fibronectin (1, 26)) and so could present multiple...
targets for alternative pathway activation. We have not yet identified specific ECM components responsible for complement activation; however, the lack of C3 deposition on purified laminin and collagen type IV indicates that these are unlikely to be involved, while C3 fixation on fibronectin and vitronectin suggests they could contribute. As some polyanions activate complement (32, 33), matrix proteoglycans are also possible candidates.

In the presence of factors B and D, bound C3b forms the C3 convertase C3bBb. Its assembly and maintenance are influenced by regulators of complement activation, both membrane-associated (decay-accelerating factor and membrane cofactor protein) and circulating in the plasma (properdin, factor H, and factor I). In the absence of soluble regulatory factors, ECM supported the assembly of a stable alternative pathway C3 convertase. However, properdin, which stabilizes the C3bBb complex (27), was required for effective reconstitution of the alternative pathway, suggesting that the C3 convertase formed in its absence was susceptible to dissociation. ECM remained capable of supporting complement activation when exposed to the full spectrum of serum-derived complement regulators; however, ECM-bound C3b was subject to the action of regulatory molecules, as evidenced by its ultimate conversion to iC3b. C3 convertases assembled under these conditions were more labile than those formed in the absence of serum regulators, as ECM preincubated with serum retained little convertase activity once serum was removed. This suggests that C3 convertases formed in serum were rapidly dissociated/inactivated, with continued convertase activity dependent on the ability to assemble new complexes. This may explain the lack of detectable terminal pathway activation, as C5 convertase activity depends on the addition of C3b to existing C3 convertase complexes (36). Taken together, these data indicate that ECM is a surface capable of supporting both C3 convertase formation and interaction with regulatory factors, and that in the presence of serum, the balance between convertase formation vs dissociation/inactivation favors net C3 convertase activity with significant C3 fixation to ECM.

Complement fixation on exposed subendothelial basement membrane in vivo could promote inflammation and vascular injury. Although many deleterious effects of complement are mediated by terminal pathway activation products (37), which we did not detect, C3 cleavage is itself associated with potentially damaging consequences. iC3b, which was generated on ECM exposed to serum, is a potent stimulus for neutrophil adhesion (2). In addition, cleavage of C3 releases the anaphylatoxins C3a and C5a. The pathophysiologic role of C3a is not fully defined; however, C3a receptors are expressed on neutrophils, monocytes, eosinophils, basophils, and mast cells (38). In addition to its well-characterized effects on vascular permeability and vasoconstriction (5), C3a modulates the expression of proinflammatory cytokines by monocytes (39), activates leukocytes (38, 40–44), and induces chemotaxis (45, 46). Focal generation of C3 cleavage products on exposed basement membrane could potentially facilitate leukocyte adhesion and migration and/or contribute to leukocyte-mediated damage to the vascular wall.

ECM-mediated complement activation may contribute to pathologic conditions involving disruption or damage to the vascular endothelium, with associated exposure of subendothelial matrix. Exposure of basement membrane in vivo has been described in a number of injurious settings. Endothelial cell retraction occurs in response to cytokines and other acute inflammatory mediators (47–51), while endothelial damage resulting from thermal injury (52), septic shock/endotoxemia (53, 54), vasculitis (55), ischemia reperfusion injury (56), and invasive procedures such as balloon angioplasty (57) would also cause exposure of ECM. Complement activation is a prominent feature of many of these conditions (58–62) and is also associated with administration of cytokines (63, 64). Although we do not have direct evidence that complement activation results from basement membrane exposure in vivo, our recent finding that decay-accelerating factor is an inducible component of endothelial-derived ECM (65) supports the view that this surface interacts with complement. The alternative pathway of complement is a phylogenetically ancient inflammatory mechanism, and many pathogenic processes arise from the subversion of normal defense mechanisms. We speculate that complement activation at a site of injury, triggered by physical disruption of the vasculature with exposure of ECM, could represent a normal hemostatic mechanism for signaling injury and inducing an appropriate inflammatory response.

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