Decay-accelerating factor is a component of subendothelial extracellular matrix *in vitro,* and is augmented by activation of endothelial protein kinase C

Elizabeth J. Hindmarsh and Rory M. Marks

Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, USA

The vasculature is protected from complement activation by regulatory molecules expressed on endothelial cells. However, complement fixation also occurs on subendothelial extracellular matrix (ECM) *in vitro,* and is initiated simply by retraction or removal of overlying cells. To investigate mechanisms controlling vascular complement activation, we examined subendothelial ECM for the presence of complement regulatory proteins. Decay-accelerating factor (DAF) was found on both human umbilical vein endothelial cells (HUVEC) and in their ECM; in contrast, membrane cofactor protein was found only on cells. ECM and HUVEC DAF were distinguishable based on several properties. While HUVEC DAF is anchored to cell membranes by a phospholipase C-sensitive glycosylphosphatidylinositol linkage, DAF was removed from ECM only by proteolytic digestion. Cytokines (TNF-α, IL-1β, IL-4) increased HUVEC DAF expression, but had minimal effect on ECM DAF; in contrast, phorbol 12-myristate 13-acetate (PMA) and wheat germ agglutinin markedly increased DAF on both HUVEC and ECM. The effect of PMA was mediated by activation of protein kinase C. The complement regulatory potential of ECM DAF was assessed by evaluating the effect of DAF-neutralizing antibodies on C3 deposition on HUVEC ECM, as well as on HeLa cell ECM, which had a considerably higher DAF content. DAF blockade enhanced C3 deposition on HeLa ECM, but had no effect on HUVEC ECM. As ECM DAF is likely to be immobile, *i.e.* able to interact only with C3 convertases forming in the immediate vicinity, its ability to regulate complement activation may be particularly density dependent, and contingent on endothelial-dependent up-regulation.

Key words: Decay-accelerating factor / Endothelium / Extracellular matrix / Complement regulators / Human

Received	3/11/97
Accepted	30/12/97

1 Introduction

Normal cells are protected from the deleterious effects of complement activation on their surface by an array of regulatory proteins, both cell surface associated and circulating in the plasma. The majority of these regulators act to prevent amplification of C3b deposition, by dissociating C3 convertase complexes or by cleaving covalently bound C3b to a convertase-inactive form. Cell surface regulators include decay-accelerating factor (DAF, CD55), which accelerates the dissociation of both classical and alternative pathway C3 convertases [1]; membrane cofactor protein (MCP, CD46), which is a cofactor for factor I-mediated cleavage of C3b to the convertase-inactive form, iC3b [2]; and CR1 (CD35), which has both decay-accelerating and cofactor activity [3]. Plasma proteins factor H and C4 binding protein (C4bp) express decay-accelerating activity towards the alternative and classical pathway C3 convertases, respectively, and also serve as cofactors for the cleavage of C3b and C4b [4].

In a recent study, we found that subendothelial extracellular matrix (ECM) generated by human umbilical vein endothelial cells (HUVEC) is a target for spontaneous

[I 17768]

Abbreviations: **C4bp:** C4 binding protein **DAF:** Decay-accelerating factor **ECM:** Extracellular matrix **GPI:** Glycosylphosphatidylinositol **MCP:** Membrane cofactor protein **PI-PLC:** Phosphatidylinositol-specific phospholipase C **SCR:** Short consensus repeat **TPCK:** L-1-tosylamide-2-phenylethylchloromethyl ketone **vWF:** von Willebrand factor **WGA:** Wheat germ agglutinin

0014-2980/98/0303-1052$17.50+.50/0

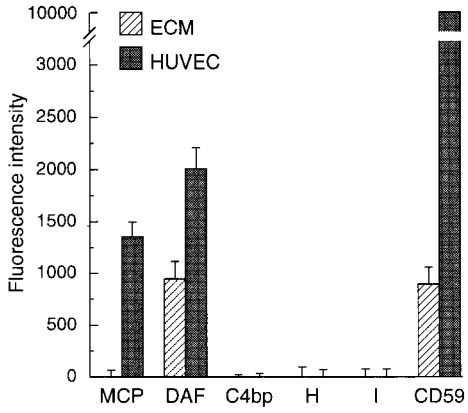

Figure 1. Comparison of complement regulatory molecules in subendothelial matrix and on the endothelial cell surface. HUVEC were cultured for 6 days past confluence, and matrices isolated as described in Sect. 4.3. Levels of MCP, DAF, CD59, C4bp and factors H and I were assessed by fluoroimmunoassay, and results expressed as the mean fluorescence intensity ± SEM of triplicate samples. Results are corrected for non-specific antibody binding.

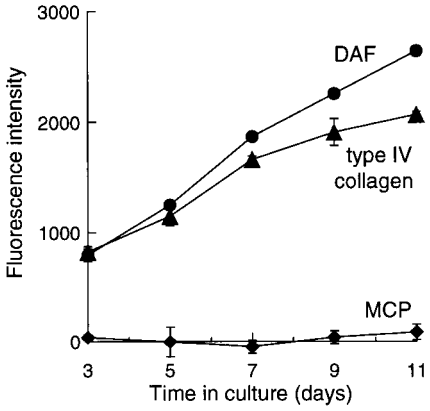

Figure 2. Time course of DAF, MCP and type IV collagen accumulation in subendothelial matrix. HUVEC were cultured for up to 11 days, with matrices isolated on days 3, 5, 7, 9 and 11. ECM components were assessed by fluoroimmunoassay. Results are expressed as the average ± range of duplicate samples, and are corrected for non-specific antibody binding.

complement activation, and supports formation of a stable alternative pathway C3 convertase*. This mechanism may provide a basis for antibody-independent complement activation in conditions characterized by endothelial disruption. HUVEC express DAF [5], MCP [6] and CD59 [7] on their surface, and secrete factors H and I [8, 9]; however, it is not known whether any of these molecules are also incorporated into the subendothelial ECM. To investigate mechanisms controlling complement activation on ECM, we assessed ECM for the presence of complement regulatory proteins, with particular emphasis on those involved in regulation of C3 convertase activity. We found that of the C3 convertase regulators, only DAF is a component of the HUVEC subcellular matrix. In addition, treatment of overlying endothelial cells with activators of PKC stimulated DAF incorporation into ECM, indicating a link between endothelial phenotype and properties of its underlying matrix.

* E. J. Hindmarsh and R. M. Marks, Complement activation occurs on subendothelial extracellular matrix *in vitro,* and is initiated by retraction or removal of overlying endothelial cells. Submitted for publication.

2 Results

2.1 DAF is a component of cell-free subendothelial matrix

HUVEC monolayers and cell-free ECM were examined for surface expression of known regulators of C3 convertase activity (Fig. 1). As expected, MCP and DAF [5, 6] were found on the HUVEC surface, but CR1 (not shown), and the soluble regulators C4bp, factor H and factor I were not detected. When ECM was examined, DAF was the only C3 convertase regulator identified. We also assayed CD59, a complement regulator that inhibits formation of the lytic membrane attack complex, as it shares with the DAF the property of being tethered to cell membranes by a glycosylphosphatidylinositol (GPI) anchor [10]. CD59 was found on both HUVEC and ECM (Fig. 1).

The DAF content of the ECM increased linearly over a culture time of 3–11 days (Fig. 2). Type IV collagen was also measured to gauge cumulative ECM generation by HUVEC; there was a strong linear correlation ($r^2 = 0.99$; three separate experiments) between increasing DAF and collagen levels over this time period. The MCP content of ECM was negligible and did not increase over time, verifying that the DAF signal was specific to isolated ECM, and not derived from any residual cellular material.

2.2 Effect of HUVEC agonists on ECM DAF content

Several endothelial agonists up-regulate DAF expression on HUVEC: phorbol esters, wheat germ agglutinin (WGA), IL-1β, TNF-α and IL-4 [11–13]. Accordingly, we examined the effect of these mediators on incorporation of DAF into ECM. Treatment with PMA and WGA increased both ECM and HUVEC DAF (Fig. 3A). In contrast, IL-1β, TNF-α and IL-4 all stimulated DAF expression on the cell surface, but had only a slight effect on ECM DAF (Fig. 3B); this trend was similar in two additional experiments (data not shown).

PMA increased both HUVEC and ECM DAF in a dose-dependent manner, with a decrease observed at concentrations of PMA > 100 nM (Fig. 4). When PMA was present for the entire incubation period, cell surface DAF reached a maximum at 20–30 h (three separate experiments), and subsequently decreased towards basal levels over the next 48 h (Fig. 5A). In contrast, ECM DAF increased over a similar period, but then remained at peak levels for the duration of the assay (Fig. 5B). Continuous stimulation with PMA was not required to induce ECM and HUVEC DAF. When HUVEC were exposed to PMA for varying periods up to 24 h, PMA withdrawn and the cells maintained in medium for the balance of the 24 h period, we found that 2 h exposure to PMA was sufficient to invoke maximum DAF expression on HUVEC (Fig. 5C), and a substantial increase in ECM DAF (Fig. 5D).

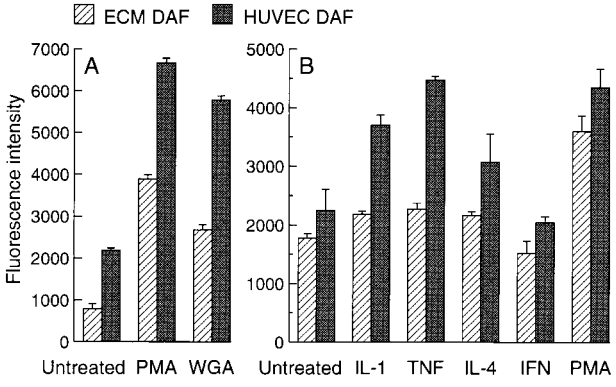

Figure 3. Effect of endothelial cell activators on HUVEC and ECM DAF. HUVEC were cultured for a total of 7 days past confluence, ending with exposure to (A) 10 nM PMA or 50 μg/ml WGA. HUVEC were treated for 24 h and 36 h for cellular and matrix measurements, respectively, to correspond with maximum DAF expression on each surface. (B) Represents a separate experiment in which HUVEC were treated with 10 ng/ml IL-1, 500 U/ml TNF-α, 100 ng/ml IL-4, 1000 U/ml IFN-γ or 10 nM PMA for 60 h. Matrices were isolated as described in Sect. 4.3, and HUVEC and ECM DAF assessed by fluoroimmunoassay. Results are expressed as the average ± range of duplicate samples, and are corrected for non-specific antibody binding.

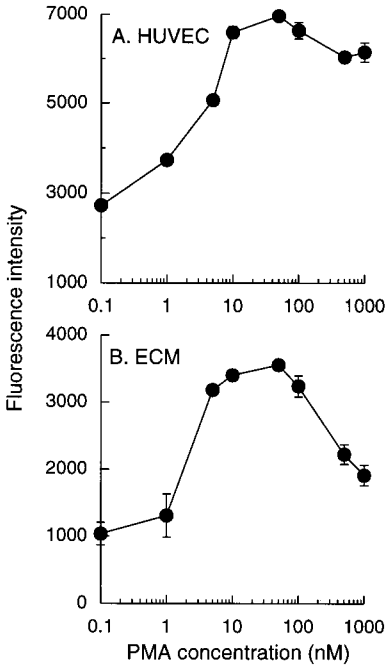

Figure 4. Dose dependence of PMA-induced DAF increase on (A) HUVEC and (B) ECM. (A) HUVEC were cultured for 5 days past confluence, then exposed to PMA for 24 h. Cell surface DAF was assessed by fluoroimmunoassay, and the results expressed as the mean ± SEM of triplicate samples. (B) In a separate experiment, HUVEC were cultured for 5 days post confluence, then exposed to the indicated concentrations of PMA for 43 h. Matrices were isolated, and DAF assessed by fluoroimmunoassay. Results are expressed as the average ± range of duplicate samples. All values are corrected for non-specific antibody binding.

PMA is a potent activator of PKC, previously shown to be responsible for DAF induction on HUVEC [11]. To assess the involvement of PKC in increasing ECM DAF, we evaluated the effects of phorbol 12, 13-dibutyrate, an analogue of PMA [14], and (–)-indolactam V, a structurally distinct, synthetic PKC activator [15, 16]. Both increased ECM DAF to a level comparable with PMA (Fig. 6). Furthermore, bisindolylmaleimide I, a selective PKC antagonist [17], inhibited the action of all three activators by approximately 80 %. These data are consistent with the PMA-induced accumulation of DAF in the ECM being mediated by activation of PKC.

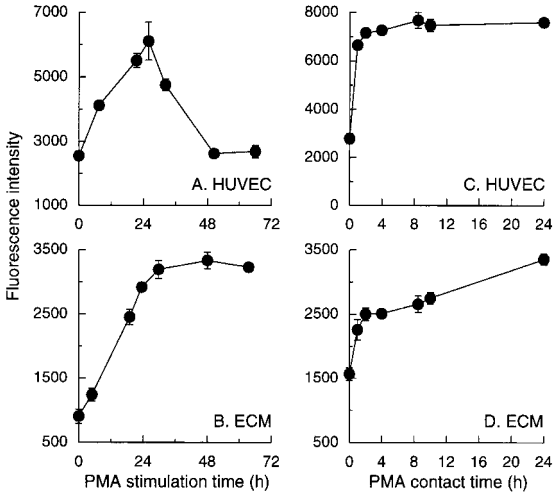

Figure 5. Time course of PMA-stimulated DAF increase on HUVEC and ECM. HUVEC were cultured for a total of 7 days post confluence, ending with exposure to 10 nM PMA for the indicated times. DAF levels on (A) HUVEC and (B) isolated matrices were assessed by fluoroimmunoassay. Results are expressed as the average ± range of duplicate samples. (C), (D) PMA exposure time required for increase in ECM and HUVEC DAF. HUVEC were cultured for 6 days past confluence, then exposed to 10 nM PMA. At the indicated times, PMA-containing medium was replaced with fresh medium (without PMA) and cells maintained for the balance of the 24-h period. DAF was then measured on (C) HUVEC monolayers and (D) isolated matrices by fluoroimmunoassay, and results expressed as the average ± range of duplicate samples. All values are corrected for non-specific antibody binding.

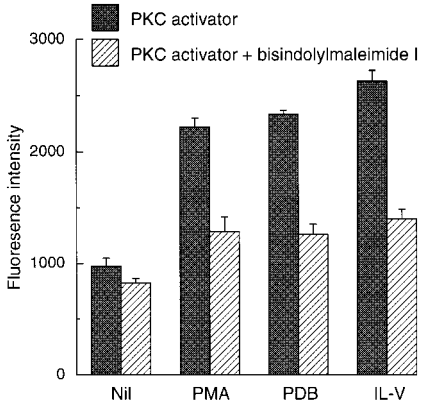

Figure 6. Effect of PKC activation/inhibition on ECM DAF. HUVEC were cultured for a total of 6 days past confluence, ending with 34 h exposure to PKC agonists PMA (20 nM), phorbol 12, 13-dibutyrate (PDB; 100 nM) or (–)-indolactam V (IL-V; 1 μM), ± the PKC inhibitor bisindolylmaleimide I (1 μM). Matrices were isolated and DAF assessed by fluoroimmunoassay. Results are expressed as the mean ± SEM of triplicate samples, and are corrected for non-specific antibody binding.

2.3 Association of DAF with ECM

DAF is attached to cell membranes via a GPI linkage, which on HUVEC can be cleaved by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) [11, 18]. As expected, exposure to PI-PLC released DAF from the HUVEC surface; however, identical treatment failed to release DAF from ECM (Fig. 7). Disruption of the ECM by digestion with collagenase completely removed type IV collagen (data not shown), but did not release DAF (Fig. 8). Similarly, the DAF content of ECM was unaffected by high ionic strength (2 M NaCl), ionic detergents (NP40, sodium deoxycholate), non-ionic detergent (Triton X-100), extremes of pH (triethylamine pH 11.5, glycine pH 2.5) or denaturants (4 M urea, 4 M guanidine chloride, 2 M potassium thiocyanate) (Fig. 8). Further, pretreating ECM with collagenase did not enhance the effect of these treatments (data not shown). DAF was, however, degraded by digestion with L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK)-trypsin and papain. Fig. 8 illustrates the effects of these agents on ECM derived from PMA-treated HUVEC; similar results were obtained using ECM prepared from untreated cells (data not shown).

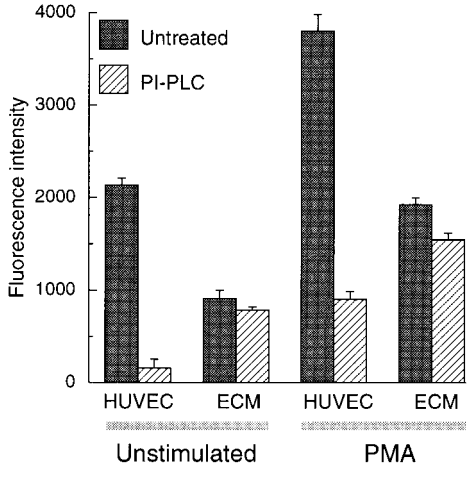

Figure 7. Effect of PI-PLC on HUVEC and ECM DAF. HUVEC were cultured for a total of 6 days post confluence, ending with or without stimulation with 10 nM PMA for 29 h (HUVEC) or 24 h (ECM). HUVEC and isolated matrices were treated with 2 U/ml PI-PLC for 1 h at 37 °C, or left untreated. DAF was assessed by fluoroimmunoassay, and results expressed as the mean ± SEM of triplicate samples, corrected for non-specific antibody binding.

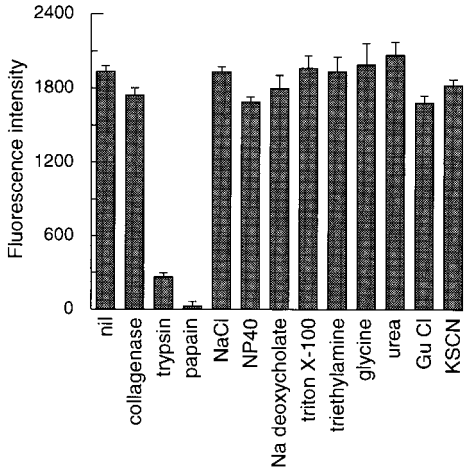

Figure 8. Effect of treatments used to remove DAF from ECM. HUVEC were cultured for 7 days past confluence, ending with 38 h exposure to 20 nM PMA. Matrices were isolated and treated with the indicated reagents: PBS, 50 U/ml collagenase, 50 μg/ml TPCK-trypsin, 25 U/ml papain, all for 90 min at 37 °C; 2 M NaCl, 1 % NP40, 0.5 % sodium deoxycholate, 1 % Triton X-100, 100 mM triethylamine (pH 11.5), 100 mM glycine (pH 2.5), 4 M urea, 4 M guanidine chloride (Gu Cl), 2 M KSCN, all for 3 h at 4 °C. Matrices were washed and DAF assessed by fluoroimmunoassay. Results expressed as the mean ± SEM of triplicate samples, corrected for non-specific antibody binding.

2.4 Functional activity of ECM-associated DAF

To determine whether ECM-associated DAF regulates complement activation, we examined the effect of a DAF-neutralizing antibody, 1H4 [19], on C3 fixation by ECM. In initial experiments, the effectiveness of 1H4 was demonstrated using HUVEC opsonized with complement-fixing rabbit antiserum. Cells were treated with 1H4, incubated with normal human serum as a source of complement, and the effect on total C3 deposition determined. Fig. 9A shows that C3 deposition was enhanced on opsonized HUVEC pretreated with mAb 1H4. 1H4 itself did not activate complement, as unopsonized HUVEC treated with 1H4 bound no more C3 than did untreated cells.

To assess the role of DAF in regulating spontaneous complement activation on ECM, ECM were pretreated with mAb 1H4, then incubated with normal human serum. 1H4 had no effect on C3 deposition on ECM derived from untreated HUVEC (Fig. 9B), nor was there a discernible effect on PMA-treated ECM (data not shown), despite the increase in ECM DAF induced by PMA (Fig. 9D). We then examined the effect of mAb 1H4 on complement fixation by the ECM generated by HeLa cells, an epithelioid carcinoma cell line with a high ECM

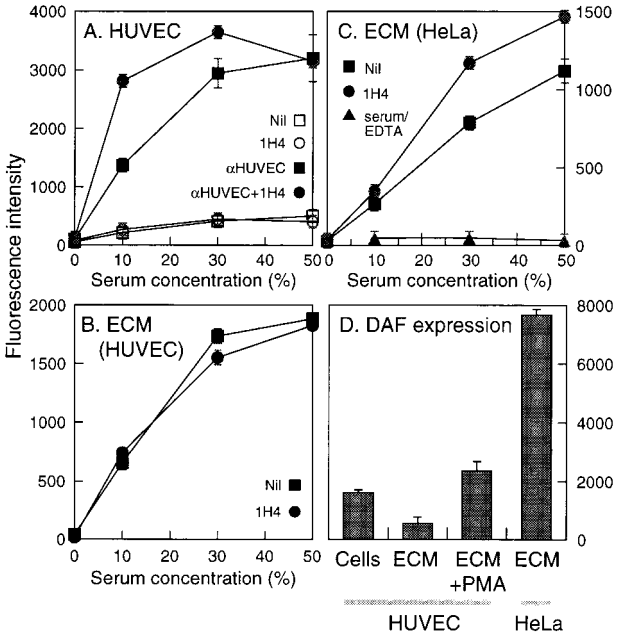

Figure 9. Effect of anti-DAF mAb 1H4 on C3 fixation by HUVEC, HUVEC-derived ECM and HeLa-derived ECM. (A) HUVEC were preincubated for 1 h at 4 °C with diluent alone (Nil), mAb 1H4, anti-HUVEC antiserum, or anti-HUVEC antiserum + mAb 1H4. (B) ECM was prepared from HUVEC cultured for 6 days past confluence, and preincubated with diluent (Nil) or mAb 1H4. (C) ECM was prepared from HeLa cells cultured for 19 days, and treated as in (B). HUVEC and ECM were washed and incubated with 50 %, 30 % or 10 % normal human serum, or equivalent dilutions containing 20 mM EDTA (serum/EDTA), at 37 °C for 15 min (HUVEC) or 45 min (ECM). Bound C3 was assessed by fluoroimmunoassay. (D) Comparison of DAF content of HUVEC, HUVEC-derived ECM (± 20 nM PMA) and HeLa-derived ECM. DAF was assessed by fluoroimmunoassay using mAb IA10. All results are expressed as the mean ± range of duplicate samples (HUVEC) or mean ± SEM of triplicate samples (ECM), and corrected for non-specific antibody binding.

DAF content; Fig. 9D indicates the relative DAF content of ECM prepared from HUVEC and HeLa cells. Pretreatment of HeLa ECM with mAb 1H4, followed by incubation with normal human serum, did enhance C3 deposition compared with the untreated matrix, consistent with HeLa-ECM DAF possessing complement regulatory activity (Fig. 9C). Serum with EDTA added was not associated with C3 deposition, indicating that C3 binding to HeLa ECM was specific and due to complement activation.

To examine whether the lack of functional activity associated with HUVEC-derived ECM DAF could be attributable to a less favorable conformation (*i.e.* inaccessible functional regions) compared with cellular DAF, we com-

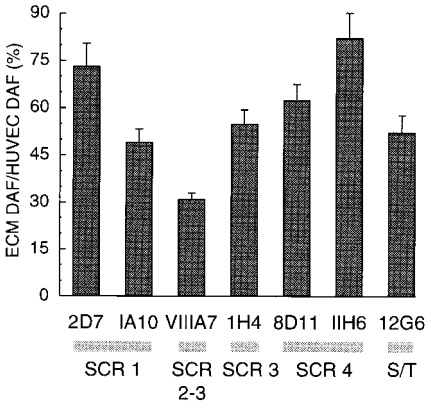

Figure 10. Comparison of anti-DAF mAb binding to HUVEC and HUVEC-derived ECM. HUVEC were cultured for 6–9 days post confluence, and ECM prepared as described in Sect. 4.3. Binding of saturating concentrations of anti-DAF mAb to intact HUVEC monolayers and ECM was assessed by fluoroimmunoassay. mAb used were: 2D7, 1H4, 8D11, 12G6 (culture supernatants, diluted 1/5) and IA10, VIIIA7, IIH6 (4 μg/ml). Results were corrected for non-specific antibody binding and expressed as percentage binding to ECM relative to HUVEC. Data shown represent the mean ± SEM of four separate experiments.

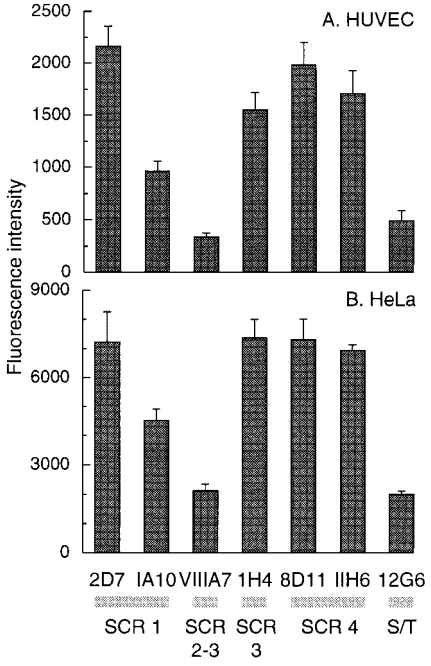

Figure 11. Binding of anti-DAF mAb to ECM derived from (A) HUVEC and (B) HeLa cells. HUVEC-derived ECM were as described in Fig. 10. HeLa cells were cultured for 7–10 days before preparation of ECM. Binding of anti-DAF mAb was assessed as indicated in Fig. 10. Results were corrected for non-specific antibody binding, and expressed as the mean ± SEM of (A) four and (B) three separate experiments.

pared the binding of a panel of anti-DAF antibodies, encompassing a range of epitope specificities, to HUVEC and HUVEC-derived ECM. Results are expressed as the ratio of antibody binding to ECM and cells (Fig. 10). Epitopes from the short consensus repeat (SCR) 1, 3, 4 and the serine/threonine-rich region [19] were detected on both surfaces; however, there was marked variation (reproducible in four experiments) in the antibody binding ratios, suggesting differences in epitope expression/accessibility between the cellular and matrix forms. ECM binding of mAb VIIIA7, recognizing an epitope encompassing SCR regions 2 and 3 [19], was particularly low relative to its binding to cells. We then compared binding of the same antibodies to ECM derived from HUVEC and HeLa cells. The absolute amounts of antibody bound were substantially higher on HeLa ECM, but the binding profiles were very similar (Fig. 11). As HeLa ECM DAF did display complement regulatory activity, this suggested that reduced accessibility of the binding region was unlikely to account for the difference in regulatory activity associated with the two types of matrix.

3 Discussion

DAF is expressed on many cell types, including peripheral blood, endothelial and other epithelial cells, and has been found in soluble form in extracellular fluids [20]. Our data demonstrate that DAF is also a component of the subendothelial extracellular matrix generated by cultured HUVEC, and is the sole representative of known regulators of C3 convertase activity present in ECM. Matrix-associated DAF was not unique to endothelial cells, as DAF was also found in HeLa cell ECM, correlating with an earlier immunohistological observation that DAF was associated with the intercellular region in a variety of tissues [21].

DAF binding within subendothelial ECM was distinct from its binding to the HUVEC surface. Release from HUVEC by PI-PLC indicated attachment via the expected GPI linkage [11]. Resistance of ECM DAF to PI-PLC does not fully exclude GPI attachment, as some modified forms of the GPI anchor are not susceptible to PI-PLC [22]; such modification in ECM-associated DAF would, however, indicate expression of a distinct ECM isoform. A non-GPI mode of attachment seems more likely, as GPI linkages anchor proteins within the lipid bilayer of cell membranes [11], a structure not characteristic of ECM, which consists of a hydrated meshwork of proteins and polysaccharides [23]. DAF was removed from ECM only by proteolysis, and was not extracted by detergents, high ionic strength solutions, or relatively harsh dissociating conditions (extremes of pH, denatur-

ants). These treatments did not release DAF even after disruption of the matrix with collagenase, which completely removed type IV collagen, an important structural component of basement membrane [23, 24]. Thus, DAF is not superficially associated with the ECM, but rather is strongly bound, possibly by a covalent linkage, or embedded within it.

DAF accumulated in the ECM in a linear fashion over time, correlating closely with accumulation of type IV collagen. This progressive increase suggested its constant transfer from HUVEC into the ECM, along with other secreted matrix proteins. ECM DAF was further increased by stimulating the overlying endothelial cells with PMA and WGA, agonists which up-regulate DAF expression on HUVEC [11, 12]. The PMA-induced increase in cell surface DAF was short-lived, consistent with the constant shedding of DAF from HUVEC reported by Tsuji et al. [18]. In contrast, PMA-induced ECM DAF was maintained at peak levels for at least the 3 day duration of the experiment, consistent with its strong association with the matrix. HUVEC activation thus imprints a lasting phenotype on the underlying matrix. Despite the similarity in initial response to PMA by both HUVEC and ECM DAF, regulation of ECM DAF content was not simply secondary to DAF expression on the cell surface. While HUVEC DAF was up-regulated by PMA, WGA and cytokines, the ECM content was increased only by PMA and WGA, indicating that elevation of ECM DAF did not necessarily accompany enhanced cell surface expression.

The mechanism for transfer of DAF from cells to ECM is not yet clear. ECM DAF could represent a cleavage product of the GPI-linked cell surface protein [18, 25] that is then incorporated into the ECM. The presence of both DAF and CD59, but not MCP, in the ECM is consistent with a mechanism involving cleavage of the GPI anchor. However, since ECM DAF did not increase under all conditions that increased cell surface DAF, this could not be occurring without a further level of regulatory specificity. Alternatively, ECM DAF may represent a distinct molecular variant. A number of DAF variants, differing in their C-terminal attachment region, have been identified [26–28]. ECM DAF may represent the hydrophilic form of DAF described by Caras et al. [26], or an as yet unidentified variant, which is released from the cell and interacts with ECM. Notably, acetylcholinesterase exists as multiple isoforms, including a globular GPI-linked form, and an asymmetric form with a collagen-like tail which noncovalently anchors the molecule within the synaptic basal lamina [29].

The wide distribution of DAF, particularly on cells associated with the vasculature, reflects its significance in protecting normal cells and tissues from attack by autologous complement. We recently found that ECM generated by HUVEC was a target for spontaneous complement fixation (E. J. Hindmarsh and R. M. Marks, submitted), raising the possibility that subendothelial matrix exposed by endothelial cell retraction or loss is vulnerable to plasma complement. Since DAF was the only known C3 convertase regulatory molecule associated with ECM, we hypothesized that it would modulate complement fixation on this surface; however, we were unable to demonstrate such an effect on HUVEC-derived ECM. We considered several possible explanations for this lack of function. DAF bound within the ECM could be partially concealed or have different conformational constraints compared with the GPI-linked cellular form, with reduced accessibility to critical functional regions. However, ECM DAF was recognized by mAb specific for SCR 1, 3 and 4, indicating that epitopes from the full-length SCR region, which includes the proposed C3 convertase recognition sites [30, 31], were present. Likewise, binding of mAb 12G6 indicated accessibility of the serine/threonine-rich region, which is also necessary for function [19]. Comparison of mAb binding to HUVEC versus HUVEC ECM indicated that epitope accessibility on the two surfaces was very different; however, the mAb binding profiles for HUVEC-ECM and HeLa-ECM were very similar to one another. As ECM DAF produced by HeLa cells did regulate complement activation, this indicated that the pattern of epitope expression associated with ECM DAF did not preclude functional activity, and that DAF can function in the context of the ECM. The major difference between ECM DAF derived from the two cell types was the DAF concentration; based on binding of mAb 1A10 (Fig. 9D), HeLa ECM contained over ten times more DAF than the basal HUVEC level in this group of experiments. The ability of DAF to dissociate C3bBb and C4b2a necessitates direct interaction with the components of these complexes. Unlike membrane-associated DAF, which is mobile within the plasma membrane, ECM DAF is likely to be fixed in position, and thus able to interact only with convertase complexes formed in its immediate vicinity. Thus, the density of DAF within the matrix could be an important determinant of whether it significantly affects C3 convertase stability.

We have demonstrated that cultured HUVEC incorporate DAF into their subcellular ECM, and that PKC activators augment this process. A recent report identifying DAF in the basement membrane of dermal microvessels indicates that subendothelial ECM found *in vivo* also contain DAF [32]; our data suggest that this is derived at least in part from the overlying endothelium. It remains to be determined whether ECM DAF is also produced by other vascular cell types (*e.g.* smooth muscle cells). Whether the local microenvironment influences the ability of cells to incorporate DAF into ECM is also an important ques-

tion. Finding that PKC activation increased ECM DAF in cultured HUVEC suggests that physiological activators of PKC [33–38] may have a similar effect *in vivo.* Based on the ability of DAF to reduce C3 fixation when present at a high density in HeLa ECM, we speculate that endothelial activation by such stimuli could release sufficient DAF into the underlying matrix to have a significant complement-inhibitory effect. *In vitro,* the composition of subendothelial ECM is greatly influenced by perturbation of overlying endothelial cells, which in turn influences its function, *e.g.* its ability to support coagulation and platelet adhesion [39–42]. Our data suggest that the susceptibility of ECM to complement activation may likewise reflect the recent state of activation of the overlying endothelium.

4 Materials and methods

4.1 Reagents

Endothelial cell growth supplement was obtained from Collaborative Research (Bedford, MA). Heparin (porcine intestinal) and tissue culture-grade gelatin (2 %) were from Sigma Chemical Co. (St. Louis, MO). All other tissue culture reagents, and human recombinant cytokines IFN-γ (7×10^6 U/mg), IL-4 (5×10^6 U/mg) and IL-1β (1.6×10^8 U/mg) were from Gibco BRL (Gaithersburg, MD). Human recombinant TNF-α (6.27×10^7 U/mg) was a gift from Genentech Inc. (South San Francisco, CA). PMA, phorbol 12, 13-dibutyrate and WGA were purchased from Sigma. (–)-Indolactam V and bisindolylmaleimide I were from Calbiochem (San Diego, CA). PI-PLC was the gift of Dr. M. Low (Columbia University, NY). Collagenase and TPCK-trypsin were from Worthington Biochemical Corporation (Freehold, NJ), and papain was from Sigma. All other chemicals used were reagent grade.

Rabbit antiserum specific for HUVEC was prepared by immunizing rabbits with a series of three intramuscular injections of 1×10^7 HUVEC over 6 weeks. Serum was isolated and pooled 3 weeks after the last injection. mAb to human DAF (clones IA10, IIH6, VIIIA7) were provided by Dr. T. Kinoshita (Osaka University, Osaka, Japan), and clones 1H4, 8D11, 11D7 and 12G6 by Dr. W. Rosse (Duke University, NC). mAb to human MCP (clone GB24) was a gift from Dr. J. Atkinson (Washington University, MO). mAb to human CD59 [clone p282 (H19)] was from Pharmingen (San Diego, CA), and goat antisera to factor H and factor I were from Quidel (San Diego, CA). Rabbit anti-human C4bp, goat anti-human C3 and murine mAb to human fibronectin were from Calbiochem. Normal goat serum was from Gibco BRL, and goat anti-human von Willebrand Factor (vWF) from Atlantic Antibodies (Scarborough, ME). mAb to human type IV collagen (clone COL-94), mouse IgG1 and IgG2a, and fluorescein-conjugated sheep anti-rabbit IgG were from Sigma. B-PE-conjugated goat $F(ab')_2$ anti-mouse IgG and donkey $F(ab')_2$ anti-goat IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

4.2 Cell culture

HUVEC were isolated from human umbilical cords [43] 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, 50 μg/ml endothelial cell growth supplement and 50 μg/ml heparin, at 37 °C under 5 % CO_2. Cells were confirmed as endothelial by typical cobblestone morphology at confluence, and binding of antibody to vWF. Experiments were performed using cells transferred to gelatin-coated 96-well flat-bottom plates at a density of 10^4 cells/well; cells were typically confluent 24 h after subculture, and were maintained for up to 10 days before use, with culture medium replaced every third day. In some experiments, cells were treated with PMA, WGA or cytokines in tissue culture medium for up to 72 h before use.

HeLa cells were obtained from the American Type Culture Collection (CCL-2) and cultured in DMEM supplemented with 10 % heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Experiments were performed using cells transferred to 96-well flat-bottom plates at a density of 7.5×10^4 cells/well; cells were maintained in culture for up to 20 days before use.

4.3 Preparation of extracellular matrix

Postconfluent HUVEC were washed with cation-free PBS, and released from the culture surface by incubation with 0.02 % EDTA in cation-free PBS at 37 °C, combined with gentle trituration. Treated wells were confirmed free of cells by phase-contrast microscopy, and shown by fluoroimmunoassay to contain ECM components type IV collagen, fibronectin and vWF. ECM were washed three times with PBS and used immediately. ECM were prepared from HeLa cells in the same manner.

4.4 Detection of complement regulatory proteins and matrix components

Complement regulatory proteins and ECM components were detected by fluoroimmunoassay. HUVEC or cell-free ECM were incubated for 1 h at 4 °C with 50 μl of primary antibody diluted in HBSS containing 5 % newborn calf serum or 0.5 % HSA. Antibodies used were: murine mAb specific for DAF (clone IA10, unless otherwise specified; 500 ng/ml), MCP (ascites, 1/200) and CD59 (10 μg/ml); rabbit antiserum to C4bp (1/200); goat antisera to factor H (1/200), factor I (1/200) and C3 (1/500); murine mAb specific for type IV collagen (10 μg/ml) and fibronectin (ascites, 1/500); goat antiserum to vWF (1/500). Control wells were incubated

with equivalent dilutions of species- and isotype-matched preparations. Wells were washed three times with HBSS/5 % newborn calf serum, incubated for 1 h at 4 °C with 50 μl of B-PE-conjugated goat F(ab')$_2$ anti-mouse IgG (10 μg/ml), donkey F(ab')$_2$ anti-goat IgG (10 μg/ml), or FITC-conjugated goat anti-rabbit IgG (10 μg/ml), then washed three times with HBSS/5 % newborn calf serum and twice with PBS. Cells were lysed with 100 μl 0.1 % SDS in 10 mM Tris-HCl. Fluorescence was quantitated using a Cytofluor 2300 fluorometer (Millipore, Bedford, MA). 530 nm excitation and 595 nm emission filters were used for B-PE detection, and 485 nm excitation and 530 nm emission filters for fluorescein. Results for control wells were subtracted from wells incubated with specific antisera.

4.5 DAF release from HUVEC and ECM

DAF was released from HUVEC by incubation for 1 h at 37 °C with PI-PLC (2 U/ml) in medium 199/1 % HSA. ECM were incubated with 50 μl of: PI-PLC (2 U/ml) for 1 h at 37 °C; collagenase (50 U/ml), TPCK-trypsin (50 μg/ml) or papain (25 U/ml), all for 90 min at 37 °C. Other treatments used were 2 M NaCl, 1 % NP40, 0.5 % sodium deoxycholate, 1 % Triton X-100, 100 mM triethylamine (pH 11.5), 100 mM glycine (pH 2.5), 4 M urea, 4 M guanidine chloride and 2 M potassium thiocyanate, all for 3 h at 4 °C. Wells were washed with PBS, and DAF quantified as described above.

4.6 Complement activation on ECM

Human serum was prepared from blood taken from healthy laboratory personnel under a protocol approved by the University of Michigan Institutional Review Board. Blood was drawn directly into glass tubes, allowed to clot for 30 min at 37 °C, the clot retracted for 30 min at 4 °C, and serum separated by centrifugation at 1800 × g for 10 min at 4 °C. Serum was used immediately or stored in single-use aliquots at −80 °C.

Activation of complement on HUVEC was induced by pretreating cells with rabbit antiserum specific for HUVEC (1/40). Opsonized HUVEC and untreated ECM were then incubated at 37 °C for varying times with 50 μl of human serum diluted with PBS. Serum containing 20 mM EDTA was used as a control. Wells were washed with HBSS/5 % newborn calf serum, and C3 deposition assessed by fluoroimmunoassay, as described above. The effect of anti-DAF mAb 1H4 on C3 deposition was assessed by preincubating HUVEC or ECM with 1H4 culture supernatant (diluted 1/5 with 0.5 % HSA) or with diluent for 1 h at 4 °C. Wells were washed with PBS, then incubated with serum as described above.

Acknowledgments: The authors thank Dr. Wendell Rosse and Sharon Hall for providing mAb 1H4, 8D11 and 11D7, Dr. Taroh Kinoshita for mAb IA10, IIH6 and VIIIA7, Dr. John Atkinson for mAb GB24, and Dr. Martin Low for PI-PLC. Dr. Marks is supported by the Pew Scholars Program, the Arthritis Foundation, and USPHS grants AI33189, AR20557, AR41703, and RR420758.

5 References

1 **Fujita, T., Inoue, T., Ogawa, K., Iida, K. and Tamura, N.,** The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J. Exp. Med.* 1987. **166:** 1221–1228.

2 **Seya, T. and Atkinson, J. P.,** Functional properties of membrane cofactor protein of complement. *Biochem. J.* 1989. **264:** 581–588.

3 **Pangburn, M. K.,** The Alternative Pathway. In **Ross, G. D.** (Ed.), *Immunobiology of the Complement System.* Academic Press, Orlando 1986, pp 45–62.

4 **Law, S. K. A. and Reid, K. B. M.,** *Complement.* IRL Press, Oxford 1988.

5 **Asch, A. S., Kinoshita, T., Jaffe, E. A. and Nussenzweig, V.,** Decay-accelerating factor is present on cultured human umbilical vein endothelial cells. *J. Exp. Med.* 1986. **163:** 221–226.

6 **McNearney, T., Ballard, L., Seya, T. and Atkinson, J. P.,** Membrane cofactor protein of complement is present on human fibroblast, epithelial, and endothelial cells. *J. Clin. Invest.* 1989. **84:** 538–545.

7 **Hamilton, K. K., Zhao, J., Rollins, S., Stewart, B. H. and Sims, P. J.,** Regulatory control of the terminal complement proteins at the surface of human endothelial cells: neutralization of a C5b-9 inhibitor by antibody to CD59. *Blood* 1990. **76:** 2572–2577.

8 **Brooimans, R. A., Hiemstra, P. S., van der Ark, A. A., Sim, R. B., van Es, L. A. and Daha, M. R.,** Biosynthesis of complement factor H by human umbilical vein endothelial cells. Regulation by T cell growth factor and IFN-gamma. *J. Immunol.* 1989. **142:** 2024–2030.

9 **Julen, N., Dauchel, H., Lemercier, C., Sim, R. B., Fontaine, M. and Ripoche, J.,** *In vitro* biosynthesis of complement factor I by human umbilical vein endothelial cells. *Eur. J. Immunol.* 1992. **22:** 213–217.

10 **Davies, A., Simmons, D. L., Hale, G., Harrison, R. A., Tighe, H., Lachmann, P. J. and Waldmann, H.,** CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. *J. Exp. Med.* 1989. **170:** 637–654.

11 **Bryant, R. W., Granzow, C. A., Siegel, M. I., Egan, R. W. and Billah, M. M.,** Phorbol esters increase synthesis of decay-accelerating factor, a phosphatidylinositol-anchored surface protein, in human endothelial cells. *J. Immunol.* 1990. **144:** 593–598.

12 **Bryant, R. A., Granzow, C. A., Siegel, M. I., Egan, R. W. and Billah, M. M.,** Wheat germ agglutinin and other selected lectins increase synthesis of decay-accelerating factor in human endothelial cells. *J. Immunol.* 1991. **147:** 1856–1862.

13 **Moutabarrik, A., Nakanishi, I., Namiki, M., Hara, T., Matsumoto, M., Ishibashi, M., Okuyama, A., Zaid, D. and Seya, T.,** Cytokine-mediated regulation of the surface expression of complement regulatory proteins, CD46 (MCP), CD55 (DAF), and CD59 on human vascular endothelial cells. *Lymphokine Cytokine Res.* 1993. **12:** 167–172.

14 **Ashendel, C. L.,** The phorbol ester receptor: a phospholipid-regulated protein kinase. *Biochem. Biophys. Acta* 1985. **822:** 219–242.

15 **Heikkila, J. and Akerman, K. E. O.,** (–)-Indolactam V activates protein kinase C and induces changes in muscarinic receptor functions in SH-SY5Y human neuroblastoma cells. *Biochem. Biophys. Res. Commun.* 1989. **162:** 1207–1213.

16 **Papadogiannakis, N.,** (–)-Indolactam V-induced mitogenesis in human/neonatal and adult T cells: lower response of neonatal cells and possible regulatory role of monocytes in protein kinase C-mediated pathways. *Cell. Immunol.* 1995. **162:** 288–294.

17 **Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. and Kirilovsky, J.,** The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 1991. **266:** 15771–15781.

18 **Tsuji, S., Kaji, K. and Nagasawa, S.,** Decay-accelerating factor on human umbilical vein endothelial cells. *J. Immunol.* 1994. **152:** 1404–1410.

19 **Coyne, K. E., Hall, S. E., Thompson, E. S., Arce, M. A., Kinoshita, T., Fujita, T., Anstee, D. J., Rosse, W. and Lublin, D. M.,** Mapping of epitopes, glycosylation sites, and complement regulatory domains in human decay accelerating factor. *J. Immunol.* 1992. **149:** 2906–2913.

20 **Lublin, D. M. and Atkinson, J. P.,** Decay-accelerating factor: biochemistry, molecular biology, and function. *Annu. Rev. Immunol.* 1989. **7:** 35–58.

21 **Medof, M. E., Walter, E. I., Rutgers, J. L., Knowles, D. M. and Nussenzweig, V.,** Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. *J. Exp. Med.* 1987. **165:** 848–864.

22 **Wong, Y. W. and Low, M. G.,** Phospholipase resistance of the glycosylphosphatidylinositol membrane anchor on human alkaline phosphatase. *Clin. Chem.* 1992. **38:** 2517–2525.

23 **Yurchenco, P. D. and Schittny, J. C.,** Molecular architecture of basement membranes. *FASEB J.* 1990. **4:** 1577–1590.

24 **Abrahamson, D. R.,** Recent studies on the structure and pathology of basement membranes. *J. Pathol.* 1986. **149:** 257–278.

25 **Metz, C. N., Brunner, G., Choi-Muira, N. H., Nguyen, N., Gabrilove, J., Caras, I. W., Altszuler, N., Rifkin, D. B., Wilson, E. L. and Davitz, M. A.,** Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D. *EMBO J.* 1994. **13:** 1741–1751.

26 **Caras, I. W., Davitz, M. A., Rhee, L., Weddell, G., Martin, D. W. Jr. and Nussenzweig, V.,** Cloning of decay-accelerating factor suggests novel use of splicing to generate two proteins. *Nature* 1987. **325:** 545–549.

27 **Nonaka, M., Miwa, T., Okada, N., Nonaka, M. and Okada, H.,** Multiple isoforms of guinea pig decay-accelerating factor (DAF) generated by alternative splicing. *J. Immunol.* 1995. **155:** 3037–3048.

28 **Spicer, A. P., Seldin, M. F. and Gendler, S. J.,** Molecular cloning and chromosomal localization of the mouse decay-accelerating factor genes. *J. Immunol.* 1995. **155:** 3079–3091.

29 **Massoulie, J., Pezzementi, L., Bon, S., Krejci, E. and Vallette, F. M.,** Molecular and cellular biology of cholinesterases. *Prog. Neurobiol.* 1993. **41:** 31–91.

30 **Kuttner-Kondo, L., Medof, M. E., Brodbeck, W. and Shoman, M.,** Molecular modeling and mechanism of action of human decay-accelerating factor. *Protein Eng.* 1996. **9:** 1143–1149.

31 **Brodbeck, W. G., Lui, D., Sperry, J., Mold, C. and Medof, M. E.,** Localization of classical and alternative pathway regulatory activity within the decay-accelerating factor. *J. Immunol.* 1996. **156:** 2528–2533.

32 **Mason, J. C., Yarwood, H., Harrison, A. A., Davies, K. A. A. and Haskard, D. O.,** Expression and regulation of complement regulatory proteins CD59 and decay-accelerating factor (DAF) on endothelial cells (EC). *Arthritis Rheum.* 1997. **40:** S77.

33 **Lee, T.-S., Saltsman, K. A., Ohashi, H. and King, G. L.,** Activation of protein kinase C by elevation of glucose concentration: Proposal for a mechanism in the development of diabetic vascular complications. *Proc. Natl. Acad. Sci. USA* 1989. **86:** 5141–5145.

34 **Kugiyama, K., Ohgushi, M., Sugiyama, S., Murohara, T., Fukunaga, K., Miyamoto, E. and Yasue, H.,** Lyso-

phosphatidylcholine inhibits surface receptor-mediated intracellular signals by a pathway involving protein kinase C activation. *Circ. Res.* 1992. **71:** 1422–1428.

35 **Siflinger-Birnboim, A., Goligorsky, M. S., Vecchio, P. J. D. and Malik, A. B.,** Activation of protein kinase C pathway contributes to hydrogen peroxide-induced increase in endothelial permeability. *Lab. Invest.* 1992. **67:** 24–30.

36 **Taher, M. M., Garcia, J. G. and Natarajan, V.,** Hydroperoxide-induced diacylglycerol formation and protein kinase C activation in vascular endothelial cells. *Arch. Biochem. Biophys.* 1993. **303:** 260–266.

37 **Sugiyama, S., Kugiyama, K., Ohgushi, M., Fujimoto, K. and Yasue, H.,** Lysophosphatidylcholine in oxidized low-density lipoprotein increases endothelial susceptibility to polymorphonuclear leukocyte-induced endothelial dysfunction in porcine coronary arteries. Role of protein kinase C. *Circ. Res.* 1994. **74:** 565–575.

38 **Xia, P., Inoguchi, T., Kern, T. S., Engerman, R. L., Oates, P. J. and King, G. L.,** Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes* 1994. **43:** 1122–1129.

39 **de Groot, P. G., Verweij, C. L., Nawroth, P. P., de Boer, H. C., Stern, D. M. and Sixma, J. J.,** Interleukin-1 inhibits the synthesis of von Willebrand factor in endothelial cells, which results in a decreased reactivity of their matrix towards platelets. *Arteriosclerosis* 1987. **7:** 605–611.

40 **de Groot, P. G., Reinders, J. H. and Sixma, J. J.,** Perturbation of human endothelial cells by thrombin or PMA changes the reactivity of their extracellular matrix towards platelets. *J. Cell Biol.* 1987. **104:** 697–704.

41 **Reinders, J. H., Vervoorn, R. C., Verweij, C. L., van Mourik, J. A. and de Groot, P. G.,** Perturbation of cultured human vascular endothelial cells by phorbol ester or thrombin alters the cellular von Willebrand factor distribution. *J. Cell Physiol.* 1987. **133:** 79–87.

42 **Tijburg, P. N. M., Ryan, J., Stern, D. M., Wollitsky, B., Rimon, S., Rimon, A., Handley, D., Nawroth, P., Sixma, J. J. and de Groot, P. G.,** Activation of the coagulation mechanism on tumor necrosis factor-stimulated cultured endothelial cells and their extracellular matrix. The role of flow and factor IX/IXa. *J. Biol. Chem.* 1991. **266:** 12067–12074.

43 **Jaffe, E. A.,** Culture and identification of large vessel endothelial cells. In **Jaffe, E. A.** (Ed.), *Biology of Endothelial Cells.* Martinus Nijhoff, The Hague 1984, p. 1.

Correspondence: Rory M. Marks, Department of Internal Medicine, Division of Rheumatology, MSRB1 Room 5520, University of Michigan Medical Center, Ann Arbor, MI 48109-0680, USA
Fax: +1-313-763-2025; e-mail: rmarks@umich.edu