DECAY-ACCELERATING FACTOR IS PRESENT ON CULTURED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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Decay-accelerating factor (DAF) is an intrinsic membrane glycoprotein of 70,000 mol wt that inhibits complement-mediated cell lysis (1–3). Recent evidence suggests that it inhibits the assembly of the C3 and C5 convertases of both the classical and alternative pathways (3–5). DAF was first described in erythrocytes, but more recently has been demonstrated in other blood cells including lymphocytes, monocytes, granulocytes, and platelets (6–8). A deficiency of DAF has been associated with paroxysmal nocturnal hemoglobinuria (PNH), an acquired hemolytic disorder (4, 9, 10). The defect leads to an enhanced susceptibility of the red cells to lysis by complement (11). In this study, we present the first evidence that DAF is present in and synthesized by human umbilical vein endothelial cells (HUVEC).

Materials and Methods

Endothelial Cells. HUVEC were obtained and maintained in culture for from one to three passages in medium 199 containing 20% human serum, 20 μg/ml crude endothelial cell growth factor, and 90 μg/ml heparin (12–15).

Preparation of Cell Extracts. Confluent HUVEC were solubilized by one of two methods. Cell extracts for immunoradiometric assay and for Western blotting were prepared from confluent cell layers. The cells were detached from culture flasks with a rubber policeman and solubilized (10^7 cells/ml) with 0.5% NP-40 in PBS containing 25 μg/ml of the synthetic elastase inhibitor Suc(OMe)-ala-ala-pro-val-MCA (Peninsula Laboratories, Inc., Belmont, CA), 0.5 mM PMSF (Sigma Chemical Co., St. Louis, MO), 2.5 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.), and 50 U/ml Trasylol (Mobay Chemical Corp., Pittsburgh, PA). The mixture was incubated for 20 min on ice and centrifuged at 12,000 *g* for 15 min at 4°C to remove insoluble materials. Cell extracts for immunoisolation of radiolabeled DAF from [^{35}S]methionine-labeled cell cultures were prepared by solubilizing confluent endothelial cell layers with 2% SDS as described previously (16).

mAb Against DAF. Three mAb with specificity for purified human DAF were used for these studies: IA10 (IgG2a), IIH6, and VIIIA7 (IgG1). The production and specificity of these antibodies are described elsewhere (8).

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Immunofluorescence Studies. First passage HUVEC were grown to confluence in microtiter wells in medium 199 containing 20% FCS. The wells were gently washed several times with serum-free medium. The cell monolayers were incubated for 1 h at 37°C with either a mixture of anti-DAF mAb or normal mouse IgG at a final concentration of 10 μg/ml each in PBS, washed three times with PBS, and then incubated with fluoresceinated goat anti-mouse IgG antibody (Cappel Laboratories, Cochranville, PA). The monolayers were again washed three times with PBS and examined with a Leitz fluorescence microscope.

FACS Analysis. $\sim 10^7$ first passage HUVEC obtained by scraping the bottom of culture flasks with a rubber policeman were resuspended in PBS, pH 7.4 containing 1% BSA, 0.1% sodium azide, and 5 mM EDTA. After washing, the cells were divided into aliquots and incubated with a mixture of mAb against DAF (5 μg/ml each) for 30 min on ice. The cells were washed three times with the same PBS and incubated with a fluoresceinated goat $F(ab')_2$ anti-mouse IgG (H and L chain-specific) (Cappel Laboratories) for 30 min on ice. After washing three times with the same PBS, the cells were analyzed by flow cytometry. Controls included normal peripheral blood cells stained with the mixture of anti-DAF mAb, as well as cells incubated with irrelevant mAb of the same subclasses as the primary antibody.

Two-site Immunoradiometric Analysis of Endothelial Cells. This was performed as described previously (8). In brief, anti-DAF monoclonal IA10 was used as a capturing antibody to coat wells of plastic plates. Cell extracts or serial dilutions of pure DAF were added to the wells. After incubation and washing, bound DAF was detected by ^{125}I-labeled anti-DAF monoclonal IIH6, which recognizes a different epitope from IA10. The amounts of DAF in the cell extracts were calculated from a standard curve obtained with purified DAF. This standard curve, in which counts bound of the revealing antibody were plotted as a function of DAF concentration, was linear up to 250 ng DAF/ml. The protein concentration was measured by the method of Lowry et al. (17) using BSA as a reference protein.

Western Blotting of Endothelial Cell Extract. Protein A-Sepharose (100 μl) was incubated with 5 ml of culture supernatant containing mAb IA10 for 1 h at room temperature. IA10-protein A-Sepharose was washed twice with PBS and then incubated with NP-40 extracts of HUVEC (20 μl beads per 1 ml of the extract) for 1 h at 4°C. The bound DAF molecules were eluted from the beads by incubation for 5 min at 80°C with 50 μl of sample buffer consisting of 5% SDS/125 mM Tris HCl, pH 6.8/10% glycerol/0.01% bromphenol blue. The eluates were subjected to SDS-PAGE (16) using 7.5% gels and transferred electrophoretically to nitrocellulose paper (18-20). DAF was detected on the nitrocellulose paper using ^{125}I-labeled IA10 followed by autoradiography.

Biosynthetic Labeling of DAF with [^{35}S]Methionine. [^{35}S]methionine-labeled DAF was isolated from HUVEC radiolabeled with [^{35}S]methionine (100 μCi/ml for 20 h) with a mixture of anti-DAF mAb, as described previously for the isolation of thrombospondin from HUVEC (16). The immunoisolated proteins were analyzed by SDS-PAGE with a 4% stacking gel and 7.5% separation gel (19), followed by autoradiography of the gel after treatment with an autoradiography enhancer (EN^{3}HANCE; New England Nuclear, Boston, MA). Molecular weights were estimated using ^{14}C-labeled marker proteins (New England Nuclear): phosphorylase (94,000), BSA (67,000), and ovalbumin (45,000).

Results and Discussion

The initial studies to determine whether DAF was present on the surface of endothelial cells were performed using indirect immunofluorescence and light microscopy. Live confluent monolayers of HUVEC were incubated with a mixture of all three anti-DAF mAb, washed, and incubated with fluoresceinated goat anti-mouse IgG. Surface fluorescence was visible on all cells in the monolayer. Control studies using normal mouse IgG as a primary antibody followed by the fluorescent second antibody were negative (data not shown).

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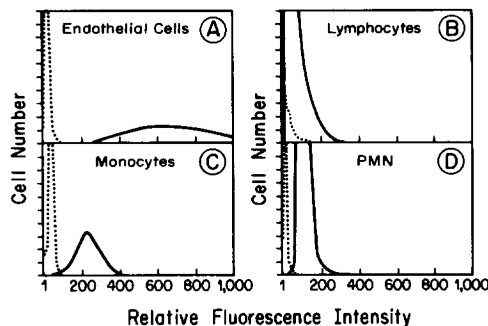

FIGURE 1. Flow cytometry. Solid lines represent fluorescence observed with anti-DAF monoclonals. Dotted lines show nonspecific fluorescence observed with irrelevant mAb of the same subclass.

When analyzed by flow cytometry (FACS), the endothelial cells displayed surface fluorescence intensity that was approximately four times that of peripheral blood monocytes and seven times that of neutrophils (Fig. 1). Control FACS studies on endothelial cells stained with irrelevant monoclonals of the same subclasses were negative.

To quantitate the number of DAF molecules per cell, a known number of endothelial cells were solubilized with NP-40 and the amount of DAF in the extract determined by double antibody RIA. An average of 3.3×10^5 molecules of DAF were present on each endothelial cell, a much higher value than that obtained for blood cells (11).

By Western blotting, endothelial cell DAF comigrated with purified erythrocyte DAF (Fig. 2). Western blots of the same samples performed with an irrelevant monoclonal of the same subclasses were negative (data not shown). To demonstrate that the DAF present in endothelial cells was synthesized by the cells, nearly confluent HUVEC were labeled for 20 h with [^{35}S]methionine and solubilized, and ^{35}S-labeled DAF was immunoisolated using a mixture of anti-DAF mAb. SDS-PAGE and autoradiography of the immunoisolate demonstrated the presence of immunoreactive DAF of mol wt 70,000 synthesized by cultured human endothelial cells (Fig. 3), showing de novo synthesis of DAF. Normal mouse IgG failed to immunoprecipitate any radioactivity.

Like peripheral blood cells, endothelial cells are continuously exposed to the plasma milieu and are susceptible to complement-mediated cell damage. Our own data demonstrates the presence and synthesis of DAF by HUVEC. This is the first evidence for the presence of DAF in cells of extra-marrow origin. DAF may have a role in protecting the endothelial cell from complement-mediated damage. Deficiency or disorders of DAF function may result in endothelial cell injury and may thus play an important role in a variety of pathologic conditions. Exposed subendothelium may lead to platelet deposition with subsequent degranulation and release of thrombogenic materials. In addition, platelet-derived growth factor released under these circumstances may lead to smooth muscle cell proliferation. Alternatively, under some conditions, the accelerated deposition of activated complement products on the endothelial cell surface may result in the recruitment of inflammatory cells and vasculitis.

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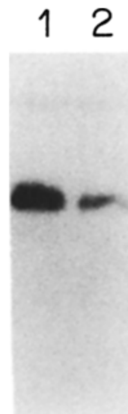

FIGURE 2. Western blotting. Immunoisolates of DAF from endothelial cell extracts or red cell stroma were subjected to SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose paper and probed with ^{125}I-labeled monoclonal IA10. (*1*) DAF from red cell stroma. (*2*) DAF immunoisolated from endothelial cell extracts.

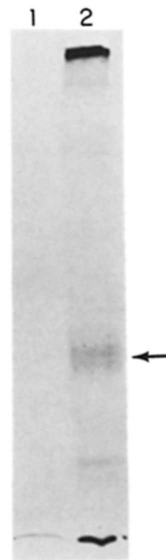

FIGURE 3. Biosynthetic labeling of DAF with [^{35}S]methionine. Nearly confluent HUVEC were labeled for 20 h with [^{35}S]methionine (100 μCi/ml). DAF immunoisolates were subjected to SDS-PAGE and autoradiography was performed. (*1*) A control immunoisolate using normal mouse IgG showing an absence of radioactivity. (*2*) Immunoprecipitated material isolated using anti-DAF mAb. A broad band is shown by the arrow, corresponding to M_r 68,000. Lower molecular weight material may represent proteolyzed DAF.

Summary

Decay-accelerating factor (DAF) has been previously described only in cells of bone marrow origin where it serves as a negative modulator of complement activation. Using mAb against human DAF, we demonstrated the presence of

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DAF in human umbilical vein endothelial cells by immunofluorescence microscopy and flow cytometry. By means of an immunoradiometric assay we detected an average of 3.3×10^5 molecules of DAF on each cell. When immunoisolates were analyzed in Western blots, endothelial cell DAF comigrated with DAF purified from normal erythrocytes. DAF was synthesized by the endothelial cells since ^{35}S-labeled DAF could be immunoisolated from HUVEC cultured in medium containing [^{35}S]methionine. This is the first evidence for the presence of DAF in cells of extra-marrow origin. DAF may protect endothelial cells from complement-mediated injury.

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