Decay-accelerating Factor Is Expressed on Vascular Smooth Muscle Cells in Human Atherosclerotic Lesions

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Abstract

Decay-accelerating factor (DAF) is a constitutively expressed plasma membrane glycoprotein on blood cells and endothelium that inhibits cell surface C3/C5 convertase formation, thus inhibiting complement activation and protecting cells from lysis by the terminal complement components. Using monoclonal anti-DAF antibodies in conjunction with anti-smooth muscle cell (SMC)-specific myosin antibodies, it was found by immunohistochemistry that vascular SMC in advanced human carotid atherosclerotic lesions express DAF antigen. The percentage of DAF-positive SMC ranged from 20 to 60% between different patient samples and SMC DAF expression was limited to SMC in the lesion proper. Normal arterial wall SMC exhibited no DAF-specific immunostaining. Essentially 100% of passaged cultured vascular SMC derived from normal human uterine artery, or from umbilical vein, expressed DAF as assessed by immunocytochemistry. A 68-kD band was observed on SDS-PAGE autoradiograms of DAF-immunoprecipitated radiolabeled cultured SMC extracts. Sensitization of rabbit erythrocytes with DAF-containing SMC extracts conferred protection against complement-mediated hemolysis in normal human serum and the protective effect could be reversed by treatment with anti-DAF antibodies. We conclude that DAF is induced on vascular SMC during atherogenesis and in culture.

Introduction

Several lines of evidence indicate that the complement system participates in atherosclerotic lesion development (1). Terminal C5b-9 complexes, a stable end product of the complement activation sequence, have been observed in lesions by immunohistochemistry (2). They have also been quantitated in human aortic lesion extracts using an enzyme-linked immunoabsorption technique (3). Antibodies recognizing native complement proteins reveal that all components required for activation are present in lesions (4, 5). Furthermore, lesion components released into the bloodstream from ulcerated plaques appear to activate complement intravascularly (6, 7), demonstrating that complement activating structures are present in plaques.

Prime candidates for lesion activating structures are hydroxyl group-bearing lipids and necrotic cells. Crystalline cholesterol, which exists predominantly in the core region of plaques (8, 9), activates complement in vitro generating C3a (des arg), C5a (des arg), and C5b-9 complexes (10). Cell organelles, such as intermediate filaments and mitochondria, are complement activators (11, 12) and would potentially be exposed to complement proteins following cell damage and necrosis (13). There is evidence that the plasma membrane under conditions of injury also becomes a complement-activating surface (14). Necrotic cells in ischemic myocardium have been observed to stain for complement components including the C5b-9 terminal complex (15, 16).

During complement activation, C3b molecules are generated that can potentially bind to cell surfaces to form C3/C5 convertases, thus amplifying the activation process and possibly leading to cell lysis via C5b-9 formation. Cell surface C3 convertase formation is inhibited on cells that bear decay-accelerating factor (DAF)[1] (17–19), membrane cofactor protein (gp 45–70) (20, 21), or complement receptor type 1 (CR1) (22, 23). This includes most blood cells (20–28). DAF is an ~ 70-kD plasma membrane glycoprotein that inhibits convertase formation by displacing C2a and Bb from activator bound C4b and C3b, respectively (18, 19). In the membranous form, it can only inhibit convertases, which result from C3b/C4b binding to the cell surface in which DAF is incorporated (29). The importance of DAF is exemplified in the acquired disorder, paroxysmal nocturnal hemoglobinuria, wherein DAF-deficient blood cells are susceptible to lysis by autologous complement (30).

Regulatory mechanisms for complement activation in the arterial wall have not yet been defined. Vascular smooth muscle cells (SMC) are virtually the only cell-type in normal arterial tunica intima and media, and whether they possess cell surface complement regulatory molecules is unknown. The presence of C5b-9 complement complexes in early as well as advanced atherosclerotic lesions suggests that complement activation is a continuous feature of lesion development. Since activation of complement with C5b-9 formation can damage or lyse cells and thus contribute to lesion necrosis, we investigated whether vascular SMC express DAF. We report that DAF is present on a population of SMC within the lesion but is not detectable on SMC of normal arterial wall. Our findings indicate that DAF expression can be induced on a normally DAF-negative cell type in a pathologic condition involving complement activation and that DAF may function to regulate complement activation in atherogenesis.

Methods

Buffers, reagents, and antibodies. Isotonic veronal-buffered saline, pH 7.4, consisted of 2.5 mM barbital, 73 mM NaCl, 2.5% dextrose, 0.1

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1. *Abbreviations used in this paper:* CR1, complement receptor type 1; CR2, complement receptor type 2; DAF, decay-accelerating factor; E^h, human erythrocytes; E^r, rabbit erythrocytes; SMC, smooth muscle cells; SMM, smooth muscle cell-specific myosin.

mM CaCl$_2$, 0.5 mM MgCl$_2$, and 1% gelatin (DGVB^{2+}). Isoionic veronal buffer for ion chelation contained 2.5 mM barbital, 146 mM NaCl, 1% gelatin, and 10 mM EDTA (GVB-EDTA), or 2 mM MgCl$_2$ with 8 mM EGTA (GVB-MgEGTA). Hanks' balanced salt solution was purchased from Gibco Laboratories (Grand Island, NY) and PBS contained 150 mM NaCl and 10 mM sodium phosphate buffer, pH 7.2. Tris-Triton buffer contained 50 mM Tris-HCl, pH 7.4; 190 mM NaCl; 6 mM EDTA; 100 U/ml Trasylol; and 2.5% Triton X-100.

Mouse monoclonal antibodies to human DAF (clones IA10 and IIH6) and SMC α-actin were generously provided by Dr. Victor Nussenzweig (New York University Medical Center, New York) (26) and Dr. Giulio Gabbiani (University of Geneva, Geneva, Switzerland) (31), respectively, and rabbit polyclonal antibodies to human SMC-specific myosin (heavy chain) (SMM) were kindly provided by Dr. David Larson (Boston University School of Medicine, Boston, MA) (32). Anti-LeuM3, a mouse monoclonal antibody recognizing the human monocyte-macrophage-specific CD14 antigen and mouse monoclonal antibodies to human complement receptors type 1 (CR1) and type 2 (CR2) and HLA-DR were purchased from Becton Dickinson & Co., Inc. (Mountain View, CA). Biotinylated anti-mouse immunoglobulin, fluorescein-conjugated streptavidin and Texas Red-conjugated streptavidin were purchased from Amersham Corp. (Amersham, UK). Rhodamine-conjugated swine anti-rabbit immunoglobulin and anti–von Willebrand factor antigen were obtained from Dakopatts (Copenhagen, Denmark). DiI-acetylated LDL was purchased from Biogenesis (Bournemouth, UK).

Vascular tissue. Atherosclerotic lesions from internal carotid arteries were obtained from patients undergoing endarterectomy surgery and nonatherosclerotic arterial tissue was obtained from the aortic base during coronary bypass surgery and from uterine arteries during hysterectomy. Tissues were transported to the laboratory in ice-cold HBSS, rinsed in PBS, embedded in OCT compound (Miles Laboratories, Inc., Naperville, IL) and snap-frozen in liquid nitrogen-cooled n-hexane. Tissue samples were stored at −70°C until frozen sections were made. Umbilical cords were obtained 24–48 h postpartum.

Plaque cell isolation. Cells were isolated from internal carotid artery atherosclerotic plaques under aseptic conditions as previously described (33). Briefly, PBS-washed arterial tissue was finely minced with scissors and then digested with 900 U/ml collagenase (type IV; Sigma Chemical Co., St. Louis, MO) in PBS containing 1 mg/ml trypsin inhibitor (Sigma), 0.4% BSA (Sigma) and 0.9 mM CaCl$_2$. Cells were separated from large debris by passing the tissue extract through a 150 μm nylon mesh filter and the cells were then plated into chamber slides (Miles Scientific) or cytospin preparations were made. Immunocytochemistry was performed on these cells as described below for frozen sections of tissue.

Immunohistochemistry. Frozen sections of arterial tissue were processed for immunohistochemistry as described (34). Briefly, ethanol-fixed tissue sections were incubated with primary antibodies and then developed with biotinylated secondary antibodies and a streptavidin-conjugated fluorescent compound. For double staining, the same tissue section was then treated with the second primary antibody and developed with a fluorescein or rhodamine-conjugated secondary antibody. Control stains were performed with nonrelevant antibodies of the same immunoglobulin subclass or by omission of the primary antibody. Optimal dilutions of SMM antibodies were determined on frozen sections of normal artery and on cultured SMC. Determination of the optimal anti-DAF antibody concentration was performed by incubating serial dilutions of antibody on cultured monocytes. The working concentration of anti-DAF antibody used (250 ng/ml) was similar to that used in a previously reported immunohistochemical study employing the same antibody (24).

Cell culture. SMC were obtained from human uterine arteries as described (35) or from umbilical veins as follows. Umbilical cords < 48 h old were cannulated, rinsed of blood with 37°C PBS, and filled with 0.1% collagenase (Type IV; Sigma) for 15 min. After several lumenal rinses with RPMI 1640 (Gibco) to remove the endothelial cells, fresh collagenase was introduced for 15 min. The liberated SMC were collected in RPMI, pelleted, and resuspended in RPMI containing antibiotics, 10% heat-inactivated FCS and 10% heat-inactivated normal human serum (35). Seeding densities were 4 × 10^5 cells/ml and medium was changed every 4 d. Cells were grown in standard tissue culture flasks (Nunc, Roskilde, Denmark) and passaged at near confluence at a 1:3 ratio using a 0.25% trypsin-EDTA solution (Gibco). Cell-type homogeneity was checked by staining cultured cells with anti-SMM; anti-von Willebrand factor, anti-LeuM3 and diI-acetylated LDL. From this assessment, cultures after third passage were found to be 99% SMC myosin positive and only a 1% contamination of endothelial cells or macrophages.

Cell labeling and immunoprecipitation. Cell surface labeling of DAF was performed using ^{125}I according to the technique of Kinoshita et al., (26). For these experiments, SMC were first removed from culture flasks with a rubber policeman, pelleted, and washed once in PBS. Approximately 10^9 cells/ml were then labeled in suspension with 1 mCi of ^{125}I using Iodogen coated tubes (Pierce Chemical Co., Rockford, IL). Human erythrocytes (E^h) were simultaneously labeled (1 mCi/2 × 10^8 E^h) to provide a positive control. After radioiodination, the cells were centrifuged through FCS and repeatedly washed until the radioactivity in the wash supernatant was < 10% of the radioactivity initially added. Cell pellets were resuspended in 400 μl of 20 mM Tris-HCl, pH 7.5, containing 1% SDS and 100 U/ml Trasylol (Bayer, Stockholm, Sweden) and treated further as described (24).

For metabolic labeling of DAF, three to six 175-mm^2 culture flasks containing SMC at near confluence were rinsed with methionine-free RPMI and the cells cultured for 26 h in methionine-free RPMI containing 50 μCi/ml [^{35}S]methionine (Amersham) and 10% heat-inactivated FCS. After three washes with complete RPMI, PBS containing 1 mM PMSF and 0.1 mM leupeptin was added and the cells detached using a rubber policeman. The pelleted cells were resuspended in 400 μl of 20 mM Tris-HCl buffer as with ^{125}I-labeled SMC and processed further as described (24).

The immunoprecipitation procedure followed was essentially that of Medof et al., (24). Cell extract supernatants (0.75 to 1.5 ml) were incubated with 300 μl of 10% protein A-Sepharose (Pharmacia, Uppsala, Sweden) in Tris-Triton buffer for 2 h at 23°C. After centrifugation, the supernatants were transferred to new tubes and incubated with anti-DAF antibody (5 μg/ml) or control antibody (5 μg/ml) for 2 h at 20°C. Fresh 10% protein A-Sepharose was then added (100 μl) for 1 h to precipitate immune complexes. After centrifugation at 10,000 g for 5 min, the precipitate was washed twice in Tris-Triton buffer and the final pellet resuspended in 50 μl of SDS-PAGE sample buffer and boiled for 10 min. The immunoprecipitated samples were subjected to 3.5%/7.5% SDS-PAGE. The gels were then fixed in 7% acetic acid for 30 min followed by incubation in Amplify (Amersham) for 20 min. Autoradiographs of dried gels were made with X-omat XAR film (Eastman Kodak Co., Rochester, NY), −70°C.

Assessment of SMC DAF function. The analysis of SMC DAF functional activity utilizing rabbit erythrocytes (E^r) as indicator cells was performed essentially as according to Medof et al., (29). E^r were washed three times in DGVB^{2+} and adjusted to 10^8 E^r/ml. SMC extract, prepared by resuspending PBS washed SMC in 20 mM Tris-HCl, pH 7.5, containing 1% SDS and 100 U/ml Trasylol, was exhaustively dialyzed against DGVB^{2+} at 4°C and diluted in DGVB^{2+} as indicated. Various dilutions of extract (1 ml final volume) or extract buffer were incubated with 1 ml of E^r for 30 min at 20°C. After three washes in DGVB^{2+}, the extract-sensitized E^r were resuspended in 1 ml of GVB-MgEGTA. 100 μl of extract sensitized E^r (10^8 E^r/ml) were then reacted with GVB-MgEGTA dialyzed pooled normal human serum for 15 min at 20°C. 1.8 ml of cold GVB-EDTA was then added to stop the reaction and after centrifugation the degree of hemolysis was assessed using a spectrophotometer set at 412 nm.

Results

DAF in atherosclerotic lesions. Atherosclerotic internal carotid arteries were obtained during endarterectomy surgery from

patients experiencing transitory ischemic attacks. These lesions represent complicated plaques. A grossly thickened tunica intima was present, usually with an irregular lumenal surface and occasional medial involvement. Lesions exhibited a fibrous cap containing variable amounts of SMC, macrophages and T lymphocytes, and a lipid-rich necrotic core (34). SMC were identified with polyclonal anti-SMC myosin antibodies (SMM) (32). The results obtained with these antibodies were confirmed with antibodies against SMC α-actin. SMM antibodies did not stain blood monocytes, 7-d-old cultured monocytes or complement receptor-positive cells in germinal centers of human lymph node. In contrast, anti-DAF antibodies reacted with all three of these cell types.

19 patient samples of carotid atherosclerotic lesions were stained with anti-DAF monoclonal antibody and all were found to exhibit DAF-specific immunofluorescence (Fig. 1). Both cellular and extracellular DAF staining was apparent. The intensity of extracellular DAF staining was generally greatest near to the vascular lumen. Cellular and extracellular DAF was limited to the area of disease involvement, whereas normal tunica media, when present in the lesion samples, was DAF-negative. Extracellular DAF antigen was granular in tissue spaces but diffuse along connective tissue fibers. Cellular DAF antigen was localized to both macrophages and SMC. DAF expressing macrophages were detected using the CD14 monoclonal antibody, anti-LeuM3, in serial sections (data not shown). DAF expression by vascular SMC was observed after double-labeling frozen sections with anti-DAF antibody and anti-SMM. Not all myosin-positive cells reacted with anti-DAF antibody (Fig. 2). The number of DAF/SMC-myosin costained cells varied between patient samples ranging from 20 to 60% of the total SMC myosin-stainable cell population. Normal arterial tissue was negative for DAF-specific immunofluorescence except at the lumenal border (Fig. 3).

Vascular SMC in atherosclerotic lesions are commonly embedded in a connective tissue sheath (36, 37). Therefore, to be certain that DAF staining was due to cell surface antigens and not molecules associated with the SMC basal lamina, carotid artery tissue samples were digested with collagenase in vitro to liberate the plaque cells (33). Isolated cells were then either stained as cytospin preparations or after a 2-d culture period (to allow adherence of live cells to a substratum). A costaining of cells with SMM and anti-DAF antibodies confirmed the results obtained with frozen sections (Fig. 4). A similar range in the number of DAF expressing SMC was obtained with this technique as compared with frozen sections. As shown in Fig. 4, DAF-positive SMC isolated from lesions often contained substantial cytoplasmic lipid deposits and not all SMC expressed DAF antigen.

DAF expression on cultured vascular SMC. Fourth to seventh passage SMC isolated from either umbilical veins or uterine arteries were assessed for DAF expression by immunocytochemistry. Essentially 100% of the cells stained for DAF in a granular pattern evenly distributed over the surface of the cells (Fig. 5).

DAF expression on cultured vascular SMC was confirmed by SDS-PAGE analysis of cell membrane immunoprecipitates. Cultured umbilical vein or uterine artery SMC surface labeled with ^{125}I and immunoprecipitated with monoclonal anti-DAF antibodies exhibited a 68-kD band on autoradiographs of SDS-polyacrylamide gels (Fig. 6). Human erythrocytes used as a positive control exhibited a DAF-specific band at 70 kD. SMC metabolically labeled with [^{35}S]methionine also yielded a DAF-specific 68-kD band (data not shown).

Functional activity of SMC DAF. To determine whether the SMC DAF antigen demonstrated by immunologic techniques exhibited functional DAF activity, a hemolytic bioassay was performed. This technique exploits the fact that rabbit
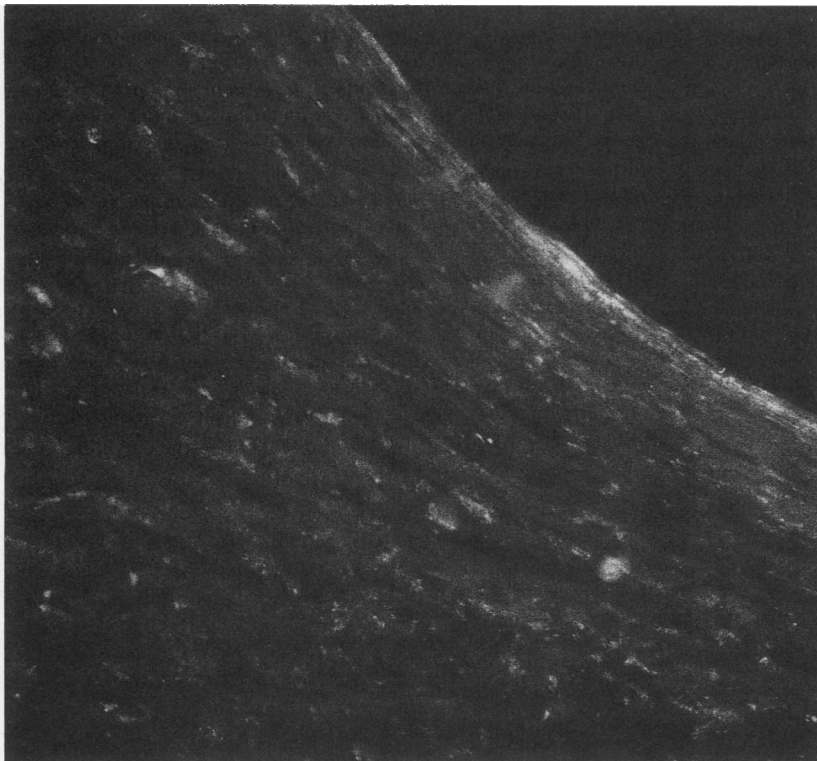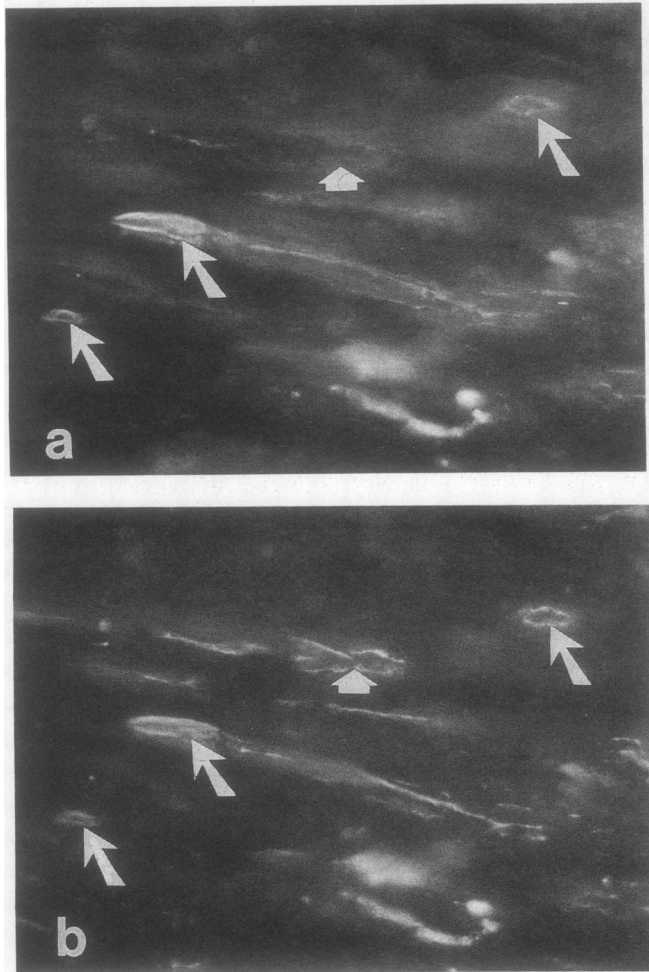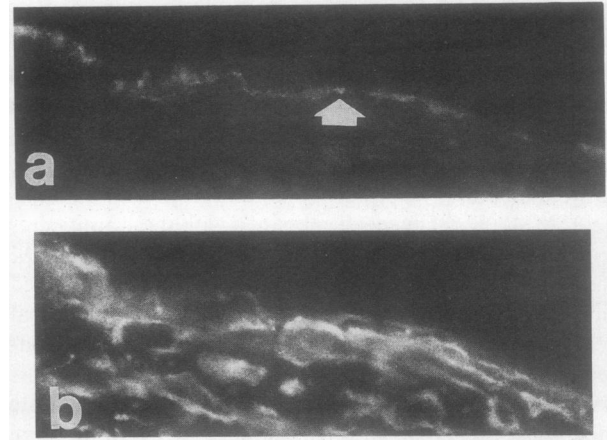

Figure 1. Immunohistochemical localization of DAF in the shoulder region of an advanced human carotid atherosclerotic lesion (×150).

Figure 2. Immunolocalization of DAF to SMC in atherosclerotic lesions by double-staining. Anti-DAF specific immunofluorescence (fluorescein) (a) and anti-SMC myosin immunofluorescence (rhodamine) (b). Arrows indicate examples of DAF-positive SMC and arrowheads indicate DAF-negative, myosin-positive cells (×300).

Figure 3. Immunofluorescent detection of DAF (a) in normal uterine arterial wall occurs only at the lumenal border (arrowhead), yet the medium is rich in SMC (b) as indicated by anti-SMC myosin staining (×300).

Discussion

DAF is a plasma membrane glycoprotein that inhibits autologous complement activation by interfering with C3/C5 convertase formation (18, 19). Obstruction of cell surface convertase formation will aid in protecting cells against terminal
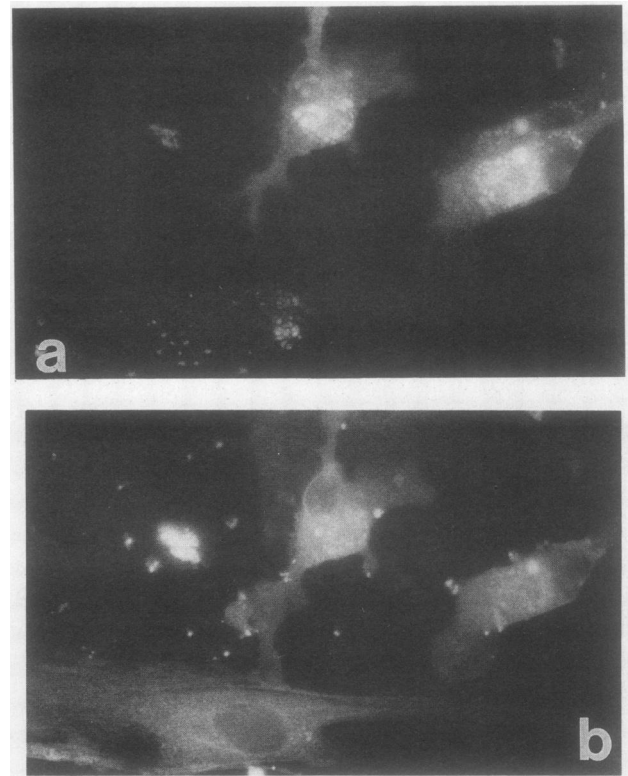
Figure 4. DAF expression by SMC isolated from carotid artery atherosclerotic lesions. Anti-DAF antibodies react with two cells in this field (a) whereas anti-SMC specific myosin stains not only the DAF-positive cells but others as well (b) (×600). Note that autofluorescent lipid droplets are collected cytoplasmically in the perinuclear region of the DAF-positive cells.

erythrocytes (E^r) are subject to alternative pathway complement mediated-lysis by normal human serum (38) and that DAF is a lipophilic molecule which can insert itself into lipid bilayers (29). Soluble human SMC DAF was obtained by extraction with 1% SDS in Tris-HCl buffer. E^r were then treated with increasing dilutions of DAF containing SMC extracts in order to produce E^r with varying amounts of human DAF incorporated into their membranes. DAF sensitized E^r were then subjected to (MgEGTA dialyzed) human serum and the degree of hemolysis assessed. As shown in Fig. 7, E^r were protected from complement-mediated lysis as a function of the amount of extract used to sensitize the cells. When E^r were sensitized with ^{125}I-radiolabeled SMC extracts, 0.2% of the radioactivity was incorporated into the cells supporting that DAF was selectively transferred as previously shown (26). Additionally, the protective effect of extract-sensitized E^r was reversed by treating the cells with anti-DAF monoclonal antibodies (Table I). Sensitization reduced hemolysis by 58%, whereas treatment of sensitized E^r with anti-DAF antibodies restored their susceptibility to complement-mediated hemolysis to within 20% of unsensitized E^r.
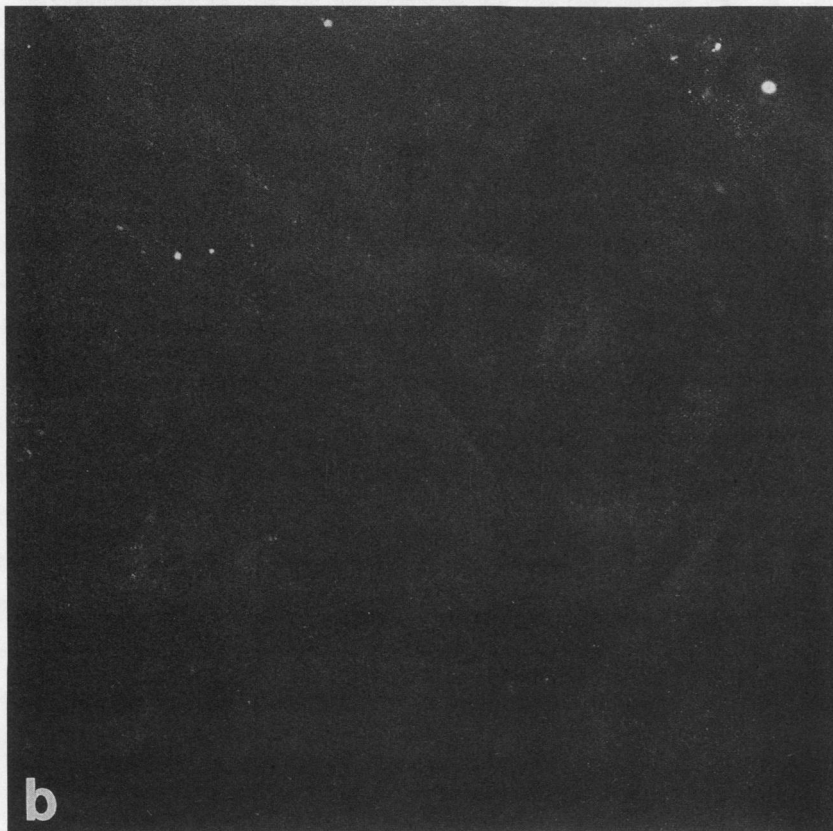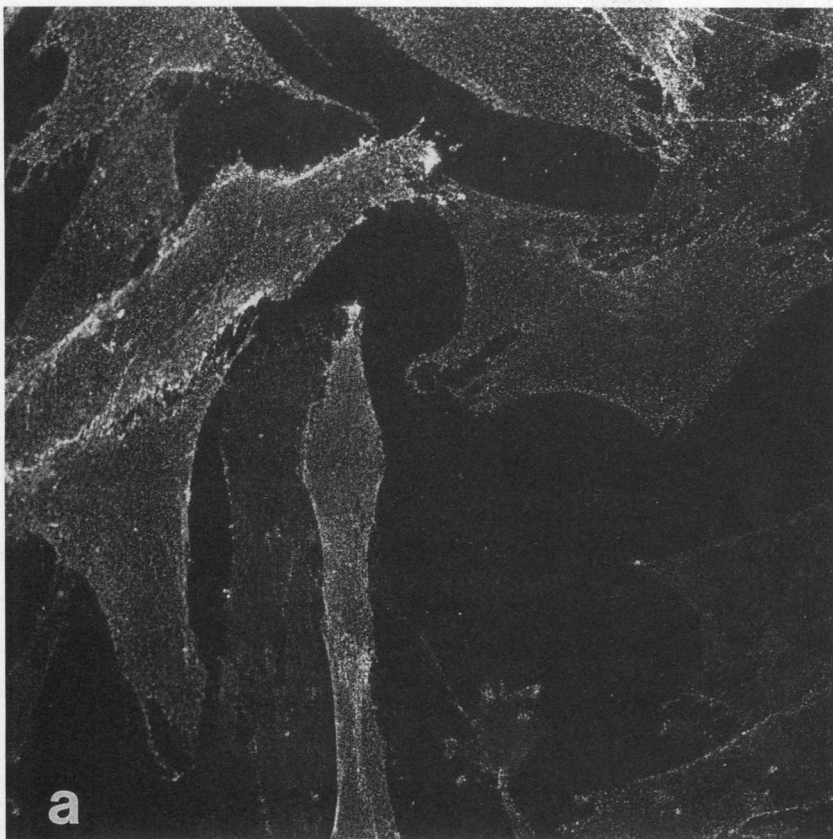
Figure 5. Immunocytochemical demonstration of DAF (*a*) on cultured SMC versus control antibody staining with anti-CR2 (*b*) (×600).
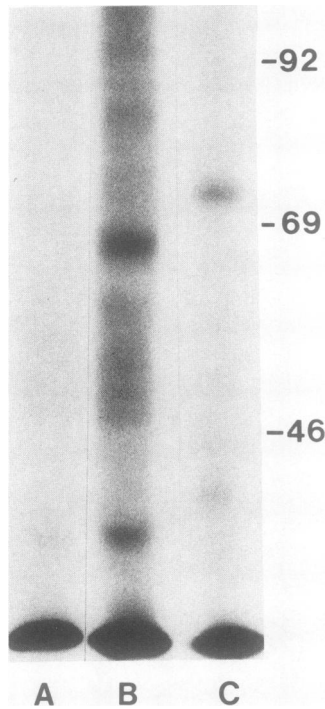
Figure 6. SDS-PAGE autoradiographs of DAF immunoprecipitated SMC extract after surface labeling with ^{125}I. Lane *a*, control precipitation with anti-CR2 monoclonal antibody. Lane *b*, immunoprecipitation with anti-DAF monoclonal antibody. Lane *c*, human erythrocyte DAF, immunoprecipitation of ^{125}I-labeled cell extract. Molecular weight marker positions shown to right.

Table I. Effect of Anti-DAF Monoclonal Antibodies on the Complement-mediated Hemolysis of SMC Extract-sensitized E^r

Experimental condition	% Hemolysis*	% Protection from hemolysis
		mean±SD‡
Unsensitized E^r + control antibodies	30	—
Sensitized E^r + antiDAF	24	18±2.6
Sensitized E^r + control antibodies	18	42±3.8

E^r (10^8/ml) were sensitized with a $1:10^3$ dilution of SMC extract, or dilution buffer (DGVB^{2+}), washed and incubated with anti-DAF monoclonal antibodies (0.25 μg/ml each of clone IA10 and IIH6) or control monoclonal antibodies of the same IgG subclass (0.25 μg/ml each of antihuman CR1 and HLA-DR) for 30 min at 20°C. After pelleting, the cells were resuspended in GVBMgEGTA at 10^8 E^r/ml and 100 μl reacted with 100 μl of varying dilutions of GVBMgEGTA-dialyzed normal human serum as shown in Fig. 7. Plots of percent hemolysis versus microliters of serum added, generated curves that were linear between 30 and 70 μl of serum added.
* % Hemolysis is given at 50 μl of serum added.
$\ddagger$ The average percent hemolysis at 40, 50, and 60 μl of serum added for sensitized E^r was compared to unsensitized E^r.

C5b-9 complex-mediated damage and downregulate the C3 amplification loop. DAF has previously been shown to be constitutively expressed on blood cells, endothelium, and epithelia (24–26, 39). Although the numbers of DAF molecules on the cell surface of neutrophils and T lymphocytes can be modulated by inflammatory mediators and mitogens (40, 41),
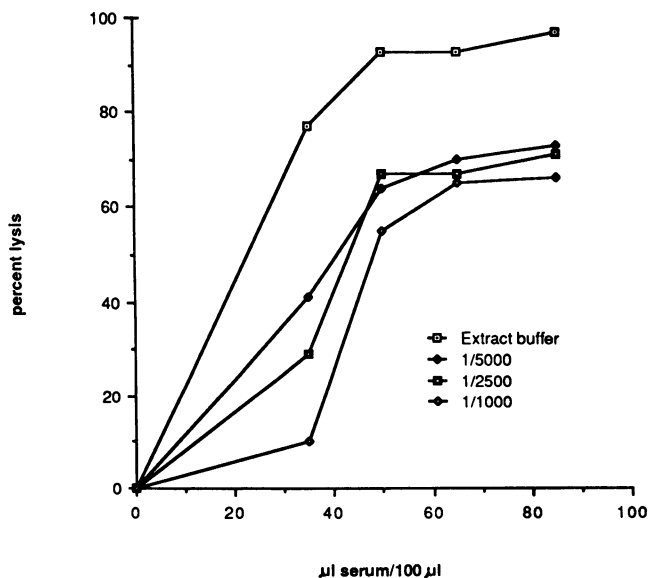

Figure 7. Functional activity of SMC DAF. Rabbit erythrocytes were sensitized with increasing concentrations of SMC extract or extract buffer ($1:10^3$ dilution), washed, and reacted with MgEGTA containing normal human serum for 15 min at 20°C. Nonlysed E^r were pelleted and the degree of hemolysis was determined spectrophotometrically (412 nm) on the supernatants.

induction of DAF expression on a DAF-negative cell type has not been described. Our immunohistochemical analysis demonstrated SMC-associated DAF antigen in atherosclerotic lesions but an absence of DAF on SMC in normal arterial wall. That DAF in lesions was present on the SMC plasma membrane and not on the surrounding basal lamina was shown by immunostaining of cells isolated from lesions by collagenase digestion. Specific induction of DAF expression is suggested by the fact that many, but not all, SMC in a lesion exhibited DAF and cultured SMC derived from DAF-negative arterial media express DAF.

In contrast to lesional SMC, essentially all SMC grown in culture demonstrated an immunoreactivity to anti-DAF antibodies. Isolation of SMC DAF by immunoprecipitation followed by SDS-PAGE analysis revealed a DAF-specific band at 68 kD. Simultaneous immunoprecipitation of E^h DAF produced a band at 70 kD. Previous studies have determined a similar 70-kD M_r for E^h (17, 25, 26). Human leukocytes and platelets, on the other hand, have a relative molecular mass in the range of 75 to 80 kD, neutrophils possess a DAF of ∼ 84 kD (25), and human endothelial cells exhibit a DAF molecule of 70 kD (39). The differences in DAF molecular weights between cell types appears to be due to variable posttranslational glycosylation of a common 46-kD precursor molecule (42).

To assess whether vascular SMC DAF possessed functional activity, E^r sensitized with SMC DAF extracts were reacted with (MgEGTA dialyzed) normal human serum and the degree of hemolysis assessed. E^r are activators of the human alternative complement pathway and are not subject to regulation by factors H and I (38). DAF is a lipophilic molecule with the capacity of inserting into lipid bilayers (29). Hence, passive transfer of DAF into E^r can be used as a means of determining the C3/C5 convertase inhibiting ability of DAF (29). It was

found that E^r treated with SMC extracts were protected from hemolysis by human serum when compared to extract buffer-treated E^r and that protection from hemolysis was related to the concentration of SMC extract used to sensitize the E^r. Furthermore, selective transfer of DAF from SMC extracts to E^r was suggested by the incorporation of radioactivity into the E^r after treatment with radiolabeled SMC extract. Also, the protection against hemolysis conferred to E^r from SMC extract exposure was reversed by reacting the sensitized E^r with anti-DAF monoclonal antibodies. The degree to which antibodies reversed the inhibitory action of DAF was comparable to that previously reported (26). We conclude from these experiments that vascular SMC possess a functional DAF species.

Complement activation in a lesion appears to be a consistent and ongoing feature of atherogenesis based on the fact that C5b-9 terminal complexes are present in early as well as advanced lesions (43). Both C5b-9 complexes and DAF are confined to the arterial region of disease involvement, raising the possibility that complement activation products are involved in the induction of DAF expression. On the other hand, since DAF is expressed on proliferating cells in culture, and SMC proliferation is a feature of atherogenesis, regulation of DAF expression may be associated with events surrounding cell proliferation.

Our results indicate that DAF can be induced on a normally DAF-negative cell type and that DAF represents a mechanism for arterial wall complement regulation, in the present instance, in terms of atherosclerotic vascular disease.

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