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

Paul S. Seifert and Göran K. Hansson
Department of Clinical Chemistry, Gothenburg University, Sahlgren’s Hospital, S-413 45 Gothenburg, Sweden

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 immunosorption 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 M Na2HPO4, 25 mM KH2PO4, pH 7.4. The antibodies were specific for DAF, GM1 ganglioside, and myosin.

Address reprint requests to Dr. Paul S. Seifert, Department of Clinical Chemistry, Gothenburg University, Sahlgren’s Hospital, S-413 45 Gothenburg, Sweden.

Received for publication 24 January 1989.

© The American Society for Clinical Investigation, Inc.
0021-9738/89/08/0597/08 $2.00
Volume 84, August 1989, 597–604

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

Vascular Smooth Muscle Cell Decay-accelerating Factor | 597
mM CaCl₂, 0.5 mM MgCl₂, and 1% gelatin (DGBV-37). Isotonic ver-
non buffer for ion chelation contained 2.5 mM barbitral, 146 mM
NaCl, 1% gelatin, and 10 mM EDTA (GVB-EDTA), or 2 mM MgCl₂
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 Nus-
zenweg (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) (SMI) were kindly provided by Dr.
David Larson (Boston University School of Medicine, Boston, MA)
(32). Anti-LeuM3, a mouse monoclonal antibody recognizing the human monoocyte-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 Dickin-
son & Co., Inc. (Mountain View, CA). Biotinylated anti-mouse immu-
noglobulin, fluorescein-conjugated streptavidin and Texas Red-conju-
gated streptavidin were purchased from Amersham Corp. (Amersham,
UK). Briefly, PBS-washed 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
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) as indicator cells
was performed essentially as described by Medof et al. (29). E were
washed three times in DGBV and adjusted to 10⁶ E/ml. SMC extrac-
ted, 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 DGBV at 4°C and diluted in DGBV as indicated.
Various dilutions of extract (1 ml final volume) or extract buffer were
incubated with 1 ml of E for 30 min at 20°C. After three washes in
DGBV, the extract-sensitized E were resuspended in 1 ml of GVB-
MgEGTA. 100 μl of extract sensitized E (10^6 E/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

P. S. Seifert and G. H. Hansson

598

Downloaded from http://www.jci.org on November 7, 2017. https://doi.org/10.1172/JCI114204
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 luminal 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 125I 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 [35S]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).](http://www.jci.org)
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 erythrocytes (E') 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' were then treated with increasing dilutions of DAF containing SMC extracts in order to produce E' with varying amounts of human DAF incorporated into their membranes. DAF sensitized E' were then subjected to (MgEGTA dialyzed) human serum and the degree of hemolysis assessed. As shown in Fig. 7, E' were protected from complement-mediated lysis as a function of the amount of extract used to sensitize the cells. When E' were sensitized with 125I-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' was reversed by treating the cells with anti-DAF monoclonal antibodies (Table I). Sensitization reduced hemolysis by 58%, whereas treatment of sensitized E' with anti-DAF antibodies restored their susceptibility to complement-mediated hemolysis to within 20% of unsensitized E'.

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).

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.
Figure 5. Immunocytochemical demonstration of DAF (a) on cultured SMC versus control antibody staining with anti-CR2 (b) (×600).
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), 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 kDa. Simultaneous immunoprecipitation of Eβ DAF produced a band at 70 kDa. Previous studies have determined a similar 70-kDa M, for Eβ (17, 25, 26). Human leukocytes and platelets, on the other hand, have a relative molecular mass in the range of 75 to 80 kDa, neutrophils possess a DAF of ~84 kDa (25), and human endothelial cells exhibit a DAF molecule of 70 kDa (39). The differences in DAF molecular weights between cell types appears to be due to variable posttranslational glycosylation of a common 46-kDa precursor molecule (42).

To assess whether vascular SMC DAF possessed functional activity, E' sensitized with SMC DAF extracts were reacted with (MgEGTA dialyzed) normal human serum and the degree of hemolysis assessed. E' 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' can be used as a means of determining the C3/C5 convertase inhibiting ability of DAF (29). It was

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>% Hemolysis</th>
<th>% Protection from hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsensitized E' + control antibodies</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>Sensitized E' + antiDAF</td>
<td>24</td>
<td>18±2.6</td>
</tr>
<tr>
<td>Sensitized E' + control antibodies</td>
<td>18</td>
<td>42±3.8</td>
</tr>
</tbody>
</table>

E' (10⁶/ml) were sensitized with a 1:10⁷ dilution of SMC extract, or dilution buffer (DGVB²⁺), washed and incubated with anti-DAF monoclonal antibodies (0.25 µg/ml each of clone IA10 and HH6) 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 10⁷ E'/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.
† The average percent hemolysis at 40, 50, and 60 µl of serum added for sensitized E' was compared to unsensitized E'.

Figure 6. SDS-PAGE autoradiographs of DAF immunoprecipitated SMC extract after surface labeling with ¹²⁵I. Lane a, control precipitation with anti-CR2 monoclonal antibody. Lane b, immunoprecipitation with anti-DAF monoclonal antibody. Lane c, human erythrocyte DAF, immunoprecipitation of ¹²⁵I-labeled cell extract. Molecular weight marker positions shown to right.

Figure 7. Functional activity of SMC DAF. Rabbit erythrocytes were sensitized with increasing concentrations of SMC extract or extract buffer (1:10⁷ dilution), washed, and reacted with MgEGTA containing normal human serum for 15 min at 20°C. Nonlysed E' were pelleted and the degree of hemolysis was determined spectrophotometrically (412 nm) on the supernatants.

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

Downloaded from http://www.jci.org on November 7, 2017. https://doi.org/10.1172/JCI114204
found that E' treated with SMC extracts were protected from hemolysis by human serum when compared to extract buffer-treated E' and that protection from hemolysis was related to the concentration of SMC extract used to sensitize the E'. Furthermore, selective transfer of DAF from SMC extracts to E' was suggested by the incorporation of radioactivity into the E' after treatment with radiolabeled SMC extract. Also, the protection against hemolysis conferred to E' from SMC extract exposure was reversed by reacting the sensitized E' 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 atherosclerosis 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.

Acknowledgments

We thank Marianne Hädén and Monika Hellstrand for technical assistance and Dr. Jan Holm and Dr. Nils Crona for providing tissue samples. Dr. Seifert was the recipient of a fellowship from the Fogarty International Center of the National Institutes of Health.

This work was supported by grants from the National Institutes of Health (HLO 7356), the Swedish National Association against Heart and Chest Diseases and the Swedish Medical Research Council (6816).

References


