α2-MACROGLOBULIN ON HUMAN VASCULAR ENDOTHELIUM

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Intravascular proteolytic enzymes participate in a variety of linked reactions which are triggered in inflammatory and hemostatic events (1–4). The mechanisms by which the action of these enzymes is limited to specific substrates, thereby sparing other proteins whose destruction would be deleterious to the organism, are not understood. Several different plasma proteins, however, function as proteolytic enzyme inhibitors in in vitro studies, suggesting that these naturally occurring inhibitors limit the digestive potential of intravascular proteases (1, 5, 6).

In this report, the localization of α2-macroglobulin (α2M) to the endothelial surface of human blood vessels and lymphatics has been demonstrated. By immunofluorescent-staining techniques, the α2M appears to form a thin, continuous lining on the luminal surface of endothelial cells. The immunologic specificity of the immunofluorescent techniques used was established by the demonstration that vessel wall staining was abolished when the antiserum was absorbed at equivalence with the purified α2M antigen. Other plasma proteins that were not detected on the endothelial cell lining included IgG, IgA, IgM, the third component of complement (C3), fibrinogen, α1-antitrypsin, and antithrombin III. These observations suggest that the association between α2M and the vascular endothelium is specific.

Prior studies have shown that the α2M modifies the enzymic activity of a number of proteases including enzymes which comprise the blood coagulation, fibrinolytic, and kinin-generating systems (1, 7–10). The demonstration that α2M is localized at the interface between the vessel wall and the circulating blood suggests that this protein may play a vital role in protecting the vascular endothelium by modulating various protease-generating reactions which take place adjacent to the endothelial surface.

Materials and Methods

Purification of Human Plasma α2M. Purification of human plasma α2M was performed as described (11). The final product formed a single precipitin arc with a mobility of an α2-globulin

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Abbreviations used in this paper: α2M, α2-macroglobulin; F-GARG, fluoresceinated GARG; FITC, fluorescein isothiocyanate; GARG, goat antirabbit IgG; PBS, phosphate-buffered saline; RAH α2M, rabbit antibody to human α2M.
after immunoelectrophoresis against rabbit anti-whole human serum. Double-diffusion analysis (12) of the purified α2M demonstrated a reaction of identity with human serum using monovalent rabbit antihuman α2M (RAH α2M). Sodium dodecyl sulfate-acrylamide gel electrophoretic analysis of the purified α2M, after disulfide bond cleavage with dithiothreitol, demonstrated a single protein band with an apparent mol wt of 185,000 as previously described for the subunit chain of α2M (7).

Preparation of α2M Antisera. Purified human plasma α2M was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). The antigen (200 μg) was injected intradermally into the shaved backs of white rabbits. A second injection (200 μg) was repeated 1 mo later. At 6 wk high titer of antibody had developed. The antiserum was passed through an affinity column (13) containing insolubilized human IgG, since some antisera demonstrated trace reactivity with this protein. This procedure removed all antihuman IgG antibody. The antiserum was then absorbed with lyophilized human type A red blood cell stroma to remove anti-A antibodies, a potential contaminant in rabbit serum. The α2M antiserum was concentrated by ultrafiltration to approximately two times the protein concentration of the starting antiserum as determined by absorbancy at 280 nm. The concentrated antiserum produced one precipitin line by double-diffusion analysis against serial dilutions of the original α2M antigen and of whole human serum. No precipitin reaction occurred between the antiserum and plasma depleted of α2M by gel-filtration chromatography (Bio-Gel A5m; Bio-Rad Laboratories, Richmond, Calif.). A reaction of identity was observed by double-diffusion analysis between purified α2M and whole human serum using the concentrated rabbit antiserum toward α2M (Fig. 1).

Rabbit Antisera. Rabbit antisera against the following human proteins were obtained from Behring Diagnostics, American Hoechst Corp., Woodbury, N. Y.: α2M, α1-antitrypsin, antithrombin III, β-lipoprotein, fibrinogen, IgG, IgA, IgM, and C3.

Preparation of Fluoresceinated Goat Antibodies to Rabbit IgG. IgG was isolated from pooled normal rabbit serum by fractionation with (NH4)2SO4 and chromatography on DEAE-cellulose columns as previously described (14). Rabbit IgG (1.0 mg) was mixed with Freund’s complete adjuvant and injected intramuscularly in a male goat. Injection was repeated after 1 mo and 2 wk later the goat was bled. Serum was harvested and tested for the presence of antibodies to rabbit IgG by immunoelectrophoresis in agarose gel. By this technique, one precipitin arc was found between goat antiserum to rabbit IgG and whole rabbit serum. The location of this precipitin arc was typical to that of IgG. This antibody was further purified by affinity chromatography using rabbit IgG coupled to Sepharose 4B (13). Goat antirabbit IgG (GARG) was eluted from the immunoabsorbent with 2 M potassium thiocyanate (15).

GARG was coupled to crystalline fluorescein isothiocyanate (FITC) according to methods described by Goldstein et al., at a ratio of 8 mg FITC/g of protein (16). Unconjugated fluorescein was removed by subsequent gel filtration chromatography using Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.).

Source of Tissue Examined by Immunofluorescence Microscopy. Tissues of adult humans were obtained at autopsy or immediately after surgical removal. Arteries with associated adventitial tissue, lymphatics, and veins were dissected from the mesenteric fat in specimens of colon or from fat and muscle in specimens of breast. In general, endothelial cells were best preserved in the surgical material.

Preparation of Tissues for Immunofluorescence Microscopy. Blocks of unfixed tissue were embedded in 7.5% gelatin and frozen according to the method of Burkholder et al. (17). Frozen sections were cut from these blocks and fixed in acetone for 10 min before treatment with serologic reagents. Tissue sections on glass slides were washed for 15 min in phosphate-buffered saline (PBS), pH 7.4. Excess PBS was wiped from the slides and a drop of RAH α2M, normal rabbit serum, or RAH α2M previously absorbed with purified α2M was applied. Rabbit antisera to a variety of other human plasma proteins were also tested on the tissue sections. The sections were incubated in a moist chamber at 37°C for 30 min. They were then washed in three changes of PBS for 15 min each. Excess PBS was wiped from the slides and a drop of fluoresceinated (F)-GARG was applied to the section and incubated at 37°C for 30 min. The slides were then washed in three changes of PBS for a total of 15 min each and mounted in phosphate-buffered glycerol-Elvanol solution (18).

Absorption Studies. RAH α2M was diluted 1:2 with PBS and 0.2-ml portions pipetted into a series of Eppendorff centrifuge tubes. To these were added serial dilutions (0.2 ml) of purified
human $\alpha_2$M, the highest concentration being 460 $\mu$g/system. The tubes were incubated with frequent shaking at 37°C for 12 h and then at 4°C for 18 h. The precipitate that was harvested by centrifugation was washed three times in PBS and its protein content determined by the Lowry procedure (19). The supernate was examined for residual antibody to $\alpha_2$M by double-diffusion analysis using purified human $\alpha_2$M as the antigen. By these methods the zones of antigen excess, equivalence, and antibody excess were determined. Portions of each supernate were used to treat tissue sections as described above in order to see if absorption of anti-$\alpha_2$M serum at equivalence with purified $\alpha_2$M would inhibit immunofluorescent staining. These preparations were compared with tissue sections treated with unabsorbed antisera to $\alpha_2$M at the same dilution as the supernate and with sections treated with pooled normal rabbit serum. Other tissue sections were treated with rabbit antisera to human IgG, IgA, IgM, C3, $\beta$-lipoprotein, fibrinogen, antithrombin III, and $\alpha_1$-antitrypsin followed by the same F-GARG.

**Immunofluorescence Microscopy and Photomicrography.** Immunofluorescence microscopy and photomicrography were performed with a Zeiss photomicroscope using an apo 40 objective (oil), a dark field condenser (1:2 to 1:4 NA), an HBO 200 W mercury vapor lamp, and a Zeiss Kg-1 absorption filter (Carl Zeiss, Inc., New York). A 1 mm thick UG-2 exciter filter and a Zeiss 41 barrier filter were also used. Photomicrographs were made on 35 mm Ansecochrome D-200 films (G A F Corp., Photo & Repro Div., New York).

**Results**

A thin, continuous band of immunofluorescence was observed on the luminal surface of endothelial cells when sections of human arteries (Figs. 2 and 4), veins (Fig. 3), and lymphatics (Fig. 6) were treated with RAH $\alpha_2$M followed by F-GARG. The immunofluorescence appeared to be localized to the glycocalyx of the endothelial cells as there was little, if any, staining of the cytoplasm. Basement membrane beneath endothelial cells, elastic lamina and myointimal and medial smooth muscle cells of arteries, smooth muscle cells of veins, and connective tissue surrounding blood and lymphatic vessels were not immunofluorescent. Elastic lamina and collagen were autofluorescent.

When sections of human liver were treated with these reagents, hepatic parenchymal cells were immunofluorescent as were the surfaces of endothelial cells lining arteries, veins, and sinusoids in the liver (Fig. 7). The intensity of immunofluorescence of liver parenchymal cells varied between tissue speci-
mens. The section shown represents the most intense immunofluorescence that we observed. When sections of human kidney were treated similarly, the endothelial surface of blood vessels was immunofluorescent but renal tubular epithelium was not stained.

Immunofluorescent staining of endothelial cells (Fig. 5) and of the cytoplasm of hepatic parenchymal cells was completely inhibited by prior absorption at equivalence of anti-α2M serum with purified α2M. Supernates from the zone of antigen excess did not stain the surface of endothelial cells, whereas supernates in the zone of antibody excess did. These absorption experiments are summarized in Fig. 8.

When sections of blood vessels were treated with rabbit antisera to human β-lipoprotein followed by F-GARG, occasionally tiny dots of immunofluorescence, probably corresponding to vesicles, were present in endothelial cells. There was no continuous line of surface staining corresponding to the endothelial surface as observed with anti-α2M.

Neither antiserum against antithrombin III nor antiserum against α1-antitrypsin reacted with endothelial cells or any other components of blood vessel or lymphatic vessel walls. Endothelial cells in normal blood vessels were not stained when sections were treated with rabbit antisera toward IgG, IgA, IgM, C3, or fibrinogen followed by F-GARG. Neither endothelium nor hepatic parenchymal cells were stained when sections were treated with pooled normal rabbit serum followed by F-GARG.

**Discussion**

In these experiments, RAH α2M stained the luminal surface of endothelial cells in sections of arteries, veins, and lymphatics by the indirect immunofluorescent technique. This antiserum also stained hepatic parenchymal cells which have previously been shown to synthesize α2M (20). The specificity of the antiserum for α2M and of the immunohistochemical localization of α2M was confirmed by immunodiffusion and immunoabsorption studies. The results of

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**Fig. 2.** Frozen section of a medium sized branch of external mammary artery treated with RAH α2M followed by F-GARG. The luminal border of the endothelium is immunofluorescent (yellow green). Underlying elastic laminae and collagen are autofluorescent (blue white). × 500.

**Fig. 3.** Frozen section of an intrahepatic vein treated with RAH α2M followed by F-GARG. The luminal border of the endothelium is immunofluorescent. Underlying connective tissue is autofluorescent. × 500.

**Fig. 4.** Frozen section of a medium sized branch of mesenteric artery treated with RAH α2M followed by F-GARG. The luminal border of an endothelial cell is immunofluorescent. Underlying elastic laminae and collagen of the arterial wall are autofluorescent. × 500.

**Fig. 5.** Serial frozen section of artery shown in Fig. 4, treated with RAH α2M previously absorbed at equivalence with α2M, followed by F-GARG. Immunofluorescence of the endothelial border is abolished. Elastic laminae and collagen are autofluorescent. × 500.

**Fig. 6.** Section of lymphatic vessel adjacent to artery shown in Fig. 4, treated with RAH α2M followed by F-GARG. The endothelium is immunofluorescent. Surrounding connective tissue is autofluorescent. × 500.

**Fig. 7.** Frozen section of liver treated with RAH α2M followed by F-GARG. Hepatic parenchymal cells are immunofluorescent. The luminal borders of endothelial cells lining hepatic sinusoids are also immunofluorescent. × 500.
these studies indicate that $\alpha_2$M is present on the luminal surface of endothelial cells of human blood vessels.

Other plasma proteins, some of which have comparable molecular weights and plasma concentrations to $\alpha_2$M were not demonstrable on endothelial cell surfaces. The plasma concentration of $\alpha_2$M and the other major plasma protease inhibitor, $\alpha_1$-antitrypsin, are similar, however, the molar ratio in plasma of $\alpha_1$-antitrypsin to $\alpha_2$M is approximately 12 to 1 (1). Thus, the absence of $\alpha_1$-antitrypsin on the vessel wall further underlines the apparent specificity of the presence of $\alpha_2$M on the endothelial cell surface. It seems likely, therefore, that $\alpha_2$M is associated with the endothelial surface in a specific manner and not as a result of passive absorption.

Recently, Mosher and Wing (21) reported synthesis and secretion of $\alpha_2$M by cultured human WI-38 fibroblasts. In the present study no immunofluorescent staining of fibroblasts in the adventitia of blood vessels from adult humans was observed. These disparate results may be due to differences between proliferating fibroblasts in tissue culture and fibroblasts in normal connective tissue.

Factor VIII antigen is synthesized by endothelial cells (22) and is stained by specific antisera in a granular pattern that appears to be localized to the cytoplasm of these cells (23). In contrast, antibodies to $\alpha_2$M stain the endothelial surface in a linear pattern without staining endothelial cell cytoplasm. These findings suggest that $\alpha_2$M may not be synthesized by endothelial cells. On the other hand, B lymphocytes, which have been reported to possess $\alpha_2$M on their
surface (24), may synthesize this protein as well (25). α₂M has also been identified in human platelet membrane and granule fractions, but not in the cytoplasm (26). The finding, as reported in the present study, of α₂M on endothelial cell surfaces extends the spectrum of proteins which are shared by both platelets and endothelial cells. Thus, both contain, in addition to α₂M, immunochemically similar smooth muscle actomyosin (27), as well as significant amounts of factor VIII antigen (28).

In conclusion, this study establishes that α₂M is located at the interface between the blood and vessel wall. Given the broad specificity of α₂M in inhibiting proteases it is likely that this protein serves as a bulwark in protecting the endothelium from a variety of potentially injurious plasma and cellular enzymes which are released in the circulation during inflammatory and hemostatic events. In addition to this protective function, membrane-associated α₂M may also modulate proteolytic reactions which occur on the surface of lymphocytes, platelets, and endothelial cells.

Summary

α₂-Macroglobulin (α₂M) has been identified on the luminal surface of endothelial cells in sections of normal human arteries, veins, and lymphatics by the indirect immunofluorescent technique. The specificity of the immunofluorescent reaction was confirmed by immunoabsorption studies. Prior absorption of the anti-α₂M antiserum by purified α₂M at equivalence completely inhibited endothelial surface as well as hepatic parenchymal cell staining. Endothelial cells in blood vessels were not stained when sections were treated with rabbit antisera toward α₁-antitrypsin, antithrombin III, IgG, IgA, IgM, C3, or fibrinogen. The location of α₂M at the surface of the vessel wall suggests that this protease inhibitor may protect the vascular endothelium from potentially injurious intravascular proteases.

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