Subunit Structure of Factor VIII Antigen Synthesized by Cultured Human Endothelial Cells

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ABSTRACT Cultured human endothelial cells were labeled with [^{3}H]leucine, and the radioactive Factor VIII antigen present in the postculture medium was isolated by double antibody immunoprecipitation and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after reduction with dithiothreitol. The Factor VIII antigen synthesized by cultured endothelial cells was found to contain the same single polypeptide subunit (mol wt 225,000) present in plasma Factor VIII antigen. These results suggest that in vivo, the endothelial cell is a major site of synthesis of circulating Factor VIII antigen.

INTRODUCTION

Purified Factor VIII is a plasma glycoprotein which, in the purified state, has a mol wt of 1.12×10^6 (1). On sodium dodecyl sulfate (SDS)[1]-polyacrylamide gel electrophoresis after reduction with dithiothreitol, purified human plasma Factor VIII migrates as a single polypeptide subunit with a mol wt of approximately 225,000 (1). Recently, we demonstrated that cultured human endothelial cells synthesize and release Factor VIII antigen ($VIII_{AGN}$) and von Willebrand Factor ($VIII_{VWF}$) into the culture media though the protein does not have Factor VIII procoagulant activity ($VIII_{AHF}$) (2, 3). In this paper we show that the $VIII_{AGN}$ synthesized by cultured human endothelial cells has the same single polypeptide subunit (mol wt 225,000) as the $VIII_{AGN}$ found in human plasma.

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[1] *Abbreviations used in this paper:* PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; $VIII_{AGN}$, Factor VIII antigen; $VIII_{AHF}$, Factor VIII procoagulant activity; $VIII_{VWF}$, von Willebrand factor; Factor VIII, protein(s) in human plasma responsible for $VIII_{AGN}$, $VIII_{AHF}$, and $VIII_{VWF}$.

METHODS

Cell culture techniques and culture media. Human endothelial cells derived from umbilical cord veins were cultured using methods and materials previously described (4). Endothelial cells were cultured in medium 199 containing 20% fetal calf serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) (Grand Island Biological Co., Grand Island, N. Y.).

Radioactively labeled $VIII_{AGN}$ synthesized by endothelial cells was prepared by culturing confluent monolayers of endothelial cells in 35-mm petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) and labeling the cells with L-[4,5-^{3}H]leucine (25 μCi/dish, sp act, 64 Ci/mmol, New England Nuclear, Boston, Mass.) for 24 h. The postculture media from five dishes were centrifuged at 40,000 *g* for 30 min at 4°C to sediment particulate debris. The supernate was made up to 40% saturation with ammonium sulfate, incubated at 20°C for 2 h and at 4°C overnight, and centrifuged at 40,000 *g* for 30 min at 4°C. The precipitate was redissolved in a total of 0.4 ml of phosphate-buffered saline (PBS, 0.145 M NaCl, 0.01 M phosphate, pH 7.4) and dialyzed for 2 days at 4°C against PBS (four 1-liter changes). Parallel experiments were also performed in which the cells were labeled with [^{3}H]leucine in the presence of either soybean trypsin inhibitor (250 μg/ml culture medium, Worthington Biochemical Corp., Freehold, N. J.) or Trasylol® (100 U/ml culture medium, FBA Pharmaceuticals, New York). In these studies the precipitate was dissolved in PBS containing inhibitor (soybean trypsin inhibitor, 250 μg/ml culture medium or Trasylol, 100 U/ml culture medium), and dialysis was performed in the presence of the inhibitor. The dialyzed preparations contained $1.3\text{–}2.7 \times 10^4$ cpm/mg protein (protein, 8.7–22.5 mg/ml).

Partially purified human plasma Factor VIII was prepared from a commercially available Factor VIII concentrate (Hemofil®, Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.). Two units of concentrate (20 ml containing 450 U of procoagulant activity) were chromatographed at room temperature on a 5×92-cm Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) column using upward flow. The column was eluted with PBS at a rate of 56 ml/h (2.8 ml/cm^2 per h), and 24-ml fractions were collected. The column void volume was determined with blue dextran 2,000 (Pharmacia Fine Chemicals). The void volume peak (four tubes containing 100 ml and 3.4 mg protein) was concentrated at 4°C to 3 ml (protein con-

centration, 0.8 mg/ml, recovery, 70% on the basis of protein) using a Pro-Di-Con® concentrating device (model 350, Bio-Molecular Dynamics, Beaverton, Ore.). On SDS-polyacrylamide gel electrophoresis, 80% of the protein in this preparation as estimated by densitometric scan moved as a single band with a mol wt of 225,000.

Antisera. Monospecific rabbit antihuman Factor VIII was obtained from Behring Diagnostics (American Hoechst Corp., Somerville, N. J.). This antibody precipitates $VIII_{AGN}$, inhibits $VIII_{AHF}$ of normal plasma, and inhibits ristocetin-induced platelet aggregation ($VIII_{vWF}$). On immunodiffusion analysis, this antibody reacted with a single line of identity when tested against normal human plasma, hemophilic plasma, and human Factor VIII concentrate (Hemofil). The antibody did not form a precipitin line with plasma from patients with severe von Willebrand's disease. The antibody also formed a line of identity with two other antisera monospecific for Factor VIII (2, 3) when all three were reacted against Factor VIII concentrate (Hemofil). The lack of a precipitin line against von Willebrand's plasma strongly suggests that the anti-Factor VIII sera did not contain contaminant antibody against cold-insoluble globulin since patients with von Willebrand's disease have normal plasma levels of cold-insoluble globulin (5). This negative finding is important because on SDS-polyacrylamide gel electrophoresis after reduction, cold-insoluble globulin also has a mol wt of approximately 230,000. Goat antirabbit γ-globulin was also obtained from Behring Diagnostics. All antisera were absorbed with aluminum hydroxide gel and heat inactivated (56°C, 30 min) before use (2).

Globulin fractions of the antiserum and normal rabbit γ-globulin were prepared as described by Kolb, Haxby, Arroyave, and Müller-Eberhard (6). The antiserum was dialyzed for 24 h at 4°C against 500 vol of sodium phosphate buffer (0.01 M phosphate, pH 7.0). After removal of insoluble protein by centrifugation at 3,000 *g* for 30 min, the dialyzed sample was applied to a 1.5 × 28.5-cm triethylaminoethyl-cellulose column equilibrated with the dialysis buffer, and the protein that did not adhere to the column was collected and concentrated by precipitation at 0°C with ammonium sulfate at 50% saturation. The precipitate was washed three times with 50% saturated ammonium sulfate, dissolved, and dialyzed against 0.15 M NaCl.

Double antibody immunoprecipitation reactions of the purified plasma Factor VIII were carried out by adding 40 μl of a γ-globulin fraction (1 mg/ml) of rabbit anti-Factor VIII to 20 μl of Factor VIII (0.8 mg/ml) and incubating for 1 h at 37°C and overnight at 4°C. 100 μl of a γ-globulin fraction (2 mg/ml) of goat antirabbit γ-globulin was then added, and the mixture was again incubated for 1 h at 37°C and overnight at 4°C. The resulting immunoprecipitate was sedimented at 12,000 *g* for 20 min at 4°C and washed with cold PBS. Double antibody immunoprecipitation of 100 μl of the fractions derived from the [^{3}H]leucine-labeled endothelial cell postculture medium was performed using whole anti-Factor VIII antiserum (50 μl) and whole goat antirabbit γ-globulin (150 μl). The incubation conditions and processing were as described above. Control experiments were performed using both normal rabbit serum and an antihuman albumin antiserum (Behring Diagnostics) in place of the rabbit anti-Factor VIII antiserum.

SDS-polyacrylamide gel electrophoresis (5% gels) was performed as described by Weber and Osborn (7). Samples for analysis were added to an equal volume of a solution containing 10 M urea, 2% SDS, and 14 mM dithiothreitol and boiled for 5 min. Immunoprecipitates for gel electrophoresis were solubilized in 100 μl of 10 M urea 2% SDS, 14 mM dithiothreitol and boiled for 5 min before electrophoresis. The gels were stained with Coomassie Brilliant Blue. Densitometric scans of gels were carried out in a Gilford model 240 spectrophotometer equipped with a gel-scanning attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and a Densicord® recorder equipped with an integrator (Photovolt Corp., New York).

The molecular weight of $VIII_{AGN}$ was determined by SDS-polyacrylamide gel electrophoresis as described (7). Molecular weight markers included phosphorylase a, mol wt 94,000 (Sigma Chemical Co., St. Louis, Mo.); ovalbumin, mol wt 43,000 (Pharmacia Fine Chemicals Inc.); chymotrypsin, mol wt 25,000 (Calbiochem, San Diego, Calif.); and α_2-macroglobulin subunit, mol wt 185,000 (Kindly provided by Dr. Peter Harpel).

After electrophoresis, gels containing radioactive material were sliced into 2-mm thick sections. The slices were placed in liquid scintillation vials, dried, and dissolved in 150 μl of 30% H_2O_2 by heating at 100°C for 2 h. 0.35 ml of distilled H_2O, 0.5 ml of 1.5 M acetic acid, and 10 ml of PCS (Amersham/Searle Corp., Arlington Heights, Ill.) were added sequentially, and the vials were counted in an Intertechnique liquid scintillation counter. (Teledyne Isotopes, Westwood, N. J.).

RESULTS

Immunoprecipitation of plasma $VIII_{AGN}$. Since endothelial cell $VIII_{AGN}$ is present in endothelial cell postculture media at very low concentrations (0.1-1 μg/ml), the antigen was isolated as a precipitate by double antibody immunoprecipitation and characterized by SDS-polyacrylamide gel electrophoresis after reduction with dithiothreitol. To demonstrate the specificity of the reaction, a control experiment using purified plasma Factor VIII was performed. Purified plasma Factor VIII (Fig. 1A) migrated as 1 band with an approximate mol wt by extrapolation of 225,000 as previously reported by others (1). When this protein was subjected to double antibody immunoprecipitation using anti-Factor VIII antibody (Fig. 1B), the isolated precipitate on SDS-gel electrophoresis contained a band of the same molecular weight as the starting Factor VIII. The two lower molecular weight bands represent the antibody H and L chains. This and other observations (see Methods) strongly suggest that the anti-Factor VIII antibody is monospecific for $VIII_{AGN}$.

Immunoprecipitation of endothelial cell $VIII_{AGN}$. $VIII_{AGN}$ synthesized by endothelial cells was identified and characterized by applying the same techniques to fractions of postculture media derived from endothelial cells cultured with [^{3}H]leucine in the presence of soybean trypsin inhibitor (250 μg/ml culture medium). Purified plasma Factor VIII on SDS-gel electrophoresis (Fig. 2A) had a mol wt of 225,000. A fraction obtained by precipitation of [^{3}H]leucine-labeled endothelial cell postculture medium with ammonium sul-

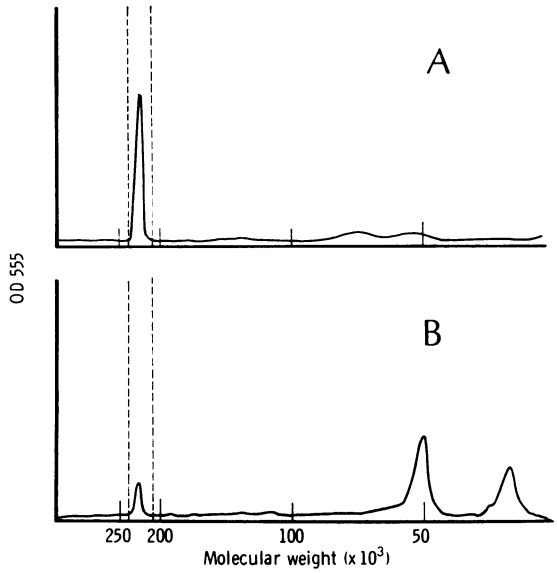

FIGURE 1 Immunoprecipitation of purified plasma Factor VIII. Densitometric scans of SDS-polyacrylamide gels of: (A) purified plasma Factor VIII (3 μg) and (B) solubilized immunoprecipitate obtained from double antibody immunoprecipitation reaction using rabbit anti-Factor VIII performed on the purified plasma Factor VIII shown in (A). 30 μl of the solubilized immunoprecipitate was applied to the gel. Coomassie Blue-stained gels with anode to the right. The vertical dashed lines enclose the plasma $VIII_{AGN}$ subunit region.

fate at 40% saturation was electrophoresed and stained for protein (Fig. 2B). This gel contained at least nine bands of different molecular weights, one of which comigrated with the plasma Factor VIII used as a molecular weight marker. A similar gel which was sliced and counted (not shown) showed four major radioactive bands one of which comigrated with the marker plasma Factor VIII. The radioactively labeled ammonium sulfate precipitated postculture fraction was subjected to double antibody immunoprecipitation. SDS-gel electrophoresis of the immunoprecipitate (Fig. 2C) revealed one major radioactive band which comigrated with the plasma Factor VIII marker. A minor band with an approximate mol wt of 95,000 was also seen. Parallel experiments were performed on [^{3}H]leucine-labeled postculture media obtained from cells grown either in the absence of any protease inhibitor or in the presence of Trasylol (100 U/ml culture medium). Identical labeling patterns were observed. Control immunoprecipitation experiments performed using either normal rabbit serum or antihuman albumin did not precipitate any labeled material.

DISCUSSION

We have previously shown that cultured human endothelial cells synthesize and release $VIII_{AGN}$ and $VIII_{VWF}$ though the cultured cells do not release $VIII_{AHF}$ (2, 3). The studies reported here demonstrate that the $VIII_{AGN}$ synthesized and released by cultured endothelial cells has the same subunit structure as the $VIII_{AGN}$ found in human plasma; both are made up of single polypeptide subunits with a mol wt of 225,000. The observation that antihuman albumin antiserum did not precipitate any labeled protein strongly suggests that Factor VIII is not related to human albumin as has been previously suggested (8). Our findings are consistent with those of Edge and Deykin who were also unable to demonstrate any relationship between Factor VIII and human albumin (9). Immunofluorescence studies have shown that in vivo, only endothelial cells and megakaryocytes and platelets possess $VIII_{AGN}$ (10). Since patients with severe von Willebrand's disease, who have very little or no plasma $VIII_{AGN}$ or $VIII_{VWF}$, do not have $VIII_{AGN}$ in their endothelial cells (11), our observations strongly suggest that endothelial cells are

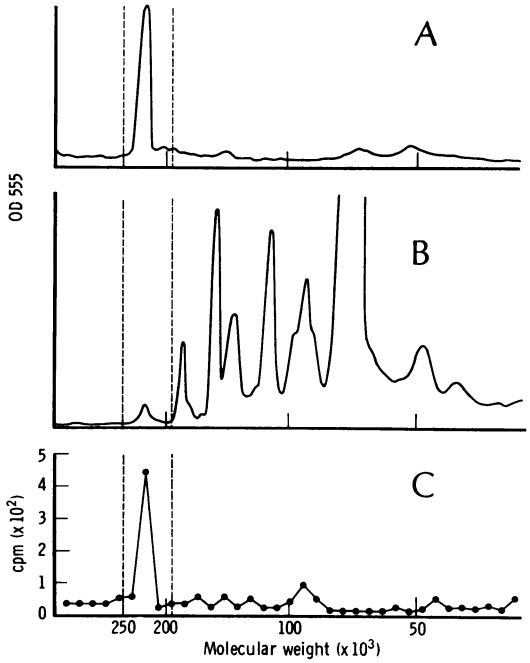

FIGURE 2 Immunoprecipitation of $VIII_{AGN}$ synthesized by cultured endothelial cells. Densitometric scans of SDS-polyacrylamide gels of: (A) purified plasma Factor VIII (4 μg); (B) fraction derived from [^{3}H]leucine-labeled endothelial cell postculture medium by precipitation with 40% saturated ammonium sulfate (16 μg); (C) distribution of radioactivity in solubilized immunoprecipitate obtained from a double antibody immunoprecipitation reaction using rabbit anti-Factor VIII performed on the ammonium sulfate precipitated radioactive postculture medium shown in (B). 60 μl of the solubilized immunoprecipitate was applied to the gel. Coomassie Blue-stained gels with the anode to the right. The vertical dashed lines enclose the plasma $VIII_{AGN}$ subunit region.

a major site of synthesis of plasma $VIII_{AGN}$ and $VIII_{VWF}$.

It is well known that $VIII_{VWF}$ is necessary for normal platelet function and that transfusion of Factor VIII concentrates to patients with von Willebrand's disease corrects their abnormal platelet function (12-15). It is also known that $VIII_{AGN}$ and $VIII_{VWF}$ are present on platelet membranes and in platelet granules (16-18). Recent studies have demonstrated a receptor for $VIII_{AGN}$ in platelet membranes and granules (19). We suggest, therefore, that endothelial cells in vivo synthesize and release molecule(s) possessing both $VIII_{AGN}$ and $VIII_{VWF}$ which are selectively taken up by circulating platelets and in a manner yet to be determined support normal platelet function.

It is not yet clear why $VIII_{AHF}$ has not been detected in media from cultured endothelial cells (2). This might be due to the lack of a necessary nutrient or precursor in the culture media. However, cells grown in culture media containing 20% human serum also do not synthesize $VIII_{AHF}$.[2] The possibility of functional heterogeneity of different endothelial cell populations must be considered. However, to date no such heterogeneity has been demonstrated with respect to $VIII_{AGN}$ (10). Transfusion of hemophilic plasma or hemophilic Factor VIII concentrates to patients with von Willebrand's disease causes an increase in the amount of $VIII_{AHF}$ over and above the amount of $VIII_{AHF}$ transfused (12, 20, 21). These observations suggest that the $VIII_{AGN}$-$VIII_{VWF}$ complex (presumably synthesized and released by endothelial cells) serves as a precursor or carrier of $VIII_{AHF}$ which develops due to interactions at a remote site, perhaps through contact with a second cell system or by modification by one or more plasma enzymes.

Both the carrier and precursor theories suggest the presence of an altered or different molecule possessing $VIII_{AHF}$. Several laboratories have previously identified subunits derived from Factor VIII that have a mol wt of 150,000, and appear to possess only $VIII_{AHF}$ and not $VIII_{AGN}$ or $VIII_{VWF}$ (22-24). Others, however, have recently shown that the $VIII_{AHF}$ of Factor VIII resides in clotting active subunits that have a mol wt of 230,000 and that either cochromatograph with or actually possess $VIII_{AGN}$ and $VIII_{VWF}$ (25-27). In addition, it has been shown by SDS-gel electrophoresis that purified clotting active Factor VIII (1), purified hemophilic Factor VIII (28), and endothelial cell $VIII_{AGN}$ all have only a single polypeptide subunit (mol wt $\sim$ 225,000). These findings suggest that if precursor or endothelial cell $VIII_{AGN-VWF}$ is a zymogen in the classic sense of requiring proteolytic activation, only a minor population of molecules (probably $\leq 1\%$) are altered at any one time and thus are not detectable by the SDS-electrophoresis systems used. Other possible alterations of a minor population of molecules which would not be detectable by SDS-gel electrophoresis but could lead to procoagulant activity are a post-translational change in the amino acid constituents of the polypeptide chain or a change in sugar composition. It is of interest in this regard that we consistently observed a minor band (mol wt, 95,000) in the radioactive immunoprecipitates. This band was present even when the cells were labeled with [^{3}H]leucine and the labeled culture media was processed in the presence of protease inhibitors. Proteolytic digestion of the $VIII_{AGN}$ in the culture medium by plasmin or trypsin (29) is not likely though proteolysis might have occurred intracellularly or might have been due to an enzyme not inhibited by the protease inhibitors used. The relationship of this minor band to $VIII_{AHF}$ is, however, unresolvable in the experimental system employed since the postculture medium did not initially have $VIII_{AHF}$.

[2] Jaffe, E. A. Unpublished observation.

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