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Synthesis of von Willebrand Factor by Cultured Human Endothelial Cells

(macromolecules and hemostasis/Factor VIII/platelets/glass bead retention/antibodies)

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ABSTRACT **Cultured human endothelial cells synthesize and secrete a protein(s) which has Factor VIII antigen but which lacks Factor VIII clot-promoting activity (*J. Clin. Invest.* 52, 2757–2764, 1973). Von Willebrand factor activity has been identified in medium from cultured human endothelial cells. This activity was demonstrated by the ability to correct the defect in platelet adhesiveness of blood obtained from patients with von Willebrand's disease. This activity also supported ristocetin-induced aggregation of washed normal human platelets. The von Willebrand factor activity from cultured endothelial cells has physicochemical and immunologic properties like those of the von Willebrand factor activity and the Factor VIII antigen present in human plasma and the Factor VIII antigen synthesized by human endothelial cells *in vitro*. Rabbit antibody to chromatographic fractions containing endothelial cell von Willebrand factor inhibits the platelet retention of normal blood in glass bead columns.**

Factor VIII (antihemophilic factor, AHF) is a plasma glycoprotein which in the purified state has a molecular weight of 1.12×10^6 (1). The partially purified protein has three distinct properties: (*a*) Factor VIII clot-promoting function ($VIII_{AHF}$), an activity which corrects the coagulation abnormality of plasma from a patient with classic hemophilia (hemophilia A); (*b*) Factor VIII antigen ($VIII_{AGN}$), an antigen identified in precipitin assays by heterologous antibodies and decreased in plasma of patients with von Willebrand's disease but normal in hemophilic plasma; and (*c*) von Willebrand factor ($VIII_{vWF}$), an activity deficient in von Willebrand's disease, which can be identified by an abnormality of the bleeding time or by *in vitro* assays of platelet function (ristocetin-induced aggregation and retention in glass bead columns). The term "Factor VIII" identifies a protein or proteins present in normal human plasma which is (are) responsible for these three activities. This operational nomenclature has recently been suggested by Weiss and coworkers (2).

We recently have demonstrated that cultured human endothelial cells synthesize $VIII_{AGN}$ though the protein does not have $VIII_{AHF}$ activity (3). In this paper we show the release of $VIII_{vWF}$ by cultured endothelial cells.

MATERIALS AND METHODS

Endothelial Cells were obtained from human umbilical cord veins and cultured as previously described (4). Post-culture medium was collected after 3 or 4 days of tissue culture, centrifuged at 25,000 × *g* for 30 min at 4°, and then stored at −20° until needed.

Abbreviations: $VIII_{AHF}$, Factor VIII clot-promoting activity; $VIII_{AGN}$, Factor VIII antigen; $VIII_{vWF}$, von Willebrand factor; PBS, phosphate-buffered saline.

Ristocetin Assay. $VIII_{vWF}$ activity was initially assayed by the ristocetin method (2, 5, 6). Washed normal human platelets were prepared using the Ardlie buffer system as previously described (7). The buffer was modified by omitting calcium and adding 1 mM adenosine to all wash solutions and increasing the total number of washes to 5. The ristocetin assay was performed in a Chrono-Log aggregometer (Chrono-Log Corp.) using 0.2 ml of washed platelets (300,000/μl), 0.2 ml of the material to be tested, and ristocetin (Abbott Laboratories) at a final concentration of 1.5 mg/ml. Platelet aggregation was expressed as the percent change in light transmission 2 min after the addition of ristocetin.

Gel Chromatography. Culture media were concentrated by adding an equal volume of saturated ammonium sulfate, mixing for 1 hr at 20°, incubating overnight at 4°, centrifuging for 30 min at 25,000 × *g* at 4°, and dissolving the precipitate in 1/20 of the original volume using phosphate-buffered saline (PBS, 0.145 M NaCl, 0.01 M phosphate, pH 7.4). This concentrate was then diluted 1:3 with PBS to reduce viscosity and samples of 10–15 ml were chromatographed on a 2.5 × 90-cm Sepharose 4B (Pharmacia Fine Chemicals) column. The separation was carried out in PBS at room temperature at an elution rate of 20 ml/hr and 6.9-ml fractions were collected. The column void volume was determined with Blue Dextran 2000 (Pharmacia). The fractions were assayed for $VIII_{vWF}$ activity by the ristocetin assay and for $VIII_{AGN}$ by radioimmunoassay (8).

Glass Bead Retention. $VIII_{vWF}$ activity was also assayed by the method of Bowie and coworkers (9) as modified by Coller and Zucker (10) with the following additional modifications: (*1*) commercially available glass bead columns holding 1.3 g of beads were used (Becton Dickinson), and (*2*) the first 3 ml of blood pumped through the column were discarded and the next 1 ml of blood was collected in $^1/_{20}$ volume of 0.1 M ethylenediamine tetraacetic acid (EDTA) and the platelets counted. Fractions were tested by adding 6.3 ml of whole blood to syringes containing 0.7 ml of test fraction, mixing the contents of the syringe, incubating at room temperature for 15 min, and then pumping the blood through the glass bead columns at 6.1 ml/min. Void volume fractions derived from pre- and post-culture media were separately pooled and concentrated approximately 50-fold by ultrafiltration, using a PM-10 membrane (Amicon Corp.). Factor VIII concentrates were added to blood as Hemofil (Hyland Laboratories, 28 units/ml).

Antibodies to proteins synthesized by cultured human endothelial cells were prepared in rabbits as previously described (7) by injecting concentrated void volume fractions

derived from Sepharose 4B chromatography. This material contained both $VIII_{VWF}$ activity and $VIII_{AGN}$. The antiserum, referred to as anti-endothelial cell VIII, was absorbed with a 2% $Al(OH)_3$ gel, heat inactivated at 56° for 30 min, and stored at −20° (3). Rabbit antibodies to highly purified human and bovine factor VIII were kindly provided by the laboratory of Dr. E. W. Davie, University of Washington School of Medicine (1, 11). Immunodiffusion studies were performed in diffusion plates containing 1% agarose, pH 7.2 (Cordis Labs.).

Clotting Assays. $VIII_{AHF}$ was assayed by a one-stage method (12) using $VIII_{AHF}$-deficient human plasma (Dade) as substrate. Pooled normal human plasma (Hyland Laboratories) was used as the standard for these assays, and $VIII_{AHF}$ values expressed as units/100 ml. One unit of $VIII_{AHF}$ is defined as that activity corresponding to 1 ml of average normal human plasma (13). The inhibition of $VIII_{AHF}$ in human plasma by the rabbit anti-endothelial VIII was assayed after equal volumes of antibody and plasma were incubated for 2 hr at 37°. This mixture was then assayed for $VIII_{AHF}$ and compared with a control in which normal rabbit serum was substituted for the rabbit anti-endothelial cell VIII.

RESULTS

Endothelial Cell $VIII_{VWF}$. Pre- and post-culture media were separately concentrated 4-fold by ultrafiltration and assayed for $VIII_{VWF}$ activity by the ristocetin assay (Fig. 1). Only the concentrated post-culture medium supported ristocetin-induced aggregation of washed normal human platelets.

The post-culture medium also contained $VIII_{VWF}$ activity when assayed for the ability to correct the defect in platelet retention of blood from patients with von Willebrand's disease. Culture medium was concentrated and chromatographed on Sepharose 4B. The void volume fractions were added to blood from three patients with von Willebrand's disease (Table 1). The concentrates from pre-culture medium had no consistent effect on platelet retention. In contrast, concentrates from post-culture media corrected the platelet retention defect to the same degree as did human Factor VIII concentrate prepared from pooled human plasma. In one patient (no. 3), the improvement in platelet adhesion to the glass beads was only partial following the addition of endothelial cell void volume material. This latter patient is unusual in that his endogenous levels of $VIII_{VWF}$ are significantly higher than his plasma level of $VIII_{AHF}$ (14).

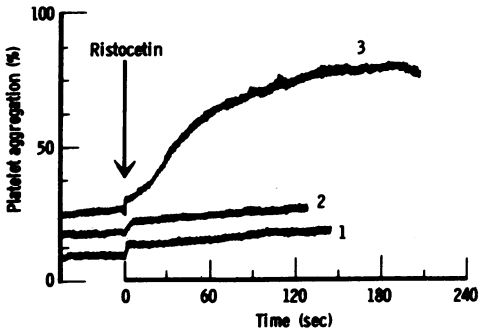

FIG. 1. Ristocetin-induced aggregation of washed normal human platelets. (*1*) 0.2 ml of platelets (300,000/μl) + 0.2 ml of Ardlie buffer; (*2*) 0.2 ml of platelets + 0.2 ml of 4-times concentrated pre-culture medium; (*3*) 0.2 ml of platelets + 0.2 ml of 4-times concentrated post-culture medium. Ristocetin was added at time 0 as 20 μl of a 30 mg/ml solution.

Properties of Endothelial Cell $VIII_{VWF}$. When post-culture medium was concentrated by ammonium sulfate precipitation and chromatographed on Sepharose 4B, $VIII_{VWF}$ and $VIII_{AGN}$ activities appeared in an identical distribution (Fig. 2). The endothelial cell $VIII_{VWF}$ activity in the void volume was resistant to $Al(OH)_3$ adsorption, was stable at −20°, but was inactivated by heating to 56° for 30 min. Prior studies (3) have shown that $VIII_{AGN}$ synthesized by endothelial cells is also resistant to adsorption by $Al(OH)_3$ and stable at −20°, but is stable to heating at 56° for 30 min. These properties are consistent with those described for similar activities present in human plasma.

Inhibition of Plasma $VIII_{VWF}$ Activity by Rabbit Anti-Endothelial Cell VIII. Antisera obtained by immunization with the concentrated void volume fraction significantly inhibited the platelet retention of normal blood in glass bead columns (Table 2). The antibody reduced the platelet retention of normal blood to levels found in severe von Willebrand's disease (Table 1). This effect was related to the concentration of antibody and was abolished by addition of concentrated human Factor VIII (Table 3).

The Inhibitory Effect of Rabbit Anti-Endothelial Cell VIII on Plasma $VIII_{AHF}$ Activity. Rabbit antiserum to endothelial cell VIII had minimal effect on the $VIII_{AHF}$ activity of normal human plasma when the two were incubated for 2 hr at 37°. When equal volumes of undiluted antiserum and human plasma were used, the residual $VIII_{AHF}$ activity was

TABLE 1. *The effect of fractions from endothelial cell culture medium on the platelet retention of von Willebrand's disease blood*

Patient	$VIII_{AHF}$ activity (units/100 ml)	Fractions added*			
		None	Concentrated pre-culture void volume	Concentrated post-culture void volume	Factor VIII concentrate
		% Platelets retained			
1	6	16	19	47	36
2	5	9	16	45	49
3	2	40	45	62	91
Normals	70–150	91 ± 4 (SD)			

* Fractions were added at 1/10 final volume in the syringe; for details see *Methods* section. The $VIII_{AGN}$ contents of these materials were: pre-culture void volume <0.2 units/100 ml; post-culture void volume—32 units/100 ml; Factor VIII concentrate (Hemofil)—2800 units/100 ml.

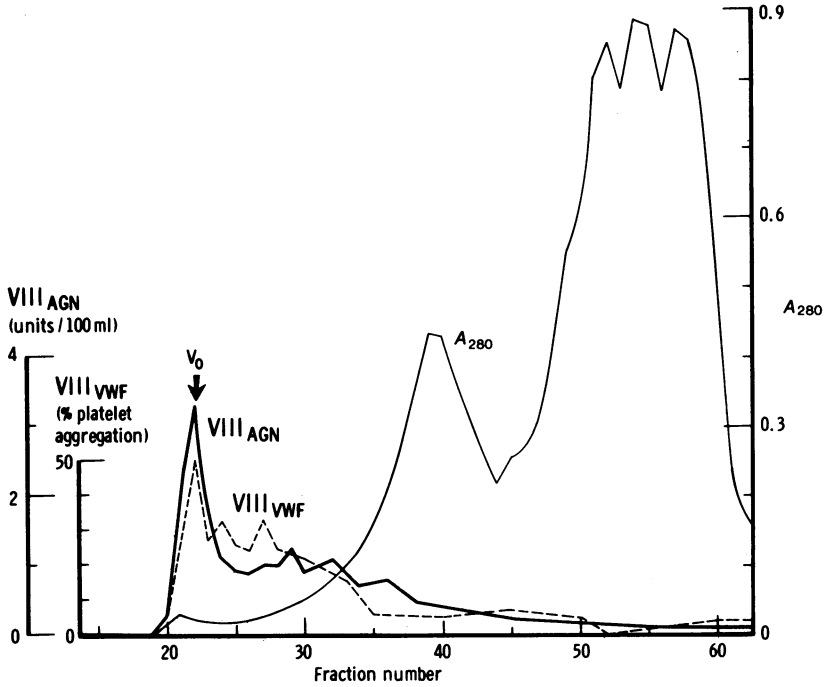

FIG. 2. Elution patterns of $VIII_{AGN}$ (*heavy solid line*) and $VIII_{VWF}$ (*dashed line*). Medium from cultured human endothelial cells was concentrated by ammonium sulfate precipitation and chromatographed on Sepharose 4B. The fractions were assayed for $VIII_{AGN}$ and $VIII_{VWF}$ activity and the absorbance (280 nm) was determined.

44% of that of a control mixture in which normal rabbit serum was substituted for the antibody. This minimal effect contrasts markedly with the ability of the rabbit anti-endothelial cell VIII to inhibit $VIII_{VWF}$ activity in normal blood at a 1:100 dilution.

Immunodiffusion Studies. On immunodiffusion analysis, rabbit anti-endothelial cell VIII and two rabbit antibodies to purified human Factor VIII (1, 8) reacted with a single line of identity when tested with a human Factor VIII preparation (Fig. 3). The fetal-calf serum used in the culture medium did not form any precipitin lines when tested with a potent rabbit antibody to bovine Factor VIII (11) or with rabbit anti-human factor VIII (8).

TABLE 2. *The effect of rabbit anti-endothelial cell VIII on the platelet retention of normal human blood*

	Materials added*				
		Normal rabbit serum	Rabbit anti-endothelial cell VIII		
	None	1:100	1:50	1:100	1:200
Subject	% Platelets retained				
1	94	91	14	22	73
2	94	91	3	0	37
3	96	96	NT†	23	NT
4	85	NT	NT	30	NT

* Materials were added at $^1/_{10}$ final volume in the syringe; for details see *Methods* section. Dilutions refer to final concentration of test material in the syringe.

† NT = not tested.

DISCUSSION

The studies reported here strongly suggest that cultured human endothelial cells synthesize von Willebrand factor. This biologic activity has been identified by two different assays: (*a*) support of ristocetin-induced aggregation of washed normal human platelets and (*b*) correction of the defect in platelet retention in glass bead columns. The physicochemical properties of this material are identical to those of plasma $VIII_{VWF}$ when examined by agarose gel filtration, adsorption to $Al(OH)_3$, and inactivation by heating to 56° for 30 min. The endothelial cell $VIII_{VWF}$ is also similar to endothelial cell $VIII_{AGN}$ and plasma $VIII_{AGN}$ in size (Sepharose gel chromatography) and resistance to $Al(OH)_3$ adsorption. However, endothelial cell $VIII_{AGN}$ and plasma $VIII_{AGN}$ are not heat sensitive.

TABLE 3. *Inhibition of rabbit anti-endothelial cell VIII*

Materials added*	% Platelet retention
None	89
Normal rabbit serum (1:100)	88
Anti-endothelial cell VIII (1:100)	42
Anti-endothelial cell VIII (1:100) plus Factor VIII†	84

* Same as Table 2.

† Hemofil = 2800 units/100 ml. Anti-endothelial cell VIII (70 μl) and Factor VIII (630 μl) were incubated together in the syringe for 30 min at room temperature. Whole normal blood (6.3 ml) was added to the syringe, the contents were mixed and incubated for 15 min at room temperature, and the test was performed.

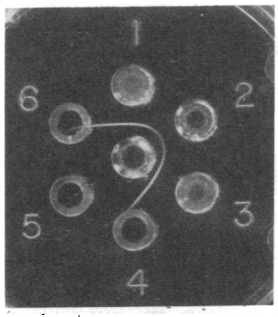

FIG. 3. Immunodiffusion analysis using three rabbit antisera. The center well contained 50 μl of Factor VIII concentrate (Hemofil, 28 units/ml). The numbered peripheral wells contained 50 μl of the following: *well 1*, rabbit anti-Factor VIII (see ref. 8); *well 2*, rabbit anti-endothelial cell VIII; *well 3*, rabbit anti-Factor VIII (see ref. 1). The photograph was taken after 24-hr incubation at room temperature.

The immunologic studies also link endothelial cell $VIII_{vWF}$ activity detected in the culture medium to endothelial cell $VIII_{AGN}$. A rabbit antiserum prepared by immunization with high molecular weight proteins from the culture medium was found to inactivate plasma $VIII_{vWF}$ activity and to precipitate plasma $VIII_{AGN}$. There was, moreover, a reaction of identity when this antiserum was tested with rabbit antibodies prepared by immunization with purified human factor VIII material that has all three properties ($VIII_{AHF}$, $VIII_{AGN}$, and $VIII_{vWF}$). Since cultured human endothelial cells synthesize $VIII_{AGN}$ and release $VIII_{AGN}$ (3) and $VIII_{vWF}$ activity into the culture medium, and since these activities have very similar physicochemical and immunologic properties, we suggest that endothelial cell $VIII_{AGN}$ and $VIII_{vWF}$ activity are synthesized and released by the cell either as one molecule or as separate subunits of a closely related, macromolecular complex (15–17). Clarification of the exact relationship between endothelial cell $VIII_{AGN}$ and $VIII_{vWF}$ must await additional purification of the newly synthesized endothelial cell protein(s).

Although the anti-endothelial cell VIII showed a strong precipitin reaction against plasma $VIII_{AGN}$ and was a strong inhibitor of plasma $VIII_{vWF}$ activity, it was only a very weak inhibitor of plasma $VIII_{AHF}$ activity. This dissociation has been previously observed in antisera from rabbits immunized with void volume concentrates derived from hemophilic plasma containing $VIII_{AGN}$ and $VIII_{vWF}$ activity but no detectable $VIII_{AHF}$ activity (18–20). Variability in $VIII_{AHF}$ inactivating properties of rabbit antisera has also been observed for animals immunized with different preparations of purified normal human Factor VIII (21, 22). The dissociation of reactivities in the rabbit anti-endothelial cell VIII corresponds to the dissociation of these properties in the endothelial cell culture medium. It is not yet clear why $VIII_{AHF}$ has not been detected in media from cultured endothelial cells (3). This may be due either to the lack of a necessary nutrient or precursor in the culture media, or to the functional heterogeneity of endothelial cells. Studies utilizing endothelial cells derived from adult veins and arteries cultured in a variety of different media may help clarify this problem. It is also possible that the endothelial cell $VIII_{AGN}$–$VIII_{vWF}$ complex serves as a precursor or carrier of $VIII_{AHF}$ activity 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.

The demonstration that endothelial cells release and probably synthesize $VIII_{vWF}$, a factor which significantly influences normal platelet function, further substantiates the importance of platelet–endothelial cell interactions in normal hemostasis (23, 24). Recent studies suggest that plasma $VIII_{vWF}$ activity is present on the platelet surface (25) and interacts with a specific platelet membrane receptor which is absent or defective in the Bernard–Soulier syndrome (26). It seems likely that circulating platelets normally adsorb $VIII_{vWF}$ activity that has been synthesized by endothelial cells and which permits platelet interaction with damaged vessel surfaces. The nature of this physiologic interaction remains to be clarified.

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