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Research Article

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Synthesis of Antihemophilic Factor Antigen by Cultured Human Endothelial Cells

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A B S T R A C T Antihemophilic factor (AHF, Factor VIII) antigen has been demonstrated in cultured human endothelial cells by immunofluorescence studies using monospecific rabbit antibody to human AHF. Control studies with cultured human smooth muscle cells and human fibroblasts were negative. By radioimmunoassay it was demonstrated that cultured human endothelial cells contain AHF antigen which is released into the culture medium. Cultured smooth muscle cells and fibroblasts did not have this property. Cultured endothelial cells incorporated radioactive amino acids into high molecular weight, AHF antigen-rich protein fractions prepared from the culture media. 7% of the radioactive amino acid counts incorporated into this material were precipitated by globulin prepared from rabbit anti-AHF whereas normal rabbit globulin precipitated only 1.5% of the counts. Although cultured endothelial cells actively synthesize AHF antigen, AHF procoagulant activity was not detected in the culture medium. Studies seeking a basis for the lack of procoagulant activity have not clarified this deficiency, but they have established that exogenous AHF procoagulant activity is not inactivated by the tissue culture system.

INTRODUCTION

The site of synthesis of antihemophilic factor (AHF, Factor VIII)[1] is not known. Previous studies have suggested the possibility that organs such as liver (1–3), spleen (1, 2, 4, 5), or kidney (6) and cells such as reticuloendothelial cells (7) or lymphocytes (8) might be sites of production; however, the evidence for these claims is indirect and is subject to several interpretations.

Recent immunofluorescence studies performed on a variety of human tissue sections, using a monospecific rabbit antihuman AHF, have demonstrated that endothelial cells contain AHF antigen (9–11). The development in our laboratory of methods for culturing and identifying human endothelial cells (12, 13) has made it possible to examine the role of the endothelial cell in AHF synthesis.

Our results show that AHF antigen, as identified by a radioimmunoassay, is found in cultured endothelial cells and in the culture medium obtained from these cells. Radioactive amino acids were incorporated into the AHF antigen present in the culture medium indicating that endothelial cells synthesize and release a protein (or proteins) that shares antigens present on normal human AHF.

METHODS

Cell culture techniques and culture media. Human endothelial cells and blood vessel medial smooth muscle cells both derived from umbilical cord veins, and fibroblasts derived from adult human abdominal skin were cultured using methods and materials described in the accompanying paper (13). Endothelial cells were cultured in Medium 199 (TC 199) containing 20% fetal calf serum (FCS), penicillin (200 μg/ml), streptomycin (200 μg/ml), and L-glutamine (2 mM) (Grand Island Biological Co., Grand Island, N. Y.) except where otherwise noted. The yield of endothelial cells per umbilical cord was variable; to assure viability, cells were plated out at $1-2 \times 10^5$ cells per 35 mm Petri dish.

Immunofluorescence studies. Specimens of umbilical cord and monolayers of cultured endothelial cells that had been cultured from 3 days to 4 wk were prepared by the methods described in the preceding paper (13). Monospecific rabbit antihuman AHF was prepared as previously described (14). Fluorescein-conjugated goat antirabbit globulin (Hyland Div., Travenol Laboratories, Costa Mesa, Calif.) was used to localize the bound rabbit anti-AHF. Before treatment with antisera, sections were immersed for 10 min in phosphate-buffered saline ([PBS] 0.145 M NaCl, 0.01 M phosphate, pH 7.0). Excess buffered saline was removed from the slide, and a drop of a 1:16 dilution of either rabbit

[1] Abbreviations used in this paper: AHF, antihemophilic factor (Factor VIII); FCS, fetal calf serum; PBS, phosphate-buffered saline; TC 199, Medium 199.

anti-AHF serum or normal rabbit serum sufficient to cover the section of tissue was added. The slide was incubated for 30 min at room temperature, and the sections were washed for 1 h at room temperature with one change of buffered saline. The staining and washing procedure was then repeated using a 1:16 dilution of the fluorescein-conjugated goat antirabbit globulin. The washed sections were covered with one drop of 10% glycerol-90% buffered saline and a cover slip and examined in a Leitz Ortholux photomicroscope (E. Leitz, Inc., Rockleigh, N. J.) using a HBO 200 W mercury vapor lamp, a UG 1 (2 mm) exciter filter, and a Leitz UV absorbing barrier filter (15). Photographs were taken using Tri-X Pan film (Eastman Kodak Co., Rochester, N. Y.).

Radioimmunoassay of AHF antigen. Radioimmunoassay of AHF antigen was performed using purified ^{125}I-labeled rabbit anti-AHF. The preparation and characterization of the antiserum and the details of the assay have been published (14). The assay sensitivity is such that AHF concentrations as low as 0.2% of those in normal human plasma can be detected. For this assay, 1 AHF antigen unit is defined as that amount of antibody-binding material present in 1 ml of a pooled normal human plasma standard, the preparation of which has been described (14). AHF antigen determinations were carried out using undiluted samples of media stored at −70°C before assay. Rabbit anti-AHF has been shown by others to have little reactivity against bovine AHF (16, 17), and there was no detectable AHF antigen in the preculture medium that contained 20% FCS (Table I). Cultured cells were detached from Petri dishes by incubation with 0.01% EDTA-0.1% collagenase (type CLS, Worthington Biochemical Corp., Freehold, N. J.) in cord buffer (0.14 M NaCl, 0.004 M KCl, 0.001 M phosphate, pH 7.4, 0.011 M glucose) for 15 min at 37°C. The cells were washed twice with diluent buffer containing 0.02% EDTA and counted in a hemocytometer. The diluent

TABLE I
AHF Antigen Content of Cultured Cells and Media

Cell type	Material tested	AHF antigen
		U/100 ml
Endothelial cells	Preculture medium (3)*	<0.2
	Postculture medium (3)	7.4±1.4‡
	Disrupted cells§	
	Supernate (3)	16.2±1.4
	Pellet (3)	13.6±1.9
Smooth muscle cells	Preculture medium (2)	<0.3
	Postculture medium (3)	<0.3
	Disrupted cells§	
	Supernate (1)	<1.0
	Pellet (1)	<1.0
Fibroblasts	Preculture medium (2)	<0.3
	Postculture medium (3)	<0.3

* Number of separate experiments.
‡ Mean ±SEM.
§ Cell suspension (15 × 10⁶ cells/ml) was frozen and thawed four times before centrifugation. The pellet was subsequently suspended in a volume of buffered saline equal to that of the supernatant fluid.

buffer contained 0.145 M NaCl and 0.01 M phosphate, pH 7.4 (PBS), 0.011 M glucose, and 0.5% bovine serum albumin (Miles Laboratories Inc., Kankakee, Ill.). The cells were resuspended at 15 × 10⁶ cells/ml in the 0.02% EDTA-diluent buffer, frozen and thawed four times, and centrifuged at 40,000 g for 30 min at 4°C. After the supernate was removed, the pellet was resuspended in a volume of 0.02% EDTA-diluent buffer equal to that of the supernate, and both samples were stored at −70°C until assayed for AHF antigen.

Protein synthesis. [³H]Leucine incorporation into trichloroacetic acid (TCA)-precipitable material as expressed in disintegrations per minute per milligram protein per hour varied substantially among cultures derived from different umbilical veins. The intraculture variation was, however, much smaller (SD-8% of the mean). Therefore, to make valid comparisons, each experiment was performed on cells cultured from a single umbilical cord. Sufficient replicate experiments were included to assure statistical validity.

Petri dishes containing 10⁶ cultured endothelial cells in the stationary phase were incubated with 5 μCi/ml of L-[4,5-³H]leucine, sp act-31.9 Ci/mmol, (New England Nuclear, Boston, Mass.). After the appropriate labeling period, the medium containing [³H]leucine was removed, and the cell monolayer was washed twice with 2 ml of ice-cold PBS containing 10⁻⁴ M unlabeled leucine. 2 ml ice-cold 10% TCA containing 10⁻⁴ M leucine was added, and the dishes were incubated for 2 h at 4°C. The dishes were washed once with cold 10% TCA containing 10⁻⁴ M leucine, twice with ethanol-ether (3:1, vol/vol), and air dried. The precipitate covering the bottom of the dish was solubilized in 1 ml 1 N NaOH at 37°C overnight. Portions of the NaOH-solubilized material were dissolved in 10 ml PCS liquid scintillation cocktail (Amersham/Searle Corp., Arlington Heights, Ill.) and counted in an Intertechnique SL30 liquid scintillation spectrometer (Intertechnique Instruments, Inc., Dover, N. J.). Counts per minute were converted to disintegrations per minute using the external standard ratio procedure. Portions of the NaOH-solubilized material were also assayed for protein by the method of Lowry, Rosebrough, Farr, and Randall (18) using bovine serum albumin as the standard. For studies with cycloheximide (Sigma Chemical Co., St. Louis, Mo.), cultures were incubated for 15 min with cycloheximide before incubation with [³H]leucine, 2.5 μCi/ml, for 2 h. Viability of cells treated in this manner was assessed by trypan blue dye exclusion (19).

Proteolytic digestion of TCA-precipitable material synthesized by endothelial cells. Cultured endothelial cells were labeled with [³H]leucine, 2.5 μCi/ml, for 24 h. The culture media was mixed with an equal volume of cold 20% TCA containing 10⁻⁴ M unlabeled leucine and incubated for 1 h at 4°C. The precipitate was washed twice with cold 10% TCA and solubilized in 6 ml 1 N NaOH. The cell monolayers were solubilized in 1 N NaOH as above. Solubilized samples were neutralized with 12 N HCl and dialyzed exhaustively against 0.01 M Tris-HCl, pH 7.8. Digestions at 37°C with trypsin (crystallized two times, salt-free, Worthington Biochemical Corp.) for 24 h and Pronase (Calbiochem, San Diego, Calif.) for 2 h, both at final concentrations of 0.2 mg/ml in 0.01 M Tris-HCl, pH 7.8, were carried out in volumes of 1 ml. At the end of the digestion, 1 mg of carrier bovine serum albumin was added followed by 0.25 ml 50% cold TCA, and the tubes were incubated for 30 min at 4°C. At the end of this period the precipitates were removed, washed once with 10% TCA, and dissolved in 0.2 ml 1 N NaOH overnight at 37°C. A

sample of this material was dissolved in PCS liquid scintillation cocktail and counted in the liquid scintillation spectrometer.

End-group analysis of [¹⁴C]leucine containing material from cultured endothelial cells. For end-group analysis, previously outlined procedures were followed (20, 21). Endothelial cells were cultured with 0.5 μCi/ml of [¹⁴C]-leucine, sp act-304 mCi/mmol, (New England Nuclear) for 24 h. The culture medium was removed and brought to 100% saturation by the addition of solid ammonium sulfate. The mixture was incubated 1 h at 20°C and centrifuged at 40,000 g for 15 min, and the precipitate was dissolved in PBS. The residual cell monolayer was detached from the Petri dish by incubation with cord buffer containing 0.01% EDTA and 0.1% collagenase for 15 min at 37°C. The cells were washed into cord buffer containing 0.2% collagenase and 0.001 M CaCl₂ and were then incubated for 15 min at 37°C. The cells were washed once in 0.2% EDTA-diluent buffer, resuspended in 3 ml of this solution, frozen and thawed three times, and centrifuged at 40,000 g for 15 min. The cell supernate was removed, and the cell pellet was resuspended in 3 ml PBS. The three samples (the protein fraction from the postculture medium and the cell-derived soluble and particulate pellet fractions) were exhaustively dialyzed against 0.05 M NH₄HCO₃ (pH 7.9). 3-ml samples of all three fractions were shaken at 22°C for 2 h with 2 ml of a 5% solution of dinitrofluorobenzene in ethanol. The samples were acidified (pH < 2) with 0.05 ml 12 N HCl, and the unreacted reagent was extracted with ether. The protein in the aqueous phase was precipitated with TCA, washed with ethanol-ether (1 : 3, vol/vol), washed with ether, and dried under nitrogen. The protein was completely hydrolyzed in 6 N HCl for 22 h at 110°C in sealed tubes. In order to extract any dinitrophenyl-leucine, the hydrolysate was shaken three times with 2 ml of ether. After evaporation, both the ether extract and the remaining aqueous material were counted in the liquid scintillation spectrometer. Complete recovery of the radioactivity in the samples was obtained.

Incorporation of radioactive amino acids into immunoprecipitable protein. For these studies, cultured cells were labeled with [³H]leucine or [³H]amino acid mixtures. High molecular weight, AHF antigen-rich fractions were prepared from the culture medium by ammonium sulfate precipitation and Sepharose 6B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) chromatography. Immunoprecipitates were formed when these fractions were incubated with unlabeled anti-AHF. The incorporation of radioactivity into these precipitates provided an index of newly synthesized AHF antigen. Control studies were carried out using cultures of smooth muscle cells.

Three separate experiments were performed, each using between 10 and 20 Petri dishes of cultured endothelial cells. The incorporation studies included both brief and prolonged incubations with labeled amino acids. In two experiments the cells were incubated for 24 h with either 2.5 μCi/ml of [³H]leucine (experiment A) or 25 μCi/ml of mixed [³H]-amino acids (experiment B), (NET-250, New England Nuclear). In a third experiment the cells were incubated in leucine-free TC 199 (Grand Island Biological Co.) containing 10% FCS and labeled for 2 h with [³H]leucine, 2.5 μCi/ml (experiment C). At the end of the incubation period the medium from 10 to 20 Petri dishes of cultured endothelial cells was pooled, mixed with an equal volume of saturated ammonium sulfate, incubated for 40 min at 20°C, and centrifuged at 12,000 g for 20 min. The precipi-

tate was washed three times with 50% saturated ammonium sulfate and then dissolved in PBS at 1/10th the original sample volume. 3 ml of this concentrated material was chromatographed on a 1.6 × 60 cm column of Sepharose 6B using barbital-buffered saline (14). The AHF antigen-rich void volume fractions were pooled and concentrated to 2 ml by precipitation with 50% saturated ammonium sulfate before dialysis against barbital-buffered saline (14).

Immunoprecipitate determinations were carried out using a globulin fraction prepared from the monospecific rabbit anti-AHF by precipitation of serum proteins with 50% saturated ammonium sulfate. Control studies were carried out using the same concentration of globulin (0.13–1.25 mg/ml) prepared in the same way from normal rabbit serum or from rabbit serum obtained from an animal that had been immunized with sulfanilic acid coupled to bovine serum albumin. $\frac{1}{10}$ ml of unlabeled rabbit anti-AHF globulin (or control globulin) was added to 0.2 ml of the concentrated, labeled culture medium proteins, and the mixture was incubated at 37°C for 1 h and at 4°C overnight. $\frac{1}{10}$ ml of undiluted goat anti-rabbit gamma globulin (Hyland Div., Travenol Laboratories) was then added, and the tubes were again incubated at 37°C for 1 h and at 4°C overnight. The resulting immunoprecipitate was washed twice with ice-cold borate-buffered saline, pH 7.85, dissolved in 2 ml NCS tissue solubilizer (Amersham/Searle Corp.), and counted in a Nuclear-Chicago (Nuclear-Chicago Corp., Des Plaines, Ill.) liquid scintillation spectrometer.

Cycloheximide inhibition of [³H]leucine incorporation into AHF antigen. Two groups of endothelial cells in leucine-free TC 199-10% FCS were labeled for 2 h with [³H]-leucine, 2.5 μCi/ml. Cycloheximide (3 μg/ml) was present during the labeling period in the culture medium of one of the groups. After the labeling media were removed, the cells were washed three times with PBS and once with TC 199-20% FCS and recultured in TC 199-20% FCS for an additional 46 h. The second culture period in the absence of both cycloheximide and labeled amino acids permitted synthesis of unlabeled AHF antigen that served as carrier protein. The media from both culture periods were combined and processed as above to determine the amounts of [³H]-leucine present in specific immunoprecipitates. Preliminary experiments using both trypan blue dye exclusion and 2 h [³H]leucine incorporation rates showed that 95% of the cultured cells remained alive and were capable of synthesizing protein after the incubation with cycloheximide.

AHF procoagulant activity assay. AHF procoagulant activity was measured by a one-stage method (22) using AHF-deficient human plasma (Dade, Miami, Fla.) as substrate. Pooled normal human plasma (Hyland Div., Travenol Laboratories) was used as the standard for these assays, and AHF values are expressed as U/100 ml. As proposed by an ad hoc committee of the National Research Council, 1 AHF unit is defined as that activity corresponding to 1 ml of average normal plasma (23). It was necessary to treat FCS to remove small amounts of residual thrombin and prothrombin before all experiments in which AHF procoagulant activity was measured. FCS was absorbed twice with sterile 2% Al(OH)₃ gel (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) incubating 0.1 ml Al(OH)₃/ml FCS for 5 min at 37°C. After centrifugation, the FCS was heated to 56°C for 30 min and sterilized by passage through a 0.22 μm Millipore filter (Millipore Corp., Bedford, Mass.). No AHF procoagulant activity could be demonstrated either in regular FCS or heated and absorbed FCS. 25% heated and absorbed FCS supported

[³H]leucine incorporation into TCA-precipitable material as well as did 20% untreated FCS. Exogenous AHF was added to cultures as Hemophil (28 U/ml, Hyland Div., Travenol Laboratories), and the concentrations were calculated from the stated AHF activity values.

In all AHF procoagulant assay experiments, Factor IX was also assayed by a modification of the one-stage method (22) using Factor IX-deficient plasma. These studies were performed to rule out the possible contribution of other procoagulant activities such as tissue factor. No activity was detected. In experiments performed to determine whether trace amounts of thrombin could activate the AHF antigen in the post-culture medium, AHF procoagulant was measured by a two-stage assay (24).

RESULTS

Immunofluorescence studies. When sections of umbilical cord were sequentially incubated with rabbit anti-AHF and fluorescein-conjugated goat antirabbit gamma globulin, the endothelial cells lining the umbilical veins and arteries were brightly stained. Blood vessel medial smooth muscle cells and fibroblasts in the adventitia and cord matrix were nonreactive. Similar studies with the cultured cells revealed intense fluorescence of the endothelial cells (Fig. 1), but cultured fibroblasts and smooth muscle cells did not stain. No cultured cells were stained when normal rabbit serum was used instead of rabbit anti-AHF. The specificity of the rabbit anti-AHF in immunofluorescence microscopy has been established by extensive absorption studies (11).

Identification of AHF antigen in culture medium and cells. No AHF antigen was present in the culture me-
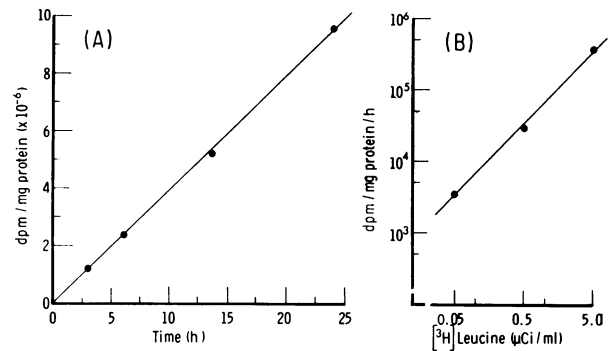
FIGURE 2(A) Time-dependent incorporation of [³H]leucine into protein by cultured human endothelial cells. (B) Dose dependent incorporation of [³H]leucine into protein by cultured human endothelial cells.

dium before incubation with cells. Postculture media (media derived from cultured cells after 72 h incubation) and cell fractions from cultured endothelial cells, smooth muscle cells, and fibroblasts were assayed for AHF antigen by radioimmunoassay (Table I). AHF antigen was detected in postculture media derived from endothelial cells; none was found in postculture media of either smooth muscle cells or fibroblasts. The release of AHF antigen into the endothelial cell culture medium was progressive over a 72 h period.

Endothelial cells and smooth muscle cells were frozen and thawed four times and separated into soluble and particulate material by centrifugation at 40,000 g. AHF antigen was present in both the soluble and particular fractions obtained from endothelial cells. Similar distribution of AHF antigen between supernatant and particulate fractions was obtained when cells were disrupted by sonication or homogenization. No antigen was detected in similar fractions obtained from smooth muscle cells.

The physicochemical properties of the tissue culture AHF antigen were like those of plasma AHF. When culture media from endothelial cells was concentrated and subsequently chromatographed using Sepharose 6B, AHF antigen was eluted with void volume fractions in a pattern similar to that of AHF from normal human plasma (14). Similarly both culture fluid AHF antigen and the AHF antigenic material of normal human plasma resisted adsorption by BaSO₄ (50 mg/ml for 10 min at 25°C) and were immunologically stable when heated to 56°C for 30 min or when incubated with 0.5, 2, or 5 U of bovine thrombin for 30 min at 37°C.

Protein synthesis by cultured endothelial cells. The following studies were performed to establish that the cultured endothelial cells synthesize protein. Cells incubated with [³H]leucine at 37°C incorporated radioactivity into TCA-precipitable cell protein. Incorporation varied linearly with time (Fig. 2A) and with [³H]leucine con-
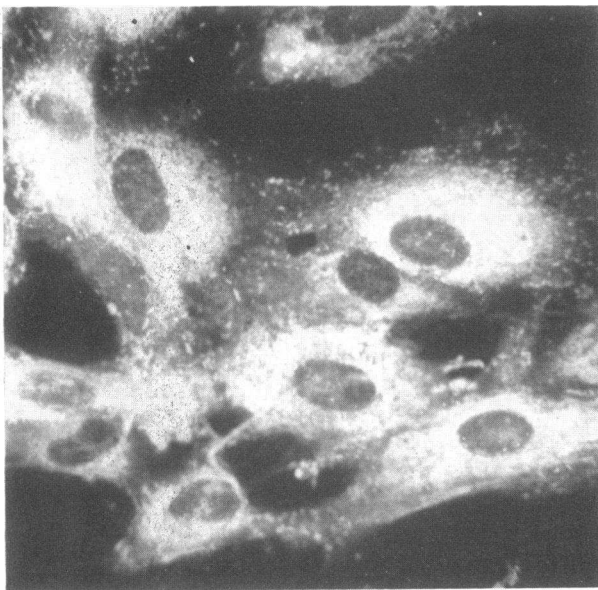

FIGURE 1 Immunofluorescence study of cultured human endothelial cells. Cells were treated with rabbit anti-AHF and then with fluorescein-conjugated goat antirabbit globulin. The cells are brightly stained (× 960).

TABLE II

Cycloheximide Inhibition of [³H]Leucine Incorporation into TCA-Precipitable Protein by Cultured Endothelial Cells

Cycloheximide µg/ml	dpm/mg protein/h	% Suppression
0	6.73×10^4	0
0.1	3.45×10^4	49
1	1.55×10^4	77
10	1.01×10^4	85
100	0.50×10^4	93

centration (Fig. 2B). No significant [³H]leucine incorporation occurred at 4°C.

The labeled TCA-precipitable materials derived from endothelial cells and culture media were digested with proteolytic enzymes to establish that the [³H]leucine was incorporated into protein. Digestion with trypsin reduced the TCA-precipitable radioactivity of cultured cells by 76% and of culture media by 65%. Digestion with Pronase reduced the TCA-precipitable material of cells and culture medium by more than 95%.

It has been shown previously that certain cells including thyroid (25), liver (26), and bacterial cells (27) may incorporate [³H]leucine into TCA-precipitable material by adding the leucine to amino-terminal ends of already existing proteins. To show that this was not the case for endothelial cells, protein containing fractions from post-culture medium and cell-derived soluble and particulate pellet fractions were reacted with dinitrofluorobenzene. After complete acid hydrolysis of the dinitrophenylated proteins, less than 3% of the total radioactivity was extracted by ether as dinitrophenyl-leucine (derived from amino-terminal leucine). These data establish that most of the leucine was incorporated within polypeptide chains. Additional evidence against terminal addition was the comparable rate of incorporation of mixed [³H]amino acids into protein by endothelial cells.

Endothelial cell protein synthesis was inhibited in the presence of cycloheximide (Table II). The viability of cells cultured with the highest concentration of cycloheximide was greater than 95% as assayed by trypan blue dye exclusion. The suppression of [³H]leucine incorporation during a 2 h incubation in medium containing cycloheximide was completely reversible, and [³H]leucine was incorporated into protein after the cycloheximide was removed.

AHF antigen synthesis. To determine if the AHF antigen present in endothelial cell culture medium was synthesized by the endothelial cells, the cultured cells were incubated with radioactive amino acids. High molecular weight, AHF antigen-rich fractions were prepared from the culture medium and the immunoprecipitable ³H-labeled protein was determined using unlabeled monospecific rabbit anti-AHF. Preliminary experiments

using unfractionated culture medium were not successful because only a small percentage of the labeled proteins was precipitable.

7% of the radioactivity present in high molecular weight fractions prepared from endothelial cell culture medium was incorporated into immunoprecipitates formed by the sequential addition of rabbit anti-AHF and goat antirabbit globulin (Table III). In contrast, 1.5% of the ³H counts were coprecipitated with the goat antirabbit globulin-rabbit globulin immunoprecipitates in control experiments carried out using the same concentration of rabbit globulin derived from either normal rabbit serum or serum from rabbits immunized with sulfanilic acid coupled to bovine serum albumin. This indicated that approximately 5.5% of the [³H]leucine present in the high molecular weight fraction was specifically incorporated into protein containing AHF antigen. No specific precipitation of labeled protein was detected when similar fractions from cultured smooth muscle cell media were tested.

Cycloheximide inhibited [³H]leucine incorporation into specifically precipitable (AHF antigen) protein by 88% (Table IV). The suppression of [³H]leucine incorporation into all high molecular weight proteins (Sepharose void volume fractions) by cycloheximide was similar, i.e., 91%. AHF antigen synthesis resumed after cycloheximide was removed, and the amount of AHF antigen produced over the 48 h period was similar for the two different experimental conditions.

AHF procoagulant activity. Procoagulant assays on culture media were feasible only if the FCS was absorbed with Al(OH)₃ and heat inactivated to remove trace amounts of thrombin and prothrombin. Endothelial cells cultured for 72 h in media containing the treated FCS released no AHF procoagulant activity (Table V).

TABLE III

Immunoprecipitation of [³H]Leucine-Labeled Proteins*

Source of culture medium	Experiment	Concentration of rabbit globulin	% cpm in Immunoprecipitate	
			Rabbit anti-AHF	Control rabbit globulin
		mg/ml		
Endothelial cells	A	0.13	7.8	2.2
	B	0.13	4.4	1.2
		0.63	6.0	0.8
	C	0.25	10.1	2.1
		1.25	6.5	1.0
			7.0 ±0.9‡	1.5 ±0.3
Smooth muscle cells	A	0.13	1.8	1.6

* High molecular weight fractions separated by agarose gel chromatography were concentrated before double antibody precipitin analysis (see Methods section for details).
‡ Mean ±SEM.

TABLE IV

TABLE IV

Cycloheximide Suppression of [³H]Leucine Incorporation

	AHF antigen, U/100 ml		[³H]Leucine, cpm/ml	
	Cycloheximide*		Cycloheximide*	
	Absent	Present	Absent	Present
High molecular weight fractions prepared by gel filtration	8.0	8.4	8.09 × 10⁴	0.76 × 10
Immunoprecipitates‡ prepared using				
(a) Rabbit anti-AHF globulin	—	—	8170	1305
(b) Normal rabbit globulin	—	—	1725	505
Specifically precipitable protein (a minus b)	—	—	6445	800

* Initial 2 h incubation of [³H]leucine in leucine-free TC 199 containing 10% FCS in presence or absence of cycloheximide. This medium was removed and subsequently added to the culture medium (TC 199 with 20% FCS with no cycloheximide or labeled amino acids) in which the cultures were incubated for an additional 46 h.

‡ High molecular weight proteins from each culture medium were incubated with either monospecific rabbit anti-AHF globulin or normal rabbit globulin, both at 0.63 mg/ml. Goat antirabbit globulin was subsequently added, and the radioactivity of the immune precipitates was determined. Values expressed are in counts per minute per milliliter of high molecular weight fractions.

However, the endothelial cells did release AHF antigen in an amount similar to that found when endothelial cells were cultured for 72 h in TC 199 containing 20% regular FCS (Table I). AHF procoagulant activity and AHF antigen were both absent in preculture media.

To determine if newly synthesized AHF was inactivated in the culture system, culture medium containing exogenous AHF (0.7 U/ml) was either incubated alone or with endothelial cells and then assayed for AHF procoagulant activity. After 72 h of incubation, AHF pro-

TABLE V

AHF Procoagulant Activity of Culture Medium

Material*	AHF procoagulant activity	AHF antigen
	U/100 ml	U/100 ml
Medium alone	<0.5	<0.5
Medium + EC‡	<0.5	7.1
Medium + exogenous AHF (0.7 U/ml)§	107	NT‖
Medium + EC + exogenous AHF (0.7 U/ml)	68	NT
Medium + exogenous AHF (0.07 U/ml)	4.5	NT
Medium + EC + exogenous AHF (0.07 U/ml)	3.4	NT

* The components were incubated together for 72 h and the supernatant culture medium assayed. For these studies, the 25% FCS added to TC 199 was adsorbed with Al(OH)₃ and heat inactivated.

‡ E.C.—Cultured human endothelial cells.

§ Exogenous AHF—Hemophil (Hyland Div., Travenol Laboratories) was added to bring the AHF activity to the specified concentration.

‖ NT, not tested.

coagulant activity was present in the media at levels consistent with the amounts of exogenous AHF added (Table V). These experiments were repeated using exogenous AHF at concentrations that more closely approximated the AHF antigen concentration of postculture media (0.07 U/ml) (Table I). Again, AHF procoagulant activity was not inactivated during the period of incubation (Table V). In a separate set of similar experiments (not shown) it was demonstrated that AHF antigen levels remained constant during the incubation period. Thus neither AHF antigen nor the AHF procoagulant activity were significantly degraded during the 72 h incubation period.

To determine whether trace amounts of thrombin could activate the AHF antigen in the postculture medium as has been described for AHF (24, 28), experiments were performed in which the postculture medium was incubated with varying thrombin concentrations (0.2–0.035 U/ml). No procoagulant activity was generated from the AHF antigen present in the postculture medium.

DISCUSSION

The studies reported here demonstrate synthesis and release of macromolecular AHF antigen by cultured human endothelial cells. These observations are compatible with the recent demonstration of AHF antigen in endothelial cells throughout the body (9–11). Control immunofluorescence and radioimmunoassay studies have established that cultured fibroblasts and smooth muscle cells do not have this capacity.

It is recognized that the validity of the radioimmunoassay and immunofluorescence studies is dependent upon the specificity of the rabbit anti-AHF. Four kinds of evidence indicate that this antibody reacts with the same molecule in human plasma as that which is responsible for AHF procoagulant activity. The most direct is the demonstration that the absorbed antiserum, which inactivates AHF procoagulant activity, forms a single line in immunodiffusion studies with AHF-rich concentrates of normal human plasma (16). Other specificities that might not be demonstrable with immunoprecipitation methods have been sought in hemagglutination inhibition experiments, but none was found (29). Thus, by immunochemical criteria, the absorbed antiserum has a single specificity. The rabbit antibody does not form a precipitin line, however, if it is tested with concentrates prepared from plasma of patients with severe von Willebrand's disease (16). The parallel reduction of AHF activity and immunoreactivity in von Willebrand's disease plasmas also suggests that the antibody reacts with the AHF molecule (14, 16). AHF procoagulant activity and the antigen identified by rabbit anti-AHF have similar physicochemical properties; an example of this third

kind of evidence for the specificity of the rabbit antibody is the similar elution pattern of the two properties when normal plasma proteins are separated by Sepharose gel filtration (14).

More direct evidence for the antibody's specificity has recently been obtained by immunizing rabbits with washed immunoprecipitates formed by incubating concentrates of normal human plasma with rabbit anti-AHF. Antibodies that inactivate AHF procoagulant activity were formed by these animals indicating that the immunoprecipitates contained proteins that had AHF determinants (30). While the relationship of the antigenic determinants detected by the absorbed rabbit antiserum to AHF procoagulant activity should continue to be examined, all available data indicate that this rabbit antibody is specific for AHF.

Although the cultured endothelial cells secrete protein that has the physicochemical and antigenic properties of AHF, no AHF procoagulant activity has been detected in the culture medium. There are several possible explanations for this marked disparity between the immunologic and functional assays:

(a) The secreted AHF might be inactivated by the tissue culture medium. Previous studies have demonstrated that AHF is rapidly inactivated by many proteolytic enzymes including thrombin (31, 32). It is possible that proteases present in the FCS or enzymes liberated by the endothelial cells might have inactivated newly synthesized AHF. To test this possibility exogenous AHF was incubated with cultured endothelial cells for 72 h, and the medium was then assayed for AHF procoagulant activity. Added AHF was not inactivated in these experiments. It seems unlikely, therefore, that the procoagulant activity of the newly synthesized endogenous AHF was destroyed in the culture medium.

(b) The immaturity of the fetal endothelial cells and/or the artificial conditions of the culture might affect AHF antigen synthesis in a way that would prevent the development of AHF procoagulant activity. Full-term newborns have normal levels of plasma AHF procoagulant activity even though AHF does not cross the placenta (33, 34). It can be assumed, therefore, that the full-term fetus can synthesize functional AHF. However, it is possible that endothelial cells in various anatomic regions are functionally heterogeneous. In addition, it is possible that the culture conditions limited AHF procoagulant synthesis. The refutation of these possibilities would require studies in which the endothelial cells were derived from adult arteries or veins as well as studies in which a variety of different culture media were utilized. These have yet to be carried out.

(c) Endothelial cells may synthesize an inactive "precursor" molecule. If this is the case, the conversion to that configuration which is necessary for AHF procoagu-

lant activity may occur at a remote site, either through interaction with a second kind of cell or by interaction with one or more plasma enzymes.

(d) Endothelial cells may normally synthesize only one component of a macromolecular complex that has AHF procoagulant activity. Several laboratories have recently identified AHF subunits when this high molecular weight protein is dissociated by high salt concentration (24, 35, 36) or by reduction and alkylation (17, 32, 37). Asymmetric dissociation of human AHF has been demonstrated by combined immunologic and procoagulant assays of AHF that has been exposed to 1 M NaCl or 0.24 M CaCl₂ (38). Nonfunctional high molecular weight material—greater than 2,000,000 daltons—is detected by AHF radioimmunoassay when AHF is dissociated by these conditions during agarose gel chromatography or sucrose density ultracentrifugation. In contrast, AHF procoagulant activity is limited to low molecular weight proteins—approximately 150,000 daltons—that cannot be detected by radioimmunoassay or electroimmunoassay (38). Human and rabbit antibodies inactivate the AHF procoagulant activity of the low molecular weight subunits, however, and are neutralized by the antigenic determinants present on the nonfunctional high molecular weight subunits (38). The presence of common antigens on the two moieties suggests a molecular relationship that remains to be fully defined.

Even though the details of AHF structure and function are not yet well understood, it is apparent that the cultured endothelial cell synthesizes a protein (or proteins) that has the same antigens as plasma AHF. These findings confirm and extend immunofluorescence studies that have localized this protein to endothelial cells in a variety of tissues and raise the possibility that the anatomical distribution of AHF antigen in cells lining vascular channels is important for its role in hemostasis. It seems likely that studies of AHF antigen production by cultured endothelial cells will prove a valuable new approach in understanding the mechanisms that control AHF synthesis and release.

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