Destruction of Factor VIII Procoagulant Activity in Tissue Culture Media

By Nancy W. Stead and Patrick A. McKee

Human endothelial cells have been reported to synthesize protein that has von Willebrand factor activity and reacts with a heterologous antibody to plasma factor VIII/von Willebrand factor (FVIII/vWF); however, no FVIII procoagulant activity has been associated with the protein produced by endothelial cells. Of its three biologic properties, only the FVIII procoagulant activity of purified plasma FVIII/vWF is rapidly destroyed by trace levels of serine proteases; despite extensive degradation of human plasma FVIII/vWF protein by proteolytic enzymes, both its vWF activity and its immunoreactivity are fairly well preserved. It is possible that endothelial cells synthesize a molecule with all the biologic properties characteristic of plasma FVIII/vWF protein and that only vWF activity and immunoreactivity are detected because protease activity in the cell culture medium destroys FVIII procoagulant activity. In order to evaluate this hypothesis, the protease activity of growth media was assessed by hydrolysis of p-nitroaniline from the tripeptide n-benzoyl-phenylalanyl-valyl-arginyl-p-nitroanilide and by destruction of the FVIII procoagulant activity of purified plasma FVIII/vWF protein added to various media. Among the growth media that had protease activity were culture medium made with fetal calf serum (FCS) and culture medium containing FCS heated to 56°C for 0.5 hr after adsorption with aluminum hydroxide. Only culture medium made with FCS heated to 66°-68°C for 2 hr did not hydrolyze p-nitroaniline from the tripeptide, and only in this medium was the FVIII procoagulant activity of purified plasma FVIII/vWF protein stable. Thus the demonstration of protease activity in growth media of endothelial cell cultures leaves open the question of whether or not endothelial cells synthesize a molecule having all the biologic properties of the plasma FVIII/vWF protein.

HIGHLY PURIFIED human plasma antihemophilic factor/von Willebrand factor (FVIII/vWF), a glycoprotein with a molecular weight in excess of 10^6 daltons, possesses three distinct properties: (1) FVIII procoagulant activity, which corrects the defect in hemophilic plasma; (2) vWF activity, as defined by its requirement for the aggregation of washed human platelets by ristocetin; (3) reactivity with heterologous antibody such that both FVIII and vWF activities are inhibited and immunoprecipitin formation occurs in agarose. There is no consensus as to whether these three properties of the FVIII/vWF protein are features of a single large molecule with a covalent subunit structure or of different noncovalently bonded components that copurify from plasma.

Cultured human umbilical vein endothelial cells have been reported to synthesize a protein that not only has vWF activity but that also reacts with heterol-
ogous antibody to human plasma FVIII/vWF protein. More recently, guinea pig megakaryocytes have been shown to synthesize a protein with vWF activity and that reacts with heterologous antibody to guinea pig plasma FVIII/vWF. However, this protein, whether from the endothelial cell or megakaryocyte cultures, did not have FVIII procoagulant activity. To some extent, these data have been used to support the theory that the protein with FVIII procoagulant activity is distinct from a larger molecule with vWF activity and the more prominent antigenic determinants.

Of its three biologic properties, only the FVIII procoagulant activity of purified plasma FVIII/vWF is rapidly destroyed by trace levels of serine proteases such as thrombin, trypsin, and plasmin; however, vWF activity and immunoreactivity are fairly well preserved despite extensive proteolytic degradation of human FVIII/vWF protein, particularly by plasmin. If in fact endothelial cells do synthesize and release FVIII/vWF protein that initially has all three biologic properties, it is tenable to suggest that the FVIII procoagulant activity is quickly destroyed by any trace proteases that might be contained in the growth medium. This is particularly reasonable when it is considered that the newly synthesized FVIII/vWF protein is present in only very small amounts and remains in contact with the growth medium for a long period of time before concentration and assay. Moreover, although the FVIII procoagulant activity might be rapidly destroyed under such conditions, the vWF activity and immunologic reactivity would likely remain functional.

The studies reported in this manuscript were designed to quantitate trace amounts of protease activity that might be present in growth medium before and after it was overlaid over endothelial cell cultures. The effect of such low levels of protease activity on the functions of added purified FVIII/vWF protein was also examined. Finally, modifications of growth medium designed to reduce protease activity were explored.

MATERIALS AND METHODS

Reagents. Reagent-grade chemicals were used without further purification. Soybean trypsin inhibitor (SBTI) was purchased from Worthington Biochemical, Freehold, N.J. Bovine pancreatic trypsin inhibitor (BPTI) was obtained from FBA Pharmaceuticals, New York, N.Y. FOY-305 inhibitor [N,N-dimethylcarbamoylmethyl-4-(4'-guanidinobenzyloxy)-phenylacetatelmethansulfonate was a gift from Dr. Kenneth Robbins, Michael Reese Blood Center, Chicago, Ill.; this synthetic substance is a reversible inhibitor of at least three serine proteases (kallikrein, trypsin, and plasmin). Disopropylphosphofluoridate (DFP) was purchased from Sigma Chemical, St. Louis, Mo. Aluminum hydroxide was obtained from Calbiochem, San Diego, Calif., as Alumina Cy Gel (grade A).

Culture medium. Fetal calf serum (FCS), medium 199 with Earle's salt (10x concentrate), basal medium Eagle vitamin solution (100x concentrate), and modified Eagle medium (MEM) amino acid solution (50x concentrate) were obtained from Grand Island Biological, Grand Island, N.Y. Dextrose was purchased from Vitarine, New York, N.Y., sodium bicarbonate from Abbott, North Chicago, Ill., l-glutamine from Schwartz-Mann, Orangeburg N.Y., N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) from ICN Pharmaceuticals, Cleveland, Ohio, morpholinopropanesulfonic acid (MOPS) from Calbiochem, La Jolla, Calif., and hydrocortisone succinate from Upjohn, Kalamazoo, Mich.

Medium 199 concentrate sufficient for a liter of culture medium was diluted to 770 ml with pyrogen-free sterile water; then 10 ml of MEM amino acid concentrate, 10 ml basal medium Eagle vitamin solution, 15 ml 8.4%, sodium bicarbonate, 20 ml 50%, dextrose, and 50 ml water containing 0.01 mole MOPS and 0.01 mole HEPEs, after adjusting to pH 7.3 with concentrated
NaOH, were added. Aliquots of this solution were stored frozen at −20°C; as required a sufficient volume for use within 1 wk was thawed and made 20% in FCS, 30 μg/ml in hydrocortisone, and 2 mM additional in glutamine. This solution was the culture medium in which endothelial cells were planted and grown.

Three modifications of culture medium were used in certain of the studies reported here: (1) culture medium containing no FCS but instead 0.05 M Tris-0.15 M NaCl pH 7.35 as a volume replacement; (2) culture medium made with FCS that had been heated to 56°C for 0.5 hr (56°-FCS), or FCS heated to 66°-68°C for 2 hr (66°-FCS); or FCS made 1.7×10⁻⁴ M in DFP 2 hr before use (DFP-FCS); (3) culture medium made with FCS that had been adsorbed two times with 2% aluminum hydroxide before heating to 56°C for 0.5 hr.¹

Pronase solution. Pronase grade B obtained from Calbiochem was dissolved to 5 mg/ml in Hanks’ balanced salt solution (HBSS) without glucose, following which the solution was incubated 2 hr at 37°C, dialyzed overnight at 4°C against 40 vol of the same solvent, sterilized by passage through a 0.22-μm Millipore filter, and frozen at −20°C until use. Cells treated with pronase in this fashion seemed to plate better, grow a little more rapidly, and look healthier. It should also be added that the use of pronase resulted in a better yield of viable endothelial cells than did trypsin or collagenase.

Preparation and culture of endothelial cells. Umbilical cords from uncomplicated normal deliveries were obtained from the Department of Obstetrics and Gynecology at Duke University Medical Center. Within 3 hr after delivery the umbilical cord vein was cannulated, filled with the pronase solution, and incubated at 37°C for 30 min. The pronase solution was collected and the vein was washed with an equal volume of culture medium pooled with the pronase solution. The endothelial cells suspended in this pool were harvested by centrifugation for 10 min at 250 g at 25°C. The cells were then washed twice with 2 ml culture medium, resuspended in 3-4 ml culture medium, and plated in one 25-cm² Falcon flask at 37°C in 5% CO₂. Thereafter the culture medium was exchanged for fresh medium every 72-96 hr.

Assay for proteolytic activity of culture medium. N-benzoyl-phenylalanyl-valyl-arginyl-p-nitroanilide HCl (S-2160) was purchased from Bofors, Molndal, Sweden. This synthetic substrate is hydrolyzed by serine proteases to release p-nitroaniline, which can then be quantitated by its absorbance at 400 nm. The specific method used in these experiments is essentially that described by Blomback et al.⁸ with the following modifications: (1) only half the volumes of reagents were used; (2) the incubation time of enzyme and substrate was increased from 30 sec to 1-2 hr, since the concentration of enzyme in the assay mixture was such that the rate of p-nitroaniline release was constant over 2 hr; and (3) protease inhibitors other than antithrombin were studied for their effect on the proteolytic activity in the culture medium. A stock solution of S-2160 tripeptide was prepared by dissolving it in water to a final concentration of 1 mg/ml. The assay buffer, 0.025 M Tris-0.025 M imidazole-0.15 M NaCl, pH 8.2, was also used as the solvent for the protease inhibitors. Selected amounts of each growth medium to be analyzed were mixed with protease inhibitors and adjusted to a total volume of 1.1 ml with assay buffer. After 0.125 ml of S-2160 solution was added, each mixture was incubated at 40°C until the reaction was stopped at 30, 60, 90, or 120 min by adding 0.15 ml glacial acetic acid. The absorbance at zero-time incubation was defined by analyzing samples to which glacial acetic acid had been added before the S-2160 tripeptide substrate so that hydrolysis did not occur.

Factor VIII procoagulant activity. The procoagulant activity of purified plasma FVIII/vWF protein that had been added to the various culture media was measured by the two-stage thromboplastin generation method of Penick et al.⁹ and by the one-stage partial thromboplastin method of Hardisty and MacPherson¹⁰ in which a 20-fold dilution of Thromboplastin in 0.15 M NaCl-0.05 M Tris pH 7.35 was used instead of inositol. Except for culture medium containing no FCS, after purified FVIII/vWF protein had been mixed with culture media containing variously treated FCS the samples to be assayed for FVIII procoagulant activity were adsorbed with 3.2% aluminum hydroxide.⁹ If the assay was to be performed immediately, the supernatant sample was kept at 4°C; however, if this was not possible, the samples were stored frozen at −70°C for up to 3 days before assay. In the latter case, appropriate controls were performed to insure that freezing and thawing did not affect the interpretation of the results.

von Willebrand factor activity. vWF activity was quantitated by the ability of a test sample to support ristocetin aggregation of washed platelets as described in reports from this labora-
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The adsorption of test samples by aluminum hydroxide did not affect the results of these assays.

Immunodiffusion. The immunoreactivity of culture medium from endothelial cells was assessed by diffusion against rabbit antiserum to purified human plasma FVIII/vWF in 1% agarose buffered with 0.15 M NaCl-0.05 M Tris pH 7.35.

Purification of plasma FVIII/vWF protein. Human FVIII/vWF protein was purified from commercial plasma concentrates as described by Switzer and McKee with the modification that the concentrated FVIII/vWF protein solution was made 1.7 x 10^{-3} M in DFP about 2 hr before being applied to the 4% agarose column. Bovine FVIII/vWF protein was a gift from Dr. Earl Davie, University of Washington, Seattle, Wash.

Partial purification of vWF protein from endothelial cells. Culture medium in which endothelial cells were grown for 72-96 hr was kept at -70°C until 500 ml was accumulated. This volume of medium was warmed to room temperature; then, after the pH was lowered to 6.7 with citric acid, it was chilled to 4°C. The acidified medium was made 4% (w/v) in polyethylene glycol 4000 (PEG) by adding chilled 40% PEG in 0.05 M Tris-0.15 M NaCl pH 6.7. After being stirred for 30 min, the solution was centrifuged at 6000 g for 30 min at 4°C. Sufficient 40% PEG was added to make the supernatant 12% in PEG; this solution was then gently stirred overnight at 4°C. The white granular precipitate that formed was separated by centrifugation in plastic bottles at 6000 g and 4°C for 30 min. The precipitate was dissolved in 5-6 ml 0.05 M Tris-0.15 M NaCl pH 7.35 to give a protein concentration of 17-25 absorbance units at 280 nm. This solution was divided into aliquots so that each amounted to ~2% of the column volume and each aliquot was filtered on a 1.3x30-cm column of 4% agarose (Biogel A-15M) in 0.05 M Tris-0.15 M NaCl pH 7.35. Three columns were developed simultaneously at a flow rate of 7 ml/hr, and 3.4-ml fractions were collected. Since most of the vWF activity eluted in the void volume, all of these fractions from the nine agarose gel filtration runs were pooled and the vWF protein was precipitated by the addition of solid PEG to a final concentration of 20%. After being stirred slowly overnight at 4°C, the precipitate was collected by centrifugation at 6000 g for 30 min at 4°C, dissolved in 2 ml 0.05 M Tris-0.15 M NaCl pH 7.35, and layered on a 1.3x60-cm column of 2% agarose (Sepharose 2B) equilibrated with the same buffer. The flow rate was 7 ml/hr, and 3.4-ml fractions were collected.

RESULTS

Synthesis by endothelial cells of a protein with vWF activity. The cells that grew in culture had the morphologic characteristics of endothelial cells and not smooth muscle as evidenced by their being polygonal and growing in monolayers with contact inhibition. With time, the culture medium overlying the cells developed an increasing level of ristocetin cofactor activity, a feature thought to be specific for the presence of live endothelial cells. For these analyses, culture medium from each of seven confluent endothelial cell cultures was analyzed for vWF activity immediately (zero time) after a complete exchange of medium and then at 24, 48, and 72 hr. Figure 1 shows that no vWF activity was detectable in any of the seven cultures at zero time. Gradually, however, the vWF activity increased in each culture to a mean of 12.3±1.8 μg/ml medium at 72 hr. In addition, the fact that the bulk of the cells in the cultures were endothelial was established by immunofluorescence. The sum of these observations is consistent with previous reports that endothelial cells synthesize a protein with vWF activity.

As described in Materials and Methods, protein with vWF activity was partially purified from endothelial cell culture media, relying mainly on successive agarose gel filtration steps. Figure 2 shows the chromatogram from the final 2% agarose gel filtration step; the void volume fractions were pooled, dialyzed.
against water, freeze-dried, and then reconstituted to 1/20 of the prelyophilization volume. Figure 3 shows that this protein solution gave an immunoprecipitin line when diffused against rabbit antiserum to purified human plasma FVIII/vWF and a reaction of identity with the purified human plasma FVIII/vWF protein. Both the protein synthesized by the human endothelial cell and human plasma FVIII/vWF showed immunoprecipitin lines of partial identity with bovine FVIII/vWF protein. Hence the protein concentrated from the culture medium is human and not bovine, which obviates the concern that FCS may have contained sufficient amounts of denatured or degraded bovine vWF protein to react with the rabbit antiserum to human FVIII/vWF.

Demonstration of enzymatic activity in culture medium before contact with endothelial cells. The hydrolysis of p-nitroaniline from the S-2160 synthetic tripeptide was used to detect trace quantities of proteolytic activity in culture medium. This method can be performed rapidly and reproducibly in the presence of protease inhibitors. Figure 4 shows that the amount of p-nitroaniline hydrolyzed from the tripeptide by the culture medium was directly proportional to the length of incubation or the volume of culture medium tested. No p-nitroaniline was cleaved when buffer was substituted for culture medium. The rate of hydrolysis of the tripeptide substrate by culture medium in the
Fig. 3. Immunodiffusion of (1) purified human plasma FVIII/vWF protein; (2) vWF-active protein recovered from culture medium overlying endothelial cells for 72 hr; (3) purified bovine plasma FVIII/vWF protein; (4) purified human plasma FVIII/vWF; and (5) protein recovered from culture medium that was never in contact with endothelial cells and that was prepared by the same fractionation method as the protein in well No. 2. Center well contains rabbit antiserum to purified human plasma FVIII/vWF protein.

Fig. 4. Proteolytic activity of culture medium containing nonheated FCS as quantitated by hydrolysis of S-2160 tripeptide. Tripeptide substrate was incubated with buffer, 0.05 ml culture medium, or 0.15 ml culture medium. Amount of p-nitroaniline hydrolyzed, ordinate; incubation time of culture medium with S-2160 tripeptide, abscissa. Mean ± SD of at least two separate experiments performed in triplicate.
experiment shown in Fig. 4 was $3.6 \pm 0.2 \times 10^{-6}$ mmole/ml culture medium/min. It is emphasized that this rate varied considerably depending on the lot of FCS used to make the culture medium, the mean rate for culture media made with three lots of FCS being $2.75 \pm 0.8 \times 10^{-4}$ mmole/ml medium/min. By comparison this amount of protease activity represents approximately 0.09 NIH unit thrombin/ml medium.

Elimination of proteolytic activity in FCS. Since culture medium without FCS did not hydrolyze the synthetic tripeptide substrate, it was concluded that any proteolytic activity present in culture medium must originate from the FCS. Therefore to eliminate the proteolytic activity in culture medium two approaches were tried: (1) to rid the FCS of proteolytic activity by heating at 56°C or 66°C before using it to prepare culture medium, or (2) to inhibit proteolytic activity by the addition of molar excesses of protease inhibitors to culture medium made with nonheated FCS.

Figure 5 shows that varying but measurable amounts of protease activity were consistently found in different lots of FCS whether heated at 56°C for 0.5 or 2 hr. In general, culture medium made with FCS heated to 56°C for 0.5 hr released $p$-nitroaniline at $\sim 30\%$ of the rate for culture medium made with nonheated FCS; for medium made with FCS heated at 56°C for 2 hr, 16% of the proteolytic activity toward the synthetic substrate was retained. Different lots of FCS had a range of hydrolytic rates of $0.36-1.8 \times 10^{-6}$ mmole $p$-nitroaniline/ml/min for culture medium containing FCS heated for 0.5 hr (mean

![Fig. 5. Effect of temperature on protease activity of growth media containing heated FCS. Amount of $p$-nitroaniline (mean ± SD) cleaved from S-2160 tripeptide substrate, ordinate; incubation time of growth medium with the tripeptide substrate, abscissa. Culture medium made with nonheated FCS, FCS heated to 56°C for 0.5 or 2 hr, or FCS heated to 66°C for 2 hr was incubated with the tripeptide. Means for five experiments performed in triplicate; SD not given because effect of incubating FCS at 56°C was dependent on lot of FCS being used.](image-url)
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1.03 x 10^-4); the value of 0.54 x 10^-6 mmole p-nitroaniline/ml/min was observed for culture media made with FCS heated at 56°C for 2 hr. Only when the FCS had been heated at 66°C for 2 hr before being used to make culture medium was the proteolytic activity completely eliminated. If heated at this temperature for a lesser period of time, trace proteolytic activity was still observed.

Because heating FCS might destroy certain substances required for optimal cell growth, attempts were made to block proteolytic activity with various protease inhibitors added to culture medium containing nonheated FCS. Based on the rate of hydrolysis of the tripeptide substrate, greater than 1000-fold molar excess of SBTI, BPTI, or FOY-305 was used in these experiments. To compare the effectiveness of each inhibitor, the rate of hydrolysis of the tripeptide by 0.15 ml culture medium was quantitated before and after the addition of the following inhibitors: SBTI, 0.46 mg; BPTI, 1.56 mg; FOY-305, 0.02 and 0.04 mg. As shown in Fig. 6, only FOY-305 substantially reduced the proteolytic activity of culture medium, the rate of 0.57±0.15 x 10^-6 mmole p-nitroaniline/ml culture medium/min being only 16% of that for culture medium without inhibitor.

Culture medium containing DFP-FCS was also assayed for protease activity toward the synthetic tripeptide. In these experiments, the average hydrolytic rate was ~10% (0.39±0.17 x 10^-6 mmole p-nitroaniline/ml culture medium/min) that of culture medium containing untreated FCS. Despite large excesses of DFP, proteolysis of the synthetic tripeptide could never be completely inhibited. Thus of the methods investigated to reduce the proteolytic activity of culture medium, only heating FCS to 66°C for 2 hr resulted in total inhibition.

Fig. 6. Effect of protease inhibitors on proteolytic activity in culture medium made with nonheated FCS. Proteolysis quantitated by the amount of p-nitroaniline cleaved from tripeptide substrate with time. Following inhibitors were used: none (---), soybean trypsin inhibitor (SBTI, ---), bovine pancreatic trypsin inhibitor (BPTI, - - - ), FOY-305 (---), and diisopropylphosphofluoridate (DFP, . . .). Values for BPTI and FOY-305 are means ±SD of three experiments with triplicate assays for each sample; those for SBTI are from one experiment using triplicate assays.
Effect of endothelial cells on the proteolytic activity of culture medium. Thus far our results indicate that proteases exist in culture medium before contact with endothelial cells. Since the cells themselves might either potentiate or reduce preexistent proteolytic activity by the synthesis and release of proteases, activators, or inhibitors, culture medium that had overlain endothelial cells was analyzed with respect to time for its ability to hydrolyze the S-2160 tripeptide. Seven confluent endothelial cell cultures, each derived from a different umbilical cord 3–24 wk earlier, were overlaid with 4 ml fresh culture medium containing unheated FCS and no added proteolytic enzyme inhibitors. Immediately after adding the medium, 1 ml was withdrawn from each culture and placed in a sterile vial. The cultures and vials were incubated at 37°C for 72 hr, at which time the medium overlying each culture was harvested separately. Each of the 14 samples was then assayed for proteolytic activity towards the synthetic tripeptide. Medium removed from the cultures before incubation at 37°C hydrolyzed an average of 2.2±0.1 × 10⁻⁶ mmol p-nitroaniline/ml culture medium/min. The culture medium that had been in contact with the endothelial cells for 72 hr did not show an increase in proteolytic activity; instead, the medium was found to have lost an average of 30% of its activity toward the synthetic substrate.

Because 66°-FCS instead of nonheated FCS might contain less nutrients for the maintenance of endothelial cell function, the effect of medium containing 66°-FCS on cell viability and production of vWF activity was examined. The facts that the cells continued to adhere to the flask, appeared viable, and continued to produce vWF activity were assumed to be indicators of viability during the 72-hr incubation; however, compared to culture medium made with nonheated FCS, the cells did not proliferate as well in culture medium made with 66°-FCS. Hence quantitation of protease activity released from endothelial cells in culture medium containing 66°-FCS was made using cultures that grew to only 25°–33° of the flask surface area. Furthermore, these cells synthesized vWF-active protein at only about 15°–20° of the rate when grown in media made with unheated FCS. Comparisons of samples of 66°-FCS culture medium before and after overlaying each of five endothelial cell cultures for 72 hr showed that none promoted the hydrolysis of p-nitroaniline from the synthetic tripeptide substrate. Thus these results are consistent with the interpretation that endothelial cells do not contribute significantly to the proteolytic activity found in culture medium in contact with endothelial cells for 72 hr. By 72 hr, preliminary assays were ambiguous with respect to the presence of FVIII procoagulant assay; this may represent a problem in concentration and handling of the harvested media. We are continuing to explore this question.

Effect of culture medium on the activities of purified plasma FVIII/vWF. These experiments were designed to assess whether the very small amounts of proteolytic activity in culture medium that had never been in contact with endothelial cells could destroy one or both of the biologic activities of added purified plasma FVIII/vWF protein. The culture media tested for an effect on FVIII and vWF activities were made (1) without FCS, (2) with FCS, (3) with 56°-FCS, (4) with 66°-FCS, or (5) with DFP-FCS. Since growth of bacteria or fungi in the culture media could conceivably give rise to proteases during the
time of incubation, each of the different culture media also contained penicillin 100 U/ml, streptomycin 100 μg/ml, and fungizone 0.25 μg/ml. After purified FVIII/vWF protein was added to give a final concentration of 0.07–0.08 mg/ml, the different culture media were incubated at 37°C. FVIII and vWF activities were determined immediately after the addition of the plasma FVIII/vWF and then at 6, 12, 24, 48, and 72 hr and expressed as a percentage of their initial values.

Figure 7 shows a comparison of the stability of FVIII procoagulant activity in culture medium made with 56°C-FCS versus that made with 66°C-FCS. The protease activity in culture medium containing 56°C-FCS progressively degraded FVIII procoagulant activity until only 26% ± 10% remained at 72 hr. In marked contrast, however, FVIII procoagulant activity was completely stable for 48 hr in culture medium made with 66°C-FCS. Moreover, 70% of the FVIII procoagulant activity was still present at 72 hr.

Different preparations of culture medium made with FCS heated to 56°C for 0.5 hr varied not only in their content of proteolytic activity, whether defined by the rate of release of p-nitroaniline from the tripeptide substrate, but also in the rate of destruction of FVIII procoagulant activity. When plasma FVIII/vWF protein was added to any culture medium made with FCS heated to 56°C for 0.5 hr, the rate of loss of FVIII procoagulant activity could be generally correlated with the level of proteolytic activity as measured by the cleavage of the tripeptide. For example, in the set of experiments shown in Fig. 7 only 12% of the added FVIII activity had been destroyed at 6 hr in culture medium that contained 25% of the initial proteolytic activity toward the synthetic tripeptide. In the experiments shown in Table 1, 72% of the initial FVIII procoagulant activity was destroyed over the same time period in culture medium that contained ~50% of its initial proteolytic activity towards the synthetic tripeptide. However, in those situations where the FVIII procoagulant activity was not destroyed, i.e., in culture medium made with 66°C-FCS, proteolytic activity
Table 1. Effects of Variously Treated Fetal Calf Sera on FVIII/vWF Protein Added to Growth Media

<table>
<thead>
<tr>
<th>Growth Media Made With</th>
<th>FVIII Activity at 6 hr With Respect to Zero Time*</th>
<th>vWF Activity at 6 hr With Respect to Zero Time†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer only</td>
<td>99%</td>
<td>110%</td>
</tr>
<tr>
<td>Unheated FCS</td>
<td>9%</td>
<td>110%</td>
</tr>
<tr>
<td>1.7 mM DFP-FCS</td>
<td>13%</td>
<td>94%</td>
</tr>
<tr>
<td>Heated FCS</td>
<td></td>
<td></td>
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<tr>
<td>56°C x 0.5 hr</td>
<td>28%</td>
<td>125%</td>
</tr>
<tr>
<td>66°C x 2 hr</td>
<td>102%</td>
<td>110%</td>
</tr>
</tbody>
</table>

*Mean of at least two experiments with duplicate assays.
†Mean of duplicate assays.

measured by the tripeptide assay was absent. Although to some extent this is still speculative, the lack of perfect correlation between the results of the two assays for proteolytic activity when 56°C-FCS was used in culture media may reflect dissimilar susceptibilities to heat denaturation for different proteases that also vary in concentration in different lots of FCS.

The data in Table 1 show the disparity between the stability of the FVIII procoagulant activity and the vWF activity of purified FVIII/vWF protein after a 6-hr incubation in culture media. Neither FVIII activity nor vWF activity was degraded in culture medium made without FCS. These data also show that when incubated in culture medium or culture medium containing a modified FCS the FVIII activity was preserved for 6 hr only in culture medium containing 66°C-FCS. Finally, these data show that vWF activity was preserved in each of the five media studied. In particular, it was preserved in culture medium, culture medium containing 56°C-FCS, and culture medium containing DFP-FCS, all of which readily inactivated FVIII procoagulant activity.

When purified FVIII/vWF protein was incubated in a culture medium that had protease activity towards the synthetic tripeptide, the apparent stability of FVIII activity was dependent on the assay used. The most dramatic example occurred when FVIII/vWF protein was incubated in culture medium made with nonheated, non-aluminum hydroxide-adsorbed FCS. As much as 210% of the initial procoagulant activity was present after a 6-hr incubation period when the one-stage assay was used to measure FVIII activity in samples not adsorbed with aluminum hydroxide. If, however, the 6-hr samples were adsorbed with aluminum hydroxide just before assay, the apparent amount of FVIII activity in the culture medium was only 20% of the initial value. When FVIII activity was measured by the two-stage method, only 55% of the initial FVIII activity remained after a 6-hr incubation in the culture medium made with the unmodified FCS; aluminum hydroxide adsorption of the samples before assay lowered its value to 8%. These findings are compatible with serum procoagulant accelerators being present and shortening the clotting time of assays designed specifically for the measurement of FVIII procoagulant activity. These findings, which certainly have been pointed out before,14 were apparent in the earliest test sample removed from the incubation mixture; if not adsorbed with aluminum hydroxide before assaying by the one-stage
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Method, this sample had 170% of the FVIII activity of a control mixture made by adding the same amount of FVIII/vWF protein to culture medium without FCS. Therefore FVIII values obtained by the two-stage method on samples adsorbed with aluminum hydroxide just before assay were considered the most reliable index of FVIII activity, since the effects of serum procoagulant accelerators, whether specific or nonspecific, are minimized.

Jaffe et al. reported that FVIII procoagulant activity of added purified plasma FVIII/vWF protein was stable for 72 hr in culture medium overlying endothelial cells. Their medium contained 25% FCS that had been adsorbed with aluminum hydroxide and then heated to 56°C for 0.5 hr; however, they followed the FVIII procoagulant activity by the one-stage assay on samples that were not readorsbed with aluminum hydroxide before performing the assay. Because our results indicated that significantly less FVIII procoagulant activity was detectable by the two-stage assay as compared to the one-stage assay and because culture medium made with FCS adsorbed with aluminum hydroxide prior to heating to 56°C for 0.5 hr retained significant protease activity towards the synthetic S-2160 tripeptide, we designed the following experiment: FCS that had been adsorbed with aluminum hydroxide and heated to 56°C for 0.5 hr was used to prepare culture medium. Purified plasma FVIII/vWF protein was added to this medium, and the mixture was immediately assayed for its level of FVIII procoagulant activity by the one- and two-stage methods before and after aluminum hydroxide readsortption of the test sample. After incubating for 72 hr at 37°C, the mixture was again assayed by the same methods. Figure 8 shows that the “apparent” stability of FVIII procoagulant activity in culture medium made with this type of FCS depended to a great extent on how the FVIII activity assay was performed. After incubation for 72 hr, the FVIII procoagulant activity appeared to be retained fully (110% ± 6%) when the one-stage method was performed on test samples not readorsbed with aluminum hydroxide. However, if the samples were readorsbed, the residual FVIII procoagulant activity was only 30% ± 2.7% of its original value. Significantly, samples assayed by the two-stage method showed that only 57% ± 5% of the initial amount of FVIII procoagulant activity was
present after a 72-hr incubation; when the sample was readorsed with aluminum hydroxide before performing the two-stage assay, this value was reduced to 32% ± 3%. It is important to note that a purified solution of plasma FVIII/vWF protein subjected to the same adsorption steps as the culture medium did not lose activity; therefore it cannot be concluded that the readorsorption step removed FVIII procoagulant activity. Instead, it would seem that certain serum procoagulant accelerators, capable of enhancing the apparent level of FVIII procoagulant activity, were removed by the aluminum hydroxide.

**DISCUSSION**

Although endothelial cells in tissue culture have been shown to synthesize and release a protein with vWF activity, FVIII procoagulant activity has never been recovered from such cultures. It is possible that the protein that has vWF activity and crossreacts with heterologous antiserum to human plasma FVIII/vWF is distinct from the one that has FVIII procoagulant activity. Alternatively, the endothelial cells might synthesize a protein that initially has both FVIII and vWF activities, but because of proteases in the culture medium the FVIII activity is rapidly degraded while the vWF activity remains. It is generally agreed that purified plasma FVIII/vWF protein and its two activities elute in the void volume when gel filtered on 4% agarose in 0.15 M NaCl. There is also consensus that the protein peak elutes in the void volume coincident with the peak of vWF activity when purified FVIII/vWF is gel filtered on 4% agarose in buffered 0.25 M CaCl₂; however, the FVIII procoagulant activity elutes much later.¹¹,¹⁵⁻¹⁹

Although these observations have been reproduced in several laboratories, their interpretation is not so uniform, and in general they have given rise to two hypotheses: Switzer and McKee" contended that the protein with FVIII procoagulant activity results from the activation in vitro of precursive FVIII/vWF protein by certain trace proteolytic enzymes (e.g., thrombin) that form during blood collection or processing. They maintained that the procoagulant-active FVIII/vWF species interacts with the 4% agarose in 0.25 M CaCl₂ and its elution is therefore delayed. They also proposed that a precursive FVIII/vWF protein is mostly degraded in vivo by trace levels of proteolytic enzymes such as plasmin with the concomitant destruction of its procoagulant potential and therefore its tendency to interact with agarose. Thus a large concentration of a minimally cleaved protein lacking procoagulant potential but retaining > 70% of its vWF activity would be generated, and this protein elutes in the void volume from 4% agarose in 0.25 M CaCl₂.¹¹ Other investigators have offered an alternative interpretation that in its simplest form states that FVIII procoagulant protein is distinct from vWF protein.¹⁵,¹⁶ In general, they propose that in vivo the FVIII procoagulant protein circulates in a noncovalently bound complex with the vWF protein; some have suggested that the two proteins become loosely associated during processing and purification. By 4% agarose gel filtration and sucrose density gradient centrifugation, several investigators¹⁵,¹⁷,¹⁹ have estimated that the FVIII procoagulant-active protein has a molecular weight of 100,000–200,000 daltons, whereas in our laboratory results obtained
by sodium dodecyl sulfate gel electrophoresis and Sephadex G-200 gel filtration suggest a molecular weight in excess of 400,000 daltons. Virtually all investigators agree that the molecular weight of the void volume protein associated with vWF activity is >10^6 daltons and that it is composed of an undetermined number of disulfide-bound subunits of ~200,000 daltons. FVIII procoagulant activity is markedly enhanced by trace amounts of thrombin or trypsin; moreover, as little as 0.05 U/ml thrombin, 0.1 U/ml trypsin, or 0.01 CTA U/ml plasmin can completely inactivate the procoagulant activity of a substantial amount of purified FVIII/vWF protein in a matter of minutes.

Switzer and McKee showed that FVIII/vWF protein, which has its procoagulant activity completely destroyed by thrombin or plasmin, retains its vWF activity as well as its reactivity to heterologous antibody to purified plasma FVIII/vWF protein.

Jaffe et al. reported that FVIII procoagulant activity did not develop in endothelial cell cultures under conditions in which the procoagulant activity of added purified plasma FVIII/vWF remained stable. Moreover, their observation that the procoagulant activity of added purified FVIII/vWF protein to the culture medium overlying cells was unaffected for prolonged incubation periods led them to conclude that protease activity did not account for the lack of endogenous FVIII procoagulant activity in endothelial cell cultures. The apparent retention of added FVIII procoagulant activity in their experiments, however, could be explained by (1) the presence of a specific or nonspecific serum procoagulant that bypasses the FVIII/vWF-IXa-X interactions or (2) the development of a balance between partially activated and inactivated FVIII procoagulant species. It also must be considered that the inability to show endogenous FVIII procoagulant activity in endothelial cell cultures might result because only a trace quantity of FVIII procoagulant protein is made and released by the very small number of endothelial cells and hence the level of protease activity required for its destruction is extremely slight and may be undetected in the usual assay. This last concern is particularly bothersome because the present methods for assaying FVIII procoagulant activity may simply not be sensitive enough to detect that level present at any given time under the usual conditions of endothelial cell culture. Obviously under the conditions the vWF activity of the protein would be essentially unaffected; hence it would increase during the 72-hr incubation period.

Our results confirm and expand the observation that endothelial cells grown in tissue culture synthesize a protein with vWF activity. Moreover, this protein reacts with an antibody to human plasma FVIII/vWF protein, and as in previous experiments by others it does not have FVIII procoagulant activity. Unlike the results of Jaffe et al., however, our data clearly indicate that proteolytic activity sufficient to destroy FVIII procoagulant activity is present in endothelial cell culture media. Such trace proteolytic activity, which we have shown by two independent methods, appears to be derived entirely from the FCS component of culture medium. The results given by the two assays always agreed with respect to detecting the presence or absence of proteolytic activity in a given culture medium; however, the rate of hydrolysis of the synthetic S-2160 tripeptide substrate did not correlate perfectly with the rate of degrada-
tion of FVIII procoagulant activity. Of the seven modifications made to rid culture media of trace levels of proteases, only culture medium containing 66° FCS had undetectable proteolytic activity as measured by the tripeptide assay or by the survival of added FVIII procoagulant activity for prolonged periods of time. The addition of SBTI, BPTI, or FOY-305 to culture medium did not completely inhibit protease activity when assayed using the tripeptide substrate. Culture medium containing nonheated FCS, DFP-FCS, 56°-FCS, or FCS adsorbed with aluminum hydroxide and heated to 56°C also hydrolyzed the tripeptide substrate. Moreover, all of these various culture media significantly degraded the procoagulant activity of added purified plasma FVIII/vWF in the period of time that the culture medium ordinarily would remain in contact with the endothelial cells. Hence our results indicate that despite excesses of various protease inhibitors sufficient proteolytic activity remains in culture media to destroy the FVIII procoagulant potential of any FVIII/vWF protein that might be released by the endothelial cells. These findings are not in accord with the conclusions of Jaffe et al. Although it is not possible to provide the exact explanation for the lack of agreement, this discrepancy between their results and ours might be explained by differences in the methods used to quantitate FVIII activity. Our results clearly indicate that it is important to adsorb test samples with aluminum hydroxide just before assaying for FVIII procoagulant activity. Otherwise serum procoagulant accelerators may confound interpretations by shortening the clotting times of the FVIII assays. We found this particularly true in the one-stage assay.

Under the conditions in our laboratory, the endothelial cell cultures consistently showed increasing levels of vWF activity during a 72-hr period. Specifically, the average vWF activity of the culture medium increased from undetectable levels at zero time to ~12.3 µg/ml at 72 hr. The development and rate of increase of vWF activity was independent of the age of the endothelial cells being monitored, averaging about the same for 2- and 5-wk-old cultures. This observation is certainly consistent with protein synthesis, as shown definitively by Jaffe et al., who found ³H-leucine in protein released by the endothelial cells into the culture medium and 5.5% of the incorporated label present in the high molecular weight fraction prepared from endothelial cell culture medium was in a protein precipitable by heterologous antibody to FVIII/vWF protein. As in the case with plasma FVIII/vWF, the vWF activity generated in endothelial cell culture media eluted in the void volume fractions from 2% agarose columns. The protein in these fractions not only had the highest specific vWF activity, as shown in Fig. 2, but also gave an immunoprecipitin line of identity with purified human FVIII/vWF protein when diffused against heterologous antiserum to human plasma FVIII/vWF. Of importance was our demonstration that a reaction of partial identity occurred between purified bovine plasma FVIII/vWF and purified human FVIII/vWF protein when antiserum to human plasma FVIII/vWF was used. Therefore the partially purified protein we recovered from the void volume fractions was of human origin and not simply degraded bovine vWF protein from the FCS.

We could not find FVIII procoagulant activity in any of our studies of culture media that had overlain endothelial cells for 72 hr. Although this result is in
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accord with the interpretation that these cells do not make the moiety responsible for FVIII procoagulant activity, our findings that culture medium, even before coming in contact with endothelial cells, contains sufficient protease activity to destroy the procoagulant potential of FVIII/vWF protein offers an alternative view. In short, we suggest that whether or not endothelial cells in fact synthesize a protein(s) with both FVIII and vWF activities remains an open question. As alluded to earlier, the problem of showing FVIII procoagulant activity in these culture media is probably compounded by the exceedingly low concentration of FVIII/vWF procoagulant-active protein to be expected in such media at any time during the incubation period if in fact it were being synthesized by endothelial cells. In this respect it is important to note that the procoagulant activity of the very large amount of purified plasma FVIII/vWF used in the experiments shown in Fig. 7—approximately six times the maximal level produced by endothelial cells—is unlikely to be degraded as fully as the trivial amounts of FVIII/vWF protein being released into the culture media at any given time during the 72-hr incubation. Hence the striking loss of procoagulant activity, although it is not destroyed completely, of the large amount of added purified plasma FVIII/vWF shown in Fig. 7 supports our proposal that the amount of protease(s) that can do this could certainly fully destroy trace amounts of "procoagulant-active" FVIII/vWF immediately upon its release into the medium.

These considerations are supported by the extrapolation that despite millions of endothelial cells lining the intact circulatory system, the concentration of circulating plasma FVIII/vWF protein is essentially at trace levels. Presently we are evaluating some of these problems; at this time, the only culture medium that reproducibly lacks protease activity and in which endothelial cells will still synthesize vWF activity is that containing 66°FCS.

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Destruction of factor VIII procoagulant activity in tissue culture media

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