Biosynthesis and Secretion of Factor VII, Protein C, Protein S, and the Protein C Inhibitor From a Human Hepatoma Cell Line

By Daryl S. Fair and Richard A. Marlar

Using specific radioimmunoassays, 8 day cultures of Hep G2 cells were shown to contain in their supernatants 16, 74, and 828 ng/ml of factor VII, protein C, and protein S, respectively. These proteins and the protein C inhibitor were functionally active, and each of these activities was neutralized by their respective polyclonal antibodies. Although vitamin K had a modest effect, warfarin decreased the activity of secreted factor VII, protein C, and protein S by 50% to 90%. Protein C and protein S antigens were reduced three- to fourfold by warfarin. The protein C inhibitor antigen and activity were unaffected by vitamin K or warfarin treatment. Intrinsic labeling and immunoprecipitation indicated that factor VII, protein S, and the protein C inhibitor were secreted as 52,000, 77,000, and 58,000 molecular weight (mol wt) proteins, respectively. Protein C was secreted as a single-chain protein of about 65,000 mol wt, indicating that all of the vitamin K-dependent proteins are translated and secreted as single-chain molecules. Each of the four proteins studied represented their plasma protein counterparts structurally, functionally, and immunologically. Thus, all of the known soluble components of the protein C pathway are produced by liver parenchymal cells.

© 1986 by Grune & Stratton, Inc.

Vitamin K-dependent proteins are characterized as glycoproteins that are carboxylated at selected glutamic acid residues (gla) by a posttranslational process involving at least two enzymes and vitamin K.2 Warfarin is an antagonist of vitamin K and inhibits this reaction. The gla residues permit the binding of calcium, which induces a conformational change in the protein and mediates its binding to negatively charged phospholipid surfaces. Most, but not all of these proteins, contain a second unique alteration, namely, the presence of a β-hydroxyaspartic acid.3 The function of this modified residue is unknown.

Factor VII, a single-chain 50,000-molecular weight (mol wt) protein containing nine gla residues,5 initiates coagulation when associated with the lipophilic, nonenzymatic cofactor, tissue factor.1 It is an active zymogen exhibiting 0.8% to 4% of the activity of its activated serine protease form.6,9 In contrast, protein C, a two-chain 62,000-mol wt molecule containing 11 gla and one β-hydroxyaspartic acid residues is an inactive zymogen.3,4,10,11 However, once activated by thrombin-thrombomodulin, it is a potent anticoagulant serine protease that inactivates factors Va and VIIIa.2

The function of activated protein C is enhanced in the presence of another vitamin K-dependent molecule, protein S.13 This single-chain 75,000-mol wt protein, which contains nine to ten gla residues and one β-hydroxyaspartic acid residue has been proposed to function as a nonenzymatic cofactor for activated protein C.3,4,13-15 Inhibition of activated protein C is by a novel, recently isolated 57,000-mol wt single-chain plasma protein that neutralizes activated protein C by forming a stable 1:1 molar complex.16,17

The liver has been shown to be the primary source of plasma proteins including those of the coagulation and fibrinolytic systems, although this conclusion was based on indirect evidence of earlier studies.18,19 With the advent of animal organ perfusion and cell culture, activity studies were used as evidence for the liver and the parenchymal cell as the principal site of synthesis of most of the plasma proteins.20-25 Because the human molecules have been isolated and well characterized, the human hepatoma cell line, Hep G2, has been used to assign the site of synthesis of most of the zymogens and inhibitors of the coagulation and fibrinolytic systems to the hepatocyte.26-30 Factor VII has been shown to be produced by rodent liver cells.21,22,31-33 It has been assumed that most of the components of the protein C system (protein C and protein S) are produced by the liver based on clinical studies.24,35 We used the Hep G2 cell line to demonstrate that factor VII, protein C, protein S, and the protein C inhibitor are synthesized by hepatocytes and that each of the molecules represents their plasma protein counterparts structurally, immunologically, and functionally. In addition, we demonstrate that protein C is secreted as a single-chain molecule, analogous to factor X.30

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line, Hep G2, was obtained from Drs Barbara B. Knowles and David A. Aden of the Wistar Institute, Philadelphia. The cells were maintained in T-75 culture flasks containing Eagle's modified medium supplemented with 10% heat-inactivated fetal calf serum (FCS) by passage of at least 10⁶ cells per flask as previously described.39 Cells were occasionally cultured for four to five days in the absence of serum to test the effects of 1 ng/mL of a warfarin derivative, 3-(a-acetonyl benzy1)-4-hydroxycoumarin (Sigma Chemical Co, St Louis) or 10 ng/mL of vitamin K (Aqua Mephyton, Merck, Sharp and Dohme, West Point, Pa) as described previously.39
Proteins. Factor VII, protein C, and protein S, and the protein C inhibitor were isolated as previously reported. All proteins were homogenous and showed the characteristics of published preparations as judged by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis with and without a reducing agent.

Antisera and neutralization. All antisera used in this study were generated in rabbits as previously reported. Supernatants from four- and five-day serum-free cultures were concentrated 20-fold by ultrafiltration. A constant volume of supernatant was mixed with equal volumes of varying concentrations of specific antibody, and after incubation for 15 minutes at 37 °C, the samples were assayed for biologic activity. Activity values were expressed as percentages of pooled normal human plasma (NHP). Immunopurification of antibodies was accomplished by affinity chromatography on columns constructed of cyanogen bromide–activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ).

Immunoprecipitation. Cultures of Hep G2 cells were grown for ten days, washed, and incubated with 4 mL of methionine-free minimum essential medium containing 1 mmol/L benzamidine, 10 μg/mL vitamin K, 1 U/mL heparin (Sigma Chemical Co.) and 150 μCi/mL of 35S-methionine (Amersham Corp). After five to six hours, the supernatants were collected and clarified by centrifugation at 100,000 g for 45 minutes at 4 °C. The supernatants were supplemented with BSA (5 mg/mL) and cleared using Staphylococcus aureus previously washed in immunoprecipitation buffer (IPB; 12.5 mmol/L borate, pH 8.3, containing 7.5 mmol/L NaCl, 0.05% Tween 20, 0.5% Triton X-100, 1% Nonidet P-40, 10 mmol/L benzamidine, 1 mmol/L PMSF, 5 μU/mL Trasylol, and 0.02% NaN3). Aliquots were incubated with immunopurified antibodies or the IgG fraction (5 to 10 μg) of normal serum or antisera to factor VII, protein C, protein S, or protein C inhibitor in the presence of 25% (vol/vol) IPB. To confirm a precipitated band as a specific protein, we included 50 μg of the purified protein to compete with the labeled molecule for the specific antibody. After incubation overnight at 4 °C, the immune complexes were collected by the addition of S. aureus, incubated for 30 minutes at room temperature and centrifuged in a microfuge for three minutes. The pellets were washed as described previously, and the SDS-soluble material was electrophoresed on SDS–9%–polyacrylamide slab gels containing 14C-protein standards (New England Nuclear, Boston) in the absence and presence of 1% 2-mercaptoethanol. Slab gels were fixed in 10% acetic acid, 25% methanol for one hour, washed extensively with water, incubated with 1 mol/L sodium salicylate for 30 minutes, dried, and subjected to autoradiography.

RESULTS

Cell extracts and supernatants from eight-day cultures were analyzed for the presence of factor VII, protein C, and protein S using sensitive and specific double-antibody–competitive equilibrium radioimmunoassays. The supernatants contained an average of 16.3, 74.0, and 828 ng/mL of factor VII, protein C, and protein S, respectively. Cell extracts were prepared by incubating 7 × 10⁶ cells in 1 mL of 1% Triton X-100 containing 10 mmol/L EDTA for 30 minutes at 37 °C and diluting to 2 × 10⁶/mL with radioimmunoassay buffer. Factor VII, protein C, and protein S were measured at 8.5, 54.5, and 48.1 ng/mL, respectively. Hence, all three proteins were detected as secreted products and intracellular proteins, provided a sufficient number of cells were used.

To determine if our radioimmunoassay could accurately quantitate the proteins present in the supernatants and cell extracts of Hep G2 cells, we compared the ability of purified proteins, NHP, and a ten-day Hep G2 supernatant to inhibit the binding of iodinated molecules to their respective anti-
bodies. Figure 1 depicts a radioimmunoassay specific for protein C and shows that the protein C in plasma secreted by the cells was immunochemically identical to the purified molecule, as evidenced by the parallel lines of inhibition. Both the plasma and the supernatant did not completely inhibit the reaction, because of the relatively low concentrations of protein C in these preparations. FCS did not compete, indicating that bovine and human protein C are immunochemically distinct. Analogous results were obtained with the radioimmunoassays for factor VII and protein S. Only protein S showed some immunologic cross-reactivity between the bovine and human molecules as reported by others and was corrected for in the quantitation. Thus, our assays could accurately measure these three proteins secreted from the cells.

To determine the secretion rates of the vitamin K-dependent proteins from the Hep G2 cells, three T-75 cultures were each seeded with one million cells to give maximal rates of secretion and incubated with 10 mL of culture medium containing serum. On even numbered days, 2 mL of culture medium was removed from each flask and replaced with an equivalent volume of fresh medium. The samples were then quantitated for factor VII, protein C, and protein S by radioimmunoassay, and these values were corrected for the dilution by added medium. Figure 2 shows the increase in each of the proteins with time of culture. However, the absolute amounts of each protein differed with factor VII the least and protein S the greatest. The maximum rates of protein accumulation determined between day 6 and 10 was 5, 25, and 170 ng/mL/d for factor VII, protein C, and protein S, respectively.

The biologic activity of the secreted proteins was analyzed using specific coagulation assays and compared to the quantity of secreted protein. Activity and antigen values were normalized to NHP for comparison (Table 1). Hep G2 cells were cultured in medium containing serum for 7 days, washed, and cultured an additional four to five days in serum-free medium supplemented with 10 μg/mL vitamin K. The supernatants were collected, clarified by centrifugation, and concentrated 20-fold by ultrafiltration. The activities measured for factor VII, protein C, protein S, and the protein C inhibitor paralleled their respective antigen levels, indicating that the secreted molecules were fully functional with specific activities (activity/antigen) of about 0.96 to 1.23. Further, the activities of each of the four proteins could be specifically neutralized, if the concentrated supernatants were pretreated with their respective antibodies before assay (Table 1). Normal IgG did not significantly affect the function of the four molecules. Thus, these four secreted proteins were biologically active, and their activity could be inhibited by specific antibodies.

Previously, we showed that vitamin K and warfarin modulated the amount of prothrombin secreted by Hep G2 cells. The effect of these agents on the quantity and activity of secreted protein C, protein S, and the protein C inhibitor were studied (Table 2). Seven-day-old cultures were washed and incubated under serum-free conditions in the absence of antigen.
Table 1. Neutralization of Functional Activities of Factor VII, Protein C, Protein S, and the Protein C Inhibitor Secreted From Hep G2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Percentage of Activity*</th>
<th>Treatment†</th>
<th>Percentage of Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VII</td>
<td>4.7</td>
<td>buffer</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>normal IgG</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-factor VII</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Protein C</td>
<td>50</td>
<td>buffer</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>normal IgG</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-protein C</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Protein S</td>
<td>75</td>
<td>buffer</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>normal IgG</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-protein S</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Protein C inhibitor</td>
<td>100</td>
<td>buffer</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>normal IgG</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-PC</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

*Activity and antigen values are presented as percentages of pooled NHP.
†Supernatants were incubated with buffer, normal, or immune IgG (40 nmol/L) for 15 minutes at 37 °C and then assayed for activity.

Serum-free culture medium from Hep G2 cells was concentrated 20-fold by ultrafiltration.

and presence of 10 μg/mL vitamin K or 1 μg/mL warfarin for an additional four days. The concentration of these proteins remained constant (protein C) or increased slightly (protein S) in the presence of vitamin K. When the cells were cultured in the presence of warfarin, a three- to fivefold reduction in the total accumulated protein C and protein S was observed by electroimmunoassay (Table 2) or radioimmunoassay (data not shown). Accumulation of the protein C inhibitor (Table 2) and the total secreted protein (data not shown) were unaffected by either treatment. Functional activity of protein C, protein S, and factor VII was decreased by 50% to 90% in the presence of warfarin, whereas the protein C inhibitor activity was not affected. Also, protein C and protein S activities were disproportionately lower than their respective antigen values. For protein C, a tenfold reduction of activity was observed compared to only a threefold reduction in antigen. The protein S activity decreased fourfold, whereas the antigen only decreased 2.5-fold in the presence of the vitamin K antagonist.

To demonstrate the synthesis of these proteins by the Hep G2 cells, cultures of 10-day-old cells were washed and incubated with methionine-free MEM containing 150 μCi/mL of 35S-methionine for six hours at 37 °C. After centrifugation, the supernatants were immunoprecipitated and electrophoresed on SDS-polyacrylamide slab gels in the absence and presence of a reducing reagent. In selected experiments, excess unlabeled protein was added to the supernatant as a competitor before immunoprecipitation. Figure 3 depicts autoradiographs of the slab gels under reduced conditions. Factor VII and protein S were precipitated as single-chain species with apparent mol wts of 52,100 ± 1,600 and 77,300 ± 1,000, respectively. The antibody to the protein C inhibitor was known to recognize at least one other plasma protein. Therefore, to identify which of the components represented the inhibitor, we included 50 μg of highly purified protein C inhibitor in the immunoprecipitation reaction and looked for the mol wt band diminished in the presence of the added protein. Of the two major bands observed in the gel, only one was displaced by added inhibitor and corresponded to an apparent mol wt of 58,000 ± 800. Two minor bands of about 98,000 and 77,000 also appeared to decrease with addition of excess protein C inhibitor. Protein C migrated as a single, diffuse component with a mol wt of approximately 65,000 ± 3,000, indicating that protein C was secreted as a single-chain molecule. The addition of

Table 2. The Effect of Warfarin and Vitamin K on the Secretion and Activity of Protein C, Protein S, the Protein C Inhibitor, and Factor VII

<table>
<thead>
<tr>
<th>Protein C</th>
<th>Protein S</th>
<th>PC Inhibitor</th>
<th>Factor VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Act*</td>
<td>Ag*</td>
<td>Act</td>
<td>Ag</td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>45</td>
<td>47</td>
<td>77</td>
</tr>
<tr>
<td>Warfarin</td>
<td>3</td>
<td>16</td>
<td>15</td>
</tr>
</tbody>
</table>

*Antigen (Ag) quantitation was by electroimmunoassay and activity (Act) was measured in coagulation or amidolytic assays as described in Materials and Methods. Values are presented as the percentage of normal human plasma for each individual protein.

T-75 flasks were cultured for seven days in medium containing 10% FCS. After washing, the cells were incubated for four days in the presence of MEM (control), medium containing 10 μg/mL vitamin K or medium containing 1 μg/ml warfarin. Supernatants were concentrated 20-fold by ultrafiltration before analysis.
excess purified protein C before immunoprecipitation prevented recovery of this band. We did not observe mol wt bands in these precipitates that would correspond to the heavy and/or light chain of protein C. For comparison, iodinated purified protein C was added to the medium and immunoprecipitated under the same conditions as the Hep G2 supernatants. The characteristic two-chain form of protein C was seen with apparent mol wts of 45,000 ± 700 and 25,500 ± 2,000 for the heavy and light chains, respectively. Although the heavy chain is clearly evident, the light chain was much fainter, indicating a disproportionate iodination of the polypeptides. However, prolonged exposure time facilitated the detection of the light chain.

DISCUSSION

The Hep G2 cell has been shown to synthesize and secrete many of the proteins thought to be derived from the liver. The molecules of the coagulation and fibrinolytic systems demonstrated to be the product of this cell reflect their plasma counterparts structurally, immunochemically, and functionally. In this report we have extended these studies to include molecules that can initiate (factor VII) and regulate (protein C, protein S, and protein C inhibitor) the coagulation process. In each case, these molecules were biologically active, neutralized by specific antibodies, modulated by known pharmacologic agents, and were structurally and immunologically similar to the molecules isolated from plasma. Thus, this study provides the first direct evidence that the soluble components of the protein C pathway are produced by the liver and confirms the synthesis of factor VII by hepatocytes.

The finding that protein C is synthesized and secreted as a single-chain molecule is consistent with recently reported data from recombinant DNA (cDNA) cloning indicating that protein C is transcribed as a single contiguous messenger RNA (mRNA), and from immunoblotting experiments suggesting that 10% of plasma protein C is present in a single-chain form. Recently, single-chain protein C has been isolated from plasma and has the same activation and enzymatic properties as their isolated plasma counterparts. Protein S and the protein C inhibitor shared the same characteristics of the secreted protein. Characteristics of the secreted protein were very similar to those of the isolated molecule with a mol wt of about 50,000 and a similar immunological profile. Extrapolation to normal tissue from the findings in a tumor cell must always be guarded; however, very similar correlations between proteins isolated from plasma and characterized from this cell line make our conclusions tenable.

Protein S and the protein C inhibitor shared the same properties as their isolated plasma counterparts. Protein S had an apparent mol wt of 75,000 to 77,000 under reduced conditions, indicating that it was synthesized and secreted as the mol wt form originally reported but was smaller than the more recently published measurements of 84,000 daltons by Dahlback. We saw no evidence of proteolysis since mol wt estimates in reduced and nonreduced gels were similar and no degradation was seen if iodinated protein S was added to cell lysates. Immunologically and functionally, the secreted molecule was identical to the isolated plasma form. Secretion of protein S from these cells appears very efficient when compared to the other vitamin K–dependent proteins. The quantity of intracellular protein S is less than that measured for prothrombin, and the rate of secretion is about 50% greater. This may be attributed to different secretion mechanisms or to differences in culture conditions (ie, serum lots) and age of the cells (about 30 more passages in this study relative to the prothrombin analysis). Both protein C and factor VII have high secretion rates relative to factor X. This may also reflect a normal process since both protein C and factor VII have shorter half-lives and, therefore, would require higher synthetic rates to maintain a steady state. The protein C inhibitor was also very similar to its recently reported plasma counterpart functionally, immunochemically, and structurally. Thus, all the soluble components of the protein C pathway are produced by the hepatocyte.

One of the principal characteristics of the family of vitamin K–dependent proteins is the effect of warfarin-like drugs on the synthesis of these molecules. Previously, we demonstrated that prothrombin and factor X, but not antithrombin III, were accumulated in the culture medium at a lower rate than untreated cells. Protein C and protein S were shown to be similarly affected, with a reduction in the amount of each protein by about three- to fourfold. Protein C inhibitor, a protein thought not to be modulated by these drugs, was unaffected in the amount of secreted antigen and activity. The functions of protein C, protein S, and factor VII were significantly decreased in the presence of warfarin, and this reduction was greater than that observed for accumulated antigen. This indicates a decrease in the specific activity of the secreted molecules and is consistent with the findings obtained from patients undergoing warfarin therapy. Vitamin K did not significantly increase production of these molecules as observed with prothrombin. It is possible that prothrombin synthesis is more sensitive to...
vitamin K or that the concentration of vitamin K in our lot of FCS was optimal to produce and maintain a maximal rate of synthesis over the serum-free culture period. This latter suggestion is supported by the production of fully active proteins by cells not supplemented with exogenous vitamin K. Thus, our results suggest that the decrease in the vitamin K–dependent proteins (measured by antigen) present in the plasma of individuals undergoing warfarin therapy can be attributed to at least a decrease in the synthetic rate of these molecules. Increases in the catabolic rates may also occur and need to be investigated.

The human hepatoma cell line, Hep G2, has been of benefit in our determining the origin of a number of plasma proteins. This cell line has been shown to synthesize and secrete most of the vitamin K–dependent proteins. Factor IX is not transcribed (P. Tolstoshev, personal communication, Transgene, Strasbourg, France, January 1985) or translated by this cell,30 and protein Z is present in culture supernatants at very low concentrations (D. Fair, unpublished observations, June, 1984). In addition, this cell has the capacity to synthesize and secrete most of the proteins of the coagulation, fibrinolytic, and protein C systems including their inhibitors. Because the cell line responds normally to known pharmacologic modulators, it provides an excellent model to investigate the molecular events underlying these measured responses.

ACKNOWLEDGMENT

We thank David Revak, Kathy McCarthy, and Marsha McDonald for excellent technical assistance and Ellen Schmeding and Joy Lozano for preparation of the manuscript.

NOTE ADDED IN PROOF

After submission of our manuscript Morito et al (Biochim Biophys Acta 844:209, 1985) published the synthesis of the protein C inhibitor by Hep G2 cells. However, these investigators were unable to detect protein C in cell supernatants in contrast to our findings.

REFERENCES

31. Prydz H: Studies on proconvertin (factor VII). V. Biosynthe-
sis in suspension cultures of rat liver cells. Scand J Clin Lab Invest 16:540, 1964

32. Babior BM, Kipnes RS: Vitamin K dependent formation of factor VII by a cell-free system from rat liver. Biochemistry 9:2564, 1970


50. Marlar RA: Single chain protein C is enzymatically similar to the two chain form of protein C. Biochim Biophys Acta (in press)


Biosynthesis and secretion of factor VII, protein C, protein S, and the Protein C inhibitor from a human hepatoma cell line

DS Fair and RA Marlar