Human Hepatoma Cells Secrete Single Chain Factor X, Prothrombin, and Antithrombin III

By Daryl S. Fair and Bruce R. Bahnak

The human hepatoma cell line, Hep G2, was analyzed for the ability to synthesize and secrete several coagulation proteins. Using specific radioimmunoassays, factor X, prothrombin, and antithrombin III were present in 8-day culture supernatants at 62, 405, and 1,220 ng/mL, respectively. Factor IX was not detected, either in supernatants or in cell extracts. Intrinsically labeled factor X was secreted as a single-chain polypeptide of 66,000 daltons, as measured by sodium dodecyl sulfate-polyacrylamide gels under nonreduced and reduced conditions. Immunoblots of Hep G2 supernatants and normal human plasma also indicate the presence of single-chain factor X. These findings support the hypothesis of a postsecretory proteolytic cleavage of factor X into the two-chain form. Prothrombin and antithrombin represented their plasma protein counterparts structurally, with molecular weights of 73,000 and 61,000, respectively. Secreted factor X, prothrombin, and antithrombin III were biologically active, as determined in coagulation or chromogenic assays, and all three activities were neutralized by monospecific antibodies. Vitamin K increased the quantity of prothrombin secreted by twofold, without affecting the rate of secretion over a five-day culture period, and had an apparent transient inhibitory effect on secretion of antithrombin III. Warfarin caused a three to fourfold decrease in the rate and quantity of secreted prothrombin, but did not affect intracellular concentrations. The intracellular and extracellular concentrations and rate of secretion of antithrombin III were not modulated by warfarin. These data suggest that the Hep G2 cell line may provide a useful model for assessing the regulation of biosynthesis and secretion of human coagulation proteins.

Prothrombin is a single-chain glycoprotein of mol wt 72,000 and contains 10 gla residues in its aminotermi

The six identified vitamin K-dependent proteins present in human plasma are zymogens of serine proteases that are characterized by unique γ-carboxyglutamic acid (gla) residues located in the amino-terminal portion of the four single-chain proteins (prothrombin, factor VII, factor IX, and protein S) and in the amino-terminus of the light chain of both factor X and protein C. These latter two proteins are composed of two chains coupled by disulfide bonds. The proposed function of the gla residues is to bind calcium, which induces a conformational change in the protein and mediates the binding of the protein to phospholipid membranes. Carboxylation of certain glutamic acid residues in these proteins occurs posttranslationally and is dependent on microsomal enzymes that require vitamin K as a cofactor. Warfarin has been shown to be an antagonist of vitamin K in vitro, and its administration in vivo decreases the activity and concentration of the family of vitamin K-dependent proteins.

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cells. In contrast, studies using rat hepatocytes or hepatoma cells have indicated that factor X and antithrombin III are synthesized in the liver. To resolve this species difference in the cellular origin of these proteins, we utilized a recently described human hepatoma cell line, Hep G2. This cell line has retained the capacity to synthesize a variety of plasma proteins, including those of fibrinolysis, coagulation, complement, lipoproteins, and others. We have quantitatively analyzed this cell line for the synthesis of prothrombin, factor X, and antithrombin III, and we show that the secreted molecules represent their plasma protein counterparts immunologically and functionally. In addition, factor X was found to be secreted as a single-chain polypeptide, in contrast to the two-chain form isolated from plasma. The secretion of these proteins can be modulated by warfarin and vitamin K.

**MATERIALS AND METHODS**

**Cell Culture**

The human hepatoma, Hep G2, was provided and characterized by Drs Barbara B. Knowles and David P. Aden of the Wistar Institute, Philadelphia, Pa.

Cells were maintained in culture by routine passage into T-75 flasks containing Eagle's minimal essential medium (MEM) and 10% heat-inactivated fetal calf serum (FCS). Depending on the experiment, cells were seeded at varying densities (0.5–2 × 10^6 cells/flask), and for kinetic studies, aliquots (0.5 mL) were removed every 2 days and stored at −20°C until analysis. In selected experiments, cells from day 10 cultures were washed 3 times with 0.01 mol/L phosphate (pH 7.4) containing 0.15 mol/L NaCl (PBS), and solubilized in 0.1% SDS and 0.1% Triton X-100. The cell extracts were dialyzed overnight against 100 vol of 0.1% Triton X-100 in PBS and assayed for specific antigen. Protein concentrations were determined by the method of Bradford, using bovine serum albumin as the standard.

For pharmacologic studies, 35-mm, 6-well plates (Costar, Cambridge, Mass) were seeded with 6.6 × 10^6 cells and grown for 7 days in MEM containing 10% FCS. The cultures were washed and incubated in serum-free MEM supplemented with glutamine. Cultures were grown in 4 mL of this medium (control), or in medium supplemented with 25 μg/mL vitamin K, (Aqua Mephyton, Merck, Sharp and Dohme, West Point, Pa), or 1 μg/mL of the warfarin derivative, 3-(α-acetyloxy benzyl)-4-hydroxycoumarin (Sigma Chemical Co, St Louis, Mo). On a given day, four tests and eight control wells were harvested and tested for the production of prothrombin and antithrombin III. All values were normalized to the quantity of either protein measured on day 1 in control cultures and are presented as the means and standard error of the means. In selected studies, cell extracts were prepared and analyzed for these proteins.

**Proteins**

Prothrombin, factor IX, factor X, thrombin, and antithrombin III were isolated as previously reported. All proteins were homogeneous, as judged by SDS-polyacrylamide gel electrophoresis in the presence and absence of a reducing agent.

**Antisera**

The antiserum to factor X has been previously characterized. For the three other proteins antisera were raised in female New Zealand rabbits injected with between 50 and 100 μg of purified protein emulsified in Freund’s complete adjuvant at multiple subcutaneous sites. Animals were challenged biweekly with 25–50 μg protein emulsified in Freund’s incomplete adjuvant. After 2 months, the antisera were judged to be of high titer and were monospecific as determined by the lack of competition of unrelated proteins in radioimmunoassays. For neutralization experiments, the IgG fraction of the immune and normal sera were isolated as previously described. Immunopurified antibodies were obtained by affinity chromatography on columns of prothrombin or factor X coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) by cyanogen bromide (CNBr) activation.

**Radioimmunoassays**

Double-antibody equilibrium radioimmunoassays were constructed for each of four coagulation proteins. Proteins were radioiodinated to a specific activity of 1–5 μCi/μg by the lactoperoxidase-glucose oxidase method (Enzymobeads; Bio-Rad Laboratories, Richmond, Calif). To 250 μL of 125I-protein ligand (0.5 mmol/L) was added 250 μL of competitor (purified plasma, culture supernatant, or cell extract) and 250 μL of rabbit antiserum diluted to give 40%–50% binding of the labeled ligand. After incubation at 4°C for 16 hours, 250 μL of goat anti-rabbit IgG was added and incubated at 4°C for 6 hours. Immunoprecipitates were collected by centrifugation at 2,000 g for 20 minutes at 4°C, and 500 μL of supernatant counted for radioactivity on an Iso-Data 20/20 series gamma counter (Iso-Data, Inc, Palatine, Ill). The buffer used for all dilutions was 42 mmol/L borate, pH 8.3, containing 25 mmol/L NaCl, 2% heat-inactivated normal rabbit serum, 1 mmol/L benzamidine, 1 mmol/L phenyl-methyl-sulfonyl-fluoride (PMSF), 10 μmol/L Trasylol (Bayer, Leverkusen, West Germany), and 0.2% sodium azide.

**Immunoprecipitation of Intrinsically Labeled Proteins**

A 10-day-old T-75 culture of Hep G2 was washed and incubated in 3 mL of methionine-free MEM for 15 minutes at 37°C. Methionine (14C; 1,400 Ci/mmol) was added to a concentration of 150 μCi/mL and incubated for 5 hours at 37°C with occasional gentle agitation. The supernatant was collected and clarified by centrifugation at 100,000 g for 30 minutes at 4°C. Direct immunoprecipitation was carried out by the addition of purified protein to 500 μL of labeled medium diluted with an equal volume of 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, 4 μmol/L Trasylol, and 0.02% NaN3) and the addition of heat-inactivated rabbit antiserum previously titrated to yield 30 μg of protein in the precipitates at equivalence. The precipitates were washed twice with IPB and solubilized by boiling for 3 minutes in 2% SDS and 1% 2-mercaptoethanol. The molecular weights of the soluble material were estimated on SDS-polyacrylamide gels, containing 7.5% acrylamide, under reduced conditions according to the method of Weber and Osborn. Transferrin (77,000) bovine serum albumin (67,000 mol wt), ovalbumin (43,000 mol wt), carbonic anhydrase (29,000 mol wt), and myoglobin (17,000 mol wt) were used as standards. The gels were sliced in 1.2-mm segments; the slices were incubated with 500 μL of 30% H2O2 at 30°C for 5 hours, and the radioactivity counted in the presence of 10 mL of Aquaphor (New England Biolabs).
Nuclear Corp, Boston) in a Beckman LS 7500 scintillation counter (Beckman Instruments, Inc, Fullerton, Calif).

Alternatively, Hep G2 factor X and pure iodinated factor X (added to the culture medium) were reacted with immunopurified antibodies to factor X. The immune complexes were collected on Staphylococcus aureus antibodies to factor X. The immune complexes were collected on Nuclear Corp. Boston) in slab gels5' under nonreduced and reduced conditions. After electrophoresis, the gels were treated with EN'HANCE (New England Nuclear), dried, and subjected to autoradiography.

**Immunoblotting**

Factor X, present in Hep G2 supernatants and in normal and factor X-deficient plasmas, was analyzed according to a modification of the method of Towbin et al.32 Normal and factor X-deficient citrated plasmas were treated with 1/10 vol of 1 mol/L barium chloride, and the protein associated with the barium citrate precipitate was eluted in 1/10 the original plasma volume with 0.2 mol/L sodium citrate, pH 7. Supernatants from Hep G2 were concentrated 75-fold by ultrafiltration on an Amicon apparatus (Amicon Crop, Danvers, Mass) using a PM-30 membrane. Purified factor X, the factor X eluted off barium citrate, and cell supernatants were electrophoresed in a 9.5% polyacrylamide slab gel containing 0.1% SDS. Normal and factor X-deficient plasmas were treated with 1/10 vol of 1 mol/L barium chloride, and citrated plasmas were treated with 1/10 vol of 1 mol/L barium chloride, and the protein associated with the barium citrate precipitate was eluted in 1/10 the original plasma volume with 0.2 mol/L sodium citrate, pH 7. Supernatants from Hep G2 were concentrated 75-fold by ultrafiltration on an Amicon apparatus (Amicon Crop, Danvers, Mass) using a PM-30 membrane. Purified factor X, the factor X eluted off barium citrate, and cell supernatants were electrophoresed in a 9.5% polyacrylamide slab gel containing 0.1% SDS, as described by Laemmli,33 and electrophoretically transferred to nitrocellulose paper using an E-C electroblot system at 300 mA for 15 hours at ambient temperature. After transfer, the paper was washed with PBS containing 1% bovine serum albumin (BSA) and 5% heat-inactivated normal goat serum (NGS) for 6 hours at ambient temperature. The paper was reacted with nonspecific antisemum to factor X diluted in this buffer for 16 hours at 4°C. After incubation, the paper was washed sequentially for 30 minutes each at ambient temperature with buffer A (0.1% BSA, 1% NGS, 0.05% Tween 20 in PBS), buffer A containing 0.5 mol/L LiCl, 0.1% SDS, and two washes with buffer A. Detection of factor X was by iodinated immunopurified goat anti-rabbit IgG, diluted to 0.2 µg/mL in buffer A. After overnight incubation at 4°C, the paper was washed as above, dried, and the radiolabeled protein was detected by autoradiography.

**Antibody Neutralization**

Hep G2 supernatants were incubated with an equal volume of 100 µg/mL of either affinity-purified anti-factor X or antiprothrombin or 1.5 mg/mL of an IgG fraction of rabbit antiserum to antithrombin III at 37°C for 30 minutes. The remaining factor X, prothrombin, or antithrombin III activities were measured in coagulation or chromogenic assays.

**Coagulation Assays**

Factor X activity was measured in the one-stage coagulation assay. To 50 µL of immunochemically depleted human factor X-deficient plasma was added 50 µL of rabbit brain cephalin (Sigma) containing 2 µg of Russell's viper venom/mL and 50 µL of 25-fold concentrated serum-free Hep G2 conditioned medium. After incubation for 1 minute at 37°C, 50 µL of prewarmed 25 mmol/L CaCl₂ was added, and the time for clot formation was measured. Prothrombin functional activity was determined in a reaction containing 50 µL of prothrombin-deficient human plasma (George King Bio-Medical, Inc, Overland Park, Kan) and 50 µL of 25-fold concentrated Hep G2 serum-free supernatant. After incubation at 37°C for 1 minute, 100 µL of rabbit brain thromboplastin containing 12.5 mmol/L CaCl₂ (General Diagnostics, Morris Plains, NJ) was added and the clotting time measured. Standard curves were constructed from dilutions of pooled normal human plasma, and 1 U of factor X or prothrombin was defined as the amount of activity of either protein present in 1 mL of pooled plasma.

**Chromogenic Assay**

Antithrombin III activity was measured by a decrease in the initial rate of thrombin cleavage of the chromogenic substrate Tos-Gly-Pro-Arg-p-nitroanilide (Chromozym-Th; Boehringer Mannheim, West Germany). To 50 µL of thrombin (3 µg/mL) was added 50 µL of heparin (4 U/mL, Sigma) and varying amounts of 96-hour Hep G2 conditioned serum-free medium. The final reaction volume was brought up to 200 µL with 0.01 mol/L Tris/HCl, pH 7.2, containing 0.15 mol/L NaCl and 0.1% bovine serum albumin. The reactants were incubated for 2 minutes at 37°C, and 100 µL of the reaction mixture was diluted with 800 µL of prewarmed 0.02 mol/L Tris/HCl, pH 8.2, containing 0.15 mol/L NaCl and 0.005 mol/L EDTA. Thrombin activity was measured with the addition of 100 µL of 1 mmol/L Chromozym-Th, and the initial rate of cleavage of the substrate at 37°C was followed by the linear increase in absorbance at 405 nm over a 5-minute period.

**RESULTS**

Supernatants and cell extracts of eight-day Hep G2 cell cultures were examined for the presence of components of the coagulation system by radioimmunoassay (Table 1). Prothrombin, factor X, and antithrombin III were present in the supernatants of the cultures at 405, 62, and 1,220 ng/mL, respectively. Factor IX was not detected at 10 ng/mL, which was the sensitivity of the assay. The mean proportion of prothrombin, factor X, and antithrombin III to the total secreted protein was 1.71%, 0.215%, and 6.07%, as measured in three separate experiments. Analysis of cell extracts indicated that factors IX and X could not be detected at a sensitivity of 10 ng/mL, whereas prothrombin and antithrombin III were present at 76.7 and 126 ng/10⁷ cells, respectively. Control experiments indicated that the extraction buffer did not interfere with the assays and that proteolysis of ³²P-prothrombin did not occur when incubated in the cellular extracts for 1 hour at 37°C.

To measure the secretion of prothrombin, factor X, and antithrombin III from these cells, it was necessary to validate the immunologic identity of the secreted

<table>
<thead>
<tr>
<th>Table 1. Coagulation Proteins in the Supernatant and Cell Extracts of Day 8 Hep G2 Cultures</th>
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<tbody>
<tr>
<td><strong>Protein</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Prothrombin</td>
</tr>
<tr>
<td>Factor IX</td>
</tr>
<tr>
<td>Factor X</td>
</tr>
<tr>
<td>Antithrombin III</td>
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</table>

*Concentrations were determined by specific double-antibody equilibrium radioimmunoassays with sensitivities of each assay below 10 ng/mL. The total secreted protein was 27 µg/mL.
†Cell extracts (475 µg cellular protein/mL) were equivalent to 10⁷ viable cells, as determined from counting parallel cultures.
proteins with their respective purified molecules. Figure 1 depicts the inhibition of binding of $^{125}$I-factor X to its antibody by the supernatant from a ten-day culture of Hep G2, purified factor X, normal human plasma, and control medium. Parallel lines of inhibition were observed with the first three competitors. Although the concentration of factor X in the culture supernatant was too low to completely inhibit the reaction, the parallel slope of the competition line indicated that the secreted molecule expressed most of the epitopes recognized by the antibody to the purified and plasma protein. Control medium containing 10% FCS did not compete, indicating that bovine factor X did not cross-react with the human molecule. Analogous results were observed for the competitive assays for prothrombin and antithrombin III.

After validating the measurements, we quantitatively determined the rate of secretion of these three proteins in culture. T-75 flasks were seeded with $5 \times 10^5$ cells, and every two days, 0.5 mL of the culture medium was removed and stored at $-20^\circ$C until assay. All three proteins increased in a near linear fashion over the ten-day culture period (Fig 2). However, the rates of secretion differed, with factor X being accumulated at the lowest rate. Prothrombin was secreted at about 7–8 times and antithrombin III at about 70 times the rate of factor X.

To determine if the rates of secretion were dependent on the initial plating density of the cultures, we inoculated T-75 flasks with 5, 10, and $20 \times 10^5$ Hep G2 cells in 15 mL of medium and measured the accumulation of factor X, prothrombin, and antithrombin III throughout 10 days of culture (Fig 3). The changes in secretion rates were parallel for the three proteins studied. Lower rates of secretion were seen with $5 \times 10^5$ cells relative to the higher plating densities, and maximal rates of secretion were reached when the cultures were seeded with $10 \times 10^5$ cells or
20 x 10^3 cells. At optimal rates, factor X, prothrombin, and antithrombin III were secreted at 8, 130, and 1,100 ng/mL/d, respectively. From these results, the remaining experiments were performed at the plating density of 10^6 cells in proportion to the culture vessel used.

Because the activities of the vitamin K-dependent proteins can be enhanced by vitamin K and reduced by warfarin, we examined the effects of these agents on the synthesis of prothrombin and antithrombin III. In preliminary experiments, we determined that the Hep G2 cells could be cultured under serum-free conditions for up to five days without affecting morphology or viability. Cells were plated in 35-mm dishes and grown for 7 days in MEM containing 10% FCS. The cells were washed and cultured for up to five days in MEM only (control) or in MEM supplemented with vitamin K or warfarin. A linear increase in the accumulation of prothrombin in the control medium was observed over the five days of culture (Fig 4A). When vitamin K was present, total prothrombin concentration increased twofold, whereas the rate of secretion was unchanged. Warfarin decreased both the rate and quantity of prothrombin accumulation 3-4-fold. When cell extracts were examined on each of the five days, warfarin did not induce a change in the intracellular prothrombin content compared to non-drug-treated controls (Table 2). However, in the presence of vitamin K, a 30%-40% reduction of intracellular prothrombin was observed. These results were constant for the five days of in vitro analysis in the presence of vitamin K. Control experiments showed that the slope of inhibition with descarboxyprothrombin in the radioimmunoassay were parallel to the regression line of competition seen with native prothrombin, validating the quantitation of the protein in our warfarin studies.

The quantity and rate of secretion of antithrombin III by the cells was not significantly affected by warfarin treatment (Fig 4B). Vitamin K, however, caused a lag in the production of antithrombin III for about three days, and then the rate of secretion approached the rate determined for the control cultures. The intracellular levels of antithrombin III were similar between warfarin-treated cells and controls (Table 2). Vitamin K-treated cells showed equivalent concentration until day 3, when lower cellular levels of antithrombin III were observed and coincided with the increased secretion of this protein.

To demonstrate direct synthesis of these three molecules, we intrinsically labeled the proteins with 35S-methionine. The Hep G2 cells were incubated in methionine-free medium and pulsed with 150 μCi/mL.

Table 2. Intracellular Concentrations of Prothrombin and Antithrombin III in Hep G2 Cells Cultured in the Presence of Warfarin and Vitamin K*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Modulator</th>
<th>Day 1†</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin</td>
<td>None</td>
<td>12.6 ± 1.1</td>
<td>15.0 ± 1.6</td>
<td>17.3 ± 1.7</td>
<td>14.4 ± 1.0</td>
<td>15.3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Warfarin</td>
<td>14.4 ± 0.7</td>
<td>14.0 ± 0.6</td>
<td>17.3 ± 2.3</td>
<td>16.4 ± 2.5</td>
<td>14.6 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Vitamin K</td>
<td>11.8 ± 0.7</td>
<td>12.0 ± 0.9</td>
<td>11.4 ± 0.4</td>
<td>17.7 ± 0.9</td>
<td>11.2 ± 0.9</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>None</td>
<td>22.9 ± 5.8</td>
<td>29.8 ± 5.5</td>
<td>53.0 ± 10.0</td>
<td>46.5 ± 10.4</td>
<td>45.4 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>Warfarin</td>
<td>26.5 ± 6.4</td>
<td>24.0 ± 3.1</td>
<td>52.2 ± 6.0</td>
<td>45.2 ± 7.1</td>
<td>29.2 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>Vitamin K</td>
<td>22.4 ± 6.6</td>
<td>28.4 ± 2.4</td>
<td>25.8 ± 2.7</td>
<td>28.3 ± 2.2</td>
<td>18.4 ± 2.5</td>
</tr>
</tbody>
</table>

*Hep G2 cells were grown in 35-mm dishes for 7 days in MEM containing 10% fetal calf serum. The cells were washed 3 times with MEM and cultured for 5 days in the presence of MEM only, or MEM containing warfarin (1 μg/mL) or vitamin K (25 μg/mL). Total cellular protein was extracted into 1 mL of 0.1% Triton X-100 and measured 0.154 ± 0.006 mg/mL (mean ± SEM, N = 60).
†Values (ng/mL) are expressed as the mean ± SEM (N = 4).
35S-methionine for 5 hours. Supernatants were clarified by centrifugation and subjected to direct immunoprecipitation. The precipitates were dissolved in SDS and electrophoresed on SDS 7.5%-polyacrylamide gels under reduced conditions (Fig 5). Factor X immunoprecipitates contained a single radiolabeled polypeptide with a molecular weight of 66,000, indicating that this molecule was synthesized and secreted as a single chain. This is in contrast to the two-chain form normally isolated from plasma. Prothrombin and antithrombin III were found to be secreted as single-chain molecules with molecular weights of 73,000 and 61,000, respectively.

Synthesis and secretion of single-chain factor X was further characterized using immunoprecipitation of 35S-methionine–labeled supernatants and of 125I–purified factor X added to medium only. After addition of anti-factor X antibody, the immune complexes were bound to Staphylococcus aureus and the labeled proteins electrophoresed in the presence and absence of a reducing agent (Fig 6). Gels run under nonreduced conditions revealed a novel band of radioactivity in the Hep G2 supernatants of 75,000 molecular weight, when immunoprecipitated with antibodies to factor X relative to nonimmune serum (Fig 6, left panel). This band migrated slightly less than the iodinated factor X isolated in an identical manner. Upon reduction, a unique band was observed that migrated with an apparent molecular weight of 65,000 when immunoprecipitated with anti-factor X antibodies compared to normal IgG (Fig 6, center panel). Iodinated factor X was separated into its characteristic heavy and light chains. If the supernatants were collected after prolonged culture (≥10 hours), the single-chain molecule (apparent mol wt of 75,000) was cleaved to the two-chain form (Fig 6, right panel), and this proteolytic event could be inhibited by benzamidine (data not shown). The heavy chain of about 50,000 mol wt was easily seen, but the light chain rarely was observed, probably because of the low occurrence of methionine in this polypeptide.

Single-chain factor X was also demonstrated in human plasma using an immunoblotting method. Barium citrate eluates from normal and factor X-deficient plasma, representing ten-fold concentrates, were electrophoresed under reducing conditions in parallel with 75-fold concentrated Hep G2 serum-free supernatant and pure factor X. After transfer to nitrocellulose, factor X was located using anti-factor X antibodies and iodinated goat anti-rabbit IgG (Fig 7). Normal plasma contained three areas that reacted with the anti-factor X antiserum and had apparent mol wt of 72,000, 63,000 and 50,000. The lowest molecular weight form was equivalent to factor X heavy chain. The middle band represented a diffuse area, whereas the higher molecular weight component represented ~10% of the total factor X-reacting material. When factor X-deficient plasma was analyzed, significant reductions in all three regions were seen, suggesting that these areas represented factor X-like molecules. Concentrates from Hep G2 cells contained about 50% single-chain and 50% two-chain factor X. The light chain of factor X was not recognized by this anti-factor X antiserum (D.S. Fair and T.S. Edgington, unpublished). Thus, a portion of both Hep G2 supernatants and human plasma contain a single-chain factor X species.

To determine if the secreted molecules were biologically active, supernatants were tested in one-stage coagulation assays. Secreted factor X and prothrombin corrected the clotting time of plasma deficient in the
Fig 6. Autoradiograms of the immunoprecipitates from [35S]-methionine-labeled Hep G2 supernatants (lanes 1, 2, 4, 5, 7–9) and [125I]-factor X (lanes 3 and 6) following incubation with normal rabbit serum (NRS) or monospecific anti-factor X serum (anti-FX). Immune complexes were collected using S aureus, and the labeled protein bands were separated on SDS-polyacrylamide slab gels in the absence (left panel) and presence (center and right panels) of 2-mercaptoethanol. The immunoprecipitates were separated in 9.5% acrylamide (left and center panels) or in a 4%–12% linear gradient of acrylamide (right panel). Standards used are indicated for the left and right panels, and the locations of the heavy (H), light (L) chain, and single-chain form (arrow) are given in the center panel. Hep G2 supernatants were collected after 5–6 hours, except for lane 9, which represented an 18-hour supernatant.

Fig 7. Immunoblot of purified, plasma and Hep G2 factor X separated by SDS-polyacrylamide gel electrophoresis under reduced conditions. After transfer to nitrocellulose, the paper was reacted with monospecific antibodies to factor X, washed, and the reacting proteins detected with iodinated goat anti-rabbit IgG. (Lane 1) 50 ng of purified factor X; (lane 2) 3 μL of Hep G2 supernatant concentrated 75-fold; (lanes 3 and 4) 2 μL of 10-fold concentrates of the barium citrate eluates from normal and factor X-deficient plasma, respectively; and (lane 6) 14C standards of bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), and cytochrome C (14,000).

Table 3. Neutralization of the Procoagulant Activities of Factor X and Prothrombin Secreted From Hep G2*

<table>
<thead>
<tr>
<th>Protein†</th>
<th>Antibody</th>
<th>Percent Relative Activity</th>
</tr>
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<tbody>
<tr>
<td>Factor X</td>
<td>Buffer</td>
<td>100</td>
</tr>
<tr>
<td>Factor X</td>
<td>Normal IgG</td>
<td>110</td>
</tr>
<tr>
<td>Factor X</td>
<td>Anti-factor X</td>
<td>19</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>Buffer</td>
<td>100</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>Normal IgG</td>
<td>99</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>Anti-prothrombin</td>
<td>18</td>
</tr>
</tbody>
</table>

*Medium from 96-hour serum-free cultures of Hep G2 was concentrated 25 times by ultrafiltration, mixed with an equal volume of Tris-buffered saline, nonimmune IgG or immunopurified antibody (100 μg/ml) and incubated for 30 minutes at 37 ºC before assay.
†Concentrations of factor X (250 ng/mL) and prothrombin (7.8 μg/mL) were measured by radioimmunoassay.
‡Factor X and prothrombin activities were equivalent to 0.02–0.03 and 0.7–0.9 U/mL, respectively.
factor X and prothrombin, respectively, but not by nonimmune IgG.

The capacity of antithrombin III to inhibit thrombin activity was determined in a chromogenic assay (Table 4). The initial rate of cleavage of Chromozym-TH by thrombin in the presence of heparin was normalized to 100%. With increasing quantities of Hep G2 serum-free supernatant, a dose-dependent inhibition of hydrolysis was observed, and this inhibition was at least partially dependent on the presence of heparin. The inhibition of thrombin was not significantly affected if the supernatant was preincubated with buffer or nonimmune IgG. However, specific antibody neutralized the inhibitory activity of antithrombin III in a dose-dependent manner. Thus, these three Hep G2-secreted molecules were biologically active, and the activity was neutralized by monospecific antibodies to each of these proteins.

**DISCUSSION**

It is generally agreed that the liver produces the majority of known plasma proteins. Formal demonstration of synthesis of some plasma proteins has been restricted to radioimmunoelectrophoresis and immunofluorescence. Implications that the liver is the site of synthesis of trace plasma proteins has, for the most part, been based on clinical data documenting reduced protein function as a manifestation of liver disease. The human hepatoma cell line, Hep G2, has morphological characteristics of liver parenchymal cells and the capacity to synthesize several plasma proteins. Many of these molecules are of known hepatic origin; and those that have been examined in detail share the same structural and functional characteristics as their authentic plasma counterparts.

Earlier studies have established that certain components of the coagulation and fibrinolytic systems are produced by this cell. We have extended these findings to include some of the vitamin K-dependent proteins and the primary coagulation protease inhibitor, antithrombin III. The two major novel findings in our study are that: (1) human factor X is secreted as a single chain protein; and (2) the secretion of prothrombin and antithrombin III can be modulated by warfarin and vitamin K.

Although factor X, prothrombin, and antithrombin III were present in the supernatant of day 8 Hep G2 cells, factor IX was not detected. Furthermore, factor IX was not present in cell extracts at an assay sensitivity of <10 ng/10^7 cells. The inability to measure this protein may be due to a deletion of the specific gene, lack of transcription of it, or the failure to translate the specific mRNA. Whereas intracellular factor X was undetectable at 10 ng/10^7 cells, both prothrombin and antithrombin III were easily measured. These concentrations were much lower than extracellular levels, suggesting that, once synthesized, each of the molecules are rapidly secreted into the supernatant at different rates or that multiple secretory pathways account for the different intracellular concentrations. Factor X, prothrombin, and antithrombin III accumulated in a linear fashion, but the concentration and secretion rate of each molecule differed. This may be due to differential rates of synthesis of these components by every cell in culture or to subpopulations of cells that have the capacity to synthesize and secrete one or more of the proteins. Because the Hep G2 cell line represents a colony of cells, immunohistochemical methods and/or cloning of the cells must be undertaken to determine these possibilities. The dependence of synthetic rates on plating density was observed and paralleled for the three proteins, suggesting that this...
finding is a general characteristic of the cells and not due to the secretion of a specific protein. Maximal rates of secretion were seen when T-75 flasks were seeded with at least $10^6$ cells in 15 mL of medium and may reflect a critical cell mass required to condition the medium or for cell contact.

Antithrombin III has been unequivocally shown to be synthesized by this cell and to have the characteristics of the molecule isolated from plasma. Immunologically, the secreted protein shared the same epitopes with the purified molecule as recognized by the antibody in competitive inhibition assays based on complete and parallel dose-dependent inhibition. Hep G2 antithrombin III was functionally active and was characteristically dependent on heparin to enhance the rate of thrombin inhibition. This inhibition could be neutralized by specific antibodies to purified antithrombin III. The intrinsically labeled and immunoprecipitated protein of mol wt 61,000 was consistent with that of purified antithrombin III. Hence, the antithrombin III synthesized and secreted by the Hep G2 appeared to be similar to the molecule isolated from plasma.

The cellular origin of antithrombin III has been controversial. Although the Hep G2 cell clearly produces this protein, immunofluorescent studies of human liver have not demonstrated this molecule. Limits of sensitivity of the immunofluorescent method used may account for the inability to demonstrate antithrombin III in human liver biopsies. Rat hepatocytes were observed to react with monospecific antibodies to rat antithrombin III using an immunoperoxidase technique, and solubilized liver microsomes contained a protein with similar epitopes to antithrombin III. Both results of Hep G2 and rat hepatocytes strongly suggest the liver as one site of synthesis of this inhibitor. Chan and Chan reported the production of functional intrinsically labeled antithrombin III from cultured human endothelial cells and, therefore, this molecule can be produced by at least two distinct cell types.

The production of prothrombin by the liver has been reported in studies using human, dog, and rat liver. Our results with the secretion of functional prothrombin from the Hep G2 cells confirms the liver parenchymal cells as a site of its synthesis. Intrinsically labeled and immunoprecipitated Hep G2 prothrombin was of the same molecular weight reported for prothrombin purified from plasma. The Hep G2 prothrombin synthesized in the medium containing vitamin K was functional, as it corrected the clotting time of prothrombin-deficient plasma and was neutralized by specific antibody to plasma prothrombin. Munns et al have also demonstrated that rat hepatoma H-35 cells secrete functional prothrombin in the presence of vitamin K.

The Hep G2 cells respond to vitamin K and warfarin by regulating the synthesis and secretion of prothrombin. Maximal accumulation of prothrombin was observed over a large dose of vitamin K (1–50 μg/mL) without an effect on cell morphology or viability. The rate of prothrombin secretion was the same as in untreated cultures, but the amount of secreted prothrombin was twice that of control. In the presence of vitamin K, the intracellular concentration of prothrombin was reduced 30%–40%, but this quantity was too low to account for the twofold increase in secretion. Similar results were reported for the synthesis of rat and human prothrombin, and our results would be consistent with the previous proposal that an increase in transcription or translation may occur when the vitamin is present for extended periods of time. Within 2–6 hours, vitamin K decreased intracellular prothrombin by increasing secretion. In our study, only the long-term culture effects of vitamin K on Hep G2 were examined, and these agreed with those of the rat H-35 results, where intracellular levels of prothrombin reached a steady state that was lower than untreated cells, and the amount of prothrombin synthesized was increased twofold.

In the presence of warfarin, both the rate and quantity of prothrombin synthesized by Hep G2 decreased three to four fold. The intracellular levels of prothrombin were identical to those of the untreated controls and suggested that the effect of warfarin may be to decrease the rate of transcription or translation rather than to increase the intracellular prothrombin pool. In individuals undergoing warfarin therapy, decreases in the concentration of the vitamin K-dependent protein antigens and their activities were reported. However, this decrease may result from either an increase in the catabolic rate of the inactive molecules or a decrease in their synthesis. Our results suggest that these decreases are more likely attributed to decreases in synthesis.

In the presence of warfarin, the rate and quantity of antithrombin III synthesized and secreted were the same as controls. These data were expected, as the concentration of antithrombin III in individuals undergoing long-term anticoagulant therapy remains stable. When vitamin K was added to the cells, a transient delay in the rate of secreted protein was observed for two to three days and then approached that of the untreated cultures. The intracellular levels of antithrombin III decreased on day 3 in response to increased secretion in the presence of vitamin K. These results indicate that vitamin K may inhibit the synthesis and secretion of antithrombin III; however, it is not
known if this inhibition is directly caused by vitamin K or by the increase in concentration of vitamin K-dependent factors. This effect may be attributed to the high concentration of vitamin K used in these long-term cultures or its differential effects on separate secretory pathways, and it merits further investigation.

Human factor X was synthesized as a single-chain precursor molecule that was subsequently processed into the two-chain form. If the Hep G2 supernatants were incubated for long periods in culture, the two-chain factor X was generated. This cleavage was not very efficient, as immunoblots of concentrated Hep G2 supernatants contained 40%-60% single-chain factor X, with the remainder being in the two-chain form. Also, factor X was detected in barium citrate eluates from normal plasma by immunoblotting, and about 10% of the factor X was in the single-chain form, which was less than that reported for rat plasma factor X. These data confirm the isolation of single-chain factor X from human and bovine plasma. Similar results were reported for rat factor X, in which a single chain was observed in liver microsomes, plasma, and secreted from the H-35 hepatoma cell line.

Recently, McMullen et al reported the amino acid sequence of the light chain of human factor X, which contained a carboxy-terminal arginyl residue, and Discipio et al documented a seryl as the amino-terminus of the heavy chain, suggesting that the two-chain molecule results from an arginyl-seryl cleavage of the single-chain precursor. Because the processing of Hep G2 factor X could be inhibited with 1 mmol/L benzamidine, the postsecretion cleavage of factor X into the two-chain form may be due to the presence of a serine protease. These data imply that a single gene, rather than two genes, encodes for one mRNA responsible for the synthesis of this protein. Because the Hep G2 cell line was derived from a malignant tumor, this interpretation must be approached cautiously. However, in vitro translation of purified normal human liver RNA and immunoprecipitation of the cell-free product indicated that factor X is indeed synthesized as a single-chain molecule (D.S. Fair and P. Tolstoshev, unpublished data). From these results, it is speculated that protein C would most likely be synthesized and secreted as a single-chain molecule and would undergo a similar postsecretion proteolytic processing into a two-chain molecule.

Rat factor X has been shown to be regulated by the presence of vitamin K; and Graves et al have suggested that all the vitamin K-dependent proteins are regulated by a common mechanism. Hep G2 factor X was not quantitated after warfarin or vitamin K treatment (the concentration secreted by the cell was too low for accurate measurement), but after 4-5 days in culture, lower levels of this protein were observed in the presence of warfarin relative to control cultures. These data are consistent with a common mechanism of regulation of the vitamin K-dependent proteins. Clearly, the Hep G2 cell has the capacity to produce a number of coagulation proteins and to respond to modulation by known pharmacologic agents. Thus, this cell line provides a useful model for assessing the regulation of biosynthesis and secretion of human coagulation proteins.

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