Biosynthesis of Factor XIII B Subunit by Human Hepatoma Cell Lines

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The plasma transglutaminase, factor XIIa (FXIIa), circulates as a zymogen containing two proteins, A and B, arranged in a noncovalent tetrameric complex, A₂B₂. Biosynthesis of plasma FXIII has not previously been demonstrated. In the present study, direct evidence has been obtained that two human hepatoma cell lines, Hep G2 and PLC/PRF/5, synthesize and secrete FXIII B protein. Secretion of the B subunit of FXIII by Hep G2 was demonstrated by immunoblotting. De novo synthesis by Hep G2 was confirmed in [³⁵S]-methionine-labeled cultures. Radiolabeled conditioned medium was concentrated, mixed (1:1) with purified B protein, and examined by crossed immunoelectrophoresis with antisera to the B subunit. The single protein precipitin arc of purified B protein comigrated with the radiolabeled FXIII from Hep G2 visualized by autoradiography, indicating both electrophoretic and antigenic identity. The data presented here represent the first demonstrations of biosynthesis of FXIII B protein by any cell type and suggest that the liver is the site of synthesis of FXIII B protein. Further analysis of concentrated Hep G2 serum-free conditioned medium (SFCM) and cell lysate by immunoblotting following nondenaturing agarose gel electrophoresis demonstrated the FXIII A protein as well as the B protein and also revealed synthesis and secretion of the A and B proteins by PLC/PRF/5. Crossed immunoelectrophoresis studies of Hep G2 SFCM and cell lysate suggest that Hep G2 cells also synthesize and secrete the plasma FXIII zymogen. With a specific radioimmunoassay for B protein, FXIII was found in Hep G2 SFCM at approximately 4 ng/mL; with an amplified rocket immunoelectrophoresis technique the level was ~5 ng/mL.

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FXIII B protein. Furthermore, preliminary studies suggest that Hep G2 and PLC/PRF/5 cells also synthesize the FXIII A protein and synthesize a FXIII zymogen identical to extracellular or plasma FXIII (A2B2).

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM), methionine-free minimum essential medium (MEM), trypsin-EDTA, Hank’s balanced salt solution (HBSS), and penicillin/streptomycin solutions were obtained from GIBCO Laboratories (Grand Island, NY); fetal bovine serum (FBS) was from Whittaker M.A. Bioproducts (Walkersville, MD). L-[35S]-methionine (1.400 Ci/mmol) was purchased from Amersham Corp (Arlington Heights, IL), and [1,25I]-protein A from DuPont NEN Research Products (Wilmington, DE). Immunoelectrophoresis buffer (1.0 mol/L barbital), acrylamide, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Richmond, CA). SeaKem Agarose ME and Gel Bond film NF were from FMC Marine Colloids, (Rockland, ME). Nitrocellulose sheets (0.2 mmol/L) were purchased from Schleicher and Stoss (Keene, NH). XAR-2 film from Eastman Kodak Co. (Rockyester, NY), and Ultrafilm from LKB (Gaithersburg, MD). Centríflo CF25 ultrafiltration membrane cones were obtained from Amicon Corp (Danvers, MA) and Pansorbin from Calbiochem Behring Corp (La Jolla, CA). Prestained protein high molwt standards were obtained from Bethesda Research Laboratories (Gaithersburg, MD) and bovine thrombin from Parke-Davis Co (Morris Plains, NJ). Phenylmethylsulfonylfluoride (PMSF), p-chloromercuribenzoate, ovalbumin, and benzamidine were obtained from Sigma Chemical Co (St Louis).

Plasma FXIII was prepared from recovered human plasma (American Red Cross Blood Program, northeast region) as previously described.30 Purified FXIII B subunit was obtained from the thrombin-treated zymogen by affinity chromatography on organomercurial agarose.31 Purified FXIII A2 was prepared from placental thrombin-treated zymogen by affinity chromatography on organomercurial agarose.31 Purified FXIII A2 and FXIII A2B2 were prepared using Na125I (0.1 mol/L barbital), acrylamide, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Richmond, CA). SeaKem Agarose ME and Gel Bond film NF were from FMC Marine Colloids, (Rockland, ME). Nitrocellulose sheets (0.2 mmol/L) were purchased from Schleicher and Schuell (Keene, NH). XAR-2 film from Eastman Kodak Co. (Rockyester, NY), and Ultrafilm from LKB (Gaithersburg, MD). Centríflo CF25 ultrafiltration membrane cones were obtained from Amicon Corp (Danvers, MA) and Pansorbin from Calbiochem Behring Corp (La Jolla, CA). Prestained protein high molwt standards were obtained from Bethesda Research Laboratories (Gaithersburg, MD) and bovine thrombin from Parke-Davis Co (Morris Plains, NJ). Phenylmethylsulfonylfluoride (PMSF), p-chloromercuribenzoate, ovalbumin, and benzamidine were obtained from Sigma Chemical Co (St Louis).

Cell culture. The human hepatoma cell line Hep G2 was provided by Drs Barbara B. Knowles and David P. Aden of the Wistar Institute, Philadelphia. PLC/PRF/5 is a tissue culture cell line derived from a human hepatocellular carcinoma established by Alexander et al.35 and was provided by Dr I.M. Arias of Tufts New England Medical Center. Both cell lines were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). To prepare large quantities of serum-free condition medium (SFCM), cells were seeded in T175 flasks at a density of 1.5 10^5 cells/flask in 50 mL of medium. When the cells had become adherent but not confluent (usually within one to two days), cultures were washed three times with 10 mL of serum-free medium and then incubated in 50 mL of serum-free medium for 72 hours. The SFCM was collected, clarified by centrifugation, concentrated 100-fold with Amicon Centríflo CF25 ultrafiltration membrane cones, and stored at 4°C or at −80°C.

Cell lysate was prepared from confluent cultures. The cells were dissociated by trypsinization, washed three times in HBSS, and lysed at a density of 10^9 cells/mL by the addition of 1% Triton X-100, 10 mmol/L Tris-HCl, 100 mmol/L NaCl, 1 mmol/L EDTA, pH 7.5, for 60 minutes at 4°C. After centrifugation for 30 minutes at 100,000 g, the supernatant was removed and assayed immediately for FXIII.

Immunoprecipitation and immunoblotting of FXIII. Hep G2 SFCM concentrated 100-fold (250 μL) was added to anti-FXIII B antiserum (10 μL) and incubated for 18 hours at 4°C. Fifty microliters of Pansorbing was added, and the immunoprecipitates were collected by centrifugation, washed in buffer (0.01 mol/L Tris HCl, 0.1% Triton X-100, 0.5% nonidet P40 (NP-40), 0.5% deoxycholate (DOC) 0.1% SDS, 0.05% Tween 20, 2 mmol/L EDTA, 1 mmol/L PMSF, 0.14 mol/L NaCl, pH 8.0), and solubilized by boiling for ten minutes in SDS-polycrylamide gel electrophoresis (PAGE) sample buffer (10% glycerol, 3% SDS, 0.025 mol/L Tris-HCl, pH 6.8, 0.01% bromophenol blue, 5% 2-mercaptoethanol). Purified FXIII A2B2, A2 or B subunit and the dissolved immunoprecipitates from Hep G2 SFCM were electrophoresed and then electrophoretically transferred to nitrocellulose.19 After the transfer, the nitrocellulose was washed with Blotto36 for 30 min, followed by 3% hemoglobin 0.01 mol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.3, and 0.1% sodium azide (Hb/PBSa, Sigma, St Louis) for 30 min then reacted with monospecific antiserum to FXIII B subunit diluted in 3% Hb/PBSa. Detection of FXIII B subunit was facilitated by the addition of 125I-protein A followed by autoradiography.

Metabolic labeling of intracellular and secreted proteins. Confluent cultures of Hep G2 cells (5 10^5 cells/T175 flask) were rinsed three times with labeling medium and incubated with 15 mL of labeling medium for 15 minutes at 37°C, 5% CO2. The labeling medium prepared from the MEM Selectam Kit (GIBCO) lacked FBS and contained 10 μmol/L rather than 100 μmol/L l-methionine. L-[35S]-methionine was added to a concentration of 100 to 150 μCi/mL, and the cells were incubated at 37°C, 5% CO2 for five hours. The conditioned medium containing the intrinsically labeled proteins secreted by the Hep G2 cells was recovered, clarified by centrifugation, and concentrated 100-fold by ultrafiltration. The cells were dissociated by trypsinization, washed three times in PBS and lysed by the addition of lysis buffer (1% Triton X-100, 0.5% DOC in PBS, 4°C, one hour). After centrifugation for 30 minutes at 100,000 g, the supernatant was concentrated 20-fold by ultrafiltration. The concentrated lysate and conditioned medium were both stored at −80°C.

Crossed immunoelectrophoresis (CIE). CIE was performed in 0.8% agarose in 0.1 mol/L barbital buffer and 2 mmol/L EDTA, pH 8.6.30 Concentratedcondition medium or the cell extract of L-[35S]-methionine-labeled cultures of Hep G2 was mixed 1:1 with either purified B protein, normal plasma, or purified FXIII A2B2. Electrophoresis in the first dimension was carried out at 10°C to 10 V/cm for two hours. Strips of agarose were then transferred to an 8% agarose gel containing 0.5% antiserum to the B subunit. Electrophoresis was carried out in the second dimension for 18 hours at 2 V/cm, 10°C. Gels were then washed in saline, rinsed, covered with filter paper, compressed, and rehydrated with saline. This washing procedure was repeated three times to remove residual, nonimmunoprecipitated proteins. Gels were then dried onto Gel Bond film and stained with Coomassie brilliant blue. The CIE gels were then subjected to autoradiography with LKB Ultraprilm.

Agarose gel electrophoresis. Agarose gel electrophoresis was performed at 10°C and 20 V/cm in 1-mm-thick gels containing 0.8% agarose for 1.5 hours in 0.1 mol/L barbital buffer and 2 mmol/L EDTA, pH 8.6.41 The proteins were transferred from the agarose gel to nitrocellulose by capillary action.41 The nitrocellulose was then treated as described above with either anti-FXIII A or B antiserum followed by 125I-protein A.
Rocket immunoelectrophoresis. Quantitation of the FXIII in Hep G2 SFCM was performed immunochemically by rocket immunoelectrophoresis coupled with an amplification procedure developed specifically for the detection of nanogram quantities of FXIII Aβ₂ antigen. Electrophoresis was carried out in agarose (1% wt/vol) containing anti-FXIII B antiserum in Tris-barbital buffer (0.03 mol/L Tris, 0.1 mol/L barbital, pH 8.6) at 5 V/cm, 10°C for 18 hours. After electrophoresis, the gel was incubated with 50 mL of 125I-protein A in 3% Hb/PBSa (150,000 cpb/mL) for one hour, covered with filter paper, and compressed with a weight. The gel was then rehydrated and washed in saline. This procedure was repeated three times to ensure the complete removal of an 125I-protein A not bound to the precipitate. The gel was dried onto a sheet of Gel Bond film and subjected to autoradiography with XAR film. Pooled human plasma, used as a standard, was assumed to contain 30 μg/mL FXIII. The precision of this method was ±5% in the height of the rocket, and the sensitivity was 0.5 ng of FXIII Aβ₂.

RESULTS

SFCM from Hep G2 cell cultures was examined for the presence of FXIII B protein by means of immunoblotting. Initially, Hep G2 SFCM was treated with Blue Sepharose (Pharmacia, Piscataway, NJ) to remove cell-secreted albumin (which caused severe distortion of the protein band in the area of the FXIII subunit) and then concentrated by ultrafiltration. By SDS-PAGE followed by immunoblotting, the B protein was detected as a very faint band in the autoradiogram, and the mobility of the subunit was similar to that of the purified polypeptide chain. However, despite removal of most of the interfering albumin, the amount of Hep G2 SFCM that could be loaded into one well of the polyacrylamide gel without band distortion limited the level of detection of FXIII B protein by this method.

To overcome these limitations, immunoprecipitation was coupled with the immunoblotting detection system. Monospecific anti-FXIII B antiserum was used to immunoprecipitate FXIII from the concentrated Hep G2 SFCM. The immunoprecipitate was then electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose, and detected with anti-FXIII B antiserum. Figure 1 shows the results of such an experiment. Lane 2 contains the immunoprecipitated material from the concentrated (100-fold) Hep G2 SFCM. Included for comparison in the gel are FXIII Aβ₂ (lane 1), FXIII A₂ (lane 3), and FXIII B protein (lane 4). These results show that the anti-FXIII B antiserum did not cross-react with the A subunit and that the mobility of the band in the immunoprecipitated material from the Hep G2 SFCM that is recognized by the anti-FXIII B antiserum is identical to that of the B subunit of FXIII. The intensity of the FXIII B subunit detected in the Hep G2 SFCM was dependent on the amount of SFCM used for the immunoprecipitation as well as the degree to which the Hep G2 SFCM has been concentrated. This method provided a means of further concentrating the FXIII B protein from the SFCM (already concentrated 100-fold) prior to electrophoresis.

Direct evidence that the FXIII detected in Hep G2 SFCM was specifically synthesized and secreted by these cells was obtained by metabolic protein labeling with L-[35S]-methionine. Demonstration that the intrinsically labeled FXIII was identical to FXIII B protein was obtained by CIE. Radiolabeled conditioned medium was concentrated 100-fold, mixed with purified FXIII B protein, and analyzed by CIE in agarose gels that contained antiserum to the FXIII B subunit in the second dimension. The protein-stained pattern is shown in Fig 2A and the corresponding autoradiograms in Fig 2B. The Coomassie staining pattern and the corresponding autoradiograms showed single immunoprecipitation arcs with identical mobility and area. Figure 2 shows that the radiolabeled FXIII present in the culture medium comigrated with purified FXIII B protein, indicating both electrophoretic and antigenic identity. These results imply that the intrinsically labeled FXIII was identical to the FXIII B protein.

Efforts to immunoprecipitate the FXIII A protein from the Hep G2 SFCM with anti-FXIII A antiserum and to
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Fig 3. Nondenaturing agarose electrophoresis of purified FXIII proteins and Hep G2 SFCM at pH 8.6: detection of FXIII B subunit. The following samples were used: (1) FXIII A_2, 0.35 μg/mL; (2) FXIII A_2B_2, 0.35 μg/mL; (3) FXIII B, 0.38 μg/mL; (4) Hep G2 100-fold–concentrated SFCM; (5) Hep G2 50-fold–concentrated SFCM; (6) PLC/PRF/5 100-fold–concentrated five-day SFCM; (7) PLC/PRF/5 136-fold–concentrated three-day SFCM; and (8) 125I-labeled FXIII B protein. Twenty microliters of each sample were loaded per slot. After electrophoresis (10 V/cm, 10°C, two hours), samples were transferred from the agarose gel to nitrocellulose paper by capillary action. The nitrocellulose was treated with anti-FXIII B antiserum followed by 125I-protein A. The bands were visualized by autoradiography. The arrow indicates the sample application well. The direction of migration of FXIII protein is toward the anode.

3, lane 1, while FXIII A_2B_2 does react, Fig 3, lane 2). The results for the control samples in lanes 1 to 3 from both Figs 3 and 4 indicate that in this system both antisera are specific for their respective FXIII subunits and do not cross-react with the other subunit. Furthermore, the antisera each react with the purified subunit as well as with the subunit in the complexed form. In Figs 3 and 4, lanes 4 and 5 contain concentrated Hep G2 SFCM (100- and 50-fold, repectively).

Fig 4. Nondenaturing agarose electrophoresis of purified FXIII proteins and Hep G2 SFCM at pH 8.6: detection of FXIII A subunit. The following samples were used: (1) FXIII A_2, 0.35 μg/mL; (2) FXIII A_2B_2, 0.35 μg/mL; (3) FXIII B, 0.38 μg/mL; (4) Hep G2 100-fold–concentrated SFCM; (5) Hep G2 50-fold–concentrated SFCM; (6) PLC/PRF/5 100-fold–concentrated five-day SFCM; (7) PLC/PRF/5 136-fold–concentrated three-day SFCM; and (8) 125I-labeled FXIII A protein. Twenty microliters of each sample were loaded per slot. After electrophoresis (10 V/cm, 10°C, two hours) the samples were transferred from the agarose gel to nitrocellulose paper by capillary action. The nitrocellulose was treated with anti-FXIII A antiserum followed by 125I-protein A. The bands were visualized by autoradiography. The arrow indicates the sample application well. The direction of migration of FXIII protein is toward the anode.

The technique of agarose gel electrophoresis under non-denaturing conditions has previously been used to study the FXIII proteins. The results indicate that the FXIII B subunit is detected by CIE with anti-FXIII A antiserum and is largely unsuccessful. In addition, when FXIII in Hep G2 SFCM was immunoprecipitated with anti-FXIII B antiserum, no FXIII A protein was detected by immunoblotting using anti-FXIII A antiserum in an SDS–polyacrylamide gel system. However, the FXIII A subunit was detected (in a degraded form in SFCM) by nondenaturing agarose gel electrophoresis coupled with immunoblotting.

The technique of agarose gel electrophoresis under non-denaturing conditions has previously been used to study the FXIII proteins. The results for the control samples in lanes 1 to 3 from both Figs 3 and 4 indicate that in this system both antisera are specific for their respective FXIII B subunits and do not cross-react with the other subunit. Furthermore, the antisera each react with the purified subunit as well as with the subunit in the complexed form. In Figs 3 and 4, lanes 4 and 5 contain concentrated Hep G2 SFCM (100- and 50-fold, repectively).
These results in Fig 3 indicate that both samples of the Hep G2 SFCM were positive for B protein. The freshly prepared Hep G2 SFCM showed a diffuse band for A protein (Fig 4, lane 5) but the Hep G2 SFCM that had been stored for several weeks at 4 °C showed a band of faster mobility, probably indicating degraded A protein (Fig 4, lane 4).

Concentrated SFCM from another human hepatoma cell line, PLC/PRF/5, was also examined in this agarose gel electrophoresis system. These cells were maintained in culture in the absence of FBS for up to five days with good viability. Figure 3, lanes 6 and 7, shows that two samples of PLC/PRF/5 SFCM were positive for FXIII B protein. In Fig 3, lane 8 shows the mobility of 125I-FXIII B protein. The PLC/PRF/5 SFCM was also positive for FXIII A protein (Fig 4, lanes 6 and 7). The fresh lysates of both cell lines were also found to be positive for A protein (Fig 4, lanes 8 and 9).

In Fig 4, lane 10 shows 125I-FXIII A2 protein. The cell lysates of both lines also showed weak bands for the B protein (data not shown).

Evidence that intrinsically labeled HepG2 SFCM and cell lysate contained FXIII identical to plasma FXIII in addition to FXIII B protein was obtained by CIE. Radiolabeled conditioned medium and cell layer extracts were concentrated 100-fold and 20-fold respectively, mixed with pooled human plasma or purified FXIII A2B2, and analyzed by CIE in agarose gels that contained antiserum to the FXIII B subunit in the second dimension. Protein-stained patterns are shown in Fig 5A and their corresponding autoradiograms in Fig 5B. Lane 1 contains Hep G2 SFCM plus normal plasma, lane 2 contains Hep G2 SFCM added to purified FXIII A2B2, and lane 3 shows Hep G2 cell lysate mixed with purified FXIII A2B2. The antiserum used in this analysis reacted with normal human plasma to form a single symmetrical peak. In plasma, FXIII is present as the A2B2 tetramer; no free B protein has been detected. In each case, the Coomassie staining pattern and the corresponding autoradiogram showed single immunoprecipitation arcs with identical mobility and area. Figure 5 shows that the intrinsically radiolabeled FXIII present in the culture medium or extracted by Triton X-100 from the cell layer comigrated with the FXIII in human plasma or with purified FXIII A2B2, indicating both electrophoretic and antigenic identity. These results suggest that this intrinsically labeled FXIII was identical to the plasma FXIII molecule.

Quantification of the FXIII in Hep G2 SFCM was facilitated by development of an amplified rocket immunoelectrophoresis procedure in which trace amounts (not detectable by Coomassie staining) of antigen–antibody immunoprecipitates in agarose are reacted with 125I-protein A and visualized by autoradiography. Concentrated (100-fold) Hep G2 SFCM was electrophoresed into a gel containing anti-FXIII B antiserum (Fig 6). Pooled human plasma was used to determine a standard curve of the height of the rocket v concentration of FXIII. The lower limit of detection of FXIII in normal plasma occurs at a 1:512 dilution, which corresponds to a lower limit of detection of 0.5 ng of FXIII A2B2 antigen. From the height of the rocket for the Hep G2 SFCM, the level of FXIII antigen in Hep G2 SFCM (unconcentrated) was determined to be approximately 5 to 10 ng/mL. SFCM from Hep G2 cell cultures was also examined for the presence of FXIII A and B subunits by
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In the means of competitive radioimmunoassay based on specific radioimmunoassays for the A and B proteins of FXIII that were developed previously,12,23 By radioimmunoassay (data not shown), the total concentration of FXIII antigen was found to be approximately 4 to 8 ng/mL in Hep G2 SFCM (unconcentrated), in good agreement with the results of the rocket immunoelectrophoresis procedure.

Altogether, these results clearly demonstrate synthesis and secretion of the FXIII B protein by both the Hep G2 and PLC/PRF/5 human hepatoma cell lines and suggest that these cells also synthesize the FXIII A protein and secrete a portion of the B protein as the plasma FXIII tetrameric complex A2B2.

DISCUSSION

The major focus of this work has been the demonstration and characterization of the FXIII synthesized and secreted by the cell line Hep G2. Hep G2 is a human hepatocellular carcinoma cell line that has morphologic characteristics similar to liver parenchymal cells and that appears to have normal biosynthetic and secretory capability.20 It has been shown to synthesize a variety of coagulation and fibrinolytic proteins, including fibrinogen,21 α2-antiplasmin,21 plasminogen activator,26 prothrombin, factor X, antithrombin III, factor V, and factor VII.22-27 The Hep G2 proteins that have been examined in detail have the same structural and functional characteristics as their native plasma counterparts. In addition, Hep G2 cells have been shown to respond to vitamin K and to warfarin in regulating the synthesis and secretion of prothrombin26 and to steroids in regulating fibrinogen synthesis.24 It is therefore clear that the Hep G2 cell line has the capacity for normal synthesis of a number of coagulation proteins and the ability to respond appropriately to modulation by known pharmacological agents. This appears to be the most carefully characterized, biosynthetically normal hepatocyte cell line available. The very low plasma concentration and long half-life of FXIII made it important to begin studies with a cell line possessing these characteristics. PLC/PRF/5 is another unrelated, human hepatocellular carcinoma cell line that, in contrast to Hep G2, shows a very different pattern of secreted proteins.20 PLC/PRF/5 does not secrete fibrinogen or albumin. From the studies described here, it is clear that both of these human hepatoma cell lines synthesize and secrete the FXIII B protein. Previously, methods similar to those used in this study were employed in analysis of the human monocyte-macrophage cell line U-93712 and human monocytes and macrophages.14 No B-chain material could be detected in these cell extracts, whereas A-chain material was readily visualized.12,14

Previously, some studies have suggested that FXIII is synthesized by hepatocytes. FXIII has been reported to be in the cytoplasm of liver parenchymal cells by both fluorescent and immunoperoxidase techniques.16,17 However, characterization of the antibodies used in these studies was not reported. In one case, all parenchymal cells appeared to be positive, raising a question of antibody specificity. Given the concentration and half-life of FXIII in plasma, one would expect cellular detection to be below the sensitivity of fluorescence microscopy. With specific antisera to A and B proteins, neither we nor others41 have been able to demonstrate convincing, reproducible staining by immunofluorescence in hepatocytes. In addition, the Hep G2 cells appear negative for FXIII by immunofluorescence studies (data not shown). This argues for the use of more sensitive biochemical methods of approach rather than the reliance on a single method of immunohistochemistry with its problems of antibody specificity and sensitivity.

To date there has been no direct demonstration of FXIII synthesis and secretion by hepatocytes. Previous evidence based on clinical data documents reduced levels of plasma FXIII concomitant with liver disease in some patients but not in others,1 but in fact, a decrease in plasma FXIII correlates with the severity of the systemic illness rather than with any specific illness.

In this work, FXIII B protein has been shown to be synthesized and secreted by a well-characterized hepatoma cell line that is known to secrete many of the proteins produced by normal, nonmalignant hepatocytes and also by a less well-characterized hepatoma cell line with a different pattern of secreted proteins. In our study, FXIII B protein has been immunohistochemically identified in the SFCM of these cell lines. This FXIII B protein was found to be antigenically identical to that of the human plasma FXIII B chain. This study represents the first detection of the B protein in any cell line by biosynthetic methods and the first biochemical demonstration of a site of synthesis of the plasma FXIII B chain.

The results of the CIE studies using normal plasma and purified FXIII A2B2, are interpreted as suggesting that the Hep G2 cell line also synthesizes and secretes at least a portion of the B protein as part of the A2B2 complex. Radiolabeling of a precipitin arc generated by CIE is not, in itself, conclusive evidence of the synthesis of a specific protein, since binding of secreted proteins to antigen-antibody precipitates or to proteins present in the normal plasma used as a carrier could give false-positive results. Experiments have shown39 that when either A or B protein is present in excess it is loosely bound to the zymogen complex. Results of our independent agarose gel electrophoresis experiments support our conclusions concerning the presence of both A and B proteins in the SFCM; however, confirmation of the presence of the FXIII A2B2 complex awaits results of additional metabolic labeling experiments.

Despite repeated efforts to determine the mol wt of the A protein synthesized by Hep G2, visualization by immunoblotting of any specific A protein band in SDS-PAGE at the appropriate mol wt was unsuccessful. Further investigation indicated that the sensitive A protein had probably undergone proteolytic cleavage in the Hep G2 SFCM. Since the specific anti-FXIII A antibodies have a decreased capacity to bind to degraded FXIII A protein (as evidenced by a lack of precipitability of activated FXIII A2 by these antibodies in both rocket immunoelectrophoresis and CIE [unpublished observations]), detection under these circumstances would be difficult. The results using nondenaturing agarose gel electrophoresis suggest A protein degradation, and possible B protein degradation, in Hep G2 SFCM upon storage. The
mobility of the degraded A protein in the agarose gel is similar to that of the thrombin-activated A protein. Evidence for the presence of proteolytic activity in Hep G2 culture medium exists from the work of others. Factor X was isolated from Hep G2 conditioned medium in both a single-chain form and a two-chain form. The postsecretion cleavage of factor X into the two-chain form was thought to be due to the presence of a serine protease in the Hep G2 conditioned medium that could be inhibited by the addition of 1 mmol/L benzamidine to the conditioned medium. Addition of several inhibitors to our cultures (such as 1 mmol/L EDTA) did not permit the detection of FXIII A protein in the SFCM by immunoblotting following SDS-PAGE but did facilitate detection of the A protein from the cell lysate in the agarose gels. FXIII is also known to be labile to degradation by granulocyte proteases such as cathepsin C. The ability to detect A protein in the SFCM (even in its degraded form) by the technique of agarose gel electrophoresis is probably due to the fact that this type of electrophoresis is performed under non-denaturing conditions. The method as described represents a form of immunochemical analysis that does not rely on immunofixation within the agarose gel or on immunoprecipitation prior to electrophoretic analysis. The absence of any SDS permits the antigen to be detected in its non-denatured state. It is clear that the anti-FXIII A antibodies are conformationally dependent.

Conclusions derived from our in vitro data may not necessarily reflect the complete in vivo scenario. Extrapolation of the synthesis of FXIII to normal human parenchymal cells on the basis of the ability of the Hep G2 cells and PLC/PRF/5 cells to secrete this protein should be made with some caution, since both cell lines originate from transformed cells. The results of the non-denaturing agarose gel electrophoresis experiments suggest that between the time of synthesis and secretion the FXIII A protein may undergo processing by Hep G2 cells, which is not equivalent to processing by PLC/PRF/5 cells. However, the evidence presented here suggests that the human hepatocyte possesses the ability to produce plasma FXIII in vivo. Recently, the primary structure of human FXIII B subunit was determined using a cDNA library prepared from human liver mRNA. Assuming the rate of secretion of FXIII by these transformed cells (0.15 ng/10^6 cells/hour) is similar to the rate of secretion by normal hepatocytes, liver synthesis is sufficient to account for the amount of FXIII present in plasma. This calculation is based on a similar calculation for factor V and assumes that the plasma volume is 2.8 L, plasma FXIII concentration is 30 μg/mL, the t1/2 of FXIII is 8.4 days, and the adult liver contains 3 x 10^11 hepatocytes. The rate of secretion of FXIII was estimated from our results for the amount of FXIII that accumulated over a three-day period from a confluent culture of the Hep G2 cells.

The control mechanisms for secretion in vivo remain to be definitely demonstrated, but present clinical evidence reflects the control of FXIII B protein expression by the levels of circulating FXIII A protein, at least in the cases of FXIII-deficient patients. In addition, patients who are congenitally deficient in FXIII have little or no detectable A protein and about 50% B protein, whereas heterozygotes have about 50% A and 80% B protein. The role of this free B protein has not been clearly defined. Current experiments are in progress to use the Hep G2 cells to address questions of metabolism as well as to elucidate the steps involved in the processing, assembly, and secretion of the FXIII A2B2 complex.

REFERENCES

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