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Research Article

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Monoclonal Anti-human Factor VII Antibodies

Detection in Plasma of a Second Protein Antigenically and Genetically Related to Factor VII

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Abstract

Several murine monoclonal anti-human Factor VII antibodies were produced using hybridoma technology. Two noncompetitive monoclonal antibodies were used to examine by Western blotting the Factor VII cross-reactive material (CRM) in normal human plasma and three commercially available congenitally Factor VII-deficient plasmas, and to construct a facile "sandwich" immunoassay for plasma Factor VII. A second, previously undescribed, form of Factor VII CRM was detected in human plasma, which on Western blotting stained with an apparent intensity 5–8% that of Factor VII. This glycoprotein, tentatively called VII*, has a molecular weight 4,500 D less than Factor VII, lacks detectable Factor VII functional activity, does not bind to barium citrate, and is not recognized by a monoclonal antibody that recognizes Factor VII but not α -chymotrypsin-treated Factor VII. VII* was not proteolytically produced from Factor VII during *in vitro* coagulation or after infusion of human Factor VII into rabbits. As determined by Western blotting, the human hepatoma cell line, HepG2, cultured in the presence of vitamin K, secreted relatively greater levels of VII* in proportion to VII (75%) than that found in human plasma. Warfarin treatment of HepG2 cells decreased the quantity of VII secreted by 77%, whereas it only inhibited the secretion of VII* by 14%. Immunologic studies of the plasmas from a patient on chronic warfarin therapy and an individual given a short course of high dose warfarin therapy corroborated the *in vitro* synthetic studies obtained with HepG2 cells. The data are consistent with the production of VII* by posttranslational, proteolytic, modification of VII, that, at least in the HepG2 cells studied, occurs intracellularly. However, other mechanisms for the production of VII*, in particular, alternative RNA splicing of the transcript from a single gene, cannot be excluded.

Introduction

Human coagulation Factor VII is a single-chain glycoprotein of ~50,000 mol wt, present in plasma in trace quantities (1, 2). It seems to be synthesized predominantly by the liver, and the production of a functional protein requires the posttranslational gamma carboxylation of specific glutamic residues located near the NH₂-terminus of the molecule. Vitamin K is required for this modification to occur, and the gamma carboxyglutamic acid residues formed play an integral role in the binding of Factor

VII and the other vitamin K-dependent coagulation proteins to phospholipid surfaces via Ca⁺⁺ bridges (3–5). These same gamma carboxyglutamic acid residues also seem to be responsible for the adsorption of the vitamin K-dependent coagulation proteins to barium salts (6).

Factor VII is a serine protease zymogen (1). It can be activated by proteolytic cleavage to a disulfide-linked, two-chain form, Factor VII_a, by Factor X_a, Factor IX_a, thrombin, and Factor XII_a or β XII_a (7). Factor VII_a possesses at least 25-fold the activity of zymogen Factor VII as measured in a functional assay (7). Whether zymogen, single-chain Factor VII possesses catalytic activity has not been established since its apparent activity by bioassay may be due to activation during the assay itself, or to trace contamination of purified preparations of Factor VII with VII_a. Indirect studies using bovine Factor VII, however, suggest that the zymogen form may have intrinsic activity (8). The enzymatic activity of Factor VII/VII_a is enormously enhanced by the presence of its lipoprotein cofactor—tissue factor. In its presence, Factor VII/VII_a can activate Factor X (the classical extrinsic pathway of coagulation) or Factor IX (9–11).

Congenital deficiencies of Factor VII are rare, with <100 cases documented in literature (12). The clinical courses in people with congenital deficiencies of Factor VII have varied from mild bleeding diatheses to major hemorrhagic disorders with hemarthrosis and intracranial hemorrhage. In addition, anecdotal reports of a possible thrombotic tendency have also appeared, and theoretical support for such events has been presented (8, 13–16).

Based on an inhibition neutralization assay using heterologous antisera to Factor VII, three immunologic variants of Factor VII deficiency have been described (17). In the VII⁻ variant, detectable cross-reacting material (CRM)¹ is absent. In VII^R, CRM is present, but reduced, and in the VII⁺ variant a normal amount of CRM is present. Recently, a radioimmunoassay for Factor VII using specific heterologous antisera has been reported (18). When this assay was applied to two commercially available Factor VII-deficient plasmas, both seemed to fall into the VII^R variant category.

Here, using two monoclonal antibodies raised against human Factor VII, we investigate the CRM status of three commercially available Factor VII-deficient plasmas. Further, the presence of an additional form of Factor VII CRM, not previously described in plasma, is documented.

1. *Abbreviations used in this paper:* Con-A, Concanavalin A; CRM, cross-reactive material; DFP, diisopropylfluorophosphate; DME, Dulbecco's modified Eagle's medium; endoglycosidase H, endo- β -N-acetylglucosaminidase; GK702 and 704, George King 702 and 704 plasma, respectively; MC, monoclonal anti-Factor VII antibody; MC-affigel, monoclonal anti-Factor VII antibody coupled with affigel 10; PAGE, polyacrylamide gel electrophoresis; VII, Factor VII CRM with the same molecular weight on SDS-PAGE as purified Factor VII; VII*, Factor VII CRM that had an apparent molecular weight 4,500 D less than purified Factor VII.

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Methods

Materials. Affigel-10 and DEAE-affigel-Blue were purchased from Bio-Rad Laboratories (Richmond, CA), and Sephacryl S-300 and Concanavalin-A- (Con-A)-Sephacrose were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Soybean trypsin inhibitor, bovine serum albumin (BSA) α -chymotrypsin, Trizma base, acrylamide and bisacrylamide, Russell's viper venom and cephalin, rabbit brain phospholipids, benzamidine, and latex beads (0.8 μ m) were bought from Sigma Chemical Co. (St. Louis, MO). Rabbit brain thromboplastin was obtained from Dade Diagnostics, Inc. (Aguada, PR). Sodium 125 I-iodide, carrier-free, was purchased from New England Nuclear (Boston, MA) and Iodo-gen was obtained from Pierce Chemical Co. (Rockford, IL). Dulbecco's modified Eagle's medium (DME), L-glutamine, penicillin/streptomycin, fetal calf serum, and horse serum were obtained from KC Biological, Inc. (Lenexa, KS). Heparin (porcine intestinal mucosal) was purchased from Organon Diagnostics (West Orange, NJ), neuraminidase was purchased from Calbiochem-Behring Corp. (La Jolla, CA), and endo- β -N-acetylglucosaminidase (endoglycosidase-H) was purchased from Miles Scientific Div., Miles Laboratories, Inc. (Naperville, IL). All other chemicals were reagent grade or better and came from Sigma Chemical Co. or from Fisher Scientific Co. (Pittsburgh, PA).

Plasmas. Pooled normal plasma (20 donors), two plasmas (GK702 and GK704) congenitally deficient in Factor VII, and plasmas congenitally deficient in Factors II, V, VIII, IX, X, XI, XII, and von Willebrand factor were obtained from George King Biomedical (Overland Park, KS). One congenitally Factor VII-deficient plasma was purchased from Dade Diagnostics, Inc. Barium-adsorbed plasma was produced from citrated plasma by adding 1/10 vol of 1.0 M BaCl₂, incubating for 1 h at 4°C, and centrifuging at 12,000 g for 10 min.

Fresh human plasma was obtained from laboratory personnel. 9 vol of blood was drawn using the two-syringe technique into 1 vol of solution containing 10 mM diisopropylfluorophosphate (DFP), 50 mM EDTA, 1,000 U/ml aprotinin, 50 U/ml heparin, 100 μ g/ml soybean trypsin inhibitor, 100 mM benzamidine, and 100 mM Tris-HCl, pH 8.0. Cells were removed by immediate centrifugation at 12,000 g for 5 min, and the supernatant plasma was collected.

Serum was obtained from whole blood (drawn in the absence of inhibitors) by incubation in a glass test tube. Serum was produced from citrated plasma (400 μ l) by adding CaCl₂ (final concentration, 25 mM) and either: (a) 100 μ l rabbit brain phospholipids (prepared as described by Sigma Chemical Co.), in a glass test tube; (b) 100 μ l crude Russell's viper venom and cephalin, or (c) 100 μ l human brain thromboplastin. After incubation for 2 h at 37°C, the clots were gently rimmed and serum was obtained after centrifugation at 4,000 g for 10 min.

Proteins. Factor VII was isolated and radiolabeled (125 I) and a crude preparation of human brain thromboplastin was prepared as previously described (1, 7, 16). Factor VII was assumed to have an extinction coefficient ($A_{1\text{ cm}}$ at 280 nm) of 11.5 (16). Thrombin was prepared from purified prothrombin (19).

To produce chymotrypsin-treated VII, Factor VII was treated with chymotrypsin at a concentration of 1% (wt/wt) in 0.1 NaCl, 0.05 Tris-HCl, pH 8.1, for 60 min at 37°C, and the reaction was stopped by the addition of DFP (5 mM). The chymolytic cleavage product was then isolated by high pressure liquid chromatography using a mono-Q column (Pharmacia Fine Chemicals, Piscataway, NJ).

Functional assays. Factor VII clotting assays were performed as previously described using GK702 or Factor VII immunodepleted plasma (see below) as substrate plasma, and human, rabbit (Dade Diagnostics, Inc.), or bovine brain thromboplastins (1). Bovine brain thromboplastin was prepared by dissolving a vial of thrombotest reagent (Nyegaard and Co., Oslo; distributed by Accurate Chemical and Scientific Corp., Westbury, NY) in 0.6 ml of water. Activities were determined by relating clotting times to a standard curve constructed using dilutions of pooled normal plasma.

Monoclonal antibodies. Hybridoma cell lines secreting anti-Factor VII antibodies were produced by using purified human Factor VII as the immunogen in Balb/c mice, fusion of immune spleen cells with the

SP2/0-Ag14 mouse myeloma cell line, and isolation and single cell lines by cloning as previously reported (20). Positive clones were selected using an enzyme-linked immunoadsorbent assay employing microtiter dishes coated with 1 μ g/ml Factor VII, spent medium, and goat anti-mouse IgG linked to alkaline phosphatase (21). Positive clones were also tested in the same manner in the presence of 10 mM EDTA to determine if divalent metal ions were required for monoclonal antibody recognition of Factor VII.

Two clones, MC1476 (E.A. 8.1) and MC1839 (E.C. 3.3), were selected for further study. Large quantities of each were produced by the induction of peritoneal tumors in Balb/c mice (20). The antibodies were purified from the subsequent ascitic fluid by ammonium sulfate precipitation and DEAE-affigel-Blue (Bio-Rad Laboratories, Richmond, CA) chromatography as described by the manufacturer.

The monoclonal antibodies were radiolabeled using Iodo-gen and carrier-free, sodium 125 I-iodide as previously described (16). The specific radioactivity of the products varied from 1,000 to 4,000 dpm/ng IgG.

Monoclonal antibodies were coupled to affigel-10 (Bio-Rad Laboratories) at a concentration of ~ 10 mg/ml packed gel as described by the manufacturer. Immobilization of monoclonal antibodies on latex beads was performed by adding 0.5 mg of monoclonal antibody to 25 mg latex beads in total volume of 5 ml of 0.1 M sodium phosphate, pH 5.5. The mixture was rocked for 2 h at room temperature. Then the beads were washed with 0.1 M sodium phosphate, pH 5.5, and resuspended to 5 ml with 0.1 M NaCl, 0.05 Tris-HCl, pH 7.5. Consistently, >90% of the monoclonal antibody bound to the beads under these conditions.

Monoclonal sandwich assay for Factor VII. To 50 μ l of citrated plasma was added 20 μ l of a 0.5% (wt/vol) slurry of latex beads to which MC1839 had been immobilized, and 20 μ l of 125 I-MC1476 (25 μ g/ml). Both additions were in a buffer consisting of 0.1 M NaCl, 0.05 M CaCl₂, 5 U/ml heparin, 1 mg/ml bovine serum albumin (BSA), and 0.05 M Tris-HCl, pH 7.5. After incubation for 1 h at room temperature the latex beads were isolated by centrifugation (12,000 g for 3 min) and the supernatant was discarded. The latex beads were then resuspended in 0.1 M NaCl, 0.01 M CaCl₂, 1 U/ml heparin, 1 mg/ml BSA, 0.05 M Tris-HCl, pH 7.5, and again isolated by centrifugation. The wash supernatant was discarded and the radioactivity was bound to the beads determined by gamma counting.

Factor VII-depleted plasma (<1% functional activity) was made by passing pooled normal plasma (George King Biomedical) through MC1476-affigel. Standard curves were constructed by reconstituting the immunodepleted plasma with purified Factor VII or mixing known quantities of normal pooled plasma and immunodepleted plasma (Fig. 1). There was a linear relationship between counts per minute and the concentration of Factor VII or pooled normal human plasma with a correlation coefficient of 0.998.

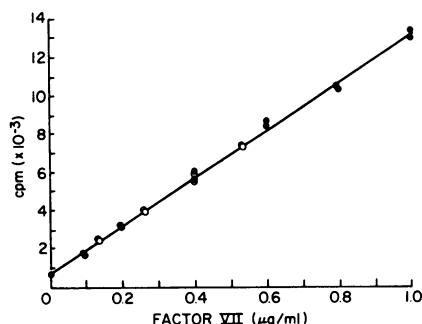


Figure 1. Monoclonal sandwich immunoassay for Factor VII. The assay was performed as described in Methods. ●, Addition of purified Factor VII to Factor VII immunodepleted plasma. ○, Mixtures of Factor VII immunodepleted plasma and pooled normal human plasma at concentrations of normal plasma of 25%, 50%, 75%, and 100%.

Western blots. Plasma samples for Western blots were prepared by initial concentration and purification of plasma Factor VII by absorption and elution from monoclonal antibodies linked to affigel-10 (MC-affigel). In the case of MC1476, 500 μ l of citrated plasma was incubated with 50 μ l of a 50% (vol/vol, in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5) slurry of MC-affigel for 1 h at room temperature. The agarose beads were isolated by centrifugation, washed once with 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, and the bound Factor VII was eluted by heating to 100°C for 5 min in 75 μ l of a solution containing 0.83 M Tris-HCl, pH 6.8, 13.3% glycerol, 2.67% sodium dodecyl sulfate (SDS), and 0.00067% bromophenol blue. The MC-affigel was removed by centrifugation, and 50 μ l of supernatant subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The procedure for MC1839 was the same with the exceptions that CaCl_2 (final concentration, 25 mM) and heparin (final concentration, 5 U/ml) were added to the citrated plasma and the wash buffer contained 10 mM CaCl_2 . The same procedures were used to prepare samples from serum except the addition of CaCl_2 and heparin were omitted.

SDS-PAGE was performed on 1.5-mm-thick slab gels using a 4% stacking gel and 10% separating gel in the buffer system of Laemmli (22). Electrophoretic transfer to nitrocellulose was performed in the buffer system of Towbin et al. (23), using a Hoefer Transfer cell with a recirculating water bath (10°C) at 60 V for 90 min.

The nitrocellulose paper was "blocked" by incubation with 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 10 mg/ml BSA, and 0.6 mg/ml bovine IgG for 1 h at room temperature. Staining was then accomplished by the addition of radiolabeled ^{125}I -monoclonal antibody at a final concentration of 0.2 $\mu\text{g}/\text{ml}$ and further incubation overnight with gentle agitation on a Junior Orbit Shaker (Lab-Line Instruments, Inc., Melrose Park, IL). The nitrocellulose paper was washed three times with 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, dried with cool air, and placed in a cassette fitted with intensifying screens (Cronex; E. I. Du Pont de Nemours & Co., Inc., Wilmington, DE) with XAR-5 film (Eastman Kodak Co., Rochester, NY) for 4–24 h at -80°C . In the case of MC1839, 10 mM CaCl_2 was also present in each buffer. For the purposes of this paper the term "Western blotting" will encompass the processes of SDS-PAGE, electrophoretic transfer of proteins from the polyacrylamide gel to nitrocellulose paper, and "staining" with radiolabeled antibody.

Infusion of VII into rabbits. 60 μg of Factor VII or ^{125}I -Factor VII (3,170 cpm/ng) in 200 μ l of 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, were infused into separate male New Zealand white rabbits (~3 kg) through the marginal ear vein of the right ear. Blood samples (1.5 ml) were drawn from the left ear into 50 μ l of 0.2 M EDTA (pH 7.0) at 8 min, 30 min, and 1, 2, 3, 4, 5, 6, and 8 h. The samples were immediately centrifuged at 12,000 g for 5 min and the plasma was frozen. The next day the plasma samples were thawed, and the Factor VII (or ^{125}I -VII) absorbed and eluted from MC1476-affigel as previously described. The radioactive samples were reduced (5% 2-mercaptoethanol) and subjected to SDS-PAGE followed by autoradiography. The nonradioactive samples were processed through Western blotting as described above, and stained with ^{125}I -MC1476.

Partial purification of Factor VII CRM in plasma. 1 U (240 ml) of fresh frozen plasma was passed through a 1-ml column of MC1476-affigel at 10 ml/h at room temperature. The column was washed with 0.1 M NaCl, 0.005 M EDTA, 0.05 M Tris-HCl, pH 7.5, and eluted with 3 M NaSCN, 0.05 M Tris-HCl, pH 7.5 containing 0.1 mg/ml BSA at a flow rate of 2 ml/h. Fractions containing VII activity were pooled (5 ml), and dialyzed against 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5. The MC1476-affigel absorbed >99% of the VII activity and after elution, recovery of activity was 90%. 2-ml fractions of this partially purified VII/VII* were then dialyzed and passed through 1-ml columns of MC1839-affigel (buffer: 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.004 M CaCl_2 , 0.1 mg/ml BSA) or Con-A Sepharose (buffer: 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.002 M CaCl_2 , 0.002 M MgCl_2 , 0.002 M MnCl_2 , 0.1 mg/ml BSA) at a flow rate of 1 ml/h. The column effluents were tested for the presence of VII CRM by Western blotting using ^{125}I -MC1476.

HepG2 cell experiments. HepG2 cells were cultured in T-75 flasks, with DME plus 4 mM L-glutamine, 10% fetal calf serum, and 1 $\mu\text{g}/\text{ml}$

vitamin K. The cells were allowed to proliferate to confluence. The medium was removed from the flasks and the adherent cells were washed gently twice with 15 ml of medium lacking fetal calf serum or vitamin K. The medium was then replaced with DME, 4 mM L-glutamine, and 1 $\mu\text{g}/\text{ml}$ vitamin K (four flasks) or DME, 4 mM L-glutamine, and 20 $\mu\text{g}/\text{ml}$ warfarin (two flasks). The cells were incubated overnight and then washed with the appropriate medium twice (15 ml each). To two of the flasks which had been incubated with vitamin K, 5 ml of the same medium was added. To the other set of two flasks that had been incubated with vitamin K, 5 ml of vitamin K containing medium plus ^{125}I -VII (0.05 $\mu\text{g}/\text{ml}$) was added. The two flasks incubated with warfarin received 5 ml each of the same medium. After incubation for an additional 24 h (37°C, 5% CO_2), the medium from each duplicate set of flasks was collected and pooled, a 1/10 volume of inhibitor cocktail (400 $\mu\text{g}/\text{ml}$ soy bean trypsin inhibitor, 0.1 M benzamidine, 0.1 M EDTA, 1 mg/ml leupeptin 0.2 M Tris-HCl, pH 8.1, DFP 20 mM) was added, and cellular debris was removed by centrifugation. The adherent cells remaining in the T-flasks were washed twice with 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.005 M EDTA, and lysed by incubation at room temperature for 1 h on a rotating shaker table in a buffer consisting of 5 ml of 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.010 M EDTA, 40 $\mu\text{g}/\text{ml}$ SBTI, 10 mM benzamidine, 100 $\mu\text{g}/\text{ml}$ leupeptin, 2 mM DFP containing 2% (vol/vol) Triton X-100. Duplicate mixtures were combined and centrifuged for 10 min at 12,000 g and the supernatants were collected. 200 μ l of a 50% slurry of MC1476-affigel was added to each sample of conditioned medium or cell lysate and the mixtures were suspended by rocking at 4°C for 18 h. The MC1476-affigel was then collected by centrifugation, washed, and eluted for SDS-PAGE and Western blots or autoradiography (^{125}I -VII) as previously described.

The same method was used to obtain samples of conditioned medium and cell lysates from HepG2 cells cultured with vitamin K for subsequent treatment with neuraminidase and endoglycosidase-H. After elution from MC1476-affigel the samples from conditioned medium and cell lysate were dialyzed against 0.02 M ammonium bicarbonate, divided into two equal portions, and lyophilized. One of the lyophilized aliquots from the conditioned medium was resuspended in 40 μ l of 0.05 M sodium acetate, pH 5.5, 0.145 M NaCl, 4 mM CaCl_2 , 50 mM benzamidine and the other was resuspended in 40 μ l of the same buffer with 1 U/ml neuraminidase. One of the lyophilized aliquots from the lysed cells was suspended in 40 μ l of 50 mM sodium citrate, pH 5.5, and the other in 40 μ l of the same buffer with 50 mU/ml endoglycosidase-H. The samples were incubated overnight under toluene at 37°C and then subjected to SDS-PAGE and Western blotting as previously described.

Results

Characterizations of antibodies. Several murine monoclonal anti-Factor VII antibodies were produced using hybridoma technology. Two, MC1476 and MC1839, were selected for further study. MC1476, an IgG₁, recognized Factor VII in the presence or absence of divalent cations, while MC1839, an IgG_{2A}, recognized Factor VII in the presence of 4 mM CaCl_2 , but failed to bind Factor VII in the absence of divalent cations (5 mM EDTA). Within experimental error, both MC antibodies recognized Factor VII and VII_a with equal affinities and neither monoclonal anti-Factor VII antibody recognized CRM in dog, cow, or rabbit plasma (data not shown).

To determine the epitope specificity of the antibodies, western blots of purified Factor VII after activation by thrombin, or treatment with chymotrypsin were performed. Analysis of thrombin-treated Factor VII after reduction and SDS-PAGE revealed the heavy and light chains of Factor VII_a and an additional degradation product (Fig. 2, lane 8). Both antibodies recognized an epitope on the light chain of Factor VII_a. MC1476 also recognized the degradation product (see Discussion). MC1839 ap-

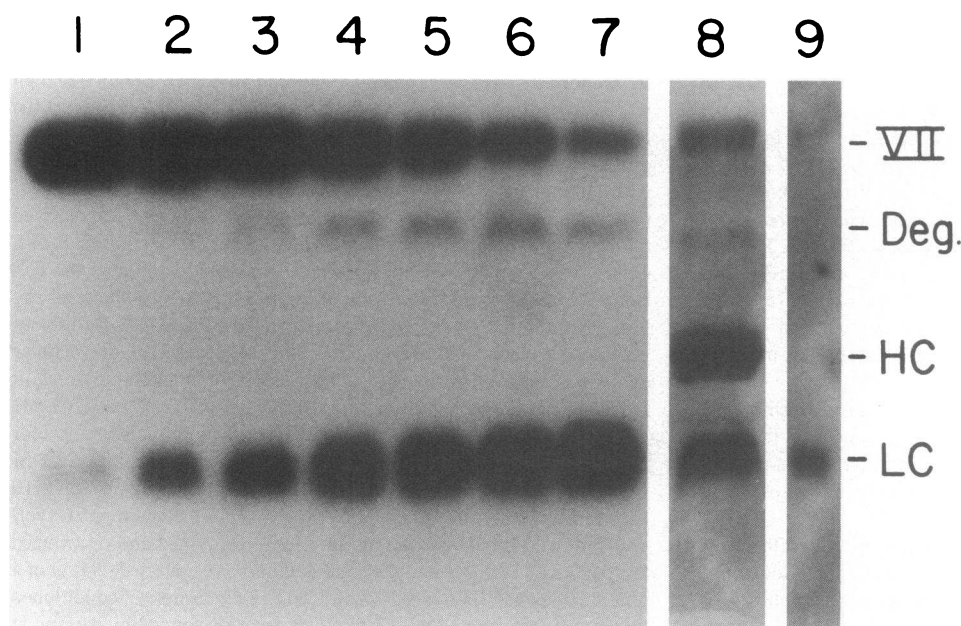


Figure 2. Factor VII light chain epitope specificity of MC1476 and MC1839. Purified Factor VII was activated by treatment with thrombin (wt/wt = 5%) at room temperature in 0.1 M NaCl, 0.05 Tris-HCl (pH 8.0). Aliquots were removed at specific times, the reaction was stopped by the addition of DFP (5 mM), the samples were reduced with 5% 2-mercaptoethanol, and subjected to SDS-PAGE. Lanes 1–7, Western blot using ^{125}I -MC1476 of Factor VII samples (5 μg) obtained at 2, 5, 10, 20, 30, 40, and 60 min after the addition of thrombin. Lane 8, Coomassie Brilliant Blue stain of Factor VII_a (15 μg) after 60 min incubation with thrombin. Lane 9, Western blot using ^{125}I -MC1839 of Factor VII_a (5 μg) at 60 min after thrombin addition. VII, purified Factor VII; Deg, degradation product; HC, Factor VII_a heavy chain; LC, Factor VII_a light chain.

peared to require an epitope very near the NH_2 -terminus since it failed to recognize chymotrypsin-treated Factor VII (Fig. 3). MC1476, on the other hand, recognized an epitope distal to the chymotrypsin cleavage site of VII. These results are consistent with the previously noted calcium dependency of binding of MC1839 since for the other vitamin K-dependent factors thus far studied, chymotrypsin cleaves a 40–44-amino acid peptide from the NH_2 -terminus of these molecules, which contains all the gamma carboxyglutamic acid residues (e.g., 24–26).

The apparent efficiency of ^{125}I -monoclonal antibody “staining” of VII or VII_a electrophoretically transferred from polyacrylamide gels to nitrocellulose was considerably decreased for both monoclonal antibodies if the proteins were reduced (5% 2-mercaptoethanol) before SDS-PAGE. Preliminary studies suggest that this phenomenon was not due to decreased transfer or absorption of protein to the nitrocellulose.

Western blots of native Factor VII in human plasmas. Technical problems arose when similar blotting experiments were attempted using samples of plasma. When sufficient quantities of plasma were subjected to SDS-PAGE ($\sim 5 \mu\text{l}$) so that adequate “staining” of the electrotransferred Factor VII could be detected, distortion of the patterns due to large quantities of extraneous proteins (especially albumin) prevented meaningful interpretation. Barium citrate adsorption of the plasma and subsequent elution of the barium citrate pellet with ammonium sulfate were found to be effective means of concentrating and partially purifying the Factor VII before SDS-PAGE and Western blotting. However, not all the Factor VII CRM present in plasma bound to barium citrate (see below).

Therefore, for the remaining studies, the plasma Factor VII was partially purified and concentrated by affinity absorption to monoclonal antibody linked to affigel-10 (MC-affigel). After elution from the gel with SDS, the proteins were subjected to SDS-PAGE, electrophoretic transfer to nitrocellulose, and “staining” with radiolabeled monoclonal antibody. Under the conditions noted in Methods, 90% of ^{125}I -Factor VII

(0.5 $\mu\text{g}/\text{ml}$) added to plasma was absorbed by MC1476 affigel, and 80% by MC1839 affigel in 1 h.

Fig. 4 shows the results of western blots using George King 702 (GK702) and 704 (GK704) and Dade Factor VII-deficient plasmas. Neither MC1476 nor MC1839 detected CRM in GK702 plasma and the amount of CRM in GK704 and Dade plasmas appeared to be reduced from that of normal human plasma. In control experiments the level of Factor VII that could be detected by these methods was determined by adding various concentrations of purified Factor VII (or pooled normal human plasma assumed to contain 100% Factor VII) to GK702 plasma. Adsorption and staining using MC1476 could detect $<0.0039 \mu\text{g}/\text{ml}$ ($<0.8\%$) Factor VII, and adsorption and staining with MC1839 $\sim 0.0313 \mu\text{g}/\text{ml}$ ($\sim 6.25\%$) Factor VII. When plasma was absorbed using MC1839-affigel and then ^{125}I -MC1476 was used to stain the subsequent blot, $0.0078 \mu\text{g}/\text{ml}$ Factor VII (1.56%) could be detected. As determined by their recognition by these two monoclonal antibodies, GK702 was CRM[−], GK704 was CRM⁺, and Dade was CRM⁺ for Factor VII (see Discussion).

The apparent functional activity of the Factor VII in each deficient plasma was evaluated using human, rabbit, or bovine brain thromboplastins (Table I). GK702 and Dade Factor VII-deficient plasmas had $<1\%$ functional activity compared with pooled normal plasma no matter which species of thromboplastin was employed. GK704 plasma, however, demonstrated considerable Factor VII functional activity when human or bovine thromboplastin was used. Using the monoclonal “sandwich” immunoassay described in Methods, GK702 plasma contained $<1\%$ (measured consistently less than the Factor VII immuno-depleted plasma), GK704 plasma contained 45%, and Dade plasma contained 36% of the Factor VII antigenic material in pooled normal human plasma.

Detection of another protein in human plasma antigenically related to Factor VII. The Western blots performed with MC1476 recognized an additional protein with a faster mobility on SDS-PAGE than purified Factor VII (Fig. 4). This band “stained”

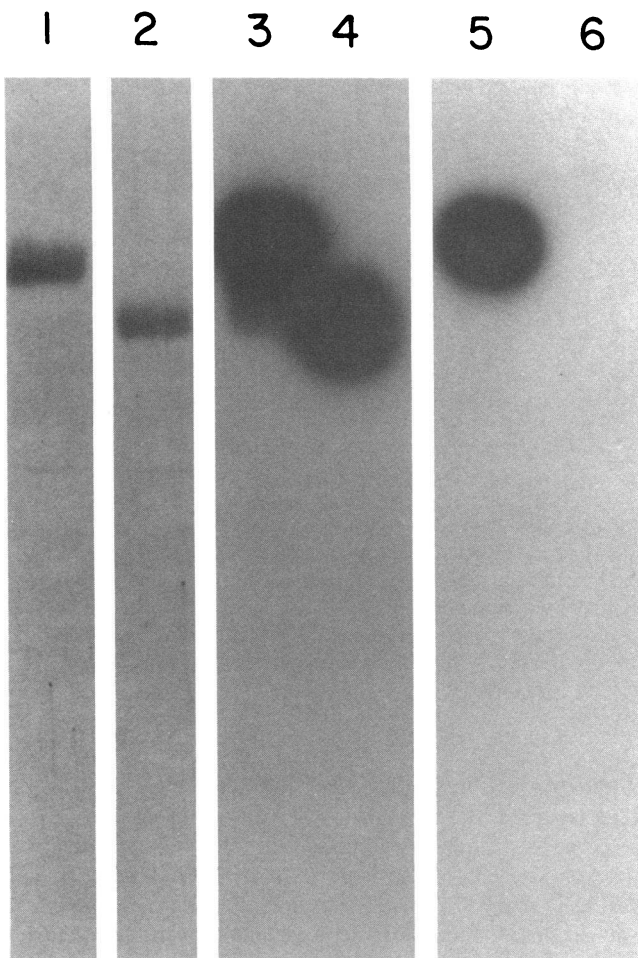


Figure 3. Recognition of Factor VII and chymotrypsin-treated Factor VII by MC1476 and MC1839. SDS-PAGE of Factor VII (2 µg), lanes 1, 3, 5; and chymotrypsin-treated Factor VII (2 µg), lanes 2, 4, 6. Coomassie Brilliant Blue staining, lanes 1 and 2. Western blot using ^{125}I -MC1476, lanes 3 and 4. Western blot using ^{125}I -MC1839, lanes 5 and 6.

with a lower intensity, and frequently appeared as a doublet with a molecular weight of $\sim 4,500$ D less than purified Factor VII. This form of Factor VII CRM, tentatively named VII*, was not detected by MC1839 (Fig. 4, B and C). Its presence was not due to proteolysis during the blotting procedure itself since fresh plasma drawn and prepared for Western blotting using a host of inhibitors (see Methods) contained comparable amounts of Factor VII* to pooled normal plasma (Fig. 4, lanes 1 and 7) and GK702 plasma reconstituted with purified Factor VII (0.5 µg/ml) (or VII_a, data not shown) and processed for Western blotting in the absence of inhibitors failed to contain detectable VII* (Fig. 4, lane 3).

Although the presence of VII* did not seem to be due to artifactual proteolysis of Factor VII during the sample preparation for blotting, it remained possible that it was the result of intravascular proteolysis. However, when GK702 plasma was reconstituted with Factor VII (0.5 µg/ml) and induced to clot in the presence of phospholipids and Ca^{++} by the addition of a glass surface, Russell's viper venom, or human brain thromboplastin, VII* was not formed. The amount of VII* in normal plasma or fresh blood was also not detectably enhanced by similar *in vitro* clotting. Further, plasmas congenitally deficient in Factors II, V, VIII, IX, XI, XII, or von Willebrand factor all contained VII*.

Next, ^{125}I -Factor VII or unlabeled VII was infused into New Zealand white rabbits and plasma samples were obtained over 8 h. The ^{125}I -VII level immediately post infusion was 0.36 µg/ml and fell with a $t_{1/2}$ of 3.2 h. During the experiments, no conversion of the Factor VII to VII* (or Factor VII_a) could be detected by autoradiography (^{125}I -VII) or Western blots (unlabeled VII) after SDS-PAGE of samples concentrated by affinity adsorption to MC1476-*affigel*.

When plasma was treated with barium citrate, most of the VII was absorbed to the barium salt while the VII* remained in the supernatant (Fig. 5, lanes 1 and 2). VII* appeared to be a single-chain protein since its mobility on SDS-PAGE was not affected by treatment with 5% 2-mercaptoethanol (Fig. 5, lanes 3 and 4). As noted previously, the efficiency of ^{125}I -monoclonal

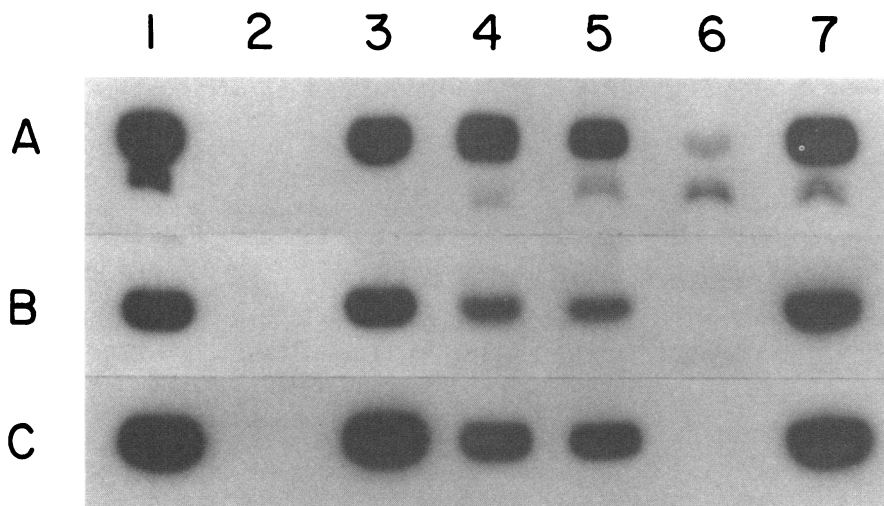


Figure 4. Western blots of Factor VII-deficient plasmas. Western blots of plasmas with monoclonal anti-Factor VII antibodies were performed as described in Methods. Lane 1, fresh normal human plasma drawn into inhibitors. Lane 2, GK702 plasma. Lane 3, GK702 plasma reconstituted with purified Factor VII (0.5 µg/ml). Lane 4, GK704 plasma. Lane 5, Dade plasma. Lane 6, plasma from a patient receiving chronic warfarin therapy. Lane 7, pooled normal human plasma. Row A, Western blot performed by immunoabsorption with MC1476-*affigel* and staining with ^{125}I -MC1476. Row B, Western blot performed by immunoabsorption with MC1839-*affigel* and staining with ^{125}I -MC1839. Row C, same blot as B after removal of ^{125}I -MC1839 by brief washing with 0.1 M NaCl, 0.05 M Tris-HCl (pH 7.5), 5 mM EDTA, and restaining with ^{125}I -MC1476. Only the portion of the Western blots containing Factor VII CRM is shown.

Table 1. Factor VII Functional and Antigenic Levels in Factor VII Deficient Plasmas

VII-deficient plasma	Functional activity			Antigen levels
	Thromboplastin source			
	Human	Rabbit	Bovine	
	%	%	%	%
GK702	<1	<1	<1	<1*
GK704	11	1.4	30	45
Dade	<1	<1	<1	36

* GK702 plasma consistently gave values lower than Factor VII immunodepleted plasma containing <1% functional activity.

antibody "staining" of Factor VII CRM was decreased if the samples were reduced before SDS-PAGE (compare Fig. 5, lanes 1 and 2 with lanes 3 and 4).

VII and VII* were partially purified by affinity chromatography on MC1476-afigel (Methods) from fresh frozen plasma (Fig. 5, lane 6). As determined by Western blotting using ^{125}I -MC1476, the passage of this material through MC1839-afigel yielded VII* and reduced amounts of VII (Fig. 5, lane 5). Both VII and VII*, however, were bound by Con-A Sepharose, suggesting the presence of carbohydrate moieties on both proteins (Fig. 5, lane 7). Western blotting using ^{125}I -MC1476 was also employed to determine the effect of chymotrypsin treatment of partially purified VII/VII* (Fig. 6). Treatment with low concentrations of chymotrypsin showed apparent cleavage of VII to a form with the same molecular weight as VII*. Higher concentrations of chymotrypsin produced further cleavage of VII and

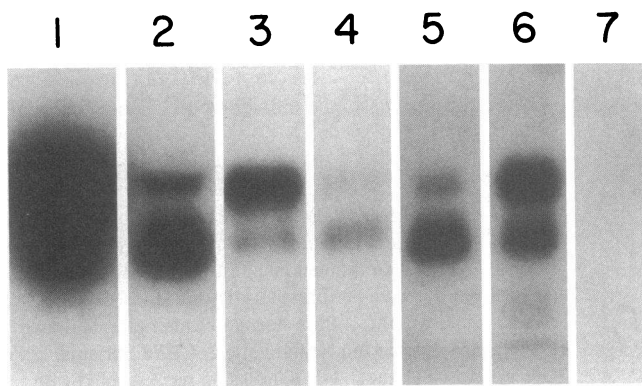


Figure 5. Western blot of Factor VII and VII*. Western blots of various samples of Factor VII and VII* were performed using ^{125}I -MC1476. Lane 1, Factor VII CRM in pooled normal plasma was immunoabsorbed with MC1476-afigel and subjected to SDS-PAGE without reduction. Lane 2, same as lane 1 except the plasma was barium citrate-absorbed before immunoabsorption with MC1476-afigel. Lane 3, same as lane 1 except sample was reduced with 5% 2-mercaptoethanol before SDS-PAGE. Lane 4, same as lane 2 except sample was reduced with 5% 2-mercaptoethanol before SDS-PAGE. (The Western blots in lanes 1-4 were autoradiographed for the same length of time.) Partially purified Factor VII/VII* was prepared and applied to a MC1839-afigel affinity column or Con-A Sepharose column as described in Methods. Lane 5, flow through from MC1839-afigel affinity column (20 μl). Lane 6, partially purified Factor VII/VII* (10 μl). Lane 7, flow through from Con-A Sepharose column (20 μl).

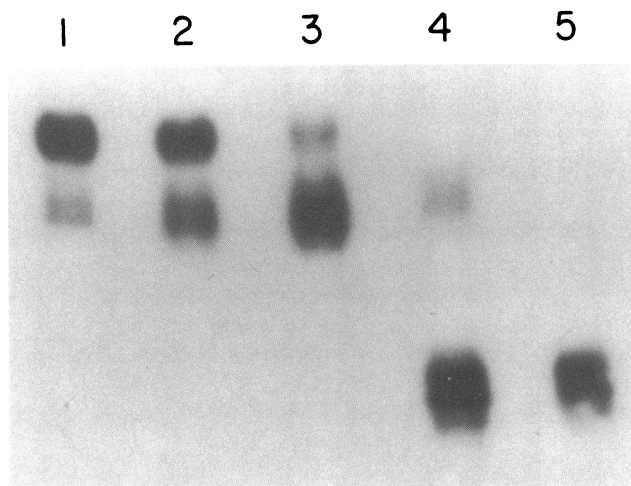


Figure 6. Chymotrypsin treatment of partially purified Factor VII/VII*. Factor VII/VII* was partially purified as described in Methods and treated with α -chymotrypsin for 30 min at room temperature. Samples were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose paper, and "stained" with ^{125}I -MC1476. Lane 1, 0.0 $\mu\text{g}/\text{ml}$; lane 2, 1 $\mu\text{g}/\text{ml}$; lane 3, 2 $\mu\text{g}/\text{ml}$; lane 4, 4 $\mu\text{g}/\text{ml}$; lane 5, 8 $\mu\text{g}/\text{ml}$ chymotrypsin.

VII* to products that had a lower molecular weight or that were not recognized by MC1476.

Since VII* did not bind to barium salts and was not recognized by MC1839, which requires the NH_2 -terminus of VII, it was likely that VII* lacked functional Factor VII activity. To confirm this, VII and VII* were separated by anion exchange chromatography. VII and VII*, which had been partially purified by immunoaffinity chromatography (MC1476-afigel, Methods), were dialyzed against 0.05 M Tris-HCl, pH 8.1, and applied to a mono-Q column, which was developed using a linear gradient from 0.05 M Tris-HCl, pH 8.1-1.0 M NaCl, 0.05 M Tris-HCl, pH 8.1. Fractions containing VII*, as determined by Western blotting using ^{125}I -MC1476, eluted before those containing VII, and contained no detectable Factor VII activity as determined by functional assay (data not shown).

Effect of warfarin therapy on plasma levels of VII and VII*

Lane 6 in Fig. 4 shows the Western blot of the plasma from a patient on long-term warfarin therapy for atrial fibrillation (prothrombin time, 28 s; control, 11 s). Interestingly, though the amount of CRM running at the same molecular weight as purified Factor VII (lane 3) is markedly reduced, the amount of VII* present is comparable to that in normal pooled plasma (lane 7) when the plasmas are absorbed and "stained" using MC1476. To further evaluate the relationship between Factor VII and VII* after following warfarin therapy, one of us (J.P.M.) was given a short course of high dose warfarin and plasma samples were obtained every 8 h. The results of Western blotting and measurements of functional Factor VII activity are shown in Fig. 7. Two sets of Western blots were performed. In the first (row A) the plasma samples were absorbed with MC1476-afigel and stained with ^{125}I -MC1476. In the second (row B) the plasma samples were absorbed with MC1839 and stained with ^{125}I -MC1476. The relative quantities of Factor VII and VII* on the Western blots were determined by laser densitometry. After the administration of warfarin, Factor VII functional activity and MC1839 detectable CRM fell rapidly to a level of $\sim 2\%$ (Fig. 7,

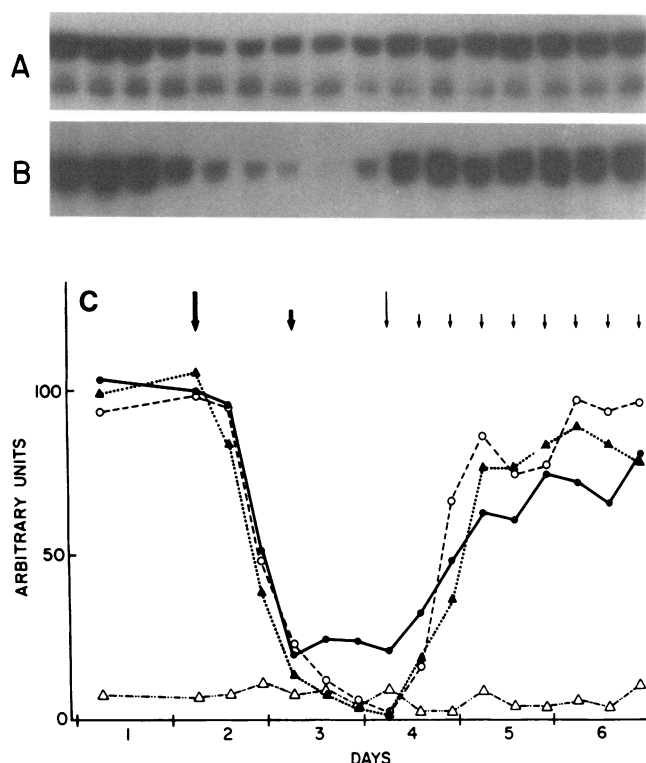


Figure 7. Effect of warfarin treatment on the levels of Factor VII and VII*. A normal individual was given warfarin (●, 60 mg; ▲, 30 mg) and subsequently vitamin K (1, 20 mg; △, 5 mg). Plasma samples were obtained every 8 h after administering warfarin. (A) Western blot, using immunoabsorption to MC1476-*affigel* and staining with ^{125}I -MC1476, of each plasma sample. (B) Western blot, using immunoabsorption to MC1839-*affigel* and staining with ^{125}I -MC1476. (Only the portion of the Western blots containing Factor VII CRM is shown.) (C) Quantitation of Factor VII/VII* CRM detected by Western blotting (determined by laser densitometry) and functional Factor VII activity. ●, VII CRM from A; △, VII* CRM from A; ○, VII CRM from B; ▲, Factor VII functional activity.

C). VII detected by MC1476, however, never fell below 20%. VII* was detectable throughout the course of the experiment, and its level relative to that of VII (as recognized by MC1476) increased from ~7% before warfarin treatment to >35% at the nadir of Factor VII functional activity.

After warfarin treatment, MC1839 did not detect the same quantity of CRM that was recognized by MC1476. This is consistent with MC1839's requirement for divalent cations and the NH_2 -terminus of Factor VII since, presumably, the Factor VII synthesized under the influence of warfarin possesses decreased or absent gamma-carboxyglutamic acid residues.

Production of VII and VII* by HepG2 cells. The human hepatocellular carcinoma cell line HepG2 has been shown to produce prothrombin and Factor X (27). Conditioned medium (24 h) and cell lysates were obtained from confluent cultures of HepG2 cells that had been grown in the presence of vitamin K or warfarin and prepared for Western blotting using MC1476 as described in Methods. To quantitate the relative amounts of VII and VII* the appropriate areas of the nitrocellulose blot were cut out and their radioactivity determined by gamma counting. The conditioned medium from HepG2 cells cultured in the presence of vitamin K contained both VII and VII* (Fig.

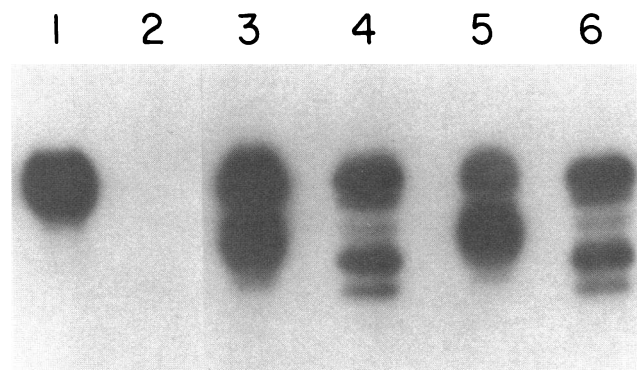


Figure 8. Factor VII CRM in conditioned medium and cell lysates from HepG2 cells. HepG2 cells were cultured in the presence of vitamin K (1 $\mu\text{g}/\text{ml}$) or warfarin (20 $\mu\text{g}/\text{ml}$) as described in Methods. Lanes 1 and 2, autoradiograph of conditioned medium and cell lysate, respectively, from HepG2 cells cultured in the presence of vitamin K and 50 ng/ml ^{125}I -VII. Lanes 3 and 4, Western blot (using MC1476) of the conditioned medium and cell lysate, respectively, from HepG2 cells cultured in the presence of vitamin K. Lanes 5 and 6, Western blot (using MC1476) of the conditioned medium and cell lysate, respectively, from HepG2 cells cultured in the presence of warfarin.

8, lane 3) and the quantity of VII* was 75% of that of Factor VII, a level considerably higher than the 5–8% noted in human plasma (Fig. 7). The conditioned medium from the warfarin-treated cells (Fig. 8, lane 5) contained markedly reduced amounts of VII (23%) but only slightly reduced amounts of VII* (86%) when compared with the conditioned medium from cells cultured in the presence of vitamin K (Fig. 8, lane 3). Western blots of intracellular material obtained by detergent lysis of the cells revealed several similar bands in both vitamin K and warfarin treated cells (Fig. 8, lanes 4 and 6). Control experiments in which ^{125}I -VII was added to the cell cultures (Fig. 8, lane 1) or to the cells at the time of detergent lysis (not shown) failed to detect proteolytic cleavage of the radiolabeled VII under the same conditions.

VII and VII* contain asparagine-linked oligosaccharides since both were bound by Con-A Sepharose (Fig. 5, lane 7). To further investigate the carbohydrate content of the Factor VII CRM, conditioned medium and cell lysates from HepG2 cells cultured in the presence of vitamin K were treated with neuraminidase (cleavage of terminal sialic residues (28) or endoglycosidase-H (cleavage of high mannose-type oligosaccharides) (29) (Fig. 9). The apparent molecular weight of both Factor VII and VII* isolated from conditioned medium was reduced after neuraminidase treatment, demonstrating that they both contain sialic acid residues. Endoglycosidase-H treatment of these secreted proteins had no effect on the Factor VII CRM, suggesting that the Factor VII and VII* asparagine-linked oligosaccharides are of the complex-type (data not shown). It is known that asparagine-linked oligosaccharides of the complex-type are initially high mannose-type structures before extensive intracellular carbohydrate processing (30). After endoglycosidase-H treatment of the cell lysate proteins, the four major bands of Factor VII CRM were consolidated into two bands suggesting that the intracellular heterogeneity of Factor VII and VII* is the result of varying degrees of oligosaccharide processing. As shown in Fig. 9, treatment of intracellular Factor VII CRM did not result in the conversion of VII to VII*.

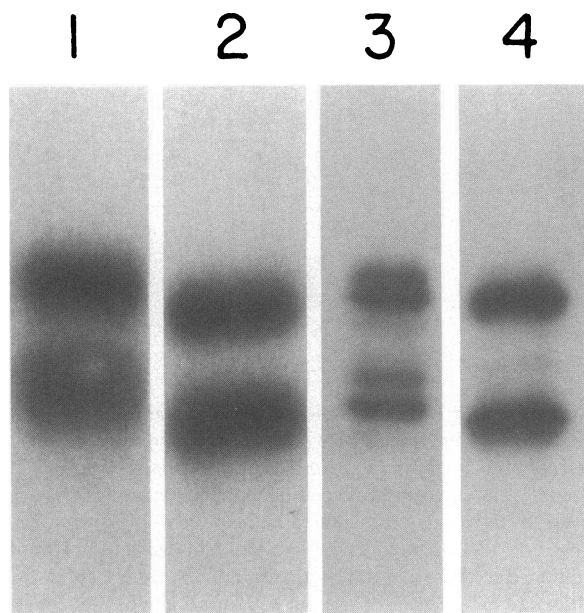


Figure 9. Neuraminidase and endoglycosidase-H treatment of conditioned medium and cell lysates from HepG2 cells. HepG2 cells were cultured in the presence of vitamin K (1 $\mu\text{g}/\text{ml}$) and the Factor VII CRM in the conditioned medium and cell lysate partially purified by immunoabsorption (MC1476-affigel) as described in Methods. Lanes 1 and 2, Western blot (^{125}I -MC1476) of conditioned medium before and after treatment with neuraminidase. Lanes 3 and 4, Western blot (^{125}I -MC1476) of the cell lysate before and after treatment with endoglycosidase-H.

Discussion

Patients with congenital Factor VII deficiency are rare and most reports have dealt with only very few patients. The presenting hemorrhagic manifestations varied widely and the clinical severity of the disease did not appear to correlate well with the measured levels of Factor VII. In a recent article, however, Mariani and Mazzucconi (17) have reviewed the experience at eight European institutions following 40 patients with Factor VII deficiency. They found that patients with Factor VII levels $\leq 5\%$ suffered hemarthroses (16/24); on the other hand, none (0/16) of the patients with Factor VII levels from >5 to 37% suffered hemarthroses. Other bleeding manifestations, however, were found in both groups of patients. They also noted no relationship between the immunologic status (CRM⁺, CRM^R, CRM⁻, as measured by inhibitor neutralization assay) of the patients and clinical symptoms.

GK702 plasma was CRM⁻, and GK704 and Dade Factor VII-deficient plasmas were CRM^R when tested using both MC1476 and MC1839. These results were confirmed when the same plasmas were tested using a third monoclonal antibody (1839 E.D. 11.3), which recognizes an epitope distal to the chymotrypsin cleavage site on the light chain of Factor VII, and similar to MC1476 detects both VII and VII* in normal human plasma (data not shown). The CRM status of patients with functional deficiencies of a particular protein, however, are classically determined through the use of polyclonal antisera. Such antisera contains multiple antibodies recognizing many separate epitopes on the protein molecule in question. Thus, an abnormal molecule lacking one or more "normal" epitopes could conceivably escape detection by monoclonal antibodies and still be recognized

by the polyclonal antisera. This may explain the apparent discrepancy concerning the CRM status of GK702 plasma between our results using monoclonal antibodies (CRM⁻, $<1\%$ antigen level) and those of Fair (18) using a polyclonal antibody (CRM^R, 12% antigen level).

The monoclonal sandwich assay for Factor VII described using MC1476 and MC1839 is simple and convenient. It is faster, and at least as sensitive as the competitive radioimmunoassay that has been previously reported (18). Since the sandwich assay employs MC1839, however, it does not detect VII* or the gamma carboxyglutamate-deficient Factor VII synthesized under the influence of warfarin (Figs. 4 and 7). Indeed, additional experiments (not shown) have confirmed a very close correlation between immunologic levels of Factor VII measured by this monoclonal sandwich assay and functional Factor VII activity after warfarin therapy in the experiment noted in Fig. 7 (correlation coefficient, 0.982).

Interestingly, GK704 plasma produced differing apparent functional levels of Factor VII depending upon the species of thromboplastin used in the assay. The species specificity of the Factor VII-tissue factor interaction is well known, and variant Factor VII molecules with different patterns of "activation" with human, rabbit, and bovine brain thromboplastins have been described (31–33). GK704 plasma expressed the greatest relative activity with bovine brain (30%) $>$ human brain (11%) $>$ rabbit brain (1.4%), a pattern similar to that reported for the Padua₁ Factor VII variant (31). Unlike GK704, however, the plasma from patients with the Padua₁ variant are CRM⁺ and yield normal levels of VII functional activity when assayed with bovine thromboplastin. It is conceivable that the donor for GK704 plasma is doubly heterozygote for the Padua₁ and an additional Factor VII variant.

Clinically, the GK704 donor has had no bleeding problems, and his coagulation abnormality was discovered as an incidental finding by laboratory testing. Both the GK702 and Dade donors have manifested a significant bleeding diathesis including hemorrhage following tooth extractions and surgery. Neither, however, has suffered hemarthrosis. None of the donors have experienced thromboembolic complications.

Unexpectedly, Western blots of plasma using MC1476 revealed two bands: a major band migrating with the same mobility on SDS-PAGE as purified Factor VII; and a minor band moving with a faster mobility. The minor band, which we have called VII*, has an apparent molecular weight of $\sim 4,500$ D less than Factor VII and "stains" with an intensity ~ 5 –8% that of the major band. Its actual concentration in plasma cannot be calculated since its affinity for MC1476 (vol/vol that of VII) is unknown. The fact that VII* was absent from GK702 plasma, which also lacks native VII, and appeared decreased in GK704 and Dade CRM^R VII deficient plasmas, is strong evidence that it is genetically related to Factor VII, and does not represent cross-reactivity of the MC1476 antibody with an unrelated plasma protein. Two additional plasmas from unrelated Factor VII-deficient donors have subsequently been tested and show the same CRM pattern as GK702 when tested by Western blotting using MC1476—that is, the lack of both VII and VII* (data not shown).

VII* was similar to chymotrypsin-treated VII in its molecular weight, its lack of functional Factor VII activity, its lack of binding to barium citrate, and its lack of recognition by MC1839, suggesting that it represented a proteolytic product of native VII which lacked the NH₂-terminal portion of the molecule con-

taining the gamma carboxyglutamic acid residues. Radcliffe and Nemerson (34) have demonstrated that treatment of bovine Factor VII with Factor X_a led to its inactivation by proteolytic degradation. This process involved the proteolytic cleavage of a 12,500-Dalton polypeptide, containing the active serine site, from the heavy chain of bovine Factor VII_a. Thus, a similar mechanism would not appear to explain the presence of VII* in human plasma. Treatment of purified human Factor VII with relatively high levels of thrombin (5% wt/wt) also produced a degradation product (Fig. 2). Its possible relationship to VII*, however, remains unclear.

Our attempts to produce VII* from purified Factor VII (or ¹²⁵I-VII) in vitro by clotting of blood or plasma, or in vivo by infusion into rabbits were unsuccessful. Furthermore, experiments studying the production of VII by human hepatoma cells suggest that if VII* is produced by proteolytic processing of VII, this occurs intracellularly. Similar apparent intracellular proteolysis of other vitamin K-dependent factors (prothrombin and Factor X) synthesized by HepG2 cells has not been described. VII* is not a precursor of VII which has not been glycosylated since it binds to Con-A Sepharose (as does VII), contains sialic acid, and exists intracellularly in forms containing high mannose-type oligosaccharides. Interestingly, while warfarin treatment of HepG2 cells inhibited the secretion of Factor VII, it seemed to have little effect upon the secretion of VII*. The immunological studies in the patient on chronic warfarin therapy (Fig. 4, lane 6) and the individual given a short course of high dose warfarin treatment (Fig. 7) corroborate this phenomenon. These immunological studies also suggest that the discrepancy between Factor VII antigen and activity measurements which has been noted in patients undergoing warfarin therapy (18, 35, 36) may not only be due to the production of Factor VII molecules lacking their full complement of gamma carboxyglutamic acids, but also may be due in part to the relative increase in VII* to VII CRM.

The ratio of VII*/VII produced by HepG2 cells (75%) was much greater than that found in normal human plasma (5–8%). Whether this discrepancy is due to differential clearance of plasma VII and VII* in vivo, the artificial in vitro culture system employed, or the phenotype of the HepG2 cells themselves, can not be determined.

Although posttranslational proteolytic degradation of VII is a likely mechanism for the production of VII*, other explanations are possible; in particular, alternative RNA splicing of the transcript from a single gene (37–40). Purification of VII* is now under way to permit further studies concerning its structure, function, and relationship to Factor VII.

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