Inhibition of Factor XIII Activation by an Anti-Peptide Monoclonal Antibody†

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ABSTRACT: As the final enzyme in the coagulation cascade, activated fibrin stabilizing factor or factor XIII catalyzes the intermolecular cross-linking of fibrin chains. To study this enzyme in plasma, we derived a monoclonal antibody (MAb 309) against a peptide sequence (NH_2-G-V-N-L-Q-E-F-C-COOH) in the thrombin activation site of factor XIII. Radioimmunoassays indicate that MAb 309 binds specifically to both platelet and plasma factor XIII. Peptide inhibition studies demonstrate that the MAb binds equally well to the factor XIII (FXIII) zymogen and the active form of FXIII (FXIIIa). In immunoblots of whole platelet lysates, MAb 309 binds only to FXIII and does not cross-react with other proteins. In saturation binding studies, the antibody shows a binding avidity of $(1.75 \pm 0.35) \times 10^9$ M^{-1}. MAb 309 also inhibited 99% of apparent FXIIIa activity in a standard transglutaminase assay. SDS–PAGE analysis of fibrin clots showed that MAb 309 inhibited fibrin γ–γ cross-linking. Moreover, MAb 309 accelerated the lysis of plasma clots, consistent with inhibition of fibrin–fibrin and fibrin–α_2-antiplasmin cross-linking. Immunoblotting experiments revealed that MAb 309 affected apparent FXIIIa activity by inhibiting the thrombin activation of the FXIII zymogen. In addition to its utility as a specific probe for the FXIII a-subunit, the strategy used to obtain MAb 309 may be used to generate MAbs that inhibit the activation of other coagulation factor zymogens.

As the final enzyme in the coagulation cascade, activated factor XIII catalyzes the intermolecular cross-linking of fibrin chains to each other and to other hemostatic proteins. Factor XIII circulates in the plasma as a heterotetrameric zymogen, a_2b_2 (M_r[1] ~310000), composed of two a-subunits (M_r ~ 75000 each) and two b-subunits (M_r ~80000 each) (Schwartz et al., 1973). Thrombin cleaves a peptide bond between Arg-37 and Gly-38, releasing a 4-kDa peptide from the amino terminus of the a-subunits and activating the zymogen ($a_2b_2 \rightarrow a_2'b_2$). This thrombin-cleaved FXIII (FXIII′) undergoes further activation in the presence of fibrin(ogen) and calcium; b-subunits dissociate from a-subunits, and the a-subunits undergo a conformational change that unmasks the active-site thiol group to yield the functional transamidase FXIIIa [$a_2'b_2 \rightarrow a_2' + b_2 \rightarrow a_2^* + b_2$ (Lorand, 1986)]. Following activation, the a-subunit remains attached to fibrin, and the noncatalytic b-subunits are released into the serum (Lorand, 1986). Dissociation of the b-subunit from the thrombin-cleaved a-subunit is the rate-limiting step in the development of enzymatic activity (Lorand, 1986). A second specific cleavage by thrombin at Lys-513–Ser-514 inactivates FXIIIa, yielding 56- and 24-kDa fragments (Schwartz et al., 1973; Takahashi et al., 1986).

FXIIIa catalyzes the formation of intermolecular γ-glutamyl-ϵ-lysyl bonds between fibrin γ-chains and between fibrin α-chains (Chen & Doolittle, 1970; Pisano et al., 1972). The γ-dimerization and α-polymerization result in a fibrin polymer which is resistant to fibrinolysis (Lorand & Jacobsen, 1962). FXIIIa also catalyzes the intermolecular cross-linking between the α-chain of fibrin and fibronectin, and between the α-chain of fibrin and α_2-antiplasmin (Tamaki et al., 1982). The cross-linking between the α-chain of fibrin and α_2-antiplasmin appears to be the fastest of these reactions (Tamaki & Aoki, 1981) and greatly increases the resistance of the clot to fibrinolysis (Aoki et al., 1983, Reed et al., 1990). FXIIIa has also been reported to catalyze the cross-linking of thrombospondin to fibrin (Bale et al., 1985) and factor V to platelet actin (Cohen et al., 1980) and myosin (Cohen et al., 1979). Studies have shown that fibrinogen accelerates thrombin cleavage of FXIII (Greenberg et al., 1986; Lewis et al., 1985). Recent experiments have indicated that plasma FXIII forms a trimolecular complex with fibrin I and α-thrombin (Naski et al., 1991). This complex accelerates the thrombin cleavage of both the fibrinopeptide B from fibrin and the activation peptide from plasma FXIII.

Monoclonal antibodies to the a-subunit of FXIII are rare, and to our knowledge, none have been extensively characterized. In order to study FXIII in plasma, we sought to produce a monoclonal antibody, MAb 309, which selectively bound to and inhibited the catalytic subunit (a-subunit) of the protein. Since FXIII, like many enzymes of the coagulation pathway, is converted from a zymogen to an active enzyme by cleavage of an activation peptide, we attempted to produce an antibody which would bind to the thrombin cleavage site

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[1] Abbreviations: FXIII, factor XIII zymogen; FXIIIa, activated factor XIII; FXIII′, thrombin-cleaved factor XIII; MAb, monoclonal antibody; M_r, relative molecular weight; GAMFAb, affinity-purified goat anti-mouse FAb; KLH, keyhole limpet hemocyanin; PPACK, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone; BSA, bovine serum albumin; TCS, thrombin cleavage site; HBSA, HEPES-buffered saline with 0.02% azide; DTT, dithiothreitol; SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; IU, international unit, SEM, standard error of the mean; TBSA, Tris-buffered saline with 0.02% sodium azide.

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of the zymogen and interfere with FXIII activation. In this study, we report the binding characteristics and functional effects of a MAb that binds to a specific peptide sequence in the thrombin activation site of FXIII.

Experimental Procedures

Materials

Materials were obtained from the following suppliers: FXIII-free fibrinogen and human plasma FXIII, American Diagnostica (Greenwich, CT); affinity-purified goat anti-mouse Fab' (GAMFAb), Cappel Laboratories (Malvern, PA); high and low molecular weight protein standards, Pharmacia (Uppsala, Sweden); prestained protein standards, Bio-Rad (Richmond, CA); BALB/C mice, Charles River (Wilmington, MA); bovine thrombin, Parke-Davis (Morris Plains, NJ); Freund's adjuvant, Difco (Detroit, MI); murine MAb isotyping kit, Zymed (San Francisco, CA); keyhole limpet hemocyanin and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), Calbiochem (La Jolla, CA); poly(vinylidene difluoride) transfer membranes, Millipore (Bedford, MA); iodoacetamide, RIA-grade bovine serum albumin, protein A, and *N,N'*-dimethylcasein, Sigma (St. Louis, MO); human tissue plasminogen activator, Genentech (South San Francisco, CA); platelet FXIII and rabbit antiserum against a-subunit of FXIII, Diagnostica Stago, France, via American Diagnostica (Parsippany, NJ). All other chemicals were of reagent grade or better. Fresh frozen plasma was obtained from random donors to the Blood Bank of Massachusetts General Hospital, Boston, MA.

Methods

Monoclonal Antibody Production. To produce antibody to the thrombin activation site of FXIII, a peptide was synthesized which mimics the amino acid sequence of the new amino terminus of the thrombin-activated a-subunit. This thrombin cleavage site (TCS) peptide had the sequence NH_2-G-V-N-L-Q-E-F-C-COOH, which duplicates the a-subunit amino acid sequence of residues 38–44. The carboxy-terminal cysteine is not part of the a-subunit sequence and was added to the peptide for the purpose of chemical coupling. The peptide was manually synthesized using a modified Merrifield method as we have previously described (Ridge et al., 1986). For the purpose of immunization, the peptide was chemically coupled to keyhole limpet hemocyanin (KLH) by the heterobifunctional cross-linking agent bromoacetic acid *N*-hydroxysuccinimide ester using a method that we have previously reported (Bernatowicz et al., 1986). After being coupled, the free peptide was separated from the KLH–peptide conjugate by extensive dialysis. The amino acid sequence of the peptide and its chemical coupling to KLH were verified by amino acid analysis on a Waters Picotag system.

CAF mice were immunized subcutaneously with 15 μg of KLH–peptide conjugate which was emulsified in complete Freund's adjuvant. The mice were given booster injections of 15 μg of KLH–peptide conjugate emulsified in incomplete Freund's adjuvant 1 month later and then once every 3 months until somatic cell fusion. Prior to somatic cell fusion, the antibody titer to the peptide was determined in a solid-phase radioimmunoassay. The TCS peptide was added to the wells of a poly(vinyl chloride) plate (10 μg/mL, 25 μL/well). After 1–2 h, the peptide was washed off, and the nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) for 1 h. Serial 10-fold dilutions of the mouse antiserum (25 μL) were added to each well and incubated for 1 h at room temperature. The wells were again washed, and 25 μL of ^{125}I-labeled goat anti-mouse Fab'_2 (^{125}I-GAMFAb), radiolabeled by the Iodogen method (Fraker & Speck, 1978), was added and allowed to incubate for 1 h. The GAMFAb was removed, and the plates were washed. The amount of bound antibody was counted in a γ-scintillation counter. The mouse with the highest antibody titer ($\sim 10^{-4}$) was used in somatic cell fusion which was performed according to the method described by Galfre and colleagues (Galfre et al., 1977). Hybridomas secreting peptide-specific antibody were selected using a reverse solid-phase radioimmunoassay with GAMFAb as described by Eshhar (1985). Hybridomas producing anti-TCS peptide antibody were then screened for their binding to purified FXIII in a solid-phase RIA using radiolabeled GAMFAb analogous to the assay described for the TCS peptide above. From these assays, MAb 309 was selected for further characterization. After cloning by limiting dilution, serotyping was performed using an isotyping kit from Zymed.

Specificity Assays. To determine the binding specificity of the antibody, a peptide was synthesized that contains the amino acids NH_2-P-R-G-V-N-L-Q-E-F-C-COOH that mimics a segment of the sequence of the a-subunit of FXIII zymogen except for the carboxy-terminal cysteine residue. This peptide was identical to the TCS peptide except for the presence of two more NH_2-terminal amino acids which are present in the zymogen and not in the activated FXIII; hence, it "spans" the thrombin cleavage site. The peptide was synthesized on a Milligen 9050 peptide synthesizer using F-moc chemistry. The peptide was determined to be homogeneous by high-performance liquid chromatography studies on a Waters 441 system. The peptide composition was confirmed by amino acid analysis on a Waters Picotag system. To ascertain whether MAb 309 was able to bind specifically to thrombin-cleaved FXIII, we compared its binding to the TCS peptide vs the spanning peptide in a radioimmunoassay. Wells of a microtiter plate were coated with the TCS peptide (25 μL of a 50 μg/mL solution) for 1–2 h. Nonspecific protein binding sites were blocked with 1% BSA for 1 h. After the wells were washed, spent hybridoma supernatants were diluted serially in culture media and added to separate wells to incubate for 1 h. The wells were then washed and incubated with ^{125}I-GAMFAb (approximately 50000 cpm) for an hour. The wells were again washed extensively, cut, and counted in a γ-scintillation counter to detect antibody binding. The dilution and hybridoma supernatant which yielded about half of maximal binding was determined and subsequently used for inhibition studies. In these experiments, increasing amounts of soluble TCS peptide or spanning peptide were mixed with MAb 309 hybridoma supernatant and added to the TCS peptide coated microtiter wells to inhibit the binding of the antibody. After incubation for 2 h at room temperature, the wells were washed, and the bound antibody was detected with ^{125}I-GAMFAb as above. The percent of inhibition was computed as the fractional binding in the presence of inhibitor compared to the binding in the absence of any inhibitor.

Avidity Measurements. To determine the avidity of MAb 309 for the TCS peptide, antigen binding experiments were performed. The TCS peptide was coupled to BSA using the heterobifunctional reagent bromoacetic acid *N*-hydroxysuccinimide ester as described above. After extensive dialysis to remove the uncoupled peptide, amino acid analysis was performed. The analysis demonstrated that 2.3 mol of TCS peptide was coupled per mole of BSA. The BSA–peptide conjugate was then radioiodinated by the chloramine-T method (Greenwood et al., 1963). The iodinated protein was separated from free iodide on a PD-10 column. The specific radioactivity was determined in triplicate by paper chromatography in 50%

ethanol/water and found to be 7.36×10^5 cpm/pmol. Equilibrium saturation binding assays were performed to determine the MAb's avidity. In these assays, MAb hybridoma supernatants were diluted in culture media to an antibody concentration below the expected K_d. The radiolabeled ligand was diluted in HEPES-buffered saline with 0.02% azide (HSBA) to concentrations approximately 10-fold above and below the K_d. Then 100 μL of the diluted antibody was mixed with 100 μL of various concentrations of radiolabeled ligand and 100 μL of HBSA in test tubes. The tubes were incubated at 4 °C overnight. Then 50 μL of goat anti-mouse antibody coupled to magnetic particles was added to each tube and allowed to incubate for 90 min at 4 °C. Two milliliters of ice-cold HBSA was added to each tube, and the bound radiolabeled ligand was separated from the free ligand by centrifugation at 3000 rpm for 15 min at 4 °C. The initial estimate of background was obtained by performing the same experiment in parallel with a control MAb 40-160 (anti-digoxin; Mudgett-Hunter et al., 1985) of the same isotype. The avidity was calculated using the Ligand program (Munson & Rodbard, 1980) on a Zenith microcomputer (McPherson, 1985).

Purification of MAb 309. Ascites were produced in pristane-primed BALB/C mice. Filtered ascites was pooled, and MAb 309 was purified on a protein A–Sepharose column. Ascites were mixed with 1.5 M glycine in 3 M NaCl, pH 8.9 (ratio 1:1), and incubated in the column for 1 h. Antibody was eluted from the column with 0.1 M sodium citrate, pH 3.5, neutralized with 1 M Tris-HCl, pH 9.1, and concentrated. To prepare Fab fragments, purified MAb 309 was dialyzed against 0.1 M sodium phosphate buffer with 0.002 M EDTA, pH 7.0. An analytical digest was performed with 20 μL of antibody (1 mg/mL), 5 μL of papain (10 μg/1 mg of Ab), and cysteine hydrochloride (50 mM) for various lengths of time at 37 °C. The reaction was stopped with 25 μL of 0.003 M iodoacetamide. The digest was analyzed by electrophoresis on 10% polyacrylamide gels. After selection of appropriate conditions, a preparative digest was performed with 5 mL of purified MAb 309 (1 mg/mL), for 3 h at 37 °C. The digest was stopped with iodoacetamide, and FAb was dialyzed against 0.005 M sodium phosphate/0.02% sodium azide, pH 8.0, and chromatographed on a DE 52 ion-exchange column.

Binding of MAb 309 to Purified FXIII. Plates were coated with 25 μL of platelet FXIII (a_2) or plasma FXIII (a_2b_2) in a concentration of 20 μg/mL. After the nonspecific protein binding sites were blocked with 1% BSA for 1 h, 25 μL of MAb 309 culture media was added to the plate for 1 h. The plates were washed, and ^{125}I-GAMFAb (50000 cpm/well) was added for 1 h. The wells were washed, cut, and counted to measure the amount of bound antibody.

Assay for Transglutaminase Activity. Human FXIIIa activity was measured by the incorporation of [^{14}C]putresceine into bovine α-casein using a filter paper assay as described by Lorand et al. (1972) with the following minor modifications. Fresh frozen human plasma, from at least four normal random donors, was used as the source of FXIII. After heat inactivation of fibrinogen, FXIII was activated by a 30-min incubation with 0.3 unit of thrombin, 3.5 mM $CaCl_2$, and 0.02 M DTT (final concentrations) at 37 °C. To determine the relative effect of MAb 309 on FXIII activation, MAb 309 or a control MAb (anti-digoxin 40-160), in a final concentration of 1 mg/mL, was added before or after the activation of FXIII by thrombin. To terminate thrombin activation of FXIII, D-phenylalanyl-L-prolylarginine chloromethyl ketone (PPACK) was added in a final concentration of 10^{-6} M. Following the activation of FXIII by thrombin, transglutaminase activity was measured by the incorporation of [^{14}C]putrescine into casein as described. After 30 min of incubation, iodoacetamide (100 μg/mL final concentration) was added to stop the reaction, and 20 μL of each sample was spotted on Whatman filter paper. The remaining assay was performed as described (Lorand et al., 1972).

Effect of MAb 309 on γ–γ Cross-Linking of Fibrinogen by FXIIIa. Placental FXIII (5 μL, 0.03 μg/mL) was preincubated with 20 μL of MAb 309 (2 mg/mL in TBSA) or TBSA alone. After 30 min, 5 μL of thrombin (0.01 unit) and 5 μL of 10 mM $CaCl_2$ were added and incubated for 0–20 min at 37 °C. Then 5 μL of FXIII-free fibrinogen (10 μg) was added. Fibrin cross-linking was stopped after 10 min by adding 10 μL of 9 M urea and immediately boiling the samples. The samples were then analyzed by SDS–PAGE on 7.5% gels under reducing conditions. Gels were stained by Coomassie Brilliant Blue and dried.

Clot Lysis Assay. Fresh frozen plasma was mixed with trace amounts of ^{125}I-fibrinogen to achieve about 20000 cpm/25 μL. Plasma (25 μL) was incubated with 25 μL of MAb 309 or control (Tris-buffered saline with 0.02% azide) for 30 min before clotting with 25 μL of 20 mM $CaCl_2$. After 30 min, the clotting was stopped with iodoacetamide (20 μL of a 1 mg/mL solution). Human tissue plasminogen activator (5 IU) was added to the clot in each tube. At 30 min, a 200-μL aliquot of supernatant was sampled and counted in a γ-scintillation counter to determine the percentage of the clot lysis. The percentage of clot lysis was computed as the quotient of the radioactivity released into the supernatant as fibrin degradation products divided by the initial radioactivity incorporated into the clot (Reed et al., 1990). The data were expressed as the mean ± standard deviation. A *t* test was used to compare the mean lysis of clots formed in the presence of MAb 309 versus the lysis of clots formed in the presence of the control MAb.

Immunoblotting experiments were performed to study the specificity of MAb 309 for binding to FXIII in platelet lysates. Platelets were isolated from 5 units of outdated platelet-rich plasma by differential centrifugation (Mustard et al., 1988). After being washed twice in HEPES-buffered saline with 1 mM EDTA (pH 7.4), the platelets were resuspended in 2 mL of HBS. Then 200 μL of platelets ($\sim 2 \times 10^{10}$ cells) was mixed with 200 μL of sample buffer (Laemmli, 1970), 20 μL of bromophenol blue–glycerol, and 10 μL of β-mercaptoethanol and boiled for 2 min. The platelet sample (20 μL) was then run on 10% polyacrylamide gels and electrophoretically transferred to poly(vinylidene difluoride) membranes (Towbin et al., 1979). The membranes were incubated with MAb 309 culture supernatant for 1 h. After the membranes were washed, they probed with ^{125}I-GAMFAb (1×10^6 cpm) to detect bound antibody. Subsequently, the membranes were washed, dried, and autoradiographed on Kodak Xomat AR films. Immunoblotting was also used to study the effects of MAb 309 on thrombin cleavage of FXIII. Purified plasma FXIII (5 μL, 0.5 μg) was preincubated with 15 μL of MAb 309 (or anti-digoxin MAb as a control) (2 mg/mL) for 30 min. The FXIII was activated by 10 μL of 0.02 IU of thrombin (in 15 mM $CaCl_2$) for 0–20 min at 37 °C. Reactions were stopped immediately by adding 10 μL of sample buffer (Laemmli, 1970) and boiling. Prepared samples were electrophoresed on 6% unreduced gels and electrophoretically transferred by the semi-dry technique (Khyse-Anderson, 1984) to a poly(vinylidene difluoride) membrane. The membranes were washed in 0.3% Tween-TBS and blocked with 1%

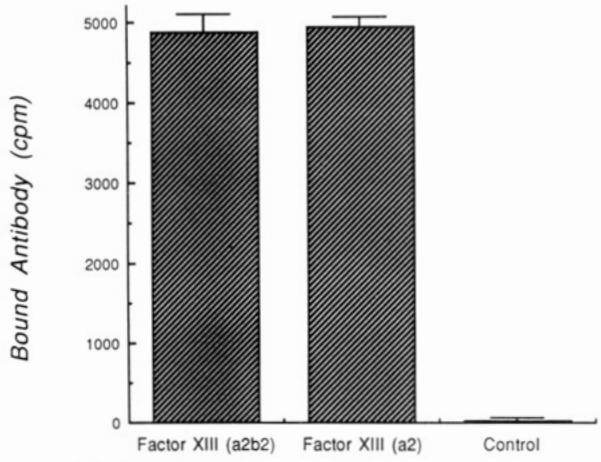

FIGURE 1: Binding of MAb 309 to purified plasma and platelet FXIII. Wells of a microtiter plate were coated with purified solutions of human plasma factor XIII (a_2b_2), platelet FXIII (a_2), or control (no FXIII). After the nonspecific binding sites were blocked with 1% BSA, MAb 309 culture medium was added to each well. After 1-h incubation, the wells were washed, and bound antibody was detected by incubation with ^{125}I-GAMFAb. The mean cpm ± SEM of bound antibody is shown.

BSA–TBSA buffer. The membrane was probed with rabbit antisera against the a-subunit of human FXIII (diluted 1:100) as a primary antibody and ^{125}I-protein A (1.5×10^9 cpm) as a detecting agent. After the membrane was washed and dried, it was autoradiographed as described above.

RESULTS

From the somatic cell fusion of splenocytes from 1 immunized mouse, 30 hybridomas were found to produce antibody to the TCS peptide. On the basis of its apparent avidity for the TCS peptide and its binding to FXIII in a solid-phase assay, MAb 309 ($Ig\gamma_1$, κ serotype) was selected for further study. Radioimmunoassays were performed to confirm that MAb 309 bound to intact, nondenatured FXIII protein. Figure 1 compares the binding of MAb 309 to purified plasma FXIII (a_2b_2), platelet FXIII (a_2, not containing b-subunit), or a control antigen (bovine serum albumin) in a solid-phase radioimmunoassay. This confirmed that MAb 309 bound to both plasma and platelet FXIII protein, indicating that the thrombin cleavage sequence was accessible to the antibody in the nondenatured zymogens. To test the binding specificity of MAb 309 for FXIII, as compared to its potential binding to other proteins, we performed an immunoblotting experiment using whole human platelets. In this experiment, the lysate from isolated, pooled human platelets was electrophoresed on polyacrylamide gels under reducing conditions. After electrophoretic transfer to poly(vinylidene difluoride) membranes, immunoblotting was performed using MAb 309 as a probe. The immunoblots (Figure 2) show that MAb binds to a single band of $M_r \sim 75\,000$, which is consistent with the a-subunit of FXIII. There was no binding to other proteins detected. Thus, these experiments using purified plasma FXIII and electrophoresed human platelet lysates confirmed the binding specificity of MAb 309 for FXIII a-subunit.

To determine whether MAb 309 bound preferentially to the amino acid sequence of the FXIII zymogen, compared to that of activated FXIII, we performed radioimmunoassays with peptides that partially mimicked these sequences. Figure 3 shows the amino acid sequence of the TCS peptide, which represents the new (thrombin-cleaved) amino terminus of the activated FXIII (GVNLQEF-C) and a peptide which "spans" the thrombin cleavage site ("S-peptide", PRGNVLQEF-C).

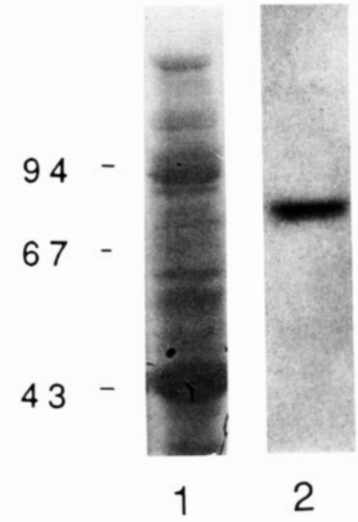

FIGURE 2: Immunoblots of platelet lysate by MAb 309. Whole platelets were isolated from outdated platelet-rich plasma and lysed. The platelet lysate was electrophoresed on 10% polyacrylamide gels and then electrophoretically transferred to a poly(vinylidene difluoride) membrane. The membrane was then divided and stained or immunoblotted with MAb 309 as described under Methods. Lane 1 shows the Coomassie Brilliant Blue staining of the membrane for protein. Lane 2 shows the immunoblot with MAb 309. The relative position of molecular weight standards ($\times 10^{-3}$) is indicated.

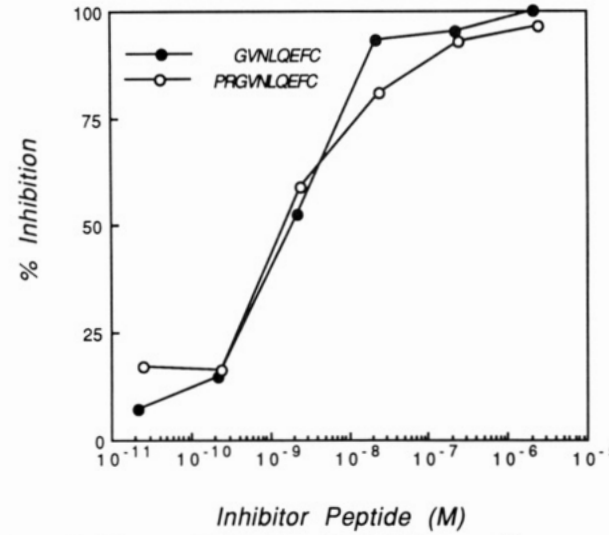

FIGURE 3: Comparative binding of MAb 309 to peptide sequences from the thrombin cleavage site of FXIII. Wells of a microtiter plate were coated with the peptide immunogen (TCS peptide) and then blocked with 1% albumin solution. Then appropriately titered MAb 309 was mixed with various amounts of peptide inhibitors—a peptide which spans the thrombin cleavage site (S-peptide, open circles) or a peptide which mimics the new, thrombin-cleaved amino terminus of FXIII′ (TCS peptide, closed circles). The peptide–MAb 309 mixture was immediately added to the wells to incubate for 2 h. After the wells were washed, the amount of bound MAb 309 was measured by incubation with ^{125}I-GAMFAb. The percent of inhibition was computed as the fractional binding in the presence of inhibitor compared to the binding in the absence of any inhibitor.

In this inhibition assay, wells of a microtiter plate were coated with the TCS peptide. Then MAb 309 culture supernatants, diluted to give approximately 50% of maximal binding, were mixed with various concentrations of the TCS or S-peptide and added to the microtiter plate wells. After 1 h of incubation, the wells were washed, and ^{125}I-GAMFAb was added to detect the bound antibody. As shown in Figure 3, both peptides inhibited the binding of MAb 309 in a parallel fashion, and at equivalent concentrations. This indicates that

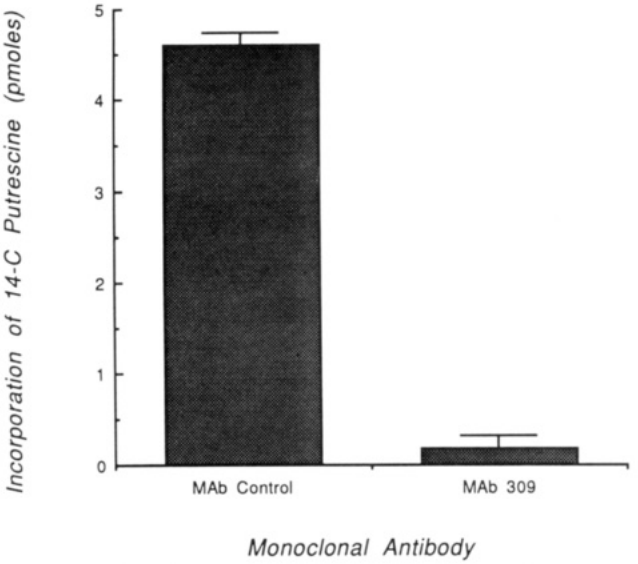

FIGURE 4: Effect of MAb 309 or control MAb 40-160 on the apparent FXIIIa activity in a transglutaminase assay (Lorand et al., 1972). Heat-treated plasma was incubated with MAb 309 (or a control antibody), and FXIII was activated with 0.3 IU of thrombin, 3.5 mM $CaCl_2$, and 0.02 M dithiothreitol at 37 °C for 30 min. FXIIIa activity was measured by the incorporation of [^{14}C]putresceine into *N,N'*-dimethylcasein.

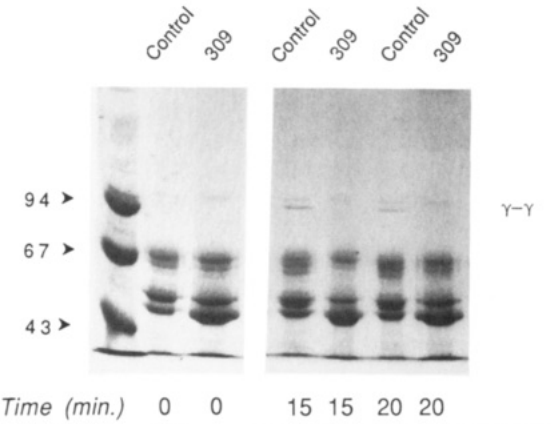

FIGURE 5: Inhibition of the γ–γ cross-linking of fibrin by MAb 309. Placental FXIII (20 μL, 0.03 μg) was incubated with 20 μL of MAb 309 (2 mg/mL in TBSA) or TBSA alone and activated with 5 μL of thrombin (total 0.01 IU) and 5 μL of 10 mM $CaCl_2$ for 0, 15, and 20 min. Then 10 μg (in 5 μL) of FXIII-free fibrinogen was added for an additional 10 min. The cross-linking was stopped by the addition of 9 M urea and immediate boiling. The samples were mixed with sample buffer, electrophoresed on 7.5% gels, and stained with Coomassie Brilliant Blue dye. On the abscissa is shown the time of thrombin activation. The relative position of molecular weight standards is indicated in the left lane with the corresponding molecular weights ($\times 10^{-3}$). Indicated on the right is the relative position of cross-linked fibrin γ–γ chains.

MAb 309 probably recognizes the amino acids distal, or C-terminal, to the thrombin cleavage site and that it does not require a free glycine N-terminus for binding. It also argues that based on sequence alone, MAb 309 binds equally well to both activated FXIII and the zymogen.

To estimate the binding affinity of MAb 309, we performed equilibrium saturation binding experiments with the TCS peptide. The binding experiments were then subjected to nonlinear least-squares analysis using the LIGAND program (Munson & Rodbard, 1980; McPherson, 1985). In these experiments, MAb 309 was found to have an avidity of (1.75 ± 0.35) × 10^9 M^{-1}.

Effects of MAb 309 on FXIII Transglutaminase Activity in Plasma. Because MAb 309 bound to the FXIII zymogen at the thrombin activation site, we hypothesized that it might inhibit the activation of FXIII by thrombin. To test this hypothesis, we measured the effects of MAb 309 or a control antibody on the cross-linking of [^{14}C]putrescine into casein by FXIIIa using a modification of the assay originally described by Lorand (Lorand et al., 1972). As Figure 4 shows, when FXIII was preincubated with MAb 309, it reduced the FXIIIa-mediated incorporation of [^{14}C]putresceine into casein by 99%. In a similar fashion, Fab fragments of MAb 309 also inhibited apparent transglutaminase activity, arguing that the inhibitory effects of the antibody were not merely due to immunoprecipitation of FXIII by bivalent antigen–antibody complexes.

Effects of MAb 309 on Fibrin Cross-Linking and Fibrinolysis. The most important natural substrate for plasma FXIII is fibrin. It is also known that in the presence of fibrin, the activation of FXIII by α-thrombin proceeds more efficiently (Naski et al., 1991). Similarly, the binding of FXIII to fibrinogen (Greenberg & Schuman, 1982) may alter the binding of the MAb to the thrombin cleavage site. To determine the effects of the MAb on fibrin cross-linking by FXIIIa we analyzed clots formed in the presence of MAb 309 or a control antibody. Figure 5 shows the reduced SDS–PAGE analysis of these clots. The left panel shows the samples which were immediately mixed together and boiled without clotting (0 min). In these unclotted samples, and all subsequent ones, there is a faint contaminating band seen at ~98 kilodaltons. Samples containing MAb 309 show a darker staining band of 50 kilodaltons due to the contribution of the reduced heavy chain of the antibody. In the clotted control samples, a band of ~95 kilodalton develops after 15 and 20 min which indicates fibrin γ–γ cross-linking. However, in the samples incubated with MAb 309, there is no evidence of γ–γ cross-linking. Thus, MAb 309 inhibited the transglutaminase activity of FXIIIa with both small artificial (putrescine) and large natural (fibrin) substrates. To investigate the effect of the MAb on FXIII in plasma, we performed fibrinolysis experiments. Fibrinolysis rates have been shown to depend on the amount of fibrin–fibrin cross-linking (γ–γ and α–α chains) and α_2-antiplasmin–fibrin cross-linking (Gaffney & Whitaker, 1979; Sakata & Aoki, 1980). In these assays, MAb 309 or no antibody was added to plasma which had been mixed with radiolabeled fibrinogen. The plasma was clotted by recalcification for 30 min at 37 °C. Tissue plasminogen activator (5 IU) was added to each tube, and the amount of clot lysis was determined after 0.5 h of incubation. Clots formed in the presence of MAb 309 lysed significantly faster (82.8% ± 1.6%) than control clots (51.4% ± 2.4%; $p < 0.005$). This indirectly indicated that MAb 309 inhibited fibrin–fibrin and fibrin–α_2-antiplasmin cross-linking by FXIIIa.

MAb 309 Inhibits Thrombin Cleavage of FXIII. MAb 309 was derived in part to inhibit the thrombin activation of FXIII. To establish that this was its mechanism of action, we studied the effect of MAb 309 or a control antibody on the rate of thrombin cleavage of FXIII. FXIII (a_2b_2) was incubated with thrombin for various lengths of time in the presence of MAb 309 or a control MAb. The reactions were stopped by the addition of sample buffer and immediate boiling. Samples were electrophoresed on 6% polyacrylamide gels and electrophoretically transferred to a poly(vinylidene difluoride) membrane. After transfer, the a-subunit of FXIII was detected by immunoblotting with a polyclonal antiserum. Thrombin-cleaved or activated FXIII a-subunit (a′) was detected by a characteristic increase in relative migration which corresponds to a decrease in molecular weight of the zymogen after release of the activation peptide (i.e., the molecular weight

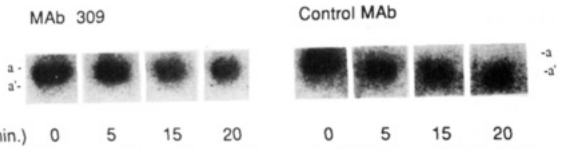

FIGURE 6: Comparative effects of a control MAb or MAb 309 on the thrombin cleavage of FXIII as a function of time. Purified plasma FXIII (5 μL, 0.5 μg) was preincubated with MAb 309 or control MAb (15 μL, 2 mg/mL) or no MAb for 30 min at room temperature and then activated by 0.02 IU of thrombin (in 15 mM $CaCl_2$) for different times as indicated. The reactions were stopped by the immediate addition of sample buffer and boiling. The samples were anlyzed by SDS–PAGE on 6% unreduced gels. The a-subunit (a) and the thrombin-cleaved a-subunit (a′) were identified by immunoblotting with polyclonal antibody against the a-subunit of FXIII, followed by ^{125}I-protein A.

decreased from 75 000 to 71 000). Figure 6 shows that in control samples after 5, 15, and 20 min of incubation with thrombin, there was an increase in relative migration of the FXIII a-subunit consistent with thrombin activation of the a-subunit. However, in samples incubated with MAb 309 and thrombin, no shift in migration of the a-subunit was seen, indicating that thrombin cleavage did not occur. Thus, MAb 309, by binding to the thrombin cleavage site, inhibited thrombin activation of FXIII.

Discussion

We have derived a monoclonal antibody against the catalytic a-subunit of FXIII to serve as a probe in biological fluids. In addition, by generating an antibody to the amino acid sequence of FXIII's thrombin cleavage site, we have sought to derive a prototype immunoinhibitor of FXIII activation. Radioimmunoassays have demonstrated that MAb 309 binds avidly to both plasma FXIII (a_2b_2) and platelet FXIII (a_2). Immunoblots of platelet lysates confirm that MAb 309 binds only to FXIII and does not cross-react with other proteins. Peptide binding studies suggest that MAb 309 appears to have the same affinity for the FXIII zymogen as it does for thrombin-cleaved FXIII. Studies performed with a modified transglutaminase assay (Lorand et al., 1972) indicated that MAb 309 was able to inhibit approximately 99% of the apparent enzyme activity of FXIIIa. Of greater importance, since thrombin cleavage of FXIII is accelerated by fibrinogen, experiments performed with purified fibrinogen showed that MAb 309 inhibited fibrin γ–γ cross-linking. In addition, inhibition of apparent FXIIIa activity was also demonstrated in plasma by the accelerated fibrinolysis of plasma clots formed in the presence of the MAb. Immunoblotting experiments confirmed that MAb 309 affected FXIIIa activity by inhibiting the thrombin cleavage step which is necessary to convert the FXIII zymogen to an active enzyme.

Although antisera to the a-subunit of FXIII have been generated, to our knowledge, MAb 309 represents the first extensively characterized MAb with this binding specificity. It has been our experience that when mice or rabbits are immunized with plasma FXIII, antibodies to the b-subunit dominate the immune response (Reed, unpublished observations). This may explain the paucity of MAbs which bind to the a-subunit of FXIII. To circumvent this problem, we immunized with a peptide which represents a potential "neoantigen" corresponding to the new amino terminus of activated FXIII after thrombin cleavage. This yielded a significant immune anti-peptide response, a large portion of which cross-reacted with the native FXIII protein.

Although acquired FXIII deficiency is rare, immunoglobulin inhibitors of FXIII have been described. These immunoglobulin inhibitors appear to have been polyclonal. Lorand [see Lopaciuk et al. (1978)] classified these inhibitors in three groups. Type I inhibitors interfere selectively with the activation of FXIII. Type II inhibitors affect the fibrin-stabilizing activity of FXIIIa, and type III inhibitors bind to fibrin and prevent fibrin from reacting properly with FXIIIa. Since FXIII activation is a sequential process which requires thrombin cleavage, dissociation of the b-subunits, and unmasking of the active site, we believe that there are at least three subtypes of type I inhibitors, corresponding to each of these steps. A number of immunoglobulin inhibitors of FXIII have been reported [see Lorand et al. (1980) for a review], but the mechanism of inhibition has been established in only a few (Table I). Three types of inhibitors have been described. Type I inhibitors inhibited the conversion $a_2b_2 \rightarrow a_2'b_2$ (MAb 309), or $a_2' \rightarrow a_2^*$ (Lopaciuk et al., 1978), or $a_2'b_2 \rightarrow a_2^* b_2$ (Lopaciuk et al., 1978; Lorand et al., 1988). Type II inhibitors inhibited a_2^* (Godal & Ly, 1977; Nakamura et al., 1988; Jansen et al., 1987; Lorand et al., 1988), and type III inhibitors inhibited the interaction of a_2^* with fibrin (Jansen et al., 1987; Lorand et al., 1988). In other studies, the mechanism of inhibition was not clearly established (Lewis, 1972; Milner et al., 1977; Rosenberg et al., 1974).

Table I: Immunoglobulin Inhibitors of FXIII[a]

type of FXIII inhibitor	references
type I	
inhibits $a_2b_2 \rightarrow a_2'b_2$	this report
inhibits $a_2 \rightarrow a_2^*$	Lopaciuk et al. (1978)
inhibits $a_2'b_2 \rightarrow a_2^* + b_2$	Lopaciuk et al. (1978); Lorand et al. (1988)
type II	
inhibits a_2^*	Godal et al. (1977); Nakamura et al. (1988); Jansen et al. (1987); Lorand et al. (1988)
type III	
inhibits reaction of a_2^* with fibrin	Jansen et al. (1987); Lorand et al. (1988)

[a] Only references with a probable mechanism of inhibition are cited. The classification of inhibitors (type I, II, or III) is based on Lorand's suggestions [see Lopaciuk et al. (1978)]. Type I inhibitors interfere selectively with the activation of FXIII. Type II inhibitors affect the fibrin-stabilizing activity of FXIIIa, and type III inhibitors bind to fibrin and prevent fibrin from reacting properly with FXIIIa.

Only a few of these immunoglobulin inhibitors have been shown to bind to the a-subunit of FXIII. (Lewis, 1972; Lopaciuk et al., 1978; Nakamura et al., 1988; Lorand et al., 1988). Of these, MAb 309 appears to be the first example of a type I inhibitor that selectively binds the thrombin activation sequence of the a-subunit of FXIII and prevents thrombin cleavage. As such, it is more accurate to classify MAb 309 as an agent that prevents thrombin from processing FXIII as a substrate. Nevertheless, our present experiments do not fully exclude that the antibody may have other inhibitory effects on FXIIIa, and studies are underway to investigate this possibility. Since MAb 309 inhibits the activation of FXIII by thrombin and thereby decreases the amount of active enzyme, we would expect that the antibody would inhibit the cross-linking of all FXIII substrates such as α_2-antiplasmin, fibronectin, factor V, etc.

During clotting, many of the coagulation factors (e.g., V, VII, VIII, IX, X, prothrombin, etc.) undergo conversion from a zymogen to an active enzyme. This activation process typically involves cleavage at a specific amino acid sequence. By generating MAb 309 against the amino acid sequence of FXIII which is cleaved by thrombin, we have been able to generate an inhibitor of FXIII activation. Because other clotting factors undergo a similar sequence of activation by specific proteases, it may be possible to generate similar

site-directed inhibitors which block the activation of other coagulation proteases. Such inhibitors could be useful for studying the regulation of clotting both in vitro and in vivo.

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