Recombinant Fibrinogen, γ275Arg ->Cys, Exhibits Formation of Disulfide Bond with Cysteine and Severely Impaired D:D Interactions

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Summary

Background and Objectives: Analysis of dysfibrinogens has provided useful information aiding our understanding of molecular defects in fibrin polymerization. We have already reported impaired fibrin polymerization in a variant fibrinogen (γArg275Cys), the Cys being located in the D:D interface. Since this substitution occurred in a heterozygous individual, interpretation of the functional analysis was complicated. We tried to resolve this complication by synthesizing a recombinant variant fibrinogen.

Methods: A variant γ-chain expression plasmid was transfected into Chinese hamster ovary cells expressing normal human fibrinogen Aα- and Bβ-chains. The recombinant variant fibrinogen (γ275C) was purified using an immunoaffinity column, and we compared its structure and functions with those of normal recombinant fibrinogen (γ275R) and plasma variant fibrinogen.

Results: Mass analyses showed the existence of disulfide-linked Cys in both patient and recombinant variant fibrinogens. Functional analyses indicated that both fibrin polymerization and γ-γ dimer formation were markedly impaired in the variant fibrinogen. The impairments were much more pronounced in γ275C than in plasma variant fibrinogen. In addition, scanning electron microscopic observation of fibrin clots made from γ275C revealed less dense fibrin fiber bundles and larger fiber diameter than in those made from γ275R, and also the existence of many aberrant fibrin fibers with tapered ends.

Conclusion: These results indicate that γArg275 has an important residue affecting the structure and function of the γ-chain C-terminal domain. However, the variant D:D interface can interact with that of the normal fibrinogen existing in a heterozygous patient with dysfibrinogenemia.

Introduction

During fibrin polymerization, thrombin cleaves fibrinogen, releasing fibrinopeptides A (FpA) and B (FpB) from the N-termini of the Aα and Bβ chains, respectively, and converting fibrinogen to fibrin monomers. Fibrin monomers polymerize spontaneously through a calcium-dependent, two-step process. The first of these steps begins with the release of FpA. This exposes the "A" site in the E domain, which interacts with the "a" site in the D domain of a neighboring molecule (the so-called D:E association), leading to the formation of half-staggered, double-stranded twisting protofibrils [1,2]. Subsequently, the release of FpB exposes the "B" site, which likely interacts with the "b" site in the D domain of another molecule to promote lateral aggregation of the protofibrils [3]. The final product is a complex, branching network of fibers. There is a constitutive self-association site in the γ-chain of each D domain that participates in the interface of two abutted D domains in the same strand of protofibrils (the so-called D:D association), independently of the D:E association [4,5].

Analysis of dysfibrinogens has provided useful information aiding our understanding of the above-mentioned molecular events, which promote thrombin-catalyzed polymerization [6-8]. The D:D association has been reported to be essential for end-to-end association of the protofibrils and to be an important determinant of the ultimate network structure of the fibrin clot [4,5]. In support of this, γArg275 mutations are the second most common mutations in dysfibrinogenemias, with the Arg being mutated to Cys (15 families), His (10 families) or Ser [9]. Besides the γArg275 mutations, 8 variants have been reported in the D:D interface and the neighboring region between γGly268 and γMet310. We have already analyzed the fibrin polymerization of a heterozygous plasma variant fibrinogen (γArg275Cys: Matsumoto III; M-III) [10] and compared it with those occurring in heterozygous γAsp364His (Matsumoto I) [11] and γAsn308Lys (Matsumoto II) [12]. These results indicated that

fibrin polymerization decreased in the order γAsn308Lys > γArg275Cys > γAsp364His, all three being decreased by comparison with normal fibrinogen. These substitutions, like most dysfibrinogens, occurred in individuals who are heterozygous for the mutation. This complicates functional analysis because fibrinogen in these individuals is a mix of normal and abnormal molecules [13,14]. We resolved this complication by synthesizing recombinant variant fibrinogen, and we examined the changes in function associated with the change in primary structure.

Here, we describe an analysis of a heterozygous plasma variant fibrinogen (M-III) and of recombinant variant fibrinogen γ275Cys, analogous to the dysfibrinogen. On the basis of our data, substitution of γ-chain residue Arg275 by Cys results in the presence of a disulfide-linked Cys and a disruption of the initial alignment of fibrin monomers into protofibrils, these changes would appear to lead to an impairment of the subsequent factor XIIIa-catalyzed γ-γ dimer formation. The impairments in recombinant γ275C fibrinogen were markedly more pronounced than those in the heterozygous plasma fibrinogen.

Materials and Methods

Preparation of plasma and recombinant variant fibrinogen. Preparation of recombinant variant fibrinogen was performed as described before [15]. To change γ275Arg to Cys, the fibrinogen γ-chain expression vector, pMLP-γ, was altered by oligonucleotide-directed mutagenesis using the selection primer 5'-TCTAGGGCCCAGGCTTGTTTGC and the mutagenesis primer 5'-TGACAAGTACTGCCTAACATA (the altered base is underlined). The expression plasmid was cotransfected with a histidinol selection plasmid (pMSVhis) into Chinese hamster ovary (CHO) cells expressing normal human fibrinogen Aα- and Bβ-chains. A selected and cloned colony was cultured in serum-free medium using 850 cm^2 roller-bottles coated with microbeads. Fibrinogen was purified from plasma or from

harvested culture medium by immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (IF-1; Iatron Laboratories, Tokyo) [16]. The fibrinogen concentration was determined from the A_{280} value, assuming that a 1 mg/ml solution has an absorbance of 1.51 [17]. The purity and characterization of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) run under either non-reducing (5% polyacrylamide gel) or reducing conditions (10% polyacrylamide gel) conditions.

Mass spectrometric analysis. Preparation of fibrinogen fragments for mass spectrometric analysis was performed as described before [18], with a minor modification. Briefly, fibrinogen (1.3 mg) was dissolved in 300µl of 6 M guanidine hydrochloride, pH 8.5, containing 10 µL 4-vinylpyridine to derivatize free sulfhydryl groups. After 30 min, protein was precipitated using 10 vol methanol. The dried precipitate was dissolved in 300 µl of concentrated formic acid containing 30 mg cyanogen bromide, incubated for 2 h at room temperature, and the digest was fractionated into 100 tubes at 2.5 mL each on a 1.6 x 100 cm of Sephadex G-50sf column in 0.2% trifluoroacetic acid. After all fractions had been monitored at 215 nm, and concentrated, they were followed by mass spectrometric analysis in a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass Voyager DE STR instrument (PE Applied Biosystems, Foster City, CA). Part of each sample was subsequently incubated overnight with mercaptoethanol (5% final concentration) at 37˚C to cleave any disulfide bonds that might be present. For all mass spectrometric analyses, 5mg/ml α-cyano-4-hydroxycinnamic acid and 0.1% trifluoroacetic acid in 37.5 % acetonitril was used as matrix.

Thrombin-catalyzed fibrin polymerization. Polymerization was followed by monitoring turbidity at 350 nm using a UV-110-02 spectrophotometer (Shimadzu Corp., Tokyo,

Japan). Reactions were performed in a final volume of 100 μl, as described elsewhere [15]. Briefly, fibrinogen (90 μl at 0.1 or 0.5 mg/ml) in 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl, with added $CaCl_2$ was mixed with human α-thrombin (Enzyme Research Laboratories, South Bend, IN) (10 μl at 0.5 unit/ml), and changes in turbidity were monitored at the ambient temperature. Three parameters (lag period, maximum slope, and Δabsorbance in 30 min) were obtained from the turbidity curves, as described elsewhere [19]. The reactions were performed in triplicate for each sample.

Factor XIIIa-catalyzed cross-linking of fibrin or fibrinogen. Factor XIII (FXIII; 50 unit/ml) (Enzyme Research Laboratories, South Bend, IN) was activated with human α-thrombin (1 unit/ml) for 60 min at 37˚C in 20 mM HEPES, pH 7.4, containing 0.12 M NaCl and 5 mM $CaCl_2$. For the studies of FXIIIa-catalyzed cross-linking of fibrinogen, thrombin (0.5 units/ml) was inhibited by hirudin (10 units/ml) prior to the addition of fibrinogen. Fibrinogen (final concentration, 0.47 mg/ml) was incubated at 37˚C with FXIIIa (final concentration, 3.3 units/ml) and human α-thrombin (final concentration, 0.07 units/ml) in the presence of 0.67 mM calcium. The reaction was stopped at various times by the addition of an equal volume of SDS-sample buffer with 2-mercaptoethanol and subsequent incubation (5 min) in a boiling-water bath. Samples equivalent to 4.7 μg of fibrinogen were separated on 8 % SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

Scanning Electron Microscopy (SEM). The SEM preparation was performed as described before [20], with a few minor modifications. Briefly, 10 μl of thrombin was added to 40μl of fibrinogen solution and mixed by repeated pipetting. Polymerization then proceeded in a moisture chamber in a 37˚C incubator for 3 h. The final concentrations of fibrinogen and thrombin were 0.40 mg/ml and 0.1 unit/ml,

respectively. The clots were fixed in 2.5% glutaraldehyde overnight, stained with 1% osmium tetroxide, critical-point dried, mounted, osmium plasma-coated at 5-nm thickness in an NL-OPC40 (Nippon Laser and Electronics Lab, Nagoya, Japan), and finally viewed on a JSM-6000F (Japan Electron Optics Laboratory Co. Ltd.). Images were taken at 3000x or 20000x using a 15.0 kV accelerating voltage. Fiber diameters were measured using a vernier caliper on a 300% enlargement from a photograph of a 20000x observation.

Results

Characterization of Plasma and Recombinant Fibrinogens.

We synthesized a recombinant variant fibrinogen with a single-amino acid substitution, γ275Cys (γ275C). Wild-type recombinant fibrinogen γ275Arg (γ275R) was also produced [15] and used as the normal control. When SDS-PAGE was run under non-reducing conditions with Coomassie staining, the fibrinogens purified from plasma and cultured media were pure, and the propositus and recombinant variant fibrinogens had the normal $A\tilde{\alpha}$, $B\tilde{\beta}$ and γ-chain components (Figure 1A). Gel resolved under non-reducing conditions revealed that each of the four fibrinogens had two broad bands corresponding to high molecular-weight and low molecular-weight fractions [21] (Figure 1B). These results demonstrated that the free sulfhydryl group in the newly substituted Cys did not form significant disulfide bonds with large molecules such as albumin and/or another variant fibrinogen, which lead to a markedly increased mobility shift in SDS-PAGE.

Mass spectrometric analysis.

To identify the modifications associated with the neo-Cys residue, peptides isolated from fibrinogens were analyzed by mass spectrometry. After protecting the free sulfhydryl, if present, by vinylpyridine treatment, fibrinogen was cleaved with CNBr

and the products separated on Sephadex G-50sf. To identify the relevant CNBr peptides, γ265Phe-310Met residues, dimeric molecules of variant γ265Phe-310Met residues, and modified peptides, all gel-filtrated fractions were analyzed by mass spectrometry. The analyzed data are summarized in Table 1.

Analysis of the M-III fibrinogen fragment revealed a normal peak, mass 5002.0, consistent with the presence of normal fragment, and an additional peak, mass 5067.9, corresponding to a variant fragment with a disulfide-linked Cys residue. Following reduction with mercaptoethanol, a new fragment of mass 4948.4, corresponding to a variant fragment with a sulfhydryl group, was present. No component corresponding to a dimeric molecule of variant fragment, a variant fragment with an attached glutathione moiety or with a pyridylethylated form was found. Analysis of the fragment of the recombinant γ275C revealed only one peak, mass 5068.0, corresponding to a variant fragment with a disulfide-linked Cys residue, and subsequent mercaptoethanol treatment reduced it to mass 4949.1, with a sulfhydryl group. On the other hand, fragments derived from normal plasma and recombinant γ275R fibrinogen exhibited masses 5002.2 and 5001.4, respectively, and these are each consistent with the presence of normal fragment. We concluded that M-III contained both normal γ265Phe-310Met(γ275Arg) and an abnormal γ265Phe-310Met (γ275Cys) with a disulfide-linked Cys residue. Thus, all the neo-Cys residues in fibrinogen M-III exist as disulfide-linked Cys, and this was confirmed by our analysis of recombinant γ275C fibrinogen.

Thrombin-catalyzed Fibrin Polymerization.

Thrombin-catalyzed fibrin polymerization (TCFP) was monitored as the change in turbidity at 350 nm, as described in Materials and Methods. Representative turbidity curves are shown in Figure 2. We found that polymerizations of M-III and γ275C fibrinogen were impaired relative to that of normal fibrinogen. Briefly, when 0.09

mg/ml fibrinogen was used γ275C did not polymerize for 6 h, but the curve for M-III showed increased turbidity after a longer lag period (more than 5 min) and with a slower rate of rise than the normal control (NC), and at 10 min the turbidity was greater for M-III than for NC. When a higher concentration of fibrinogen was used (0.45 mg/ml), M-III and γ275C started polymerization after lag periods of 6 and 12 min, respectively, and the M-III turbidity curve crossed those for NC at 10 min, while the γ275C curve crossed that for γ275R at 30 min. These aberrant TCFP patterns for M-III and γ275C fibrinogen were similar to those reported for Matsumoto II [10] and recombinant γ308K fibrinogen [manuscript submitted].

FXIIIa-catalyzed cross-linking of fibrin or fibrinogen.

Cross-linking of fibrin α- and γ-chains was performed in the presence of factor XIII and thrombin, and the reaction products were analyzed by SDS-PAGE, as described in Materials and Methods. After a 1-min reaction with NC fibrinogen, the γ-γ dimer band was weakly evident, and after 5 min two α-polymer bands were evident (Figure 3A; NC). During longer incubation periods (from 1 to 20 min), these products increased with time, while the α- and γ-chain monomer bands decreased. Formation of γ-γ dimer and α-polymer from γ275R fibrinogen was almost the same as for NC (Figure 3A; γ275R). For M-III fibrinogen, the γ-γ dimer and α-polymer bands were evident after 1 and 20 min, respectively (Figure 3A; M-III). In contrast, for γ275C fibrinogen the γ-γ dimer was evident after 20 min, while α-polymer bands were not evident for 60 min (Figure 3A; γ275C). To confirm the role of D:D interactions in polymerization and to assess the influence of the substitution *per se*, we examined the FXIIIa-catalyzed cross-linking of fibrinogen. After FXIII had been activated with thrombin, hirudin was added to inhibit thrombin-catalyzed fibrinopeptide release and fibrin polymerization, and then the enzyme mixture was added to fibrinogen solution. After a 0.5 h reaction, the $\tilde{\gamma}\gamma$ dimer and α-polymers were seen for both NC and M-III

fibrinogen (Figure 3B; NC and M-III). In the case of γ275R fibrinogen (Figure 3B; γ275R), again the γ-γ dimer formation started at 0.5 h, but formation of α-polymers was slightly delayed starting at 1 h. In contrast, for γ275C fibrinogen neither the γ-γ dimer nor α-polymers were evident for 24 h (Figure 3B; γ275C). We conclude that the delay in γ-γ dimer formation between fibrin monomers that we observed with the variant fibrinogen was due to impaired alignment of the fibrin monomers.

Scanning Electron Microscopy (SEM) of Fibrin Clots and Fibers.

To clarify the difference in the ultrastructure of the fibrin clot between variant and normal fibrinogens, we observed fibrin clots under the SEM. The clots prepared from M-III fibrinogen with the aid of thrombin differed in ultrastructure from those prepared from NC fibrinogen, the density of the bundles of fibrin fibers being less in the former than in the latter case (Figures 4A and B) and the fiber diameter being larger in the former (158 ± 55 nm) than in the latter (134 ± 30 nm) (Figure 4B). In addition, fibrin bundles derived from M-III fibrinogen appeared "frayed" around the major fiber in these clots and there was a marked increase in the number of fibers with abrupt terminations (by comparison with NC fibrin architecture) (Figure 4B). The clots prepared from γ275C fibrinogen with the aid of thrombin differed markedly in ultrastructure from those prepared from γ275R fibrinogen: *viz.* in the former, there were many aberrant fibrin fibers with tapered ends and the fiber diameter was much larger (366 ± 69 nm) than in the case of γ275R (142 ± 28 nm) (Figure 4B).

Discussion

Our results indicate that the substitution of the γ-chain residue Arg275 by Cys results in the presence of a disulfide-linked Cys and a disruption of the initial alignment of fibrin monomers into protofibrils, and that these changes lead to an impairment of the subsequent FXIIIa-catalyzed γ-γ dimer formation. Since the plasma

fibrinogen purified from the heterozygous proband with dysfibrinogenemia generally contains three types of molecules (normal homodimer, variant homodimer, and heterodimer) and the expected distribution of these three molecules is 1:1:2 according to Mendelian genetics [13,14] without unique dysfibrinogens such as Aα16Arg->Cys [22] as Bβ14Arg->Cys [23] etc, we can not know the precise function of a fibrinogen consisting only of a variant γ-chain and resulting in fibrin clot structures. To address this problem, we synthesized recombinant γ275C fibrinogen using CHO cells and compared it with heterozygous M-III plasma fibrinogen.

Because the post-translational nature of neo-Cys residues in abnormal fibrinogens is variable, we examined the nature of this neo-Cys by SDS-PAGE analysis under non-reducing conditions and by mass spectrometric analysis. The former revealed that the neo-Cys residue was not disulfide-bridged to other mobility-shifting proteins, such as albumin. Mass analysis of CNBr-cleaved fibrinogen M-III and CNBr-cleaved recombinant γ275C demonstrated that in these, this neo-Cys residue was entirely disulfide-bridged to a free Cys. Previously, neo-Cys residues in abnormal fibrinogens have been found either as free sulfhydryls or disulfide-bridged to other molecules such as albumin, Cys, or a second abnormal fibrinogen molecule [18]. In the γ-chain residue of γTyr280Cys (Banks Peninsula) [24] and in that of γSer358Cys (Milano VII) [25], the neo-Cys residue exists as a free sulfhydryl and disulfide-bridged to albumin, respectively. In the case of γArg275Cys, only dysfibrinogen Osaka II [26] out of 12 families [9] has been demonstrated by mass analysis to possess a disulfide-bridging Cys residue. Unfortunately, we have no convincing explanation for the variability in the post-translational nature of the neo-Cys residues in abnormal fibrinogens.

Here, we found that the thrombin-catalyzed fibrin polymerization curve obtained using a high concentration of M-III or recombinant γ275C fibrinogen was unusual, each curve having a prolonged lag period and a slow rate of rise, but with a Δabsorbance in 30 min that was larger than (M-III) or almost same as (γ275C) that for

NC. As we anticipated, the impairment was much more pronounced for recombinant γ275C fibrinogen than for heterozygous plasma fibrinogen from the M-III proband. These results indicate the M-III plasma fibrinogen contains heterodimers and that after thrombin stimulation, these can polymerize with both the normal homodimers and the variant homodimers. We have previously observed similar polymerization curves for fibrinogen Matsumoto II [10] and recombinant γ308Lys [manuscript submitted], but not for either fibrinogen Matsumoto I [11] or Kosai/Ogasa (BβGly15Cys) [20]. These results also indicate that polymerization curves for dysfibrinogens with impaired D:E interaction or lateral aggregation are significantly different from that for a dysfibrinogen with impaired D:D interaction like that described here.

Because the γ-chain residue Arg275 locates at the so-called 'D:D interface' and plays a central role in the initial alignment of fibrin monomers into protofibrils, we guessed that FXIIIa-catalyzed γ-γ dimer formation would be markedly disturbed in the case of γ275C (versus M-III plasma fibrinogen). Our results show that γ-γ dimer formation was almost the same for M-III fibrin as for the normal control, a finding consistent with one described by Mosesson et al. [5]; besides, that for γ275C was markedly more disturbed than that for γ275R. Contrary to our expectations we found that the D:D interface of the variant fibrinogen could interact with that of the normal fibrinogen present in the plasma of the proband with heterozygous dysfibrinogenemia. Our present data do not clarify whether the impairment in γ275C might be caused by substitution of the Arg residue *per se* or following neo-Cys residue disulfide-bridging to Cys. Since two other dysfibrinogens with a γ275 residue, γArg275His [27] and γArg275Ser [28], exhibit an impairment of fibrin polymerization similar to that shown by γArg275Cys, substitution of γArg275 might itself markedly affect both the initial alignment of fibrin monomers and the subsequent fibrin polymerization. The reported high resolution structures show that each γArg275 residue at the D:D interface has a different set of contacts (*viz.* γArg275 in one molecule interacts with γTyr280 in a

second molecule, while γArg275 in the second molecule interacts with γSer300 in the first molecule) [29]. The above also demonstrates that γArg275 is one of the most important residues in the D:D interface.

Our scanning electron microscopic observations of fibrin clots made from recombinant γ275C revealed an extensively branched fiber network and a lower fiber density than in the case of γ275R, and the fiber bundle was 2.6 times thicker than for γ275R, although many fibers had tapered ends. Weisel and Nagaswami [30] analyzed the effects of thrombin concentration on clot structure and turbidity curves. They showed that with smaller amounts of thrombin, increasing the thrombin concentration led to decreases in average fiber-bundle size, lag period, and maximum final turbidity, while the maximum rate of turbidity development increased. These differences in clot structure and turbidity curves between higher and lower concentration of thrombin are consistent with our observations for γ275R and γ275C fibrinogens. In addition, our SEM images of fibrin clots and bundles made from γ275C fibrinogen were similar to the images of those made from fibrinogen Tokyo II observed by Mosesson et al [5]. However, our images of fibrin clots and bundles made from M-III fibrinogen somewhat differed from those from fibrinogen Tokyo II. Namely, the number of fibrin bundles with abnormal ends in M-III fibrin clots was less than those from fibrinogen Tokyo II. These discrepancies might have been caused by the procedures used in sample preparation for SEM observation. Although we can not explain how a markedly affected D:D interaction in γ275C might lead to thicker fiber bundles, at least the D:D interface and γArg275 appear not to be essential for lateral aggregation during normal fibrin polymerization. Actually, previous X-ray crystallographic analysis has predicted that lateral aggregation takes place between the $\tilde{\gamma}$-chain of neighboring molecules (γ350-360 and γ370-380), and also between the $\tilde{\beta}$-chain of neighboring molecules (β330-375) [31].

In conclusion, in fibrinogen the substitution of γ-chain residue Arg275 by Cys

results in the presence of a disulfide-linked Cys and a disruption of the initial alignment of fibrin monomers into protofibrils, and these changes lead to an impairment of the subsequent FXIIIa-catalyzed γ-γ dimer formation. The impairment or abnormality was much more pronounced for recombinant γ275C fibrinogen than for heterozygous plasma fibrinogen. These results indicate that γArg275 has an important residue affecting the structure and function of the γ-chain C-terminal domain. However, the variant D:D interface can interact with that of the normal fibrinogen present in the plasma of a proband with heterozygous dysfibrinogenemia.

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References

1 Hantgan RR, Hermans J. Assembly of fibrin. A light scattering study. *J Biol Chem* 1979;**254**:11272-81.

2 Budzynski AZ, Olexa SA, Pandya BV. Fibrin polymerization sites in fibrinogen and fibrin fragments. *Ann NY Acad Sci* 1983;**408**:301-14.

3 Blomback B, Hessel B, Hogg D, Therkildsen L. A two-step fibrinogen-fibrin transition in blood coagulation. *Nature* 1978;**275**:501-5.

4 Matsuda M, Baba M, Morimoto K, Nakamikawa C. "Fibrinogen Tokyo II" an abnormal fibrinogen with an impaired polymerization site on the aligned DD domain of fibrin molecules. *J Clin Invest* 1983;**72**:1034-41.

5 Mosesson MW, Siebenlist KR, DiOrio JP, Matsuda M, Hainfeld JF, Wall JS. The role of fibrinogen D domain intermolecular association sites in the polymerization of fibrin and fibrinogen Tokyo II (γ275 Arg->Cys). *J Clin Invest* 1995;**96**:1053-8.

6 Henschen A, Kehl M, Southan C. Genetically abnormal fibrinogens -Strategies for structure elucidation, including fibrinopeptide analysis. *Curr Probl Clin Biochem* 1984;**14**:273-320.

7 Cote HCF, Lord ST, Pratt KP. γ-chain dysfibrinogenemias: molecular structure-function relationships of naturally occurring mutations in the γ chain of human fibrinogen. *Blood* 1998;**92**:2195-212.

8 Matsuda M, Sugo T. Structure and function of human fibrinogen inferred from dysfibrinogens. *Int J Hematol* 2002;**76** Supplement 1:352-60.

9 http://www.geht.org/pages/database__fibrino__uk.html.

10 Terasawa F, Okumura N, Higuchi Y, Ishikawa S, Tozuka M, Ishida F, Kitano K, Katsuyama T. Fibrinogen Matsumoto III: a variant with γ275 Arg->Cys (CGC->TGC) -Comparison of fibrin polymerization properties with those of Matsumoto I (γ364 Asp->His) and Matsumoto II (γ308 Asn->Lys). *Thromb Haemost* 1999;**81**:763-6.

11 Okumura N, Furihata K, Terasawa F. Nakagoshi R, Ueno I, Katsuyama T. Fibrinogen Matsumoto I: A γ364 Asp->His (GAT->CAT) substitution associated with defective fibrin polymerization. *Thromb Haemost* 1996;**75**:887-91.

12 Okumura N, Furihata K, Terasawa F, Ishikawa S, Ueno I, Katsuyama T. Fibrinogen Matsumoto II: γ308 Asn->Lys (AAT->AAG) mutation associated with bleeding tendency. *Br J Haematol* 1996;**94**:526-8.

13 Hogan KA, Lord ST, Okumura N, Terasawa F, Galanakis DK, Scharrer I, Gorkun OV. A functional assay suggests that heterodimers exist in two C-terminal γ-chain dysfibrinogens: Matsumoto I and Vlissingen/Frankfurt IV. *Thromb Haemost* 2000;**83**:592-7.

14 Okumura N, Terasawa F, Fujita K, Fujihara N, Tozuka M, Koh C-S. Evidence that heterodimers exist in the fibrinogen Matsumoto II (γ308N->K) proband and participate in fibrin fiber formation. *Thromb Res* 2002;**107**:157-62.

15 Okumura N, Gorkun OV, Lord ST. Severely impaired polymerization of recombinant fibrinogen γ-364 Asp->His, the substitution discovered in a heterozygous individual. *J Biol Chem* 1997;**272**:29596-601.

16 Takebe M, Soe G, Kohno I, Sugo T, Matsuda M. Calcium ion-dependent monoclonal antibody against human fibrinogen; preparation, characterization, and application to fibrinogen purification. Thromb Haemost 1995;**73**:662-7.

17 Mihalyi E. Physicochemical studies of bovine fibrinogen. IV. Ultraviolet absorption and its relation to the structure of the molecule. *Biochemistry* 1968;**7**:208-23.

18 Lounes KC, Lefkowitz JB, Henschen-Edman AH, Coates AI, Hantgan RR, Lord ST. The impaired polymerization of fibrinogen Longmont (Bβ166Arg->Cys) is not improved by removal of disulfide-linked dimers from a mixture of dimers and cysteine-linked monomers. *Blood* 2001;**98**:661-6.

19 Furlan M, Rupp C, Beck EA. Inhibition of fibrin polymerization by fragment D is affected by calcium, Gly-Pro-Arg and Gly-His-Arg. *Biochim Biophys Acta* 1983;**742**:25-32.

20 Hirota-Kawadobora M, Terasawa F, Yonekawa O, Sahara N, Shimizu E, Okumura N, Katsuyama T, Shigematsu H. Fibrinogens Kosai and Ogasa: Bβ15Gly->Cys (GGT->TGT) substitution associated with impairment of fibrinopeptide B release and lateral aggregation. *J Thromb Haemost* 2003;**1**:275-83.

21 Holm B, Nilsen DWT, Kierulf P, Godal HC. Purification and characterization of 3 fibrinogens with different molecular weights obtained from normal human plasma. *Thromb Res* 1985;**37**:165-76.

22 Miyata T, Terukina S, Matsuda M, Kasamatsu A, Takeda Y, Murakami T, Iwanaga S. Fibrinogens Kawaguchi and Osaka: an amino acid substitution of Aα arginine-16 to cysteine which forms an extra interchain disulfide bridge between the two Aα chains. *J Biochem (Tokyo)* 1987;**102**:93-101.

23 Pirkle H, Kaydewitz H, Henschen A, Theodor I. Substitution of Bβ14 arginine by cyst(e)ine in fibrinogen Seattle I. In: *Fibrinogen 2. Biochemistry, physiology and clinical relevance.* Lowe GDO, Douglas JT, Forbes CD, Henschen A, eds. Elsevier Science Publishers, Amsterdam 1987; 49-52.

24 Fellowes AP, Brennan SO, Ridgway HJ, Heaton DC, George PM. Electrospray ionization mass spectrometry identification of fibrinogen Banks Peninsula (γ280Tyr ->Cys): a new variant with defective polymerization. *Br J Haematol* 1998;**101**:24-31.

25 Steinmann C, Bogli C, Jungo M, Lammle B, Heinemann G, Wermuth B, Redaelli R, Baudo F, Furlan M. A new substitution, γ358 Ser -> Cys, in fibrinogen Milano VII causes defective fibrin polymerization. *Blood* 1994;**84**:1874-80.

26 Terukina S, Matsuda M, Hirata H, Takeda Y, Miyata T, Takao T, Shimonishi Y. Substitution of γArg-275 by Cys in an abnormal fibrinogen, “fibrinogen Osaka II”.

Evidence for a unique solitary cystine structure at the mutation site. *J Biol Chem* 1988;**263**:13579-87.

27 Borrell M, Gari M, Coll I, Vallve C, Tirado I, Soria JM, Sala N, Munoz C, Oliver A, Garcia A, Fontcuberta J. Abnormal polymerization and normal binding of plasminogen and t-PA in three new dysfibrinogenaemias: Barcelona III and IV (γArg275->His) and Villajoyosa (γArg275->Cys). *Blood Coagul Fibrin* 1995;**6**:198-206.

28 Mimuro J, Kawata Y, Niwa K, Muramatsu S, Madoiwa S, Takano H, Sugo T, Sakata Y, Sugimoto T, Nose K, Matsuda M. A new type of substitution for γArg-275 in fibrinogen Kamogawa I characterized by impaired fibrin assembly. *Thromb Haemost* 1999;**81**:940-4.

29 Everse SJ, Spraggon G, Veerapandian L, Riley M, Doolittle RF. Crystal structure of fragment double-D from human fibrin with two different bound ligands. *Biochemistry* 1998;**37**:8637-42.

30 Weisel JW, Nagaswami C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. *Biophys J* 1992;**63**:111-28.

31 Yang Z, Mochalkin I, Doolittle RF. A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides. *Proc Natl Acad Sci USA* 2000;**97**:14156-61.

Table 1. Mass spectrometric analysis of CNBr-treated fragments from γ-chain of fibrinogen

Fragment corresponding to positions	calculated mass	observed mass			
		normal control plasma	Matsumoto III plasma	recombinant γ275Arg	recombinant γ275Cys
265-310, normal	5001.4	5002.2	5002.0	5001.4	NF
265-310, abnormal, with pyridylethylated Cys	5053.4	NF	NF	NF	NF
265-310, abnormal, with additional, disulfide-linked Cys	5067.4	NF	5067.9	NF	5068.0
265-310, abnormal, with disulfide-linked glutathione	5253.6	NF	NF	NF	NF
265-310, abnormal, with disulfide-linked abnormal 265-310	9896.7	NF	NF	NF	NF
265-310, abnormal, after disulfide cleavage	4948.3	NF	4948.4	NF	4949.1

NF: not found

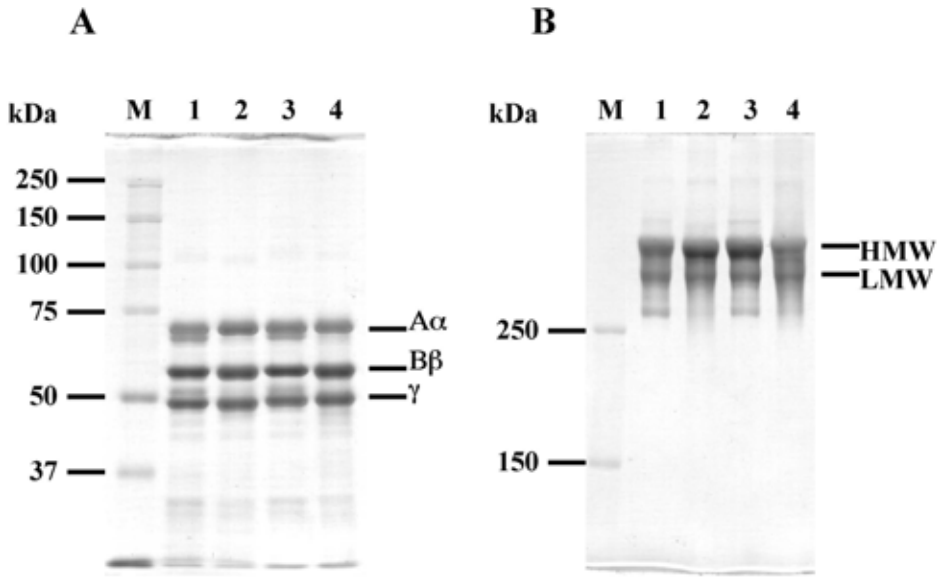


Figure 1. **Characterization of plasma and recombinant fibrinogens.** Coomassie-stained 10% SDS-PAGE run under reducing conditions (A) or 5% SDS-PAGE run under non-reducing conditions (B). Normal control (lane 1) and Matsumoto III plasma fibrinogen (lane 3), and recombinant wild-type γ275R (lane 2) and variant γ275C fibrinogen (lane 4). HMW and LMW, high molecular-weight and low molecular-weight fibrinogen, respectively.

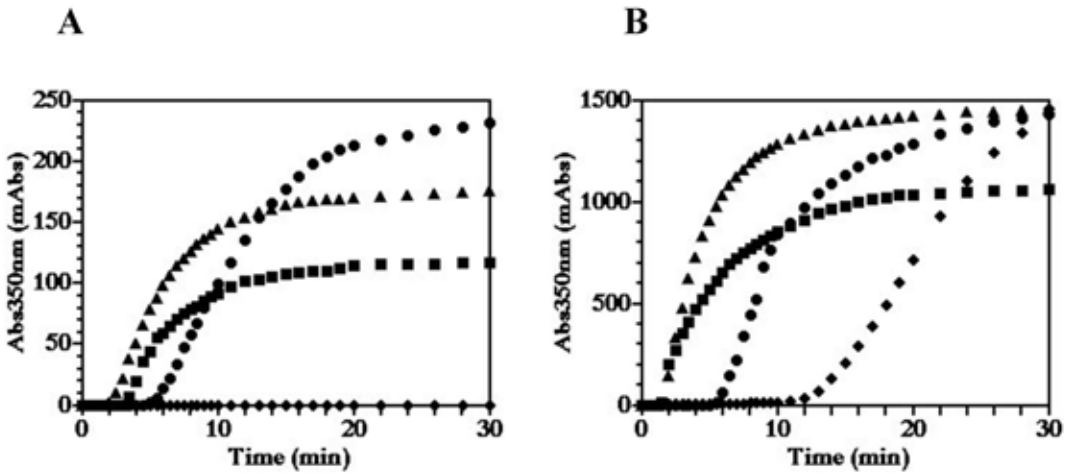
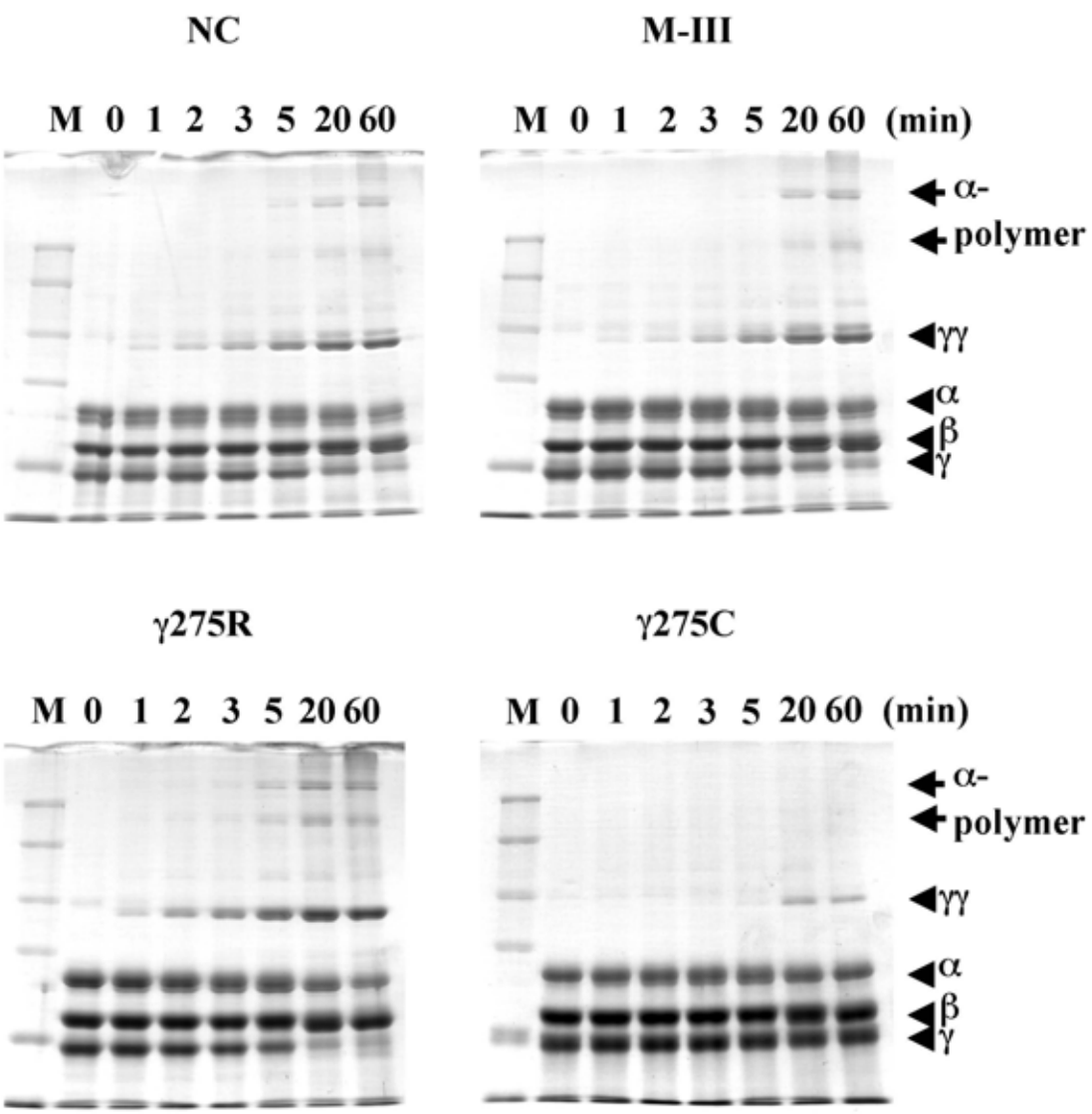


Figure 2. Thrombin-catalyzed fibrin polymerization. Polymerization of fibrinogen (0.09 mg/ml, A ; 0.45 mg/ml, B) was initiated with thrombin (0.05 U/ml), and the change in turbidity with time was followed at 350 nm for normal control (■), Matsumoto III plasma (●), recombinant γ275R (▲), or γ275C (◆) fibrinogens in 20 mM HEPES, pH 7.4, 0.12 M NaCl.

A


NC
M-III
M 0 1 2 3 5 20 60
M 0 1 2 3 5 20 60 (min)
α-
polymer
γγ
α
β
γ
γ275R
γ275C
M 0 1 2 3 5 20 60
M 0 1 2 3 5 20 60 (min)
α-
polymer
γγ
α
β
γ

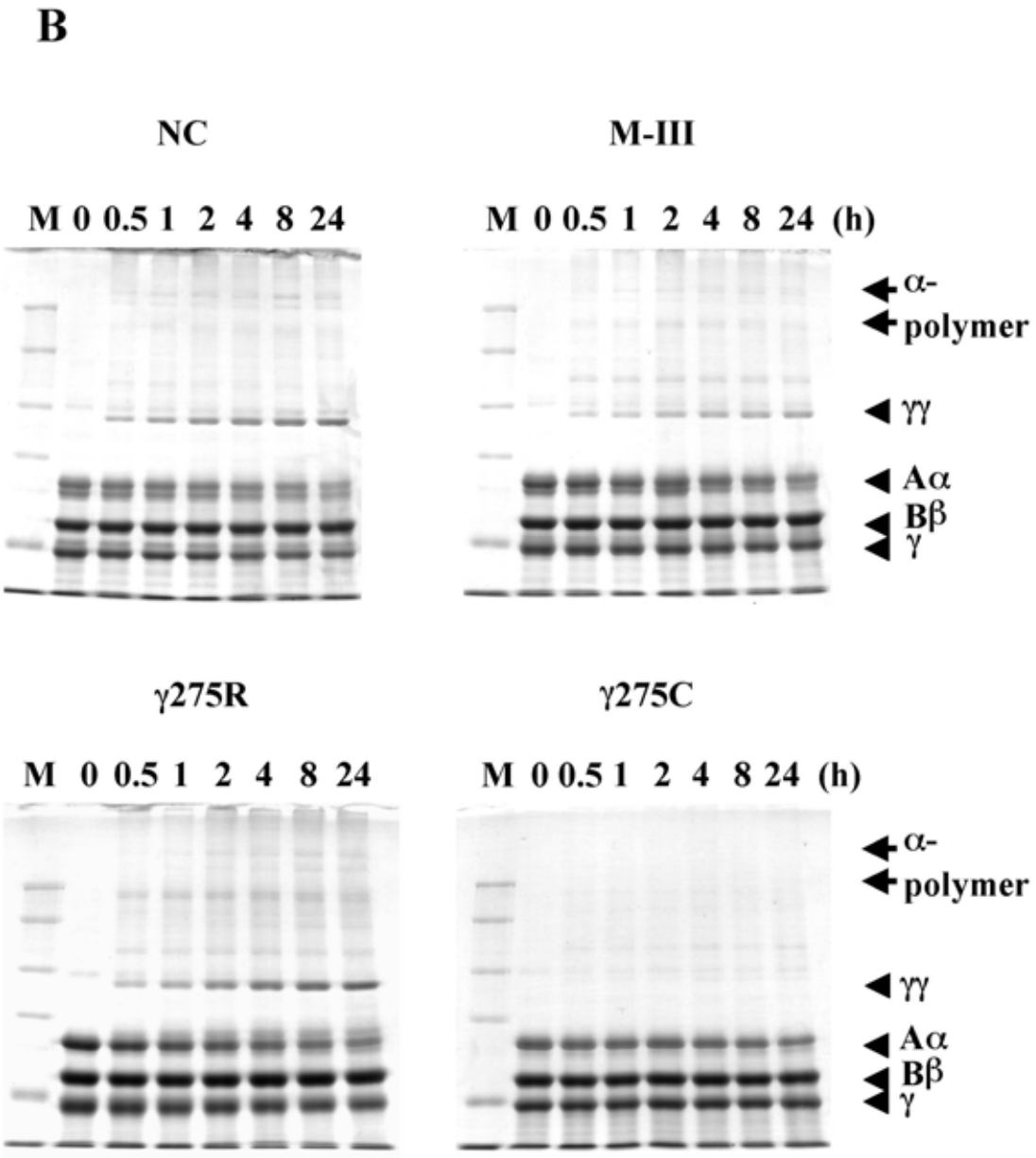
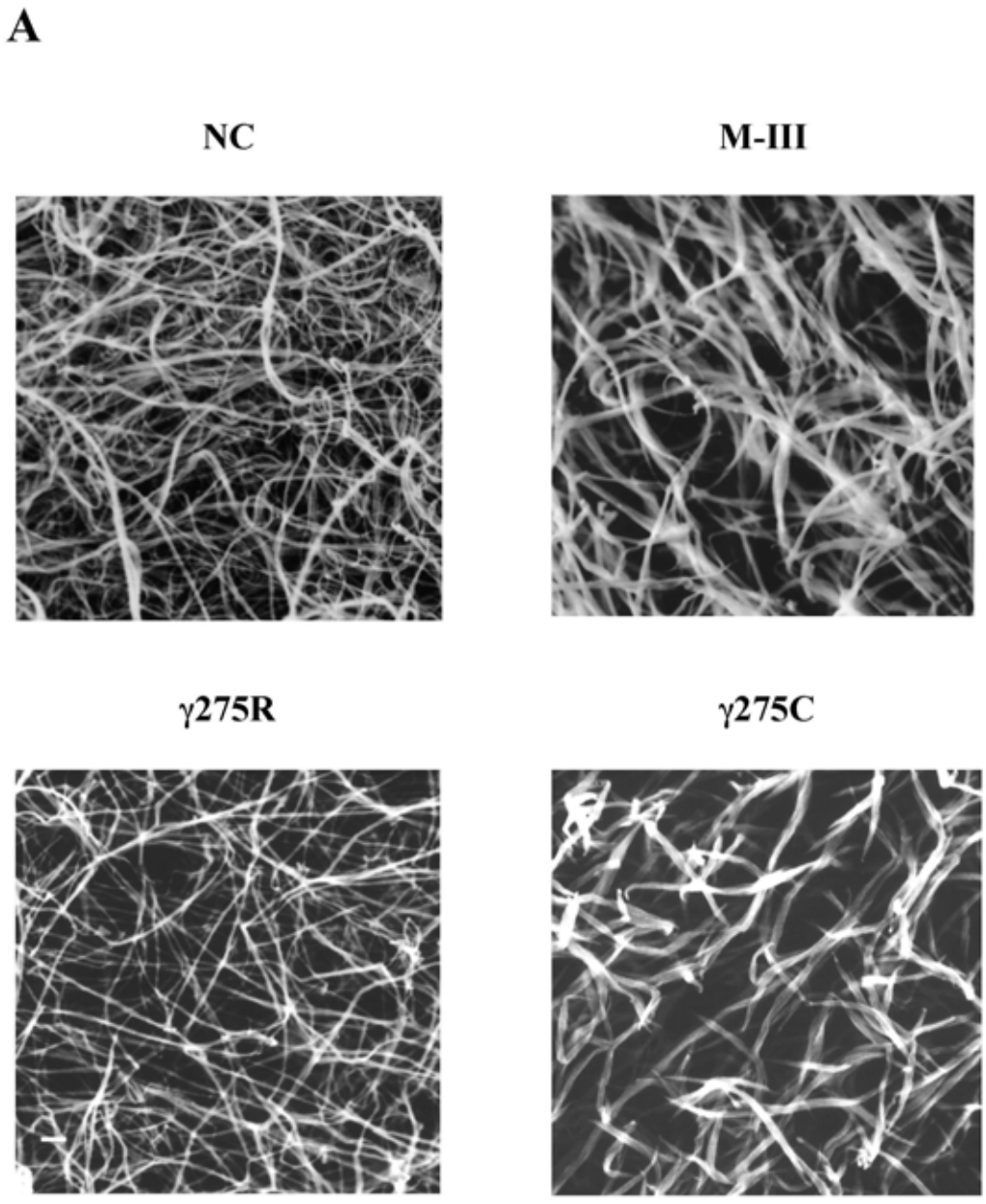


Figure 3. FXIIIa-catalyzed cross-linking of fibrin or fibrinogen. Time-dependent FXIIIa-catalyzed cross-linking of fibrin (A) or fibrinogen (B) was examined, 8% SDS-PAGE being run under reducing conditions with Coomassie Brilliant Blue staining, as described in Material and Methods. The individual fibrinogen chains (Aα, α, Bβ, β, γ, cross-linked γ-γ dimer, and cross-linked α-chain polymers) are indicated on the right

side of the gels. The molecular markers (250, 150, 100, 75 and 50 kDa; from top to bottom) were run in lane M.



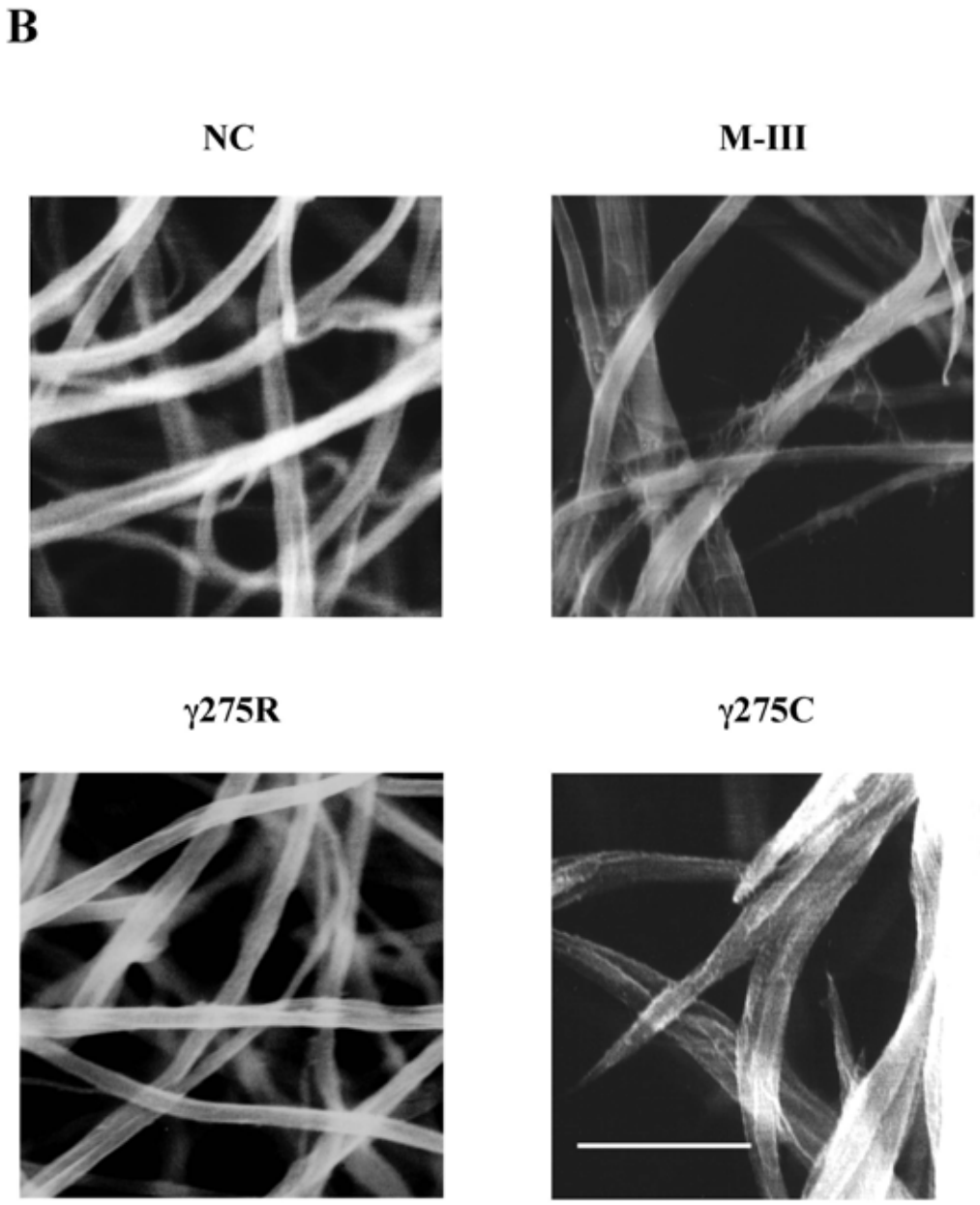


Figure 4. Scanning electron microscopy of fibrin clots formed using thrombin. All samples were prepared as described in Material and Methods. Micrographs in A and B were taken at 3000x and 20000x, respectively. Bar**,** 1 μm.