Citrullinated fibrinogen shows defects in FPA and FPB release and fibrin polymerization catalyzed by thrombin

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Abstract

Background: Antibody-antigen complexes formed by IgG autoantibodies against citrullinated proteins and citrullinated forms of the α- and β-chains of fibrin in rheumatoid synovial tissue play a key role in the pathophysiology of rheumatoid arthritis.

Methods: Recombinant fibrinogen was citrullinated by rabbit skeletal muscle peptidylarginine deiminase so that we could analyze the function of citrullinated fibrinogen. Namely, thrombin-catalyzed fibrin polymerization and fibrinopeptide release, protection against plasmin digestion, and factor XIIIa-catalyzed cross-linking of fibrin or fibrinogen were performed.

Results: Strong citrullination of the Aα- and Bβ-chains and weak citrullination of the γ-chain were detected by an anti-modified citrulline detection kit. Citrullinated fibrinogen did not release FPA or FPB by thrombin catalyzation and no thrombin-stimulated conversion of fibrinogen into fibrin occurred. The citrullination of fibrinogen did not affect the three functions of the C-terminal γ-chain, “a-hole”, low affinity Ca binding, and γ-γ cross-linking.

Conclusion: Our functional analyses demonstrated that no thrombin-stimulated conversion of fibrinogen into fibrin occurred, because citrullinated fibrinogen did not release FPA or FPB after thrombin catalyzation. Our results and those of other reports suggest that citrullinated fibrin and fibrinogen are present in the synovium and might both be associated with the pathophysiology of RA.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unclear etiology that is characterized by chronic joint inflammation and eventually leads to joint destruction and dysfunction. IgG autoantibodies against citrullinated proteins (ACPA) are highly specific to RA and are present in the serum of 75 % of patients with RA [1]. Recently, it was demonstrated that the citrullinated forms of the α- and β-chains of fibrin correspond to the major antigenic targets of antifilaggrin autoantibody in rheumatoid synovial tissue [2].

Fibrinogen is a 340 kDa glycoprotein that circulates in the blood at 2-4 g/L and is composed of a pair of Aα-, Bβ-, and γ-chains. During coagulation, thrombin cleaves fibrinogen, releasing fibrinopeptide A (FPA) and B (FPB) from the N-termini of the Aα- and Bβ-chains exposing the “A-knob” and “B-knob”, respectively, and converting fibrinogen to fibrin monomers. Fibrin monomers polymerize spontaneously through a two-step process [3]. The first step is the formation of double-stranded protofibrils by so-called “A-knob” - “a-hole” interaction and “D-D” interaction [3,4], and the second step is the lateral aggregation of the protofibrils [3,4]. During fibrin assembly, Factor (F) XIIIa cross-links individual fibrin molecules and finally forms a complex, stable, insoluble, branching network of fibrin fibers and bundles of fibers.

In this study, fibrinogen was citrullinated *in vitro* by rabbit skeletal muscle peptidylarginine deiminase, so that we could analyze the function of citrullinated fibrinogen, namely, thrombin-catalyzed fibrin polymerization and fibrinopeptide release, protection against plasmin digestion, and factor XIIIa-catalyzed cross-linking of fibrin or fibrinogen were performed.

2. Materials and Methods

2.1. Materials.

All chemicals were of a reagent grade and were purchased from Sigma (St. Louis, MO), unless otherwise noted. Human γ-thrombin and factor XIII were purchased from Enzyme Research Laboratories (South Bend, IN).

2.2. Preparation of recombinant fibrinogen and its citrullination.

The construction of mutant expression vectors and the synthesis and purification of the recombinant fibrinogens have been described previously [5]. Briefly, recombinant fibrinogens were expressed in Chinese hamster ovary (CHO) cells cultured in serum free medium. Fibrinogen was purified from harvested culture medium by ammonium sulfate

precipitation and subsequent immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (IF-1, Iatron Laboratories, Tokyo). Fibrinogen was eluted with 5 mM EDTA before the fractions were pooled and dialyzed against 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4, and 0.12 M NaCl (HBS). Dialyzed samples were aliquoted and stored at -80°C. The fibrinogen concentration was determined from the A_{280} by assuming that a 1 mg/ml solution has an absorbance of 1.51.

Citrullination was performed with rabbit skeletal muscle peptidylarginine deiminase 2 (PAD2, 7 unit/mg fibrinogen) in 0.1 M Tris-HCl (pH 7.4), 0.5 M NaCl, 10 mM $CaCl_2$, and 0.5 mM dithiothreitol (DTT) for 2 h at 37°C [6]. Non-treated fibrinogen and citrullinated fibrinogen are designated as Fbg and C-Fbg, respectively, and control non-citrullinated fibrinogen (NC-Fbg) was incubated in buffer alone. To remove DTT, C-Fbg and NC-Fbg were dialyzed four times against HBS.

The purity and characterization of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), run under either nonreducing (7% polyacrylamide gel) or reducing (10% polyacrylamide gel) conditions. This was followed by Coomassie Brilliant Blue R-250 staining or immunoblot analysis and development with an anti-modified citrulline detection kit (AMC) (Upstate, Waltham, MA) [6]. This kit contains anti-modified citrulline polyclonal antibody and detects ureido group adducts, regardless of neighboring amino acid sequences. Briefly, chemical modification of citrulline residues was performed with 2,3-butanedione monoxime and antipyrine in a strong acid.

2.3. Thrombin-catalyzed fibrin polymerization.

Polymerization was followed by measurement of 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 [5]. Briefly, fibrinogen (90 μl at 0.2 mg/ml) in HBS with 1 mM $CaCl_2$ was mixed with human α-thrombin (10 μl at 0.5 unit/ml), and changes in turbidity were monitored at ambient temperature [5]. The reactions were performed in triplicate.

2.4. Thrombin-catalyzed fibrinopeptide release.

Fibrinogen (0.45 mg/ml) in HBS was incubated with human α-thrombin (2.5 unit/ml) at 37ºC for 3 h, fibrinopepetides were released, and the reactions were stopped by increasing the temperature to 100ºC. After centrifugation, the supernatants were analyzed by

high-performance liquid chromatography (HPLC) using a Cosmocil 5C18P column (Nacalai Tesque, Inc, Kyoto, Japan; 4.5 x 150 mm) as previously described with minor modifications [5]. Briefly, the buffer system consisted of solvent A (0.025 M ammonium acetate adjusted to pH6.0 with phosphoric acid) and solvent B [50% acetonitril for HPLC (Wako Chemical Co, Osaka, Japan) and 50% 0.05 M ammonium acetate, pH6.0]. The gradient was 18-38% B in 40 min and the flow rate was 1.0 mL/min.

2.5. Protection against plasmin digestion of fibrinogen.

Fibrinogen (0.2 mg/mL) in HEPES buffer containing 5 mM EDTA, 5 mM $CaCl_2$, or 2 mM Gly-Pro-Arg-Pro-peptide supplemented with 1 mM EDTA was incubated with 0.2 casein-unit/mL plasmin for 4 h at 37 ºC [7]. The reactions were stopped by the addition of an equal volume of sodium dodecyl sulfate (SDS)-sample buffer and heating at 100 ºC for 5 min. The plasmin digests were analyzed on 8 % SDS-PAGE under reducing conditions, and the gels were stained with Coomassie brilliant blue.

2.6. Factor XIIIa-catalyzed cross-linking of fibrin or fibrinogen.

Factor XIII (FXIII; 50 unit/ml) was activated with human α-thrombin (1 unit/ml) for 60 minutes at 37°C in HBS with 5 mM $CaCl_2$. To examine the cross-linking of fibrin, it (final concentration: 0.3 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 HBS and 0.67 mM calcium [8]. To examine the cross-linking of fibrinogen, hirudin (10 units/ml) was added to thrombin-activated FXIIIa prior to incubation with fibrinogen (final concentration of FXIIIa: 8.3 units/ml) [8]. The reactions were stopped at various times by addition of equal volumes of SDS-sample buffer and 2-mercaptoethanol and incubation (5 min) at 100ºC. Samples equivalent to 4.5 μg fibrinogen were separated on 8 % SDS-PAGE under reducing conditions and were stained with Coomassie Brilliant Blue R-250.

2.7. Scanning Electron Microscopy (SEM).

The SEM preparation was performed as described before [5], with a few minor modifications. Briefly, 10 μl of thrombin were added to 40μl of fibrinogen solution and were mixed by repeated pipetting. Polymerization then proceeded in a moisture chamber in an incubator at 37˚C for 4 h. The final concentrations of fibrinogen and thrombin were 0.16 mg/ml or 0.08 mg/ml and 0.05 unit/ml, respectively. The clots were fixed in 2.5% glutaraldehyde overnight, stained with 1% osmium tetroxide, freeze dried (JFD310 freeze

drying device; Japan Electron Optics Laboratory Co. Ltd., Tokyo, Japan), 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.

3. Results

3.1. SDS-PAGE analysis for citrullinated fibrinogen.

To avoid contamination of the purified fibrinogen by plasminogen, factor XIII, and other plasma proteins, we used genetically synthesized recombinant protein in serum free medium. C-Fbg was similar in mobility to NC-Fbg during nonreducing SDS-PAGE analysis, whereas under reducing conditions, the Aα- and Bβ-chains of C-Fbg had markedly and slightly slower mobility, respectively, than NC-Fbg. Namely, the relative molecular mass of the citrullinated Aα-chain was 98 kDa (31 kDa higher than the normal Aα-chain), and that of the citrullinated Bβ-chain was 59 kDa (2 kDa higher than the normal Bβ-chain) (Fig 1). A similar mobility shift in citrullinated Aα-chains has already been observed by other researchers [2, 9-11]

To demonstrate the citrullination of fibrinogen, blots were visualized by an anti-modified citrulline detection kit (Upstate, Waltham, MA) [6]. Fibrinogen (under nonreducing conditions) and the Aα- and Bβ-chains of C-Fbg (under reducing conditions) showed strong citrullination, moreover, the γ-chain and some degradation products derived from fibrinogen and/or its peptides were weakly citrullinated (Fig 1).

3.2. Thrombin-catalyzed fibrin polymerization and thrombin-catalyzed fibrinopeptide release.

Representative curves of thrombin-catalyzed fibrin polymerization are shown in Figure 2A. NC-Fbg polymerized to almost the same extent as Fbg, whereas C-Fbg did not polymerize after 10 h stimulation. To examine the influence of C-Fbg on the polymerization of NC-Fbg, a mixing experiment of NC-Fbg and C-Fbg was performed. Representative curves showed that C-Fbg did not inhibit the polymerization of fibrin monomers derived from NC-Fbg (Fig. 2A).

To analyze the cause of the absence of fibrin polymerization for C-Fbg, thrombin-catalyzed fibrinopeptide release was examined. A representative separation of fibrinopeptides by reversed phase high performance liquid chromatography is shown in Figure 2B. NC-Fbg released FPA, FPB, and des-Arg-FPB, but C-Fbg did not release any fibrinopeptides.

3.3. Protection against plasmin digestion of fibrinogen and FXIIIa-catalyzed cross-linking of fibrin or fibrinogen.

To analyze the other functions of the C-terminal module of the fibrinogen γ-chain, the "a-hole", and the low affinity Ca-binding sites, protection against plasmin digestion of C-Fig was performed [7]. In this assay, in the presence of 5 mM $CaCl_2$ or 2 mM Gly-Pro-Arg-Pro (GPRP) peptide (the "A-knob" peptide mimic), C-Fbg was completely protected against plasmin digestion of fragment D1 into D3, and our findings were similar to those for NC-Fbg (Fig. 3). These results demonstrate that neither the binding of the GPRP peptide to the "a-hole" nor the binding of Ca ions to the low affinity Ca-binding sites in the C-terminal module of the γ-chain are affected by the citrullination of fibrinogen [7].

In addition, cross-linking of fibrin α- and γ-chains was performed in the presence of FXIIIa and thrombin, and the reaction products were analyzed by SDS-PAGE. For NC-Fbg (Figure 4A), the γ-γ dimer and α-polymer bands appeared at 2 and 5 min, respectively. For C-Fbg (Figure 4B), the γ-γ dimer and Aα-polymer bands were evident after 60 min. Since the slower rate of cross-linking of fibrin might reflect the complete impairment of fibrin polymerization, the cross-linking of fibrinogen Aα- and γ-chains was performed in the presence of hirudine as a thrombin inhibitor, i.e., in conditions without fibrin polymerization. Of interest, for C-Fbg (Figure 4D), the γ-γ dimer and Aα-polymer bands appeared at 30 min, increased in intensity in a time dependent-manner, and were almost identical to those of NC-Fbg (Figure 4C). Specifically, the observed cross-linking of C-Fbg in the presence of thrombin reflected fibrinogen cross-linking.

3.4. Scanning Electron Microscopy (SEM).

It is well known that the diameter of fibrin fibers increases proportionally to fibrinogen concentration [12]. Since the turbidity change caused by thrombin-catalyzed fibrin polymerization of a mixture of NC-Fbg (0.09 mg/ml) and C-Fbg (0.09 mg/ml) was larger than that for control non-citrullinated fibrinogen alone (0.09 mg/ml) (Fig 2A), we observed

fibrin clots by SEM to clarify the incorporation of C-Fbg into the fibrin fibers formed from NC-Fbg. SEM observation showed that the fiber diameter was 97 ± 23 nm (n=60) for 0.08 mg/ml of NC-Fig and 95 ± 22 nm (n=60) for the mixture of 0.08 mg/ml of NC-Fig and 0.08 mg/ml of C-Fig, and these values were significantly (p<0.001) lower than that for 0.16 mg/ml of NC-Fig (114 ± 22 nm, n=60) (Fig 5). These results indicate that C-Fbg does not participate in fibrin fiber formation.

Discussion

To confirm the citrullination of arginine residues of fibrinogen by PAD2 *in vitro*, we performed SDS-PAGE and subsequent Western blot analysis using anti-modified citrulline polyclonal antibody. These analyses demonstrated that both the Aα- and Bβ-chains of fibrinogen were strongly citrullinated and that the γ-chain was weakly citrullinated. Our results agree with Nakayama-Hamada's analysis [10]. Interestingly, the citrullinated Aα-chain had a markedly higher molecular mass than the normal Aα-chain under reducing SDS-PAGE conditions, but citrullinated fibrinogen did not under non-reducing SDS-PAGE conditions. These observations have been already reported by others [2, 9-11]. Although the degree of our and Zhao's observations [11] were marked, Masson-Bessiere's [2] was medium and Takizawa's [9] and Nakayama-Hamada's [10] were mild. Citrullination of arginine residues might lead to slower mobility shifts during SDS-PAGE analysis, and the degree of this change is dependent on the number of citrullinated residues on the Aα-chain because Arg makes up 40 of its 610 residues [10]. Furthermore, Nakayama-Hamada *et al.* [10] and Suzuki *et al.* [6] also observed an increase in molecular mass for citrullinated filaggrin and type I collagen α1- and α2-chains. These observations suggest that citrullination of arginine residues decreases the positive charge, follows conformational change, and leads to a mobility shift on SDS-PAGE [13-15].

Our functional analyses demonstrated that citrullinated fibrinogen does not release FPA or FPB by thrombin catalyzation, and we observed no conversion of fibrinogen into fibrin. These results suggest that both Aα-16Arg and Bβ-14Arg are citrullinated by PAD2 *in vitro* and so thrombin can not cleave Aα-16Arg-17Gly or Bβ-14Arg-15Gly bonding. Citrullination at Aα-16Arg and Bβ-14Arg have been already reported by Nakayama-Hamada *et al.* [10]. Most recently, Nakayama-Hamada *et al.* [16] reported that no FPA or FPB release and no polymerization of citrullinated fibrinogen were observed after thrombin stimulation. Moreover, they also demonstrated that citrullinated fibrinogen

and a synthetic peptide that mimics citrullinated FPA inhibit thrombin-catalyzed fibrin polymerization. The latter result is incompatible with our present results. Their citrullination was performed by human PAD4 and ours was performed by rabbit PAD2; however, we cannot explain the cause of the differences in the results. Whereas, our functional analyses demonstrated that the three functions of the γ-chain C-terminal (γC) module, the so-called "a-hole", low affinity Ca binding, and γ-γ cross-linking, were not affected in citrullinated fibrinogen. The γ-chain has only 10 Arg residues out of its 411 residues, and there are no Arg residues in the above three sites located in the γC module (140 – 411 residues). These results are consistent with the markedly weaker citrullination of the γ-chain in our Western blot analysis and a report that no ACPA epitopes are present on the γ-chain [17].

Citrullinated α- and β-chains of fibrin in synovial membranes of RA have been identified as targets of ACPA [2], and citrullinated fibrinogen is detected in RA synovial fluid [9]. Recently, immune-complexes containing citrullinated fibrinogen have been detected in 50% of anti-cyclic citrullinated peptide antibody-positive RA patients' sera [18]. Our present results suggest that both citrullinated fibrinogen and fibrin are present in the synovium. As the expression of PAD2 and PAD4 has been demonstrated in the synovial tissue of RA patients [19], the fibrinogen in the extravasated plasma that passes into the synovium might be deiminated at residues Aα16Arg and Bβ14Arg, resulting in citrullinated fibrinogen. In addition, elevated levels of thrombin in the RA synovial fluid were demonstrated by measurement of thrombin-antithrombin III complexes [20]; therefore, some fibrinogen (non-citrullinated fibrinogen at residues Aα16Arg and Bβ14Arg) might be converted into fibrin by thrombin and deposited on synovial tissue, before finally being deiminated by PAD2 and PAD4. The roles of synovium-based citrullinated fibrinogen and fibrin in the pathogenesis of RA disease are controversial; however, there is the possibility that both are associated with the onset and/or pathophysiology of RA. Citrullinated fibrinogen is reported to be an immunogenic molecule for ACPAs production [21-23], and citrullinated fibrin is a target of ACPAs [2] (but citrullinated fibrin is not specific to RA [24]) and antigen-antibody conflict induces and activates the proinflammatory effects in RA [24]. Finally, our results show that citrullinated fibrinogen is defective in FPA and FPB release and fibrin polymerization catalyzed by thrombin; however, we can not explain the pathological function of this phenomenon.

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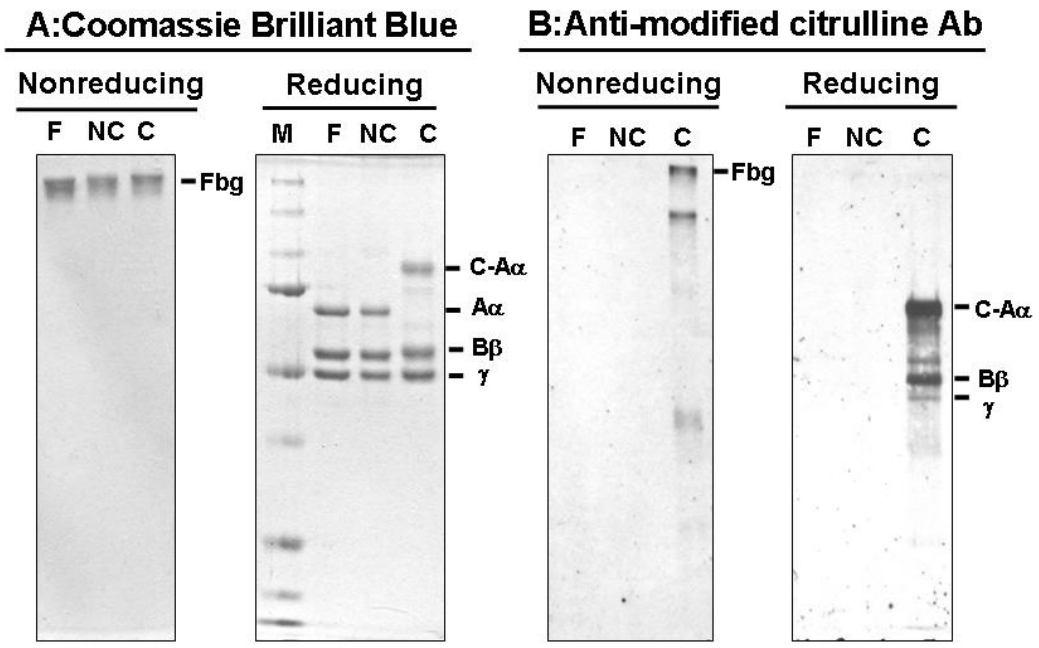


Figure 1. SDS-PAGE a**nalysis for citrullinated recombinant fibrinogen. (A)** Coomassie brilliant blue-stained 10% SDS- PAGE run under non-reducing conditions and 7% SDS-PAGE run under reducing conditions, **(B)** using an anti-modified citrulline detection kit-visualized nitrocellulose membrane transferred from 10% SDS- PAGE run under non-reducing conditions or 7% SDS-PAGE run under reducing conditions; non-treated recombinant fibrinogen (lane F), control non-citrullinated fibrinogen (lane NC), citrullinated fibrinogen (lane C), and the molecular markers (lane M, from upper to lower: 250, 150, 100, 75, 50, 37, and 25 kDa). C-Aα: citrullinated Aα-chain.

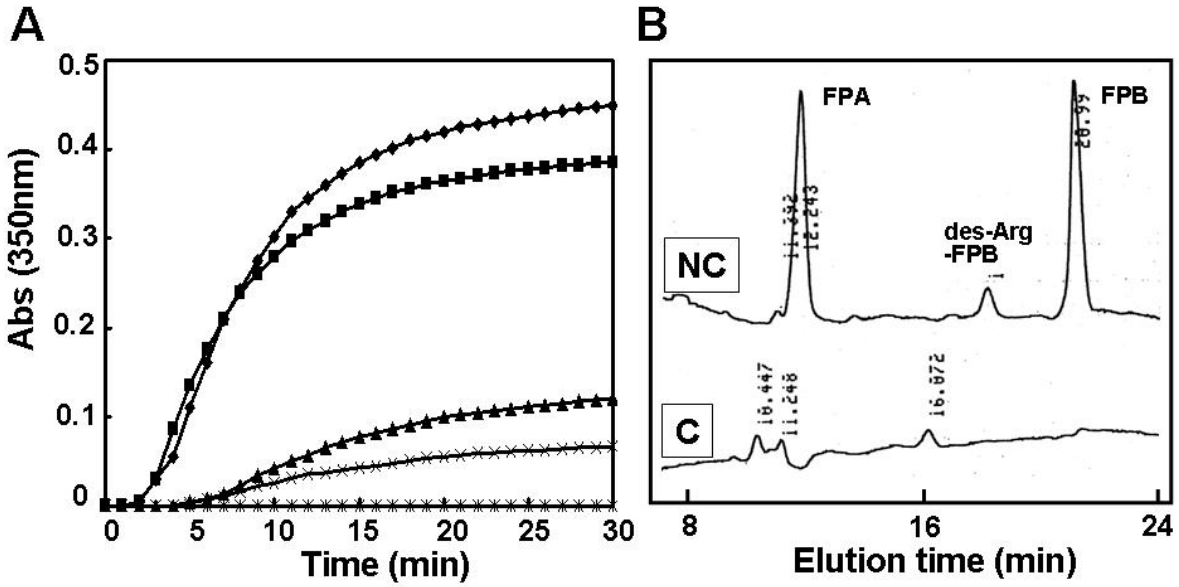


Figure 2. Thrombin-catalyzed fibrin polymerization and fibrinopeptide release of citrullinated recombinant fibrinogen. (A) Polymerization of fibrinogen (0.18 or 0.09 mg/ml) was initiated with thrombin (0.05 U/ml), and the change in turbidity at 350 nm was followed. Representative polymerization curves of non-treated recombinant fibrinogen (0.18 mg/ml; ◆), control non-citrullinated fibrinogen (0.18 mg/ml; ■), citrullinated fibrinogen (0.18 mg/ml; ∗), a mixture of non-citrullinated (0.09 mg/ml) and citrullinated fibrinogen (0.09 mg/ml) (▲), and control non-citrullinated fibrinogen (0.09 mg/ml; ×). **(B)** Fibrinogen (0.45 mg/ml) in HBS was incubated with human α-thrombin (2.5 unit/ml) at 37ºC, fibrinopepetides were released, and the reactions were stopped by incubation at 100ºC. Representative separation of fibrinopeptide by reversed phase high performance liquid chromatography is shown between 8 and 24 min (acetonitril 11.5 – 15 %); control non-citrullinated fibrinogen (NC), citrullinated fibrinogen (C). NC-Fbg released FPA, FPB, and des-Arg-FPB.

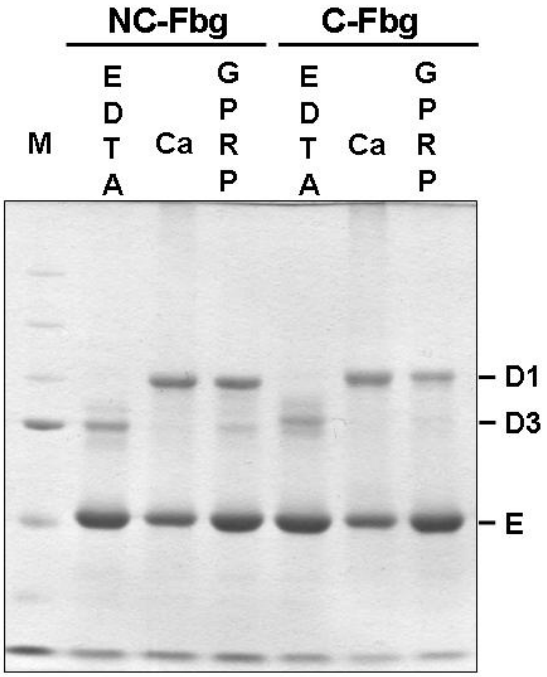
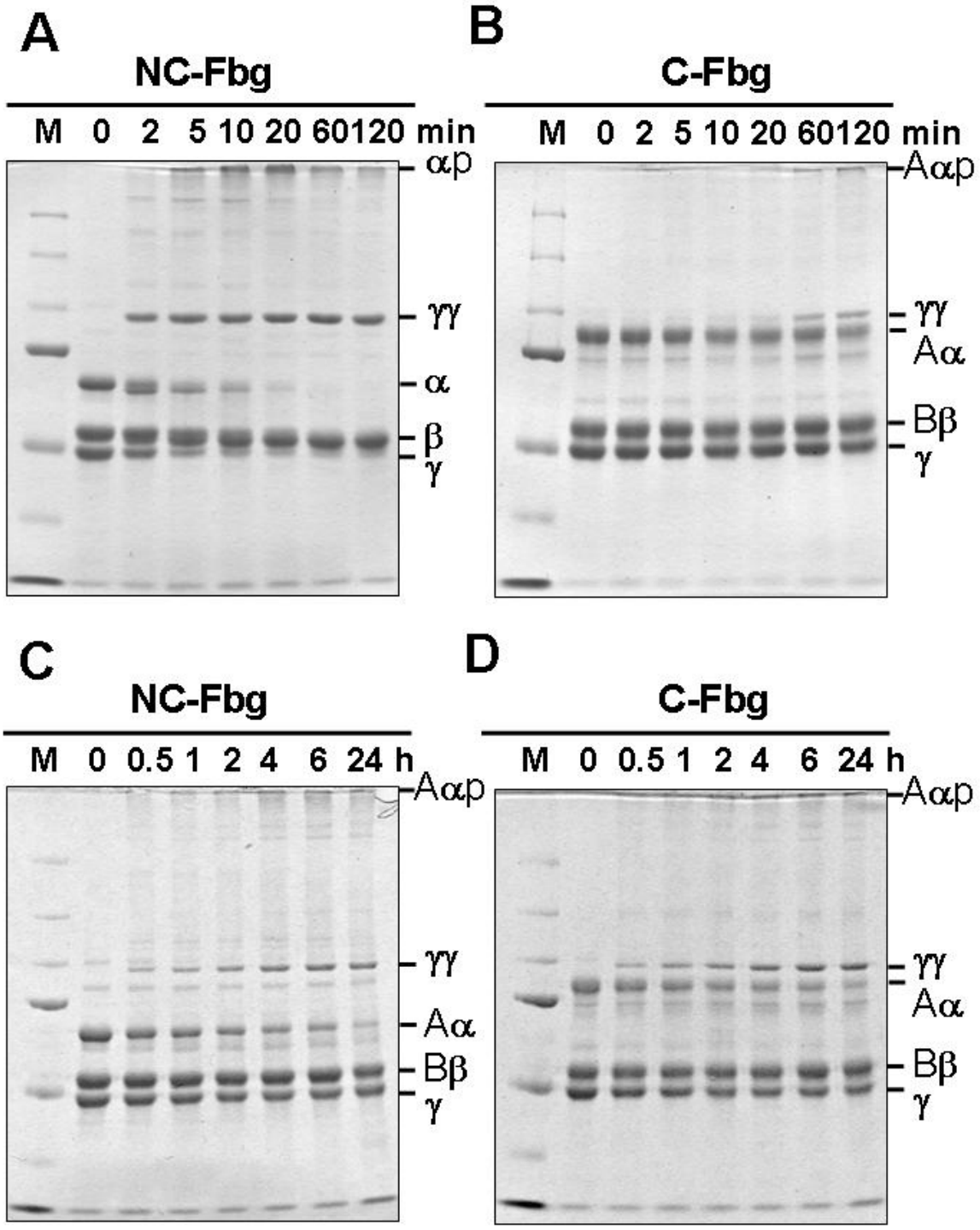


Figure 3. Protection against plasmin digestion of fibrinogen. Control non-citrullinated fibrinogen (NC-Fbg) and citrullinated fibrinogen (C-Fbg) were digested in HEPES buffer containing 5 mM EDTA (lane EDTA), 5 mM $CaCl_2$ (lane Ca), or 2 mM Gly-Pro-Arg-Pro peptide supplemented with 1 mM EDTA (lane GPRP). The plasmin digests were analyzed on 8 % SDS-PAGE under reducing conditions, and the gels were stained with Coomassie brilliant blue. Lane M: molecular markers (see Fig 1 legend).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Bβ
γ

Figure 4. FXIIIa-catalyzed crosslinking of citrullinated fibrinogen. FXIIIa-catalyzed crosslinking of control non-citrullinated fibrinogen (NC-Fbg) **(A)** and citrullinated fibrinogen (C-Fbg) **(B)** was performed in the following conditions: 0.3 mg/ml of fibrinogen (final concentration) was incubated at 37°C with FXIIIa (3.3 units/ml) and α-thrombin (0.07 units/ml) in HBS and 0.67 mM calcium. FXIIIa-catalyzed crosslinking of control non-citrullinated fibrinogen (NC-Fbg) **(C)** and citrullinated fibrinogen (C-Fbg) **(D)** in the presence of the thrombin inhibitor hirudin was performed with 8.3 units/ml of FXIIIa. Samples equivalent to 4.5 μg fibrin were separated on 8 % SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue. The reduced chains (Aα, α, Bβ, β, γ, cross-linked γ–γ dimer: γ–γ, cross-linked α-chain polymer: αp, and cross-linked Aα-chain polymer: Aαp) are indicated on the right side of the gels. Lane M: molecular markers (see Fig 1 legend).

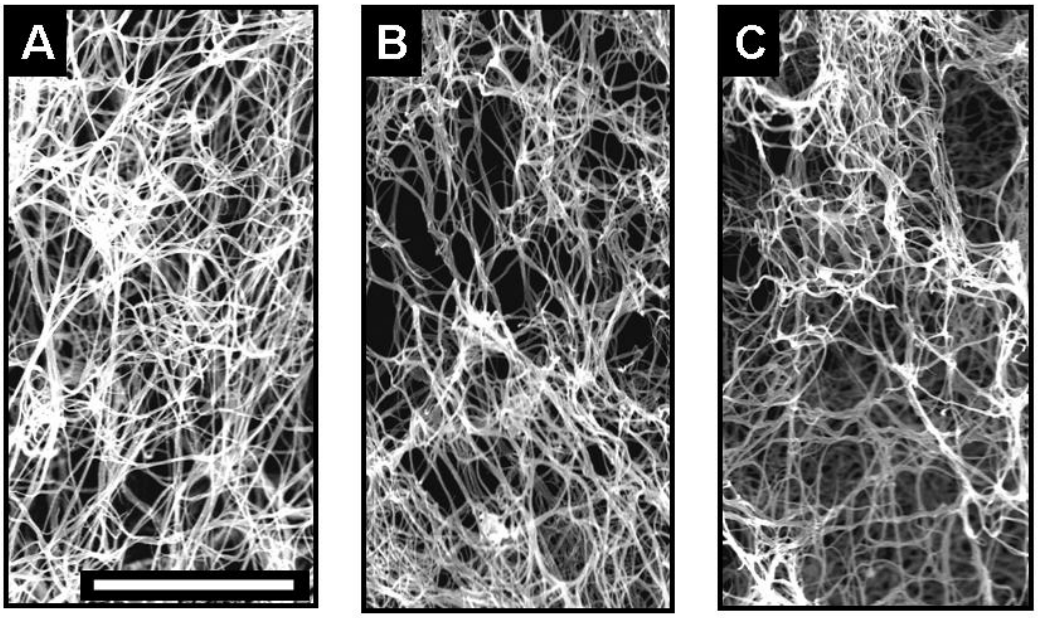

Figure 5. Scanning electron microscopic images of a mixture of citrullinated and non-citrullinated fibrinogen. The SEM preparation was prepared from 0.16 mg/mL **(A)** or 0.08 mg/mL **(B)** control non-citrullinated fibrinogen (NC-Fbg) and a mixture of NC-Fbg (0.08 mg/mL) and C-Fbg (0.08 mg/mL) (NC-Fbg + C-Fbg) **(C)** and was viewed on a JSM-6000F. The images were taken at 3000x with a 15.0 kV accelerating voltage. The white bar represents 10 μm.