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Evidence that α2-Antiplasmin Becomes Covalently Ligated to Plasma Fibrinogen in the Circulation: a New Role for Plasma Factor XIII in Fibrinolysis Regulation

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Abstract

Summary. *Background*: Plasma alpha$_2$-antiplasmin (α_2AP) is a rapid and effective inhibitor of the fibrinolytic enzyme plasmin. Congenital α_2AP deficiency results in a severe hemorrhagic disorder due to accelerated fibrinolysis. It is well established that in the presence of thrombin-activated factor XIII (FXIIIa), α_2AP becomes covalently ligated to the distal α chains of fibrin or fibrinogen at lysine 303 (two potential sites per molecule). Some time ago we showed that α_2AP is covalently linked to plasma fibrinogen . That singular observation led to our hypothesis that native plasma factor XIII (FXIII), which is known to catalyze covalent cross-linking of fibrinogen in the presence of calcium ions, can also incorporate α_2AP into fibrinogen in the circulation. *Results and Conclusions*: We now provide evidence that FXIII incorporates I^{125}-labelled α_2AP into the Aα-chain sites on fibrinogen or fibrin. We also measured the content of α_2AP in isolated plasma fibrinogen fractions by ELISA and found that substantial amounts were present (1.2–1.8 moles per mole fibrinogen). We propose that α_2AP becomes ligated to fibrinogen while in the circulation through the action of FXIII, and that its immediate presence in plasma fibrinogen contributes to regulation of *in vivo* fibrinolysis.

Introduction

Plasma alpha$_2$-antiplasmin (α_2AP) is a rapid and effective serine protease inhibitor (serpin) of the fibrinolytic enzyme plasmin[1-4]. When this inhibitor is absent from plasma, as occurs in congenital homozygous α_2AP-deficiency, a severe hemorrhagic disorder results that is characterized by increased susceptibility of intravascular thrombi to fibrinolysis[5-7]. The fibrinolytic defect is reversible by the addition of α_2AP. It is well known that in the presence of thrombin-activated factor XIIIa (FXIIIa) the inhibitor becomes covalently ligated ('cross-linked') to a single site, lysine 303, on the fibrin- or fibrinogen Aα-chain[8, 9]. Unbound α_2AP inhibits tPA-induced fibrinogenolysis; however, only after it has been incorporated into fibrin via FXIIIa, is α_2AP an effective inhibitor of fibrinolysis[10-13]. In addition, plasmin that becomes bound to fibrin is protected from inhibition by unbound α_2AP[14], thus emphasizing the importance of prior α_2AP incorporation into fibrin(ogen) for effectively mediating inhibition of fibrinolysis. Finally, α_2AP is incorporated into fibrin at only 20% to 30% of the potentially available lysine 303 sites[15-17], suggesting that the unreactive sites may already be occupied or otherwise unavailable for ligation.

There are two forms of α_2AP in human plasma, a full length 464-residue protein with amino-terminal methionine (Met-α_2AP) that accounts for ~30% of the total, the remainder being composed of a shortened 452-residue form with amino-terminal asparagine (Asn-α_2AP)[18, 19]. The proportions of the two forms of α_2AP in plasma are related to the R/W6 single nucleotide polymorphism, with R being associated with higher Asn-α_2AP levels[20]. Incorporation of Asn-α_2AP into fibrin is 3–13 times greater than with the full length precursor form, Met-α_2AP[21, 22], but otherwise both forms display the same inhibitory activities.

A plasmin inhibitory activity like that of plasma α_2AP was discovered in fibrinogen many years ago[23], although it was not identified as α_2AP until much later when immunochemical analyses showed that α_2AP was a constituent of normal fibrinogen as well as a dysfibrinogenemic fibrinogen (fibrinogen Cedar Rapids)[24]. These observations combined with prior knowledge that native non-thrombin-activated plasma factor XIII (FXIII) can efficiently introduce covalent cross-links into fibrinogen as well as fibrin in the presence of calcium ions[25], and the retrospective insight that α_2AP is incompletely incorporated into fibrin by FXIIIa[15-17], suggested to us that the α_2AP found in plasma fibrinogen might have been incorporated in the circulation through the action of FXIII. In this present study we show that substantial amounts of α_2AP are present in circulating fibrinogen, and we present evidence that this process is mediated by plasma FXIII. We also introduce the concept that ligation of α_2AP to plasma fibrinogen prior to initiation of clotting serves an important role in down-regulating the rate of fibrinolysis.

Materials and methods

Chemicals and reagents were of the highest purity available. Trasylol (aprotinin) was obtained from Miles Inc. (Kankakee, IL, USA), and DE-52 cellulose was from Whatmann Inc. (Clifton, NJ, USA). Human α–thrombin (3188 μ mg^{-1}) was obtained from Enzyme Research Laboratories (South Bend, IN, USA), and fibrinogen was isolated from human citrated plasma pools (obtained through the BloodCenter of Wisconsin) by glycine precipitation followed by sub-fractionation to 'fraction I-2' as described[26]. Most Aα-chains in fraction I-2 fibrinogen are full length and therefore they contain the α_2AP ligation site at Aα303. This plasma fraction was further separated into fibrinogen 1 (γ_A,γ_A) and fibrinogen 2 (γ_A,γ′) by ion exchange chromatography[27]. Des-αC fibrinogen (old terminology, 'fraction I-9D') was produced by limited plasmin digestion of fibrinogen[28], lacked ∼390 residues of the C-terminal region of the Aα chain[29, 30], and therefore lacked the sequence containing Aα303. Fibrinogen fraction I-2 from the fibrinogen Cedar Rapids proposita, which previously had been demonstrated to contain α_2AP[24], was also analyzed. FXIII was prepared from pooled plasma[31] and assayed as FXIIIa on a FXIII-free fibrin substrate (fibrin 1) in the presence of 10 mm $CaCl_2$ as described[25, 32]. FXIII concentrations were determined spectrophotometrically at 280 nm, using an absorbance coefficient ($\mathrm{A}^{1\%}_{1\mathrm{cm}}$, 280 nm) of 13.8[33]. The specific activity was 2100 to 2300 Loewy u mg^{-1}. Normal plasma levels of factor XIII are 80–110 Loewy u mL^{-1}. Recombinant Asn-α_2AP comprised of 464 AA, was prepared as described[34], and had a mass of 50 583 Da. A portion of this material was labeled with ^{125}I by a lactoperoxidase method[35], and stored at a stock concentration of 0.87 mg mL^{-1} (17 μm).

Incorporation of α_2AP into fibrinogen or fibrin by FXIII

For investigating FXIII-mediated incorporation of α_2AP, FXIII-free fibrinogen 1 (8.8 μm, final) in 50 mm Tris, 100 mm NaCl, 5.0 mm $CaCl_2$, 0.4 mm DTT, pH 7.4, was mixed with ^{125}I-labeled α_2AP (1.0 to 6.0 μm), and ligation initiated at room temperature by adding 100 Loewy u mL^{-1} FXIII (final). After 6 h incubation, the reaction was terminated by adding an equal volume of 2-fold concentrated Laemmli buffer containing 1%β-mercaptoethanol, and the products of the reaction were analyzed by SDS-PAGE on 9% polyacrylamide gels employing a discontinuous buffer system[36]. Dried Coomassie Brilliant Blue-stained gels were subjected to autoradiography using Kodak X-Omat film. Autoradiograms were digitized on a CanoScan 9950F flat bed scanner (Canon USA Inc, Lake Success, NY, USA), and α_2AP-containing bands quantified using Image J (http://rsb.info.nih.gov/ij/). Results from stained gels were normalized against the Bβ region of the gel whereas results from autoradiograms were normalized against known quantities of ^{125}I-labeled α_2AP run on identical gels. Control samples included ^{125}I-labeled α_2AP, fibrinogen 1, and fibrinogen 1 plus FXIII without ^{125}I-labeled α_2AP. To determine the amount of ^{125}I-labeled α_2AP that could be incorporated into cross-linked fibrin by FXIIIa, identical samples were prepared and the reaction initiated by adding 100 Loewy u mL^{-1} FXIII and 0.5 u mL^{-1}

thrombin. For determining incorporation rates, FXIII-free fibrinogen 1 (8.8 μm final) in 50 mm Tris, 100 mm NaCl, 5.0 mm $CaCl_2$, 0.4 mm DTT, pH 7.4, was mixed with ^{125}I-labeled α_2AP (1.0 or 5.0 μm), and the reaction initiated by adding 100 Loewy u mL^{-1} FXIII or FXIIIa (containing 0.5 u mL^{-1} thrombin). At selected intervals of up to 18 h the incubation was terminated by adding an equal volume of 2X Laemmli buffer containing 1%β-mercaptoethanol, and the products of the reaction were analyzed as described above.

Immunoassay of fibrinogen and α_2AP

Fibrinogen concentrations were determined by ELISA[37] using biotinylated rabbit anti-human fibrinogen IgG (Dako, Carpinteria, CA, USA) for tagging the fibrinogen that had been bound to wells. Antigen loading, equilibration and processing conditions were the same as described below for α_2AP detection. Fibrinogen fraction I-2 (>97% coagulable) was used for constructing a standard curve at test concentrations ranging from 20 to 0.0125 μg mL^{-1}. In order to measure α_2AP in fibrinogen-containing samples, we applied fibrinogen at concentrations that were five to eight times higher (2.5–20 μg mL^{-1}, 0.74–5.9 nm) than those usually used for obtaining a linear plot[37]. Thus, the amount of fibrinogen bound to the plate was determined from a non-linear portion of the fibrinogen calibration curve.

To measure α_2AP in fibrinogen-containing samples by ELISA, we first constructed an α_2AP calibration curve as follows: Asn-α_2AP (0.125–1.5 μg mL^{-1}) was added to immunoplates (MG Scientific, Pleasant Prairie, WI, USA), incubated overnight at 4°C, washed with PBS, blocked with 2% non-fat dried milk in PBS for 1 h at room temperature, and rinsed with 200 μL PBS-Tween 20 (0.05%). Washed plates were then treated with goat anti-human α_2AP (Nordic Immunology, Tilberg, The Netherlands) at 1:5000 dilution in PBS-Tween 20, incubated for 1 h at 37°C, and washed four times with PBS-Tween (0.05%). Horseradish peroxidase-labeled rabbit anti-goat IgG (Zymed, South San Francisco, CA, USA) at 1:15 000 dilution was added, incubated for 1 h at 37°C, and washed with PBS-Tween. After the final rinse, 100 μL o-phenylenediamine (OPD) solution (Zymed, South San Francisco, CA, USA) in 0.22 m citric acid, 0.05 m sodium phosphate, pH 5.0 buffer, was added and incubated in subdued light at room temperature for 10–15 min. The reaction was terminated with 50 μL 2 N H_2SO_4 and the plate read at 490 nm on a Versamax Plate Reader. For determining the α_2AP content in fibrinogen-containing specimens, replicate wells were processed exactly as described above for α_2AP calibration. The amount of α_2AP in fibrinogen was expressed as a molar ratio.

Results

Incorporation of α_2AP radioactivity into fibrinogen or fibrin

Earlier studies had demonstrated that native FXIII (FXIII) was able to cross-link fibrinogen in the presence of calcium ions[25], and these findings prompted us to investigate whether FXIII might also incorporate α_2AP into fibrinogen without requiring thrombin activation. Incubating fibrinogen at a physiological concentration (8.8 μm) with ^{125}I-labeled α_2AP (1–6 μm) plus physiological concentrations of FXIII (100 Loewy units mL^{-1}), as assessed from autoradiograms of SDS-PAGE gels, showed new radio-labeled bands (Fig. 1, panel A). These corresponded to an α_2AP/Aα-chain heterodimer (‘α_2AP/Aα chain’), an α_2AP/Aα-Aα chain heterotrimer (‘α_2AP/Aα dimer’) and an α_2AP/Aα-chain heteropolymer (‘α_2AP/Aα polymer’). In the Coomassie stained gel, we found new bands corresponding to the radiolabeled α_2AP/Aα heterodimer and α_2AP/Aα-chain heteropolymer positions, plus non-radiolabeled γ-dimers (panel B). The α_2AP/Aα-Aα chain heterotrimer position was faintly stained but readily detected in the autoradiogram.

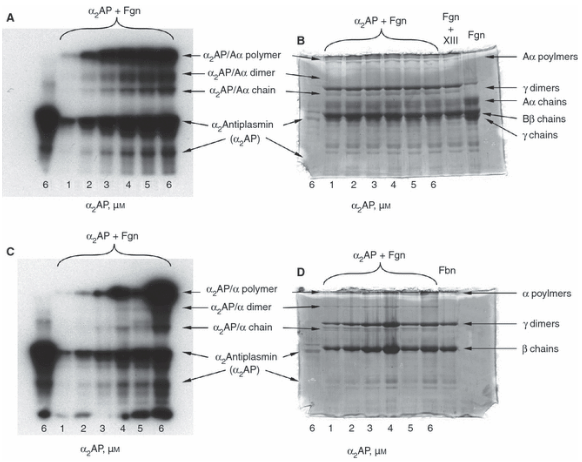

Figure 1 Incorporation of I^{125}-labeled α_2AP into fibrinogen in the presence of FXIII (panels A and B) or, after adding thrombin to form FXIIIa and fibrin (panels C and D). Autoradiograms are shown in panels A and C and corresponding Coomassie blue stained gels in B and D).

Adding thrombin to the reaction mixtures to convert fibrinogen to fibrin and to activate FXIII to FXIIIa (Fig. 1, panels C and D) resulted in similarly higher molecular weight stained bands, but the vast majority of radioactive α_2AP had been incorporated into the α-polymer region rather than the α_2AP/α heterodimer or heterotrimer positions (panel C). Figure 2 shows densitometric scans of the Fig. 1 panels A and C gels. It is evident that the radioactivity incorporated into fibrinogen or fibrin increased proportionately with the concentration of α_2AP, and that incorporation into fibrin was slightly higher than for fibrinogen at any given α_2AP concentration. At the highest α_2AP concentration that we studied, only about 40% of the potential α_2AP sites had been labeled.

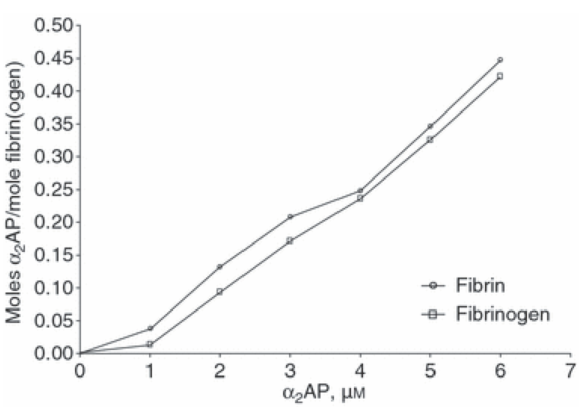

Figure 2 Molar ratio of α_2AP incorporation into fibrinogen (FXIII) or fibrin (FXIIIa) as a function of the concentration of α_2AP (μm). Data points are derived from densitometric scans of the gels in Fig. 1, panels A and C.

We also studied time-dependent incorporation of radioactivity into fibrinogen at α_2AP concentrations of 1 and 5 μm (Fig. 3) as well as into fibrin. At the lower α_2AP concentration, radioactively-labeled α_2AP/Aα chain polymers were detected in fibrinogen within 5 min (Fig. 3, panels A and B), and to a similar extent in fibrin (gels not shown). Inspection and densitometric scans of these gels revealed that incorporation into either fibrinogen or fibrin began to plateau at about 30 min. At 5 μmα_2AP, incorporation of radioactivity began to plateau at 15 min (Fig. 3, panels C and D) for fibrinogen as well as fibrin (gels not shown). Densitometric scans of the fibrinogen or fibrin SDS-PAGE gels (Fig. 4) indicated that maximal incorporation of radioactivity was about 0.25 moles per mole for fibrinogen, and slightly greater for fibrin (~0.30 moles per mole).

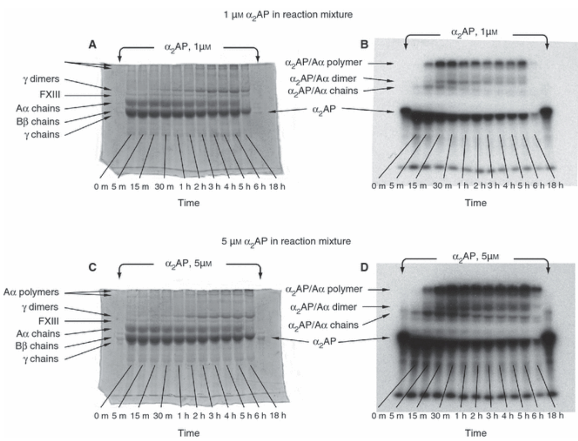

Figure 3 Incorporation of α_2AP into fibrinogen at 1 and 5 μmα_2AP, as a function of time in minutes (m) or hours (h). Stained gels, left. Autoradiograms, right.

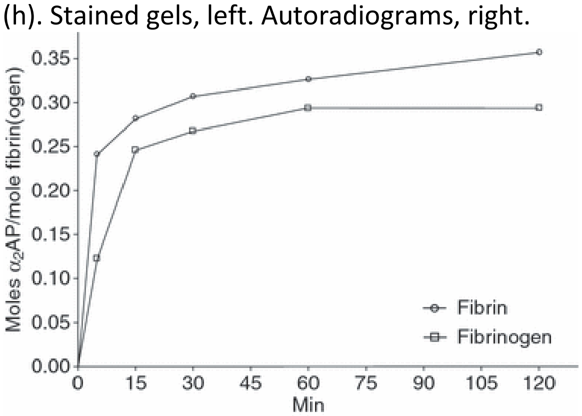

Figure 4 Molar ratio of α_2AP incorporation into fibrinogen by FXIII or into fibrin by FXIIIa as a function of time (min). The reaction is presented at an α_2AP concentration of 5 μm. The data points for fibrinogen are based upon the experiment shown in Fig. 3, whereas the gel for fibrin is not shown.

Fibrinogen α_2AP content

The α_2AP content in plasma fibrinogen fraction I-2 from four separate pools of normal citrated plasma and that from the proposita of the Cedar Rapids dysfibrinogenemia (γ275R to C)[24] was assessed by immunoassay and is represented as the molar ratio of α_2AP to fibrinogen (Table 1). Given the fact that there is a single site at Aα303 on each Aα chain to which α_2AP can become ligated[8, 15-17, 38], the maximum ratio of α_2AP to fibrinogen is two. With the sole exception of des-αC fibrinogen, the negative control, which lacks C-terminal portions of Aα chains containing the α_2AP binding site, each of the fibrinogen fractions tested contained α_2AP. In every case, the α_2AP:fibrinogen ratio was between one and two, indicating that considerable amounts of α_2AP had been incorporated into plasma fibrinogen, a result that is consistent with earlier observations of the presence of α_2AP in plasma fibrinogen[23, 24]. As discussed in the next section, the α_2AP content of Cedar Rapids fibrinogen was the same or possibly even lower than that in normal fibrinogen, and thus did not account for delayed fibrinolysis that has been observed in this fibrinogen[24].

Table 1. α_2AP/fibrinogen molar ratio

Fibrinogen fraction	***n***	**Ratio**	**Range**
Normal fraction I-2	4	1.5 ($\bar{X}$)	1.25–1.8
Cedar Rapids fraction I-2	1	1.2	–
Des-αC fibrinogen	1	0	0

Discussion

A plasmin inhibitory activity corresponding functionally to α_2AP was discovered in plasma fibrinogen many years ago[23], although it was not specifically identified as α_2AP until years later, when it was shown by immunochemical analysis to be a constituent of normal fibrinogen as well as a dysfibrinogenemic fibrinogen, fibrinogen Cedar Rapids[24]. This discovery suggested that α_2AP might have been incorporated covalently into fibrinogen through the action of a calcium ion-dependent transglutaminase like FXIII, which circulates with fibrinogen in plasma. This postulation, combined with prior knowledge that 'native' FXIII is an active enzyme that efficiently introduces covalent cross-links into fibrinogen or fibrin molecules[25], prompted us to investigate this possibility in detail. Our present experiments confirm that α_2AP is a covalently bound constituent of plasma fibrinogen, that it is present in substantial amounts, and that plasma FXIII can mediate its incorporation into fibrinogen. These findings support the hypothesis that α_2AP becomes ligated to plasma fibrinogen in the circulation, and is very likely to have been incorporated through the action of circulating FXIII.

We were particularly interested in quantifying the α_2AP content in the Cedar Rapids dysfibrinogen[24], not only because members of this kindred had experienced severe thrombophilia associated with delayed fibrinolysis, but also because Cedar Rapids fibrinogen had been the index case for measuring the presence of α_2AP in plasma fibrinogen; we were still seeking to explain that phenomenon in terms of the α_2AP content. Our present results confirm those in the first study, and indicate that the α_2AP content of Cedar Rapids fibrinogen is the same or possibly even lower than that in normal fibrinogen, and thus cannot alone account for delayed fibrinolysis[24]. Another explanation will be required.

Although it is well established that in the presence of thrombin-activated FXIIIa, α_2AP becomes ligated to the fibrin or fibrinogen Aα-chain[8, 9, 15-17, 38], it was not known prior to this study that native FXIII, in addition to introducing covalent cross-links into fibrinogen or fibrin in the presence of calcium ions[25], can also incorporate α_2AP into fibrinogen. The rate of α_2AP incorporation into fibrinogen by FXIII was nearly as rapid as the rate of incorporation into fibrin by FXIIIa. These findings provide an attractive mechanistic explanation for why circulating fibrinogen contains α_2AP. Furthermore, as the content of α_2AP in fibrinogen is so relatively high (i.e. 1.2–1.8 moles per mole fibrinogen (Table 1) it readily explains why we (this study) as well as previous investigators found that the number of available sites on Aα-chains for α_2AP incorporation were only 20–30% of those potentially available[15-17]. In contrast to the relatively high content of α_2AP that we found in plasma fibrinogen few, if any, intermolecular fibrin(ogen) γ chain cross-links are found in normal plasma, although substantial amounts of these products occur under pathological circumstances, including subjects with familial Mediterranean fever[39] and disseminated intravascular coagulation syndromes[40]. Thus, the effective down-regulation of FXIII-mediated fibrin(ogen) cross-linking in blood contrasts with the relatively high content of α_2AP in plasma fibrinogen, implying that this interaction is less effectively regulated.

Our present estimations for the α_2AP content in fibrinogen are between 1.2 and 1.8 moles per mole fibrinogen. Since the plasma fibrinogen level is ~9 µm, the plasma concentration of α_2AP that has been incorporated in the fibrinogen compartment lies between 11 and 16 µm. The reported level of plasma α_2AP is, however, only ~1 µm (17 *inter alia*), a value that vastly underestimates the total amount of α_2AP in plasma. The lower estimate is derived from radial immunodiffusion or rocket immunoelectrophoresis measurements that would be mainly if not solely, a measure of 'free'α_2AP, because these techniques are not likely to have taken into account the relatively large amount of more slowly diffusing fibrinogen-bound α_2AP. Thus, except for this present study and two earlier ones that focused on the inhibitor content of fibrinogen itself (23, 24), the existence of α_2AP covalently bound to fibrinogen in plasma has been overlooked.

Congenital homozygous α_2AP deficiency causes a severe hemorrhagic disorder characterized by increased susceptibility of intravascular thrombi to fibrinolysis[5-7], underscoring the importance of α_2AP for down-regulating fibrinolysis. Observations on this catastrophic hemorrhagic condition have not, however, addressed an obverse possibility, namely that incorporation of α_2AP into plasma fibrinogen prior to the initiation of clotting and subsequent fibrinolysis, serves an important role in its own right in down-regulating the rate of fibrinolysis. This notion is supported by the fact that α_2AP is an effective inhibitor of fibrinolysis only after it has been incorporated into fibrin[10-13]. The extent to which the α_2AP incorporation into fibrinogen varies from individual to individual remains to be investigated, but it is tempting to speculate that the degree of α_2AP incorporation plays an important role in the fibrinolytic response *in vivo*. In summary, we propose that incorporation of α_2AP into circulating fibrinogen prior to initiation of blood clotting plays an important role in down-regulating fibrinolysis, thus suggesting a new role for plasma FXIII in regulation of fibrinolysis.

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