Evidence for Intramolecular Cross-Linked Aα·γ Chain Heterodimers in Plasma Fibrinogen[†]

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ABSTRACT: A peptide band of ∼105 kDa migrating near the γ dimer position of disulfide bond reduced human plasma fibrinogen prepared from fresh single donor or outdated plasma was identified by SDS−PAGE. The band, amounting to ∼2% of the total Aα/γ chain population, was thrombin and plasmin sensitive and reacted with antibodies to Aα or γ chains but not with antibodies to Bβ chains, plasminogen, or factor XIII. Amino acid sequencing revealed a double sequence corresponding to that of Aα and γ chains, indicating that the band consists of covalently cross-linked Aα·γ chain heterodimers. Aα·γ heterodimers were identified as a component of monomeric fibrinogen by two-dimensional SDS−PAGE and by SDS−PAGE analysis of the monomer fraction isolated by gel sieving chromatography, thus indicating that Aα·γ heterodimers arise by *intramolecular* Aα/γ chain cross-linking.

In the presence of factor XIIIa, fibrin assembly is accompanied by intermolecular covalent cross-linking, in which ϵ-amino (γ-glutamyl) lysine isopeptide bonds are introduced between appropriately positioned donor lysine and acceptor glutamine residues (Pisano et al., 1968; Matačiĉ & Loewy, 1968). Cross-linking occurs initially between γ chains to form γ dimers. Concomitantly, albeit at a slower rate, cross-linking occurs among α chains (McKee et al., 1970; Folk & Finlayson, 1977) and to a minor extent between α and γ chains (Mosesson et al., 1989; Amrani & Mosesson, 1989; Shainoff et al., 1991; Grøn et al., 1993). Continued factor XIIIa activity among the existing γ dimer population results in slow progressive evolution of γ chain multimers (Mosesson et al., 1989; Shainoff et al., 1991; Siebenlist & Mosesson, 1992). XIIIa can also cross-link fibrinogen (Kanaide & Shainoff, 1975; Mosesson et al., 1996), which forms a translucent, nonsynergizing gel, concomitant with γ dimer formation. γ chain multimers and cross-linked α−γ hybrid chains have also been detected in XIIIa cross-linked fibrinogen polymers (Murthy & Lorand, 1990; Shainoff et al., 1991).

Fibrinogen and fibrin can also be cross-linked by tissue transglutaminase, which follows a different course than that of XIIIa (Chung, 1972; Purves et al., 1987; Murthy & Lorand, 1990; Shainoff et al., 1991). α chains become cross-linked more rapidly than γ chains, and both α and γ chains become incorporated into high molecular weight polymeric structures. There is no direct evidence that γ dimers, per se, are formed (Purves et al., 1987; Shainoff et al., 1991), since γ chains become preferentially incorporated into α-γ hybrids early in the cross-linking reaction (Murthy & Lorand, 1990; Shainoff et al., 1991).

Recent studies have shown that erythrocyte transglutaminase can cross-link fibrinogen (Murthy et al., 1991), although this type of cross-linking reaction is not known to occur *in vivo* in circulating fibrinogen molecules. In this report, we demonstrate the existence of a subpopulation of native fibrinogen molecules containing intramolecular cross-links between Aα and γ chains, probably mediated *in vivo* by a tissue transglutaminase.

METHODS

All chemicals and reagents were the highest grade available from commercial sources. Human fibrinogen (greater than 98% coagulable) was isolated from pooled citrated plasma by glycine precipitation (Kazal et al., 1963) and subfractionated to fraction I-2 by an established procedure (Mosesson & Sherry, 1966). Fibrinogen concentrations were determined spectrophotometrically at 280 nm using an absorbance coefficient ($A_{cm}^{1\%}$) of 15.1 (Mosesson & Finlayson, 1963). Peak 1 (γA, γA) and peak 2 (γA, γ') fibrinogen were prepared from fraction I-2 by chromatography on DE-52 (Whatman Inc., Clifton, NJ) (Finlayson & Mosesson, 1963; Mosesson et al., 1972). Single donor plasma was obtained by contrifugation of blood collected into acid−citrate−dextrose (9:1) supplemented with a variety of protease/transglutaminase inhibitors [PMSF (0.5 mM, final); aprotinin (15 KIU/mL, final) (Trasylol, Mobay Chemical Corp., New York); PPACK (2.5 μM, final) (Calbiochem, San Diego, CA); N-ethylmaleimide (10 mM, final); EDTA (5 mM, final)]. These inhibitors were added to all solutions employed during fibrinogen isolation. Fibrinogen was labeled with ^{125}I using the iodine monochloride method (McFarlane, 1963), resulting in a labeling ratio of 0.6 atom of ^{125}I per fibrinogen molecule.

Factor XIII was purified from pooled human plasma (Lorand & Gotoh, 1970); its specific activity ranged from 2000 to 2500 Loewy units/mg (Loewy et al., 1961). Factor XIII was activated to factor XIIIa by addition of human α-thrombin (10 units/mL) (Enzyme Research Inc., South Bend, IN) (Kanaide & Shainoff, 1975). Plasminogen was

isolated from human plasma by affinity chromatography on lysine−Sepharose (Robbins & Summaria, 1976), activated to plasmin by incubation with streptokinase (1:800 molar ratio) for 20 h at room temperature (Robbins & Summaria, 1970), and assayed colorometrically using Kabi S-2251 (Chromogenix, Mölndal, Sweden).

For analysis of thrombin or plasmin susceptibility, fibrinogen (1 mg/mL) in 50 mM Tris and 100 mM NaCl, pH 7.4, was clotted by the addition of thrombin (0.5 unit/mL) and incubated at room temperature for 1 h or digested with plasmin (0.1 unit/mL) for up to 360 min at room temperature. Digestions were terminated by adding Laemmli sample buffer containing reducing agents.

SDS−PAGE was performed as described by Weber and Osborn (1969) for tube gels (3 mm diameter) or on 1.5 mm slab gels using the method of Laemmli (1970). Five percent polyacrylamide gels were used for reduced samples and 7.5% or 9% gels for disulfide bond reduced samples. Fibrinogen fractions from a gel sieving column were analyzed on 8−25% gels using the Phast Gel System (Pharmacia, Piscataway, NJ) and stained with 0.5% Coomassie Brilliant Blue R250 (Sigma Chemical Co., St. Louis, MO). Immunoblotting was carried out on samples that had been separated by SDS−PAGE (Laemmli, 1970), electroblotted onto nitrocellulose paper, and probed with polyclonal rabbit antibodies raised against the Aα, Bβ, and γ chain of fibrinogen, factor XIII, or plasminogen (Dako Corp., Carpinteria, CA) followed by ^{125}I-labeled goat anti-rabbit IgG.

Size exclusion chromatography on 50−100 mg fibrinogen samples was performed on a tandem pair of 1.5 × 90 cm columns that had been equilibrated in either 50 mM Tris, 100 mM NaCl, 0.5 mM PMSF, and 5 KIU/mL aprotinin, pH 7.4, buffer or the same buffer containing 500 mM NaCl and developed at a flow rate of 15 mL/h.

For amino acid sequencing, reduced fibrinogen chains were separated on 7.5% polyacrylamide gels (Laemmli, 1970), the regions of interest were excised from the gel, and the protein was recovered by electroelution. The pooled and concentrated samples were reelectrophoresed, electroblotted onto a poly(vinylidene difluoride) membrane (Matsudaira, 1987), excised, and then subjected to automated sequencing on a model 477A Applied Biosystems pulsed liquid-phase sequencer with a model 120A on-line phenylthiohydantoin amino acid analyzer.

RESULTS

Identification of a 105 kDa Peptide in Fibrinogen. During studies originally designed to explore the cross-linking reaction between fibrin(ogen) and factor XIIIa, a radioactive band was noted on autoradiographs of reduced un-cross-linked ^{125}I-labeled fibrinogen (Figure 1, lane A). This band migrated close to the γ dimer position corresponding to a molecular mass of ~105 kDa. This unanticipated band could also be detected on Coomassie Blue stained SDS−PAGE gels of reduced fibrinogen (Figure 1, lane B). However, unlike γ dimers, the 105 kDa band was thrombin sensitive, as assessed by the faster migration rate in fibrin samples compared with fibrinogen (Figure 2). The band was also plasmin sensitive, disappearing from its SDS−PAGE position in the same time frame as monomeric fibrinogen chains (Figure 3). On the basis of densitometric analysis of Coomassie blue stained gels, this band represented ap-
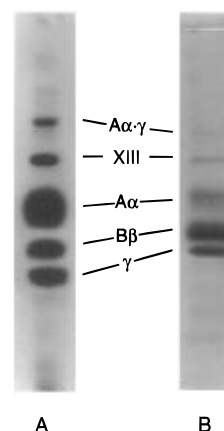

FIGURE 1: SDS−PAGE of fibrinogen demonstrating the presence of the 105 kDa band in fibrinogen. Lane A is an autoradiograph of reduced un-cross-linked ^{125}I-labeled fibrinogen separated on 9% polyacrylamide gels. Lane B contains the same amount of fibrinogen separated under similar conditions and stained with Coomassie Blue R250. The differences in mobility are due to slight differences in the time of electrophoresis. The positions of the relevant fibrinogen chains and factor XIII are indicated. The location of the 105 kDa band is designated by Aα·γ.
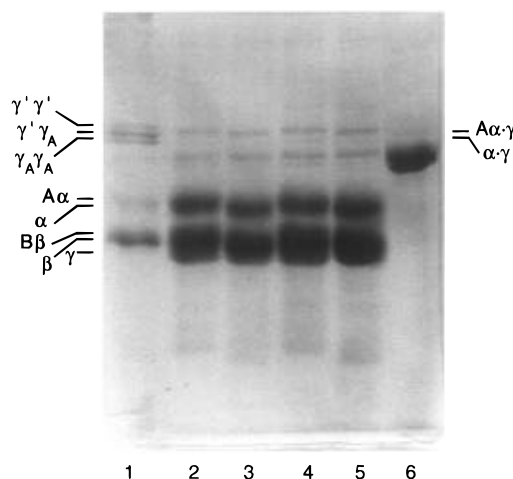

FIGURE 2: Thrombin sensitivity of the 105 kDa band. Aliquots of fibrinogen before and after thrombin treatment (0.5 unit/mL for 1 h at room temperature) were separated on 7.5% polyacrylamide gels and stained with Coomassie Blue R250. Lanes 1, cross-linked peak 2 fibrin; 2 and 4, fibrinogen; 3 and 5, fibrin; 6, factor XIII. The positions of the relevant fibrinogen chains and factor XIII are indicated. The location of the 105 kDa band in fibrinogen is designated by Aα·γ and as α·γ in fibrin.

proximately 2% of the total Aα and γ chain monomer population.

The peptide chain composition of the 105 kDa band in fibrinogen (peak 2) was explored by Western blotting of reduced fibrinogen samples. The band reacted against antibodies to Aα or γ chains (Figure 4) but did not react with antibodies against Bβ chains, factor XIII, or plasminogen. The Aα·γ identity of the 105 kDa band was confirmed by amino acid sequence analysis of material electroeluted from SDS−PAGE gels. Multiple amino acids were found at each step (Table 1). The most prominent amino acids corresponded to the γ chain sequence, whereas the others indicated the presence of Aα or α chain sequences (Table 1). About 60% of the non-γ chain amino acids were derived from the intact Aα chain (Ala$_1$-Asp$_2$...), and approximately 15% came from a minor Aα chain population lacking the N-terminal Ala residue (Asp$_2$-Ser$_3$...). The

Table 1: Amino Acid Sequence Analysis of the 105 kDa Band[a]

chain	step													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Predicted Sequence													
Aα[b]	Ala	Asp	Ser	Gly	Glu	Gly	Asp	Phe	Leu	Ala	Glu	Gly	Gly	Gly
α	Gly	Pro	Arg	Val	Val	Glu	Arg	His	Gln	Ser	Ala	Cys	Lys	Asp
γ	Tyr	Val	Ala	Thr	Arg	Asp	Asn	Cys	Cys	Ile	Leu	Asp	Glu	Arg
	Amino Acids Found/Yield (pmol)													
Aα[b]	Ala	Asp	Ser	Gly	Glu	Gly	Asp	Phe	Leu	Ala	Glu	Gly	Gly	Gly
	12	21	21	20	8	25	26	10	10	15	11	12	12	10
α	Gly	Pro	Arg	Val	Val	Glu	Arg	His	Gln	Ser	Ala	X	Lys	Asp
	12	7	3	9	5	8	4	8	5	8	8		6	9
γ	Tyr	Val	Ala	Thr	Arg	Asp	Asn	X	X	Ile	Leu	Asp	Glu	Arg
	31	34	27	29	16	28	29			20	19	21	17	17

[a] The 105 kDa band was separated from other fibrinogen bands by SDS−PAGE under reducing conditions. Appropriate regions of the gel were excised and protein was collected by electroelution. Concentrated samples were rerun on SDS−PAGE, transferred to poly(vinylidene difluoride) membrane, and sequenced on a model 477A Applied Biosystems pulsed liquid-phase sequencer. The amino acid recovery (picomoles) at each cycle is indicated below the residue. [b] A minor Aα chain variant (≈15%) beginning at position 2 (Asp) was present in the samples. The sequence data for this variant was included with the intact Aα chain by putting it in register beginning at position 2.
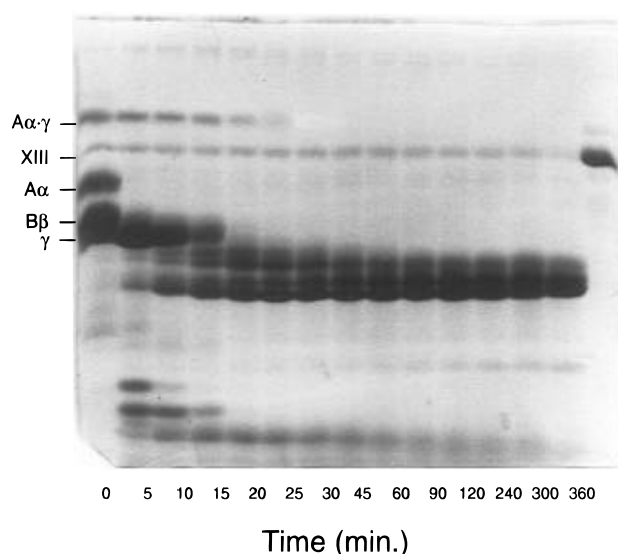

Time (min.)

FIGURE 3: Plasmin sensitivity of the 105 kDa band. Aliquots of fibrinogen were treated with plasmin (0.1 unit/mL) for up to 360 min at room temperature, electrophoresed on a 9% polyacrylamide gel under reducing conditions, and stained with Coomassie Blue R250. The positions of the relevant fibrinogen chains and factor XIII are indicated. The location of the 105 kDa band is designated by Aα·γ.
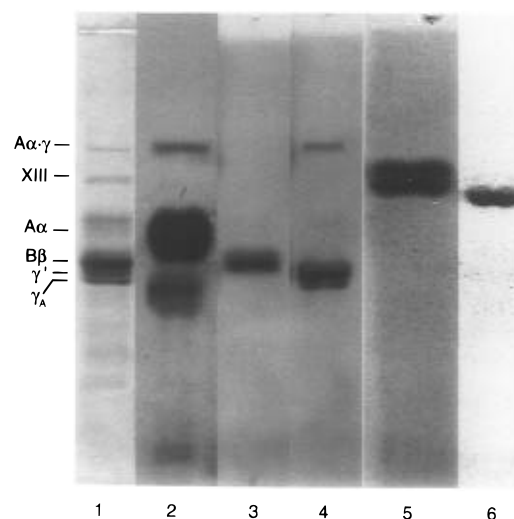

FIGURE 4: Western blots of fibrinogen. Aliquots of fibrinogen were separated on gels under reducing conditions, electroblotted onto nitrocellulose paper, and probed with rabbit antibodies raised against human fibrinogen Aα, Bβ, or γ chains, human factor XIII, or human plasminogen followed by ^{125}I-labeled goat anti-Aα; 3, anti-Bβ; 4, anti-γ; 5, anti-factor XIII; 6, anti-plasminogen. The positions of the relevant fibrinogen chains and factor XIII are indicated. The location of the 105 kDa band is designated by Aα·γ.

remaining amino acids corresponded to the fibrin α chain (Gly$_{17}$-Pro$_{18}$...). The total recovery of A(α) chain amino acids approximated the amount recovered from the γ chains. These findings further suggested that a small proportion of the Aα·γ chains had been converted to fibrin α·γ.

Intramolecular Nature of the Aα·γ Band. In nonreduced fibrinogen fraction I-2 preparations, monomeric fibrinogen and the factor XIII A and B subunits were detected, but a band migrating in the 105 kDa position was absent (Figure 5, horizontal tube gel). When a gel of unreduced fibrinogen was run in a second dimension under reducing conditions, the 105 kDa Aα·γ band appeared in the region corresponding to fibrinogen monomer, indicating that it was covalently incorporated into a monomeric form of fibrinogen (Figure 5, slab gel).

We attempted to separate the 105 kDa band from the fraction I-2 fibrinogen by size exclusion chromatography in 50 mM Tris and 0.1 or 0.5 M NaCl, pH 7.4, buffers on the premise that the fibrinogen preparations might contain small

amounts of multimeric cross-linked fibrinogen or fibrin molecules. Under either buffer condition, fibrinogen was separated into two fractions: a minor early eluting peak and a major peak of fibrinogen monomer (Figure 6). SDS−PAGE of peak fractions (Figure 6, inset) revealed that the early eluting peak contained multimeric cross-linked fibrinogen or fibrin formed through γ chain cross-linking. The fibrinogen monomer peak expressed the 105 kDa Aα·γ band.

The method of plasma collection from which the fibrinogen was isolated had little, if any, effect on the occurrence of the Aα·γ band. Plasma from fresh single donor blood that had been collected into acid−citrate−dextrose supplemented with PMSF, aprotinin, PPACK, *N*-ethylmaleimide, and EDTA was subfractionated from buffers containing these inhibitors in all solutions. SDS−PAGE analysis demonstrated the presence of the Aα·γ band (Figure 7, lane 1) in about the same amounts as had been found in fibrinogen isolated from outdated plasma (cf. Figure 1). When a fibrinogen sample prepared from fresh single donor plasma was chroamatographically subfractionated into peaks 1 and
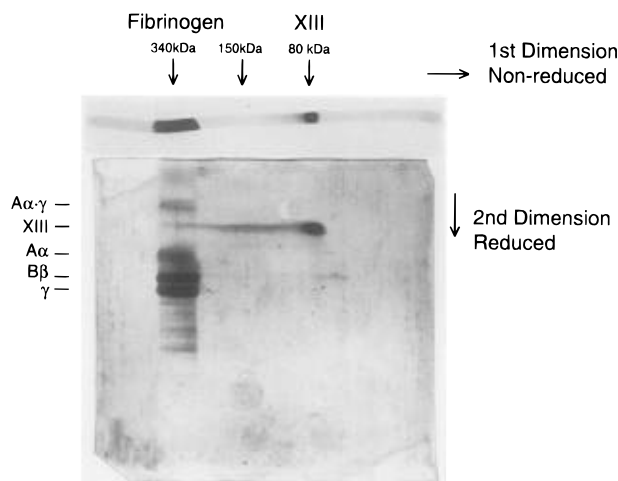
FIGURE 5: Two-dimensional SDS−PAGE of fibrinogen. In the first dimension fibrinogen was separated on a 5% gel under nonreducing conditions (horizontal tube gel). For the second dimension, the tube bel was placed on top of a 9% gel and electrophoresed under reducing conditions. Protein bands were visualized by staining with Coomassie Blue R250. The positions of the relevant fibrinogen chains, factor XIII, and the $A\alpha{\cdot}\gamma$ band are indicated.
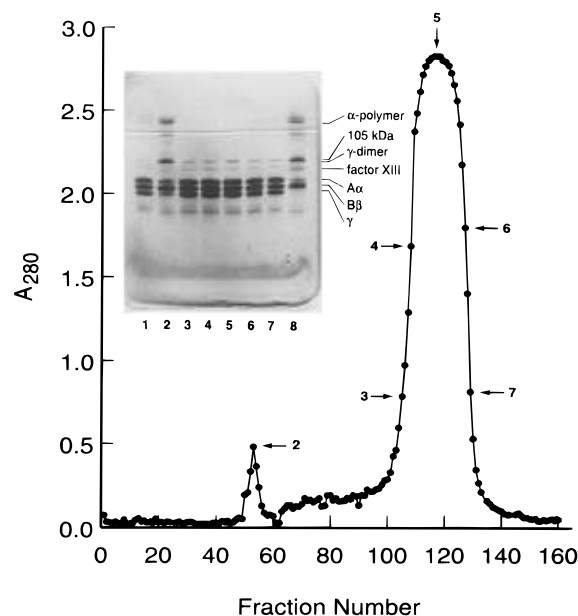

FIGURE 6: Size exclusion chromatography of fibrinogen. Fibrinogen was loaded onto a pair of columns containing Sepharose 4B-CL equilibrated in 50 mM Tris, 100 mM NaCl, 0.5 mM PMSF, 5 KIU/mL Trasylol, pH 7.4. A fast eluting minor peak and a slower eluting major peak were resolved. The inset to this figure is an 8−25% Coomassie Blue stained Phast gel of selected fractions. Lanes: 1, starting fibrinogen; 2−7, column fractions as indicated by arrows; 8, factor XIIIa cross-linked fibrin. The positions of the relevant fibrinogen chains, factor XIII, and the $A\alpha{\cdot}\gamma$ band are indicated.

2, the $A\alpha{\cdot}\gamma$ band was found in both peak fractions, with a somewhat higher proportion in the peak 2 fibrinogen (Figure 7, lanes 2 and 3).

DISCUSSION

A band corresponding to the $A\alpha{\cdot}\gamma$ chain fibrinogen band has been observed previously by other investigations but was not recognized to consist of cross-linked $A\alpha{\cdot}\gamma$ chain heterodimers. Grøn et al. (1993) noted that the 105 kDa band reacted with antibodies directed against $A\alpha$ or γ chains,
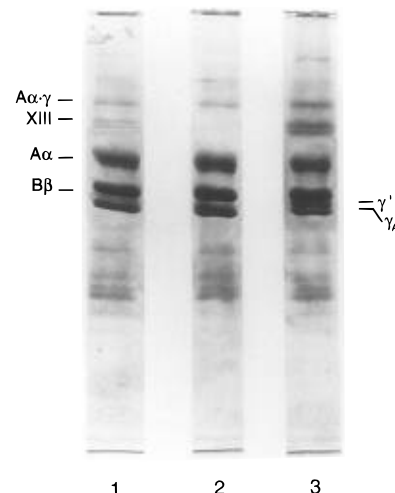

FIGURE 7: Identification of the $A\alpha{\cdot}\gamma$ band in fibrinogen isolated from fresh plasma. Single donor plasma was collected into acid−citrate−dextrose (9:1) supplemented with a variety of protease/transglutaminase inhibitors (see Methods). These same inhibitors were present in all solutions employed during fibrinogen isolation. An aliquot of fraction I-2 fibrinogen (lane 1) was separated into peak 1 (lane 2) and peak 2 (lane 3) fibrinogen by chromatography on DE-52. The positions on this Coomassie Blue stained gel of the relevant fibrinogen chains, factor XIII, and the $A\alpha{\cdot}\gamma$ band are indicated.

but they did not analyze the band in further detail and believed that it consisted of γ dimers superimposed upon partially degraded cross-linked $A\alpha$ chains. Our present experiments show that intramolecular cross-linked $A\alpha$ and γ chains are present in circulating monomeric fibrinogen molecules. The $A\alpha{\cdot}\gamma$ heterodimers are present in a small proportion of circulating fibrinogen molecules amounting to approximately 2% of the $A\alpha/\gamma$ chain population.

The $A\alpha{\cdot}\gamma$ heterodimers are presumably formed by the action of a transglutaminase. Murthy et al. (1991) recently found that transglutaminase from erythrocytes catalyzed the formation of intermolecularly cross-linked fibrinogen polymers *in vitro*, suggesting to us that a tissue-type transglutaminase is responsible for formation of these complexes in circulating fibrinogen *in vivo*. However, a possible role for factor XIIIa cannot be completely discounted, since both forms of factor XIII have been shown to produce cross-linked $A\alpha{\cdot}\gamma$ chains in fibrin and fibrinogen (Mosesson et al., 1989; Amrani & Mosesson, 1989; Shainoff et al., 1991; Grøn et al., 1993). Similarly, the region(s) of the $A\alpha$ chain that become(s) cross-linked to the γ chain is (are) unknown as is the chain which contributes the lysine donor and glutamine acceptor groups.

Several plausible scenarios can be suggested to explain how $A\alpha{\cdot}\gamma$ complexes form *in vivo*. Fibrinogen has been shown to bind covalently to hepatocytes and endothelial cells in a calcium-dependent manner (Barsigian et al., 1987, 1988; Greenberg et al., 1987; Martinez et al., 1989; Barsigian & Martinez, 1990). Tissue transglutaminase evidently is the processing enzyme mediating the binding because there was preferential cross-linking of $A\alpha$ chains over γ chains and γ dimers were not observed. Cross-linking at the cell surface, in which the $A\alpha$ chain reacts internally with a neighboring γ chain before it binds to the cell, could lead to circulating monomeric fibrinogen containing intramolecular $A\alpha{\cdot}\gamma$ complexes. Alternatively, these complexes could form in the circulation when factor XIII becomes activated or whenever

a transglutaminase is released from a cell such as an erythrocyte (even through normal senescent erythrocyte processing). Pathological conditions causing hemolysis and/or cell damage could also result in increased levels of Aα·γ heterodimers in circulating fibrinogen.

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