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Cross-linked Aα·γ chain hybrids serve as unique markers for fibrinogen polymerized by tissue transglutaminase

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ABSTRACT **Notwithstanding the high degree of amino acid sequence homologies between human factor $XIII_a$ on the one hand and intracellular transglutaminases (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) from guinea pig liver or human erythrocytes on the other, we find that the two sets of enzymes differ remarkably in the mode of cross-linking the same protein substrate—i.e., human fibrinogen. In the program of polymerization with factor $XIII_a$, production of the known γ-γ′ homologous chain pairs is the dominant feature, whereas with either intracellular transglutaminase, a series of hitherto unidentified Aα·γ hybrid chain combinations, designated $A\alpha_p\gamma_q$ (p and q = 1, 2, 3 . . .), is generated and practically no γ-γ′ dimers are formed. Two-dimensional electrophoresis is particularly useful for demonstrating the production of $A\alpha_p\gamma_q$ structures by protein staining as well as by immunoblotting against specific antibodies to the Aα and γ chains of fibrinogen. These findings should aid in deciding whether the direct cross-linking of fibrinogen by transglutaminase might contribute to thrombotic processes in addition to the thrombin- and factor $XIII_a$-dependent pathway of clot formation.**

Clotting time is usually not delayed in the various bleeding disorders caused by the malfunctioning of the factor XIII (fibrin-stabilizing factor) system in plasma (1). Thus, though the thrombin and Ca^{2+}-activated form of this zymogen (called factor $XIII_a$) is critical to strengthening of the clot network by generating N^{ϵ}-(γ-glutamyl)lysine side-chain bridges between associated fibrin units, when acting in the normal mode, factor $XIII_a$ does not function as a true cross-linking enzyme (2). Clotting fibrin, rather than the soluble fibrinogen molecule in plasma, is its natural substrate. Nevertheless, factor $XIII_a$ can still react with fibrinogen, albeit at a slower rate than with fibrin (3).

By contrast, the related tissue transglutaminases (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) are highly efficient in catalyzing the direct cross-linking of proteins from solutions, with coagulation of lobster fibrinogen and that of seminal vesicle secretion proteins in the prostatic fluid of rodents as prime examples (4–6). Our laboratory has been concerned for some time as to what could happen if intracellular transglutaminase, due to excessive hemolysis of erythrocytes as an example, were discharged into the human circulation under pathologic circumstances (7, 8). Since currently available diagnostic tests might not be specific for detecting the fibrinogen polymers cross-linked by a tissue type of transglutaminase, we searched for markers that could serve the purpose of distinguishing these structures from the ones produced by the action of factor $XIII_a$. In this paper, we report the presence of unusual cross-linked forms of Aα·γ hybrid chain combinations, designated $A\alpha_p\gamma_q$, in the former polymer which are virtually absent in the latter.

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MATERIALS AND METHODS

The contents of one vial of human fibrinogen (40 mg; IMCO, American Diagnostica, Greenwich, CT) were taken up in 3 ml of buffer (50 mM Tris·HCl, pH 7.5/1 mM EDTA) and were dialyzed against 4 liters of the same buffer for 16 hr at 4°C. Sediment was removed by centrifugation, and the concentration of fibrinogen (9.3 mg/ml) was estimated by using $A_{280}^{1\%}$ (1-cm cell) = 15.1 (9). Human plasma factor XIII and human erythrocyte and guinea pig liver transglutaminases were purified by John Colaluca and David Schilling to apparent SDS/PAGE homogeneity based either on published procedures (10–12) or by methods developed in our laboratory. Concentrations of factor XIII (3.3 mg/ml of 50 mM Tris·HCl, pH 7.5/1 mM EDTA/10 units of Trasylol per ml) and of the liver enzyme (1.1 mg/ml of 50 mM Tris·HCl, pH 7.5/1 mM EDTA) were obtained by measuring absorbancies at 280 nm (13, 14); that of the erythrocyte enzyme (1.7 mg/ml of 20 mM imidazole·hydrochloride, pH 6/1 mM EDTA/ 1 mM dithiothreitol/0.35 M KCl/10 units of Trasylol per ml/5% glycerol) was estimated by using the BCA (bicinchoninic acid) protein assay kit (Pierce). Factor XIII was stored at 4°C, and the intracellular transglutaminases were stored at −80°C.

Activation of the purified factor XIII zymogen by thrombin was carried out just prior to the cross-linking experiments. About 0.1 mg (*ca.* 30 μl) of factor XIII was treated with *ca.* 2.5 units (5 μl) of human α-thrombin (a gift from John W. Fenton III, New York State Department of Health, Albany) in a solution of 175 μl of 50 mM Tris·HCl (pH 7.5) at room temperature (≈24°C) for 25 min, at which time thrombin action was quenched by adding 20 μl of 20 units of hirudin (Sigma).

Cross-linking of fibrinogen was typically performed at 37°C in reaction mixtures of about 0.1 ml containing ≈8 μM fibrinogen, 0.1 M NaCl, 50 mM Tris·HCl (pH 7.5), 20–40 μg of one of the three enzymes per ml, and 5 mM $CaCl_2$, added last. In the controls, 5 mM EDTA was used instead of $CaCl_2$. The cross-linking reactions were stopped at various times by adding 10 μl of 0.1 M EDTA.

Agarose (2%)/SDS (0.1%) electrophoresis (*ca.* 24°C) under nonreducing conditions (15) was carried out on protein samples (*ca.* 30 μg) after treatment at 37°C with 6 M urea/2% SDS for 30 min. SDS (0.1%)/PAGE (5% acrylamide) analysis (*ca.* 24°C) was performed as described by Weber and Osborn (16) on samples of *ca.* 40 μg, following a 30-min treatment of the proteins at 37°C with 40 mM dithiothreitol/6 M urea/2% SDS. Two-dimensional electrophoresis utilized the procedure of O'Farrell (17) with Ampholine (LKB) of pH 3.5–10 in the first dimension. Separation in the second dimension was by SDS/PAGE as indicated above. Electroblotting to nitrocellulose (0.2 μm, Schleicher & Schuell) was carried out with a LKB model 2005 electroblotting unit for 2 hr at 4°C by the method of Towbin *et al.* (18) in 25 mM Tris/192 mM glycine, pH 8.3/20% (vol/vol) methanol. Protein staining of the gels was with 0.025% Coomassie brilliant blue R (in 10% acetic

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acid/20% methanol) and of the nitrocellulose transblots was with 1% amido black (in 10% acetic acid/50% methanol).

For purposes of immunostaining, a rabbit antiserum against the Aα chains (residues 241–476) and a mouse monoclonal antibody against the γ chains (residues 392–406) of fibrinogen were provided by Joan Sobel of the College of Physicians and Surgeons, Columbia University, and by Bohdan J. Kudryk of the New York Blood Center. The antiserum against Aα (H51-8) was diluted 1:120,000, and the antibody against γ (6/20/5) was diluted 1:420,000 into phosphate-buffered saline. Vectastain ABC kits (Vector Laboratories) were used for the peroxidase-based staining of immunoblots (19).

RESULTS AND DISCUSSION

SDS electrophoresis in agarose was used under nonreducing conditions (15) to prove that multimers of fibrinogen of various degrees of polymerization were produced when either factor $XIII_a$ or an intracellular transglutaminase was added to a clear solution of the monomeric protein substrate (Fig. 1). These polymerization reactions were accompanied by a readily measurable increase in the turbidity of the solution (90° scattering; data not shown). The SDS/PAGE profiles, obtained under disulfide-reducing conditions, revealed that the nature of the polymerization process of transglutaminase was quite different from that brought about by factor $XIII_a$ (Fig. 2). Instead of the characteristic appearance of the *ca.* 90-kDa γ-γ′ chain dimers with factor $XIII_a$ (13), the intracellular enzyme gave rise to a principal band of about 100 kDa and several other bands at higher mass values that were not present in factor $XIII_a$-polymerized fibrinogen. Our observation with purified transglutaminase supports the findings of Martinez *et al.* (20), who used an umbilical vein endothelial cell extract for effecting the cross-linking of fibrinogen.

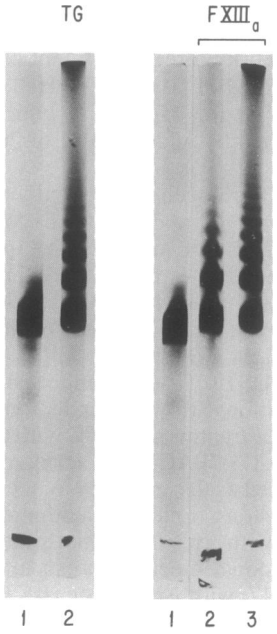

FIG. 1. Production of cross-linked multimers of fibrinogen upon exposure of this protein substrate to an intracellular transglutaminase (TG) or to factor $XIII_a$ ($FXIII_a$) as demonstrated by nonreducing SDS electrophoresis in agarose. Starting fibrinogen controls are shown in lanes 1. (*Left*) Lane 2: fibrinogen monomer and multimers after 15 min of reaction with liver transglutaminase (20 μg/ml). (*Right*) Lanes 2 and 3: fibrinogen monomer and multimers after 15 and 30 min of reaction with factor $XIII_a$ (20 μg/ml), respectively.

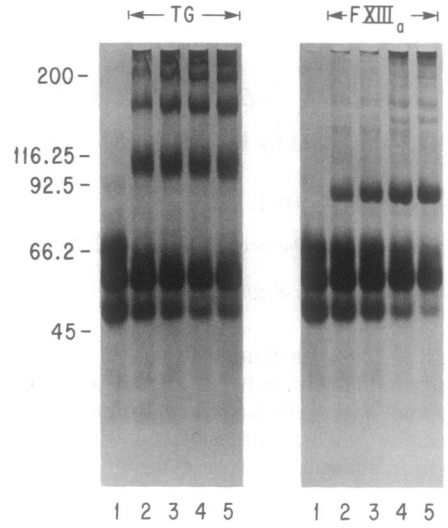

FIG. 2. Differences in the SDS/PAGE protein profiles of fibrinogen treated with intracellular transglutaminase (TG) or with factor $XIII_a$ ($FXIII_a$). Molecular mass markers are given on the left in kDa. The chain pattern of starting fibrinogen exposed to either enzyme in the presence of 5 mM EDTA is shown in lane 1. The other lanes pertain to samples of reaction mixtures containing 5 mM Ca^{2+} and exposed to transglutaminase (*Left*) or factor $XIII_a$ (*Right*) as in Fig. 1, for periods of 15, 30, 60, and 120 min (lanes 2, 3, 4, and 5, respectively).

Immunostaining of the gel transblots with antibodies specific against regions of the Aα (residues 241–476) and γ (residues 392–406) chains of the fibrinogen molecule (Fig. 3) indicated that the ≈110-kDa band and some of the higher mass bands with transglutaminase as the cross-linking enzyme were hybrid combinations of Aα and γ chains—e.g., ≈110 kDa = Aα + γ = 66.2 kDa + 47 kDa.

Two-dimensional electrophoresis was then used, coupled with immunostaining of the transblots, to further delineate the differences in the mode of polymerization of fibrinogen with factor $XIII_a$ and with intracellular transglutaminase. As seen in Fig. 4, this seemed to be, indeed, the best procedure for distinguishing the two polymeric structures. The control fibrinogen (Fig. 4 *Left*) gave a fairly compact spot for the γ chain but multiple spots for the Aα and Bβ chains, which were resolved by isoelectric focusing in the first dimension. Immunostaining with antiserum to Aα showed the presence of partially degraded Aα chains with molecular mass values in SDS electrophoresis (second dimension) overlapping the Bβ chain spots. The preparation also contained a small amount of γ-γ′ dimers, as shown by staining with the anti-γ chain antibody. The two-dimensional patterns obtained after the cross-linking of fibrinogen with either factor $XIII_a$ (Fig. 4 *Center*) or the intracellular transglutaminase (Fig. 4 *Right*) differed markedly not only from the above control but also from each other. Salient differences between the latter two are as follows: Products of homologously cross-linked γ chains [i.e., γ-γ′ and, perhaps, a $(\gamma\text{-}\gamma')_2$ structure] were found in significant amounts only when fibrinogen was treated with factor $XIII_a$ and not with transglutaminase. The isoelectric points of these homologously cross-linked products appeared to be similar to that of the single γ chain, itself. By contrast, the polymerization of fibrinogen with the intracellular enzyme gave rise to a series of spots migrating to a less basic isoelectric point. Moreover, since these spots cross-reacted with antibodies to both the Aα and γ chains of fibrinogen, they must represent hybrid, covalent chain combinations, which we designate $A\alpha_p\gamma_q$ (where p and q = 1, 2, 3 . . .).

Although the figures used as illustrations relate to experiments with the liver enzyme, virtually identical one-

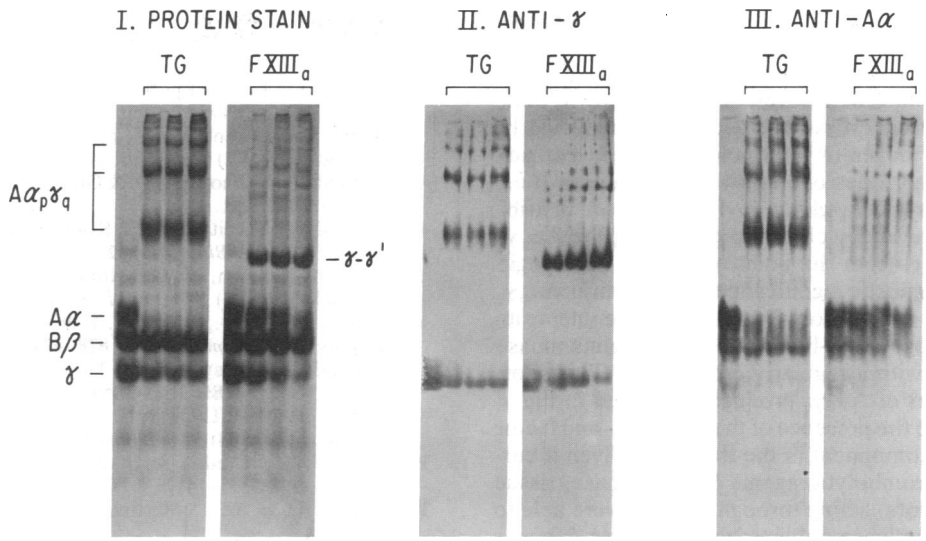

FIG. 3. Immunostaining of SDS/PAGE transblots of transglutaminase (TG)-polymerized fibrinogen reveals covalent Aα·γ hybrid chain combinations (designated Aα$_p$γ$_q$) essentially absent from factor XIII$_a$ (FXIII$_a$)-treated fibrinogen where the γ-γ' combination predominates. Nitrocellulose blots were stained with amido black (*Left*) (set I) or immunostained with the use of antibodies against the γ chains (*Center*) (set II) and Aα chains (*Right*) (set III) of human fibrinogen. The first lane in each gel shows fibrinogen exposed to either enzyme as indicated in the presence of 5 mM EDTA (Aα, Bβ, and γ chains are marked). The other lanes (from left to right) pertain to samples treated with transglutaminase or factor XIII$_a$ for 30, 60, and 120 min.

dimensional SDS/PAGE and two-dimensional electrophoretic results were obtained with the human erythrocyte transglutaminase. These intracellular transglutaminases share major sequence homologies at their active centers and elsewhere with the *a* subunit of factor XIII (21); yet we find that they catalyze the cross-linking of the same fibrinogen molecule by an entirely different program of polymerization: γ-γ' dimerization predominates with factor XIII$_a$ and Aα$_p$γ$_q$ hybrid chain oligomerization with either of the tissue transglutaminases.

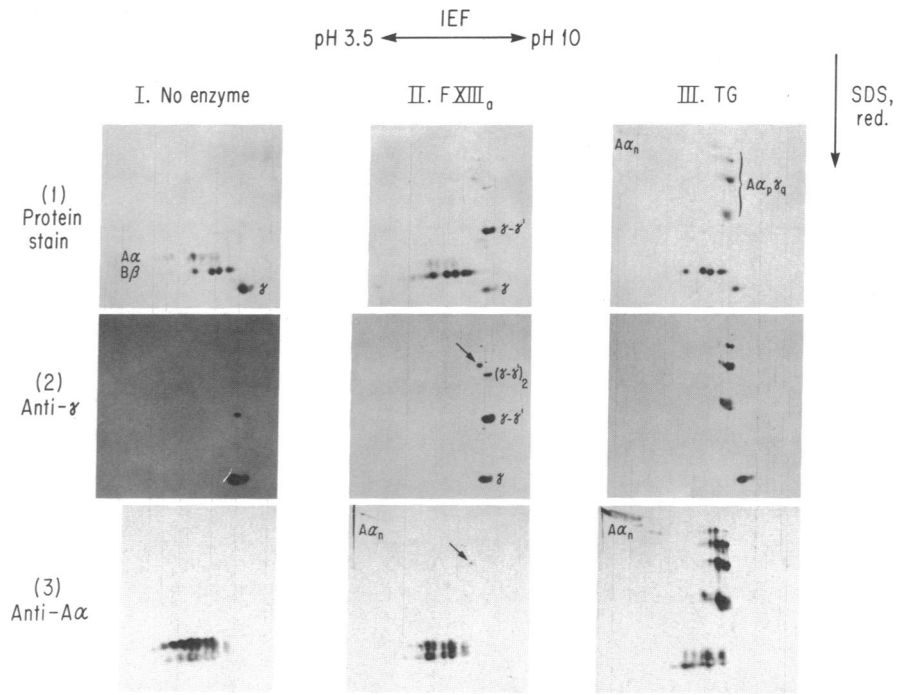

FIG. 4. Two-dimensional electrophoresis highlights a series of unique Aα$_p$γ$_q$ hybrid chain combinations produced in the reaction of human fibrinogen with an intracellular transglutaminase (TG). For isoelectric focusing (IEF) in the first dimension, a buffer range from pH 3.5 (left) to pH 10 (right) was used; separation in the second dimension, vertically, was carried out in SDS under reducing conditions. (*Left*) Set I shows the patterns for the control fibrinogen substrate. (*Center* and *Right*) Sets II and III present the profiles for fibrinogen treated with factor XIII$_a$ (FXIII$_a$) and with transglutaminase, respectively. The nitrocellulose transblots were stained with Amido Black (*Top*) (row 1) or were developed by immunostaining using either an antibody to the γ chains (*Middle*) (row 2) or to the Aα chains (*Bottom*) (row 3) of fibrinogen. Cross-linked chain pairs of γ-γ' characterize the action of factor XIII$_a$ on fibrinogen (set II), whereas Aα$_p$γ$_q$ hybrids predominate when this protein is polymerized by transglutaminase (set III). Aα$_n$ homopolymers (seen in the upper left hand corners of plates in sets II and III, row 3) were found with transglutaminase as well as with factor XIII$_a$. The arrows in set II, rows 2 and 3, mark a spot possibly corresponding to a minute amount of cross-linked Aα·γ hybrid even in the factor XIII$_a$-treated fibrinogen.

Apart from the absence of γ-γ' cross-linking, formation of the $A\alpha_p\gamma_q$ type of covalent chain combinations is, thus, the distinguishing hallmark of the action of an intracellular transglutaminase on fibrinogen. As yet, it is not known what proportion of the hybrid cross-links occurs intramolecularly within the same fibrinogen or intermolecularly between adjacent fibrinogens: nor does one know how the donor (i.e., ε-lysyl) and acceptor (i.e., γ-glutaminyl) residues are distributed between the $A\alpha$ and γ chains in generating the $A\alpha_p\gamma_q$ structures. However, from the point of view of pathologic assessment, the diagnostic significance of the hybrid $A\alpha_p\gamma_q$ chain combinations must be obvious. If fibrinogen deposits were produced through cross-linking with a transglutaminase and were mixed in with a regularly cross-linked fibrin clot, analysis of the series of $A\alpha_p\gamma_q$ products described in Fig. 4 *Right* would indicate the presence of the thrombin- and factor $XIII_a$-independent component in the thrombus. Even if currently employed thrombolytic agents (streptokinase, tissue plasminogen activators) acting through plasmin were able to digest the transglutaminase-polymerized fibrinogen deposits with some degree of efficiency, the resulting split products must be different from those derived from the factor $XIII_a$-cross-linked fibrin. Dosage dependence, kinetics of digestibility with plasmin and the nature of split products from such mixed thrombi should be important topics for future investigations. Furthermore, if transglutaminases released from broken erythrocytes, for example, can be shown to play a role in the inclusion of fibrinogen polymers into thrombi, perhaps as they age, attention might also be given to inhibitors of cross-linking as adjuvants of thrombolytic therapy. Cross-linked fibrinogen polymers might also be generated elsewhere, perhaps in heterologous combinations with other proteins (22).

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