CROSSLINKING OF HUMAN FIBRIN: EVIDENCE FOR INTERMOLECULAR CROSSLINKING INVOLVING α-CHAINS

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1. Introduction

In the presence of activated factor XIII (fibrin stabilizing factor) fibrin is crosslinked by the formation of peptide bonds between ε-amino groups of certain lysine residues and γ-amino groups of glutamines to the extent that approximately two moles of ε(γ-glutamyl)lysine are formed per 330,000 g of fibrin [1]. Chen and Doolittle [2, 3] have presented data demonstrating that such peptide bond formation occurs between two γ-chains and have also suggested the possibility of crosslinking between α- and γ-chains. Lorand et al. [4] have reported that the primary crosslinkage bond occurs between α- and γ-chains, forming an α-γ hybrid dimer. Working with bovine fibrin, Takagi and Iwanaga [5] have found evidence only for a γ-γ crosslink. There appears to be general agreement that β-chains are not involved in the crosslinking of human fibrin. Recently McKee et al. [6] reported data which suggested that a crosslinkage occurred between α-chains of human fibrin as well as between the γ-chains.

We have also confirmed the formation of γ-chain crosslinks, and in this report we present evidence for the formation of a polymer from crosslinked human fibrin consisting only of α-chains. Free α-chains from non-crosslinked, reduced, alkylated fibrin and α-polymer from crosslinked, reduced, alkylated fibrin were purified by carboxymethylcellulose chromatography and examined by fingerprinting and polyacrylamide electrophoresis. The results show that the crosslinked α-chains have a fingerprint pattern nearly identical to that of the free α-chain and may have a molecular weight greater than 340,000.

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2. Materials and methods

2.1. *Purification of fibrinogen and preparation of crosslinked and non-crosslinked fibrins*

Human fibrinogen, fraction 1–4 was prepared from fraction 1–2 [7]. The coagulability of this material, determined spectrophotometrically, was 98%. This fibrinogen preparation also contained sufficient factor XIII to ensure the formation of crosslinked fibrin under appropriate conditions. One gram of this material, dissolved in 50 ml of 0.3 M NaCl, was diluted 20-fold with 0.05 M tris–0.1 M NaCl, pH 7.35. For the preparation of crosslinked fibrin 10 ml of 1 M $CaCl_2$ were added, and for the preparation of non-crosslinked fibrin 10 ml of 10% EDTA were added in place of $CaCl_2$. To initiate clotting bovine thrombin was added to the fibrinogen mixture to a concentration of 0.5 NIH U/ml. The mixture was incubated for 4 hr at 37°, after which the resulting clot was removed and the excess fluid expressed. The fibrin was finely divided and suspended in 2 liters of 1% monochloroacetic acid in the case of crosslinked fibrin or distilled water for non-crosslinked fibrin and stirred for 16 hr. The fibrins were finally washed extensively with distilled water and freeze-dried.

2.2. *Preparation of reduced, carboxymethylated fibrin (S-CM derivatives)*

Separate samples of both crosslinked and non-crosslinked fibrins were suspended in 8.4 M guanidine

hydrochloride at pH 8.5, reduced with dithiothreitol, and alkylated with tritiated monoiodoacetic acid. Amino acid analysis demonstrated complete reduction and alkylation of all half-cystine residues [8].

2.3. *Separation of the chains of crosslinked and non-crosslinked fibrins*

The S-CM derivatives were subjected to ion-exchange chromatography on carboxymethylcellulose (Whatman CM-52) with a continuous sodium acetate gradient (0.05 M, pH 4.8 to 0.25 M, pH 5.5) containing 8 M urea. During fractionation the protein absorbance was monitored with an ISCO ultraviolet analyzer at 280 nm. From each fraction a 100 μl aliquot was mixed with 1.9 ml of 10% acetic acid and 10 ml of scintillation fluid, consisting of 100 g naphthalene and 5 g PPO per liter of dioxane, and radioactivity was measured in a Beckman liquid scintillation system. The fractions were dialyzed exhaustively against distilled water and freeze-dried.

2.4. *Fingerprinting*

Two-dimensional peptide mapping (fingerprinting) was performed on tryptic hydrolysates of fractions from chromatography of the two types of fibrin [9]. Samples were digested with TPCK-trypsin (enzyme: substrate, 1:50) and were then subjected to thin-layer electrophoresis in the first dimension, followed by ascending chromatography in the second dimension. The dried plates were developed with ninhydrin.

2.5. *Polyacrylamide electrophoresis*

Analytical polyacrylamide gel electrophoresis of the fractions of crosslinked and non-crosslinked fibrins was performed in 10%, 7.5%, and 5% gels [10]. The electrophoresis was run in a Shandon apparatus with 75 mm tubes. Samples were dissolved in 10 M urea, electrophoresed in 5% acetic acid, and stained with Coomassie blue.

3. Results

The results of chromatographic separation of the S-CM derivatives of crosslinked and non-crosslinked fibrins on carboxymethylcellulose are shown in fig. 1. Non-crosslinked fibrin (fig. 1a) was separated into four components, corresponding to γ-chain (peak I),

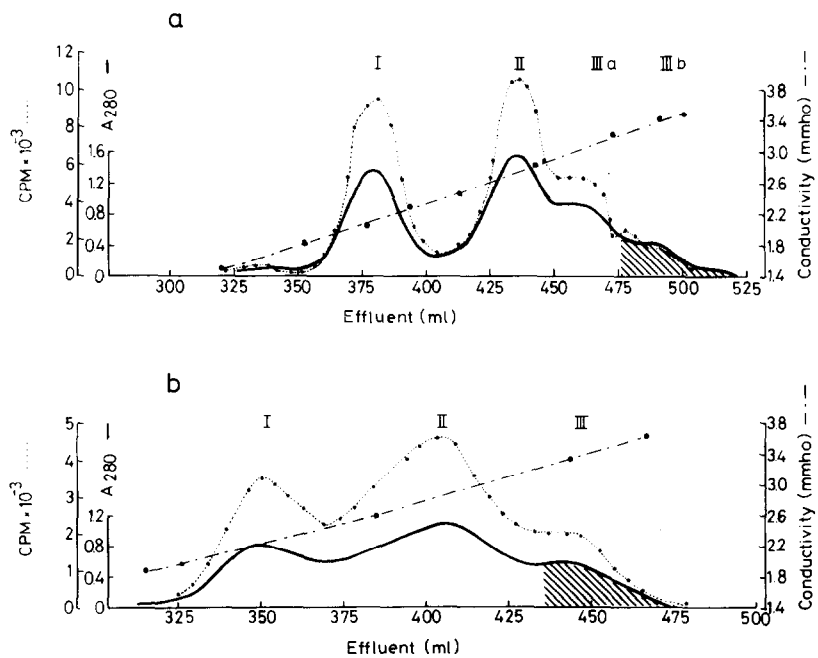

Fig. 1. Carboxymethyl-cellulose chromatography of (a) reduced, alkylated, non-crosslinked fibrin and (b) reduced, alkylated, crosslinked fibrin with a continuous sodium acetate gradient containing 8 M urea.

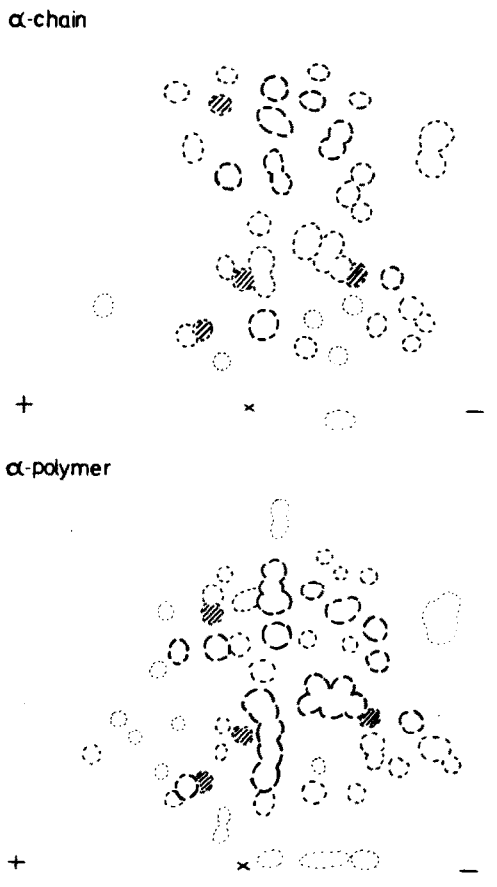

Fig. 2. Fingerprints on thin-layer starch plates of tryptic hydrolysates of free α-chain from non-crosslinked fibrin and α-polymer from crosslinked fibrin. Peptides were separated by electrophoresis at 300 V and 15 mA for 1.25 hr at pH 5.5 and ascending chromatography in *n*-BuOH–pyridine–HAc–H_2O (15:10:3:12, v/v).

β-chain (peak II), and two α-chain peaks (peak IIIa and b); while crosslinked fibrin (fig. 1b) was separated into three major components. In both cases chromatographic separation was confirmed by radioactivity measurements. Material eluting from each column from 3.2 to 3.6 mmho conductivity (peak IIIb and peak III) corresponding to free α-chains for non-crosslinked fibrin, was pooled, as indicated by the shaded area in the chromatograms, desalted and freeze-dried for further analysis.

Characterization of the crosslinked material eluting in the α-chain region was performed by two-dimensional peptide mapping of tryptic hydrolysates of this material and the free α-chain from non-crosslinked fibrin. In fig. 2 are shown the fingerprints of purified α-chain from non-crosslinked fibrin and of the crosslinked material eluting from the column in the α-chain region (fig. 1b, peak III). Heavier outlines indicate those spots which stained most intensely with ninhydrin reagent. Spots which appeared yellowish with ninhydrin are indicated by shading. It can be seen that the four yellow spots appeared in the same relative position in both fingerprints and that there are numerous other spots which were identical in the two fingerprints.

Electrophoretic patterns in 7.5% polyacrylamide gels of the materials applied to the columns and the fractions which were pooled are shown in fig. 3. The unfractionated, S-CM derivatives of non-crosslinked fibrin (fig. 3a) gave three major bands, corresponding to the α-, β-, and γ-chains. Microheterogeneity of the α-chain was also observed, since it could be separated into two to five smaller bands. It can be seen clearly that the protein eluting from the CM-cellulose column at 3.2 to 3.6 mmho from non-crosslinked fibrin had an electrophoretic mobility identical to α-chain when compared to the unfractionated starting material (fig. 3a and b). Similar comparison of the fraction obtained from chromatography of the S-CM derivatives of crosslinked fibrin with the unfractionated material (fig. 3c and d) demonstrated the appearance of a high molecular weight substance which did not penetrate the gel under the conditions of electrophoresis. It can also

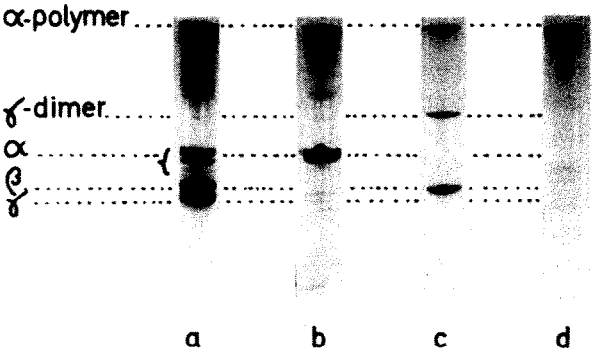

Fig. 3. Analytical disc electrophoresis in 7.5% polyacrylamide at acid pH. Samples were electrophoresed for 2.5 hr at 2.5 mA per tube. (a) reduced, alkylated, non-crosslinked fibrin; (b) purified α-chain from non-crosslinked fibrin; (c) reduced, alkylated, crosslinked fibrin; (d) purified α-polymer from crosslinked fibrin.

be seen that in the unfractionated, crosslinked material (fig. 3c) the free α- and γ-chains have disappeared while the β-chain retains an electrophoretic mobility identical to the β-chain of non-crosslinked fibrin. In addition the unfractionated, crosslinked fibrin contains the γ-γ dimer, as previously described by others [3, 6]. Electrophoresis in gels containing 10% and 5% polyacrylamide gave similar results.

4. Discussion

Activated factor XIII functions as an aminoacyl transferase, catalyzing the formation of peptide bonds in fibrin and thereby strengthening the fibrin matrix. Recent interest has been focused on the determination of which fibrin chains are involved in these crosslink bonds and whether the crosslink is intramolecular or intermolecular. Chen and Doolittle [3] have located crosslinks in the γ-γ dimer near the *C*-terminal ends of two γ-chains. In this report evidence is presented for the formation of α-chain crosslinks, in addition to the γ-γ dimer.

The α-chain crosslinked material was purified from reduced, alkylated, crosslinked, human fibrin by carboxymethylcellulose chromatography. The identity of the crosslinked material, which eluted from the column at the same conductivity range as free α-chains from non-crosslinked fibrin, was confirmed by fingerprint analysis. Comparison of the fingerprints of the crosslinked material and of the free α-chains showed that the general characteristics of the two were identical, with yellow spots and other unique groups of spots appearing in the same relative positions on both fingerprints. Comparison of the fingerprint of the crosslinked α-chains with fingerprints of purified β-chains and purified γ-chains showed considerable differences in both cases. Further evidence confirming the identity of the crosslinked material with α-chains was provided by *N*-terminal and amino acid analyses. Glycine was found to be the only *N*-terminal amino acid in the crosslinked material, which eliminated the possibility of γ-chains being in this material since fibrin has glycine as the *N*-terminal residue for α- and β-chains and tyrosine for the γ-chains. Total amino acid analysis of the crosslinked material showed that it was nearly identical in composition to free α-chains. Thus the crosslinked material can be identified as consisting only of α-chains.

It was further found that the crosslinked α-chains would not penetrate during disc electrophoresis on 10, 7.5, or 5% polyacrylamide gels. Under the same electrophoretic conditions fibrinogen, with an estimated molecular weight of 340,000, will enter such gels. Therefore, the α-chain crosslink material appears to be larger than fibrinogen and is probably an α-polymer. Since the α(A)-chain of fibrinogen has been shown to have a molecular weight of 68,000 to 76,000 by SDS-polyacrylamide electrophoresis [6], the α-polymer should consist of a minimum of five to six α-chains. Thus it appears that α-chain crosslinking is an intermolecular event involving several fibrin monomers. Further experiments are in progress to determine the nature of the crosslinks in the α-polymer.

Acknowledgements

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