Studies of Platelet Fibrinogen From a Subject With a Congenital Plasma Fibrinogen Abnormality (Fibrinogen Paris I)

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Fibrinogen from normal platelets and plasma was compared with material derived from a subject with the congenital fibrinogen abnormality termed fibrinogen Paris I. The molecular abnormality in this subject is characterized by the presence (in plasma molecules) of relatively large mutant γ-chains (γ-Paris I) that replace 50% or more of the normal γ-chain population and that, unlike normal γ-chains, do not form covalently linked γ-dimers in the presence of plasma transglutaminase (factor XIIa). Extracts of washed or washed and trypsin-treated platelets that had been lysed in the presence of 8 M urea were subjected to CM-cellulose chromatography in the presence of 8 M urea. The fibrinogen-containing chromatographic peak from each sample was then concentrated and analyzed by dodecyl sulfate gel electrophoresis. All unreduced samples (5% polyacrylamide gels) possessed a fibrinogen band migrating in the same position as plasma fibrinogen. Unstained gel slices containing platelet fibrinogen were reduced with dithiothreitol and again subjected to dodecyl sulfate gel electrophoresis (10% polyacrylamide gels). All such samples showed bands corresponding in position and in relative staining intensity to the Aα, Bβ, and γ-chains of normal plasma fibrinogen, findings confirming the observation that platelet fibrinogen is not significantly different in size from that in plasma. The band corresponding to the γ-Paris I chain was not observed in any sample. The absence of mutant γ-chains from platelet fibrinogen molecules derived from the Paris I subject is confirmatory evidence that platelet and plasma fibrinogen pools are separate from one another. The results also indicate that the subject's platelet and plasma fibrinogens, at least as far as their γ-chains are concerned, are not assembled from identical gene products. It remains to be shown whether this finding reflects a more general situation regarding hepatic and platelet fibrinogen from normal individuals.

There is abundant evidence indicating the existence of a platelet fibrinogen pool that is separate from that in plasma1,8 (inter alia). Nevertheless, certain fundamental aspects of the nature of platelet fibrinogen, particularly those relating to its size and to whether it is genotypically identical to hepatic (plasma) fibrinogen, are still the subjects of spirited debate.9,10 Although several studies have indicated that the molecular weight of platelet fibrinogen is the same as that from plasma,9,11,13 significant physiochemical,13 bioochemical,13,14 immunochemical,15 and functional13,14 differences have been identified. These differences notwithstanding, Doolittle et al.9 on the basis of analyses of subunit composition,
crosslinking characteristics, and the amino acid composition of fibrinopeptides released by thrombin, have concluded that platelet and hepatic fibrinogen are identical gene products. This conclusion implies that the observable differences cited above between platelet and hepatic fibrinogen reflect posttranslational modifications of otherwise identical gene products. On the other hand, Ganguly and coworkers have been steadfast in their contention that intact platelet fibrinogen has a lower molecular weight than plasma fibrinogen. They have taken this as evidence of differences in the structural genes governing the synthesis of the two types of fibrinogen.

The evidence concerned in framing these arguments is complex and often conflicting, but the apparent contradictions can be reduced to two major issues regarding: (A) possible differences in molecular size and (B) interpretation of compositional and functional differences. Experiments bearing directly on these issues are quite limited. In one such report, Soria et al. have analyzed platelet fibrinogen from a subject with a congenital plasma fibrinogen abnormality termed fibrinogen Metz. Fibrinogen Aα chains from their subject’s platelets did not express the charge anomaly found in the plasma fibrinogen Aα chains. However, it is not yet clearly established whether the Aα chain anomaly of fibrinogen Metz is attributable to the mutation of a structural gene.

We have been studying various aspects of the structural abnormality of a congenitally abnormal fibrinogen molecule termed fibrinogen Paris I. This structural anomaly is characterized by the presence of a mutant chain, termed γ-Paris I, that replaces normal γ-chains. Family studies indicate that the inheritance pattern is autosomal and that the only living subject is heterozygous with respect to the defect. Consistent with this, roughly one-half or more of all γ-chains in the subject’s plasma fibrinogen are of the mutant variety. The γ-Paris I chains can easily be distinguished from normal γ-chains by dodecyl sulfate gel electrophoretic analysis since: (A) the γ-Paris I chain is somewhat larger than the normal γ-chain and (B) unlike normal γ-chains, γ-Paris I chains do not form covalently linked dimers when crosslinked in the presence of plasma transglutaminase (factor XIIIa).

There is good evidence that γ-Paris I chains have structural overlap with normal γ-chains* and that the abnormality of the γ-Paris I chain involves the amino acid sequence in the COOH-terminal region of the chain (i.e., that portion of the γ-chain contributing to the structure of plasmic fragment D). Although the exact location and sequence of the abnormality is not yet known, it seems evident that the difference between γ-Paris I and normal γ-chains reflects mutation of a structural gene. Given these facts, it appeared to us that comparative studies of platelet and plasma fibrinogen from the fibrinogen Paris I subject presented an opportunity to shed light on the broader question of the genetic nature of platelet fibrinogen. In this article we report results indicating that our subjects’ platelet and plasma fibrinogen, at least as far as their γ-chains are concerned, are not assembled from identical gene products.

*In recent unpublished experiments, we have obtained evidence that a cyanogen bromide fragment from the S-carboxymethyl γ-Paris I chain, corresponding to its NH₂-terminal segment, is the same as that derived from the normal γ-chain.
PLATELET FIBRINOGEN

MATERIALS AND METHODS

Preparation of Platelet Protein Extracts and Plasma Fibrinogen

On 2 different occasions, 9 parts of blood from the dysfibrinogenemic subject (M. T.) or from normal control subjects was collected into 1 part 0.13 M sodium citrate solution containing aprotinin (Iniprol, Choay Laboratories, Paris), 5000 U/ml. The results were the same for each occasion. Platelet-rich plasma was obtained by centrifugation at 100 g for 15 min; this material was then subjected to centrifugation at 1000 g for 20 min. The resulting platelet button was washed 3 times with a Tris-EDTA buffer that had been prepared by adding 1 part of 0.077 M EDTA solution to 49 parts of a 0.015 M Tris buffer (pH 7.4) containing 0.138 M NaCl and 0.05 M KCl.

A portion of these washed platelets was suspended in the Tris-EDTA buffer at approximately the same volume as that of the original platelet-rich plasma and then subjected to trypsin digestion at room temperature for 15 min with TPCK-trypsin (Worthington Biochemical Corp., Freehold, N.J.; final concentration, 1 µg/ml) that had been dissolved in a 0.03 M sodium phosphate buffer, pH 7, containing 0.15 M NaCl. Following the incubation period, aprotinin (2500 U/ml, final concentration) was added to inhibit the trypsin, and the mixture was then subjected to centrifugation for 15 min at 1000 g. The resulting platelet button was washed once with the Tris-EDTA buffer to which aprotinin had been added (2000 U/ml, final concentration).

Owing to the limited quantity of the subject's platelets that were available to us for this study, we selected 8 M urea as the solvent for extracting and processing platelet proteins in order to prevent proteolytic enzyme activity at all phases and to minimize losses of the desired platelet fibrinogen molecules. Accordingly, protein extracts were prepared from washed or trypsin-treated platelets that had been suspended in a freshly deionized (Biores AG-501 x 8, Bio-Rad Laboratories, Richmond, Calif.) 8-M urea solution (containing 1 mM HCl) at the same volume as that of the original platelet-rich plasma, and then subjected to 3 freeze-thaw cycles.

Plasma fibrinogen from the fibrinogen Paris 1 subject was prepared from citrated platelet-poor plasma by the glycine precipitation procedure of Kazal et al.25 Fibrinogen fraction 1-4 from normal subjects was prepared from citrated platelet-poor plasma by a modification of the technique of Blombäck and Blombäck.27 For certain experiments, fibrinogen was radioiodinated with ¹²³I (carrier-free, New England Nuclear, Boston, Mass.) by the iodine monochloride method of McFarlane.28 The iodine content of the labeled fibrinogen was less than 0.5 atom/molecule. Radioautography of vertical slices of acrylamide gels containing this material was carried out by exposure to standard x-ray film.

Processing and Analysis of Platelet Protein Extracts

Platelet extracts in 8-M urea were first subjected to linear gradient elution column chromatography on a CM-cellulose (CM-23, Whatman Biochemicals, Ltd., Springfield Hill, Mardston, Kent) column, 0.9 × 15 cm, that had been equilibrated with an 8-M urea, 0.1-M acetic acid-Tris, pH 4.1, buffer. The amount of protein applied was estimated by spectrophotometry at 280 nm, assuming an absorbance coefficient (A_m) of 15. The column was developed with a linear gradient consisting of 100 ml of the pH 4.1 buffer and 100 ml of a 0.1-M acetic acid–Tris limit buffer of pH 6.2.30 Fractions of 4.0 ± 0.2 ml were collected. Three major protein peaks were pooled, dialyzed against 10% acetic acid, and then freeze-dried. For analysis by immunodiffusion against anti-human fibrinogen serum (Organon Technika, St. Denis, France), this material was dissolved in a 0.015 M Tris-buffered solution, pH 7, containing 0.15 M NaCl and 4 M urea. As assessed by this type of analysis, only chromatographic peak 3 contained fibrinogen.

Unreduced material from the fibrinogen-containing chromatographic peak was subjected to electrophoresis for 3 hr at 8 mA/gel in dodecyl sulfate-containing gels (5% polyacrylamide).29 Certain samples were stained with Coomassie brilliant blue in order to locate the position of the fibrinogen band. For analysis in a second electrophoretic dimension after disulfide bridge reduction,22,23 unstained gels from the same run were sliced into 3-mm sections. Fibrinogen-containing slices were ground up into fine pieces in the presence of approximately 100 µl of a 0.1-M sodium phosphate buffer, pH 7, containing 8 M urea and 70 mM dithiothreitol. The mixture was heated in a boiling water bath for 2 min and then incubated overnight at 37°C. Following this period of incubation, each reduced sample was placed on the surface of a 10% polyacrylamide gel containing Na dodecyl sulfate and then subjected to electrophoresis for 3 hr at 8 mA/gel. The gels were eventually stained with Coomassie brilliant blue.
RESULTS

Electrophoretic Analysis of Unreduced Platelet Fibrinogen

When subjected to dodecyl sulfate gel electrophoresis, unreduced plasma fibrinogen (fraction I-4) can usually be resolved into two major fibrinogen bands, termed I and II (Fig. 1). Band I reflects the presence of intact fibrinogen molecules, whereas material migrating within band II includes fibrinogen species that have undergone some proteolytic catabolic attack involving loss of regions containing the COOH-terminus of Aα chains. The same band pattern has been found for plasma fibrinogen from the Paris I subject. The same sort of electrophoretic analyses were carried out on chromatographic fractions from the various platelet extracts. Only gels of material from chromatographic peak 3 possessed a band corresponding to the position occupied by plasma fibrinogen, a finding consistent with results of immunodiffusion analyses that also indicated the presence of fibrinogen only in this peak. Unlike the case for plasma fibrinogen, the fibrinogen from platelet-derived samples showed no tendency to be resolved into two bands—most material migrated in a position corresponding to the band I position.

Electrophoretic Analysis of Reduced Platelet Fibrinogen

The electrophoretic gel pattern obtained from reduced samples of chromatographic peak 3 showed several bands in addition to those attributable to the normal fibrinogen subunit chains. A band corresponding to the γ-Paris I position was not evident in any. However, the complex band pattern rendered interpretation somewhat ambiguous in terms of unequivocally identifying the band(s) attributable to the fibrinogen subunit chains. Owing to the small amounts of fibrinogen-containing platelet material that were available from the Paris I subject, it was impractical to attempt further purification of the fibrinogen in this chromatographic peak by additional chromatographic or fractionation procedures. Instead, we elected to analyze the fibrinogen band from the peak 3 samples by...
subjecting unstained fibrinogen-containing gel slices to dodecyl sulfate gel electrophoretic analysis in a second dimension (in 10% polyacrylamide gels) after disulfide bridge reduction (Fig. 2).

In this system, slices containing plasma fibrinogen Paris I revealed a gel pattern characteristic of the Aα, Bβ, γ-Paris I, and γ-chains known to comprise these molecules. Normal plasma fibrinogen or platelet fibrinogen from normal subjects or the Paris I subject (washed or trypsin-treated platelets) showed bands only in the Aα, Bβ, and γ-chain positions. There was no suggestion of material migrating in the γ-Paris I position. The gel of a mixture of platelet fibrinogen with a sample containing a source of the Paris I chain (gel 6) clearly showed the band corresponding to γ-Paris I. Electrophoretic analyses of a reduced plasma fibrinogen Paris I sample (added as a source of the γ-Paris I chain) that had been diluted progressively (1/2, 1/4, 1/8, etc.) with normal fibrinogen, indicated that had γ-Paris I chains amounted to 6% or more of the total γ-chain population in the platelet samples, they would have been detected readily.

Radioautography of 125I-Labeled Fibrinogen

The fact that the platelet fibrinogen samples we examined were indistinguishable (whether reduced or unreduced) from intact plasma fibrinogen molecules indicates that our method of isolating platelet fibrinogen in the presence of 8 M urea virtually eliminates the possibility of significant proteolytic attack on platelet fibrinogen molecules during their isolation. To reinforce this conclusion, we carried out the following additional experiment to show that γ-Paris I chains had not been selectively degraded during processing (Fig. 3). 125I-labeled normal fibrinogen or Paris I plasma fibrinogen was added to washed normal platelets prior to lysis in the presence of 8 M urea. The platelet mixture was then processed in the usual way and the reduced extracts, containing both platelet protein and the added 125I-fibrinogen, were subjected to dodecyl sulfate gel electrophoresis and the gels eventually analyzed by radioautography. The radioautogram of material recovered from the various extracts yielded a band pattern that was the same as that of the starting material.
DISCUSSION

The results of the present investigation provide confirmatory evidence for the existence of an intraplatelet fibrinogen pool that is separate from that circulating in plasma. First, the absence of detectable θ-Paris I chains from platelet fibrinogen molecules derived from the Paris I subject is evidence that plasma fibrinogen molecules, per se, are not an appreciable constituent of extracts of well washed platelets. Second, extracts of washed platelets, which contain virtually no fibrinogen on their surfaces,273536 or washed platelets incubated with trypsin prior to lysis, still contained fibrinogen. Moreover, the platelet fibrinogen within these extracts, as assessed by Na dodecyl sulfate gel electrophoresis of unreduced (Fig. 1) or reduced samples (Fig. 2), was the same size as “nontabolized” fibrinogen molecules prepared from plasma. The last results also confirm several other studies, indicating that platelet and nascent hepatic fibrinogen do not differ significantly in size.91113

Doolittle et al.9 concluded that platelet and hepatic fibrinogen were identical gene products. This conclusion seems to have been premature, since it was based only on similarities in size, crosslinking behavior, and in the amino acid composition of fibrinopeptides released by thrombin. Ganguly and coworkers16-18 have argued that platelet fibrinogen is produced by a unique genetic mechanism. Their argument was based mainly on observations of significant differences in molecular size. Their findings of size differences are not supported by other studies,1113 including that of Doolittle et al.9 or by our present experiments. Nevertheless, our present results, as well as data from other reports,561316 are consistent with their conclusion regarding the unique genetic origin of platelet fibrinogen.

The fibrinogen Paris I anomaly evidently reflects a genetic mutation involving synthesis of hepatic fibrinogen molecules. The mutant θ-chains that replace 50% or more of the θ-chains found in Paris I plasma fibrinogen19,20 were not detectable in the subject’s platelet fibrinogen. Although not the only plausible explanation, the apparent absence of these chains from the subject’s platelet fibrinogen molecules can be explained by differences in the structural gene(s) controlling synthesis of platelet and plasma fibrinogen θ-chains. This explanation is not unprecedented. Elzinga et al.37 have shown a difference in the amino acid sequence of human platelet actin compared with that derived from cardiac muscle. Storti and Rich38 have provided evidence that chicken brain and muscle actins are coded by different messenger RNAs.
In related experiments, Soria et al.\textsuperscript{14} found that platelet fibrinogen from their subject with a congenital plasma fibrinogen abnormality (fibrinogen Metz) did not express the A\textalpha{} chain charge anomaly characterizing the plasma fibrinogen molecules. If it can be established that the fibrinogen Metz molecules arise via mutation of a structural gene, these results would also support the notion that there are genetic differences between platelet and hepatic fibrinogen.

Among alternative explanations for our findings are the possibilities that the mRNA for \gamma{}-Paris I chains, destined for incorporation into platelet fibrinogen, is unstable, or that \gamma{}-Paris I chains are produced but not incorporated into platelet fibrinogen molecules. Thus, differences in the programming and/or regulation of identical structural genes\textsuperscript{39} could explain our results. Whatever the mechanism may prove to be, our data on the Paris I subject indicate that his platelet and plasma fibrinogen, as least as far as their \gamma{}-chains are concerned, are not assembled from identical gene products. It remains to be shown whether this finding reflects a more general situation regarding hepatic and platelet fibrinogen from normal individuals.

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