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FIBRINOGEN AND PLATELET AGGREGATION.
ROLE OF THE GLYCOPEPTIDIC PART AND OF THE FIBRINOPEPTIDE B.
Description of a new technique of fibrinoglycopeptide isolation.

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SUMMARY :

In order to determine the active groups of the fibrinogen molecule in ADP induced aggregation, various cleavage fragments of fibrinogen were tested on plasma protein-free platelets. An original technique is described for the isolation of fibrinogen glycopeptides. The glycopeptides thus obtained exert an inhibition on platelet aggregation by ADP in the presence of fibrinogen, when incubated previously with the plasma protein free platelets. The carbohydrate fraction seems thus to have an important role on ADP platelet aggregation.

The N. DSK and E fragments are inactive as cofactors of ADP induced aggregation.

It is suggested that the N-terminal part of the Bβ chain does not have an important role in the cofactor activity of fibrinogen. Moreover, the importance of an intact fibrinogen molecule is underlined.

The role of fibrinogen in ADP induced platelet aggregation has ben known for a long time. However, its mechanism is not yet clear, and more particularly, the part of the molecule involved is not determined. Niewiarowski and al. (1) have shown that degradation of fibrinogen by plasmin induces a loss of platelet cofactor activity. Only the early X product remains active. On the other hand, the degradation of the carbohydrate fraction, either by selective oxidation with periodic acid (2), or by neuraminidase treatment (3) induces a loss of activity in platelet aggregation.

These facts demonstrate that the fibrinogen molecule must be intact and that its carbohydrate fraction has an essentiel role in aggregation, either directly, or indirectly, by conferring to fibrinogen a tertiary structure necessary to its activity.

To demonstrate the role of the glucidic fraction, we have isolated with our own technique the glycopeptides of fibrinogen, and then studied their

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effect on platelet aggregation induced by ADP. Furthermore, the platelet aggregation cofactor activity of fibrinogen from acquired dysfibrinogenemia containing a high level of sialic acid was studied. These acquired dysfibrinogenemia are observed in some cases of severe hepatic disease (advanced cirrhosis, hepatoma and severe jaundice). They are characterized by a prolonged plasma clotting time induced by thrombin or reptilase (4, 5). The impairment in fibrin formation is due to a gross defect of fibrin monomer aggregation resulting in the formation of a translucent clot. Two types of biochemical abnormalities of fibrinogen have been described : either an increase in the sialic acid content of the molecule (6, 7) or a degradation leading to a fibrinogen molecule in which the Aα chain is shorter than normal (8). In this study, abnormal fibrinogens rich in sialic acid but without degradation of the Aα chain were used, so as to examine only the role of the glucidic fraction.

MATERIALS AND METHODS

Reagents :

- Human fibrinogen was purified by precipitation from normal plasma and plasma of patients with acquired dysfibrinogenemia, according to the method described by Blomback (9). Thrombin clottability of the purified fibrinogens was 95 %. For platelet aggregation studies, the fibrinogen was dissolved in NaCl solution of variable concentrations adjusted in order to obtain an osmolarity of 300 milliosmoles determined using an Halbmikro osmometer.

- Agarose 2 % : Sepharose 2B purchased from Pharmacia, Pronase from Calbiochem : The enzyme had an activity of 45,000 P.U.K./g.

- Fibrinogen degradation product E was obtained according to Soria and Fabiani procedure (10). N. DSK was prepared according to Blomback and al (11) and kindly supplied by Dr. Hessel (Pr. Blomback Laboratory, Karolinska Institutet, Stockholm).

- Carboxypeptidase B was purchased from Boehringer, Dansyl chloride from Calbiochem and Standard dansyl amino acids from Nutritional Biochemical Corporation.

Methods

- Study of fibrinogens from dysfibrinogenemia associated with liver disease

a) Verification of the integrity of the molecule by sodium dodecyl sulphate polyacrylamide gel electrophoresis of reduced fibrinogen (12). The amount of Aα - Bβ and γ chains was determined using ISCO model 659 gel scanner attached to a model UA-4 absorbance monitor.

b) Neuraminic acid content : the samples were hydrolysed with sulfuric acid for various lengths of time at 100° C, and the neuraminic acid content was determined colorimetrically with thiobarbituric acid (13).

- Isolation of platelets : The platelet gel filtration procedure as described by Levy-Toledano (14) was used to obtain platelets separated from plasma proteins.

- Isolation of the fibrinogen glycopeptides : 50 mg of normal human fibrinogen were dissolved in 5 ml of distilled water and then incubated with 3.5 mg of pronase during 48 hours at 37° C (15). The pH of the reaction was 7.4. The degradation products obtained in these conditions were unclottable by thrombin. The hydrolysate was filtered through a column of Sephadex G 25

(100 cm x 3 cm^2). The elution buffer consisted of 0.15 M. NH_4HCO_3. The optical density of the eluate was recorded at 280 nm, and the eluate was recovered in 3 ml fractions. At the same time, the glycopeptidic part was detected using the resorcinol-HCl method (16). Then the fraction containing the carbohydrate moiety was ultrafiltered using Centriflo membrane cones type CF 25 (Amicon) thus eliminating peptides of more than 25,000 M.W., which might contaminate the glycopeptide part. These operations were repeated 6 times to obtain the glycopeptides corresponding to 300 mg of fibrinogen. The glycopeptide fraction thus isolated was freeze dried. At this stage, several peptides were revealed by the analysis of N-terminal amino acids. A second purification was therefore necessary : the freeze dried peptides were dissolved in 0.3 ml of phosphate buffer (0.01 M - pH 6.5) and further purified by chromatography on a D.E.A.E. cellulose (Whatman D.E. 52 cellulose) column (5 cm x 0.2 cm^2) using successively for elution 5 ml of the following phosphate buffers pH 6.5, of increasing molarity 0.01 M - 0.05 M - 0.125 M - 0.200 M - 0.280 M - 0.360 M 0.400 M. The eluate was run out in fractions of 0.3 ml. The analysis for carboydrates was performed using the resorcinol-HCl procedure on 0.07 ml of each fraction in micro test tubes. The glycopeptide fraction was present in the fraction eluted by the buffer 0.01 M - pH 6.5. The fractions eluted by buffers of higher ionic strenght did not contain carbohydrate and were discarded. In a third step, to eliminate the phosphate, the fraction containing the glycopeptides is passed through a Sephadex G 25 column (10 cm x 0.5 cm^2) using 0.15 M NH_4HCO_3 as elution buffer. The fraction containing the carbohydrate was freeze dried and then submitted to the following controls :

- high voltage electrophoresis : was performed in a CAMAG electrophoresis apparatus on 2043 b Mg 1 Schleicher and Schull paper with pyridine - acetic acid - water (100 : 4 : 896, V/V) pH 6.4 buffer, 110 volts/cm during 20 minutes. For staining, fluorescamine was used (17).

- end group determinations : N terminal amino acids were determined using the dansylation method described by Hartley (18). Positive identification of the dansylated amino acids was obtained through bi-dimensional polyamide chromatography using a new solvent system (19). The C terminal ends were studied by digestion of the peptides with Carboxypeptidase B in 0.2 M sodium Phosphate pH 8.6. The enzyme-substrate **ratio** was 150 U.I. of carboxypeptidase per micromole of substrate. Digestion was stopped by pipetting aliquot volumes into test tubes containing the exact amount of sulfosalicylic acid to obtain a final concentration of 12.5 % (W/V). Test tubes were centrifuged and the supernatants analyzed for free amino acids with the amino acid analyzer.

- amino acid analysis : amino acid analysis was carried out in a Labotron liquimat F analyser after hydrolysis of peptides with constant boiling HCl at 110°C during 22 hours.

- Compared effect of glycopeptides and normal fibrinogen on platelet aggregation : The glycopeptides were dissolved in the Tris-saline buffer used for the gel filtration of platelets. Gel filtered platelet aggregation was investigated by the method described by Levy-Toledano (14). The aggregation was measured photometrically using a Labintec-France aggregometer and recorder. Gel filtered platelets (4 x 10^8 cells/ml) were suspended in Tris-saline buffer. Composition of reaction mixtures we used are described in Table II and Table III Aggregation was determined and results expressed as percentage of aggregation obtained in the presence of fibrinogen and buffer under the same conditions of incubation.

Compared effect of normal and abnormal fibrinogens on platelet aggregation : 0.2 ml gel filtered platelet suspension (4 x 10^8 cells/ml) were added to 0.1 ml Tris-saline buffer and 0.1 ml normal or abnormal fibrinogen solution at various concentrations. The extent of ADP induced platelet aggregation was determined.

Table I : Results of amino acid analysis of isolated glycopeptides

Asp. 1.550 nanomole	Gly. 0.87 nanomole	Leu. 0.06 nanomole
Thr. 0.16	Ala. 0.11	Lys. 0.87
Ser. 0.21	Val. 0.09	Arg. 0.87
Glu. 0.88	Ile. 0.03	

RESULTS

I - Analysis of the glycopeptides

a) Amino - acid analysis : Results are shown in Table I. The stoechiometry is self evident : Asp 2, Glu 1, Gly 1, Lys 1, Arg 1 and glucosamine was present but not quantitatively determined.

b) N-terminal amino acids : Isolated glycopeptides gave after dansylation and hydrolysis three fluorescent spots : dansyl-aspartic acid, dansyl-glycine and ε dansyl-lysine.

c) C terminal amino acids : Carboxypeptidase B released lysine and arginine from the glycopeptide fraction.

d) High voltage electrophoresis : The glycopeptide fractions were separated at pH 6.4 into two distinct spots : one nearly neutral, the other exhibiting anodal migration.

II - Effect on ADP induced platelet aggregation of several cleavage fragments of fibrinogen

It can be seen from Table II that ADP does not aggregate gel filtered platelets in the absence of fibrinogen. The glycopeptide solution is unable to induce platelet aggregation in the presence of ADP. Similarly, N. DSK and fragment E at 1 or 5 mg/ml do not act as cofactors.

Furthermore, (Table III), the glycopeptide solution does not have an inhibitory effect on aggregation in the presence of fibrinogen, when the glycopeptide solution is not preliminarly incubated with the gel filtered platelet suspension.

On the other hand, when the platelet gel suspension is incubated for 10 minutes at room temperature with the glycopeptide solution before the addition of fibrinogen, an inhibitory effect on ADP aggregation in the presence of fibrinogen is noted. The extent of the inhibition seems dependent on the length of incubation of the platelets with the glycopeptides and on the quantity of glycopeptides added.

After incubation with platelet suspension, N. DSK does not exert any significant inhibitory effect on ADP induced aggregation.

Table II : Effect of different fragments of fibrinogen on ADP induced platelet aggregation

REAGENTS TESTED 0.2 ml platelets + 0.1 ml buffer in the presence of :	Aggregation by ADP of gel filtered platelets in the presence of cleavage products of fibrinogen (percentage of aggregation obtained in the presence of fibrinogen)
0.1 ml fibrinogen (5 mg/ml)	100
0.1 ml buffer	<10
0.1 ml glycopeptide (0.5 mg/ml)	<10
(1 mg/ml)	<10
0.1 ml N. DSK (1-5 mg/ml)	<10 - 14
0.1 ml F.D.P.E. (1 mg/ml)	10

+ Mean value of 5 assays

III. - Effect on ADP induced platelet aggregation of abnormal fibrinogens containing high level in sialic acid

For this study, we used normal fibrinogen and abnormal fibrinogens containing a high level of sialic acid and without any degradation of the Aα chain, as it was checked by SDS gel electrophoresis of the 3 dissociated chains of fibrinogen.

When fibrinogen is not degraded, a parallelism is observed (specially with low concentrations) between fibrinogen activity as cofactor action in platelet aggregation and level of sialic acid in the fibrinogen molecule (table IV) : thus, the first abnormal fibrinogen tested contains a level of sialic acid 34 % higher than that of normal fibrinogen ; the aggregation it induces is 25 % or 32 % more important than that obtained by normal fibrinogen at 0.125 or 0.20 mg/ml. The second abnormal fibrinogen tested shows a 69 % increase in sialic acid levels, when compared to the control. This abnormal fibrinogen at 0.125 mg/ml produces a 72 % more platelet aggregation cofactor activity than for the normal fibrinogen used at the same concentration.

DISCUSSION

Human fibrinogen is a glycoprotein of 340,000 molecular weight containing two major glycopeptides. The glycopeptides are present on the Bβ chain and γ chain (20). Removal of sialic acid and destruction of the carbohydrate moiety by selective periodate oxidation induce a loss of activity of the fibrinogen on ADP induced platelet aggregation (2, 3). Furthermore, the plasmin degradation of fibrinogen destroys the action of this protein on platelet aggregation as

Table III : Inhibiting effect of fibrinogen glycopeptides on ADP induced platelet aggregation

MIXTURE	Aggregation by ADP of gel filtered platelets in the presence of fibrinogen and cleavage products of fibrinogen (percentage of aggregation obtained in the presence of buffer and fibrinogen)*
0.2 ml platelets 0.1 ml fibrinogen (5 mg/ml) 0.1 ml of the following reagent	
- Buffer	100
- Glycopeptides (0.5 mg/ml)	75 - 100
(1.0 mg/ml)	75 - 100
- N. DSK (1.0 mg/ml)	100
0.2 ml platelets 0.1 ml of the following reagent (incubation for 10 min before adding 0.1 ml fibrinogen 5 mg/ml)	
- Buffer	100
- Glycopeptides (1.0 mg/ml)	26
- N. DSK (1 mg/ml)	89
0.2 ml platelets 0.1 ml of the following reagent (incubation for 6 min before adding 0.1 ml fibrinogen 5 mg/ml)	
- Buffer	100
- Glycopeptides (0.5 mg/ml)	45

* Mean value of 5 assays

shown by Niewiarowski and al (1). Only the fragment X stage 1 promotes ADP induced platelet aggregation. Thus, Niewiarowski suggested that the amino terminal part of the Bβchain could have a role in platelet aggregation. We therefore compared the action of N. DSK with that of fragment E, because N. DSK, unlike fragment E, of comparable structure, possesses fibrinopeptide B (21, 22).

Table IV : Aggregation cofactor activity of fibrinogens from acquired dysfibrinogenemia containing high level of sialic acid. Comparison with normal fibrinogen

% of sialic acid in the fibrinogen molecule tested	Extent of ADP induced gel filtered platelet aggregation° in the presence of normal or abnormal fibrinogen used at a final concentration (mg/ml) of		
	0.75	0.20	0.125
0.52*	47		
0.55*	54	36	29
0.73**	70	48	36
0.92**	70	-	50

* Normal fibrinogen ; ** fibrinogen from acquired dysfibrinogenemia

° Mean of 3 assays

Both have been shown inactive in platelet aggregation. This leads us to think that the N terminal part of the Bβ chain does not have an important role in the cofactor activity of fibrinogen.

In a second phase, in order to evaluate the role of the glucosidic moiety of fibrinogen, we studied the activity of the isolated glycopeptides of fibrinogen on ADP induced platelet aggregation, and the activity of purified fibrinogens containing various amounts of sialic acid.

I. - Activity of glycopeptides on ADP induced platelet aggregation

Glycopeptides of the fibrinogen molecule were obtained by a personal technique. From N terminal amino acid determinations, it can be concluded that the glycopeptidic fraction contains essentially two peptides. Such a hypothesis is substantiated by the results of high voltage electrophoresis. Results concerning the peptidic nature of these glycoproteins are in good correlation with those obtained by Henschen (24), who has isolated glycopeptides from fibrinogen after tryptic degradation, by affinity chromatography using concanavalin-Sepharose. However, because of the different nature of the proteolytic enzyme used, the size of our glycopeptides is smaller than that of glycopeptides obtained by Henschen.

From their results, and our amino acid analysis, we can assume that the sequence is for the first glycopeptide As(x)-Lys, and for the second Gly-Glu-As(x)-Arg.

We have shown that the isolated glycopeptides have no cofactor aggre-

gating activity on gel filtered platelets in the presence of ADP.

However, the incubation of filtered platelets with glycopeptides induces an inhibition of ADP platelet aggregation in the presence of fibrinogen. Thus, one can consider the hypothesis that glycopeptides might bind to platelet membrane receptors, preventing the later action of fibrinogen. But, it cannot be concluded that this inhibitory effect of the glycopeptides is due to a blockage of the sepcific sites for the biological function of fibrinogen on the platelet membrane.

The inhibiting doses of glycopeptides are very high compared with the quantity of glycopeptides present on fibrinogen, at concentrations used for platelet aggration (10 times more, when one considers the molecular weight of glycopeptides and fibrinogen, as it was shown by Mester and al (15). However, it is possible that intact fibrinogen plays the role of "conformer" faciliting the binding of glycopeptides on the platelet membrane.

In accordance, Niewiarowski and al (1) indicate that the intact fibrinogen molecule is essential for ADP induced platelet aggregation : indeed they have shown that a mixture of the 3 chains of fibrinogen, undegraded but dissociated and carboxymethylated, looses also its aggregation cofactor activity.

II. - Relationship between ADP cofactor activity and sialic acid content of fibrinogen

For this study, we used 2 normal fibrinogens (containing 0.52 and 0.55 % sialic acid) and 2 fibrinogens from acquired dysfibrinogenemia without any degradation of the Aα chain (containing 0.73 and 0.92 % sialic acid).

Our results show that when the content of sialic acid is higher than normal, fibrinogen is more active in ADP induced gel filtered platelet aggregation.

IN CONCLUSION

We can suggest that :

- The tertiary structure of the fibrinogen molecule is essential for its function as cofactor in ADP induced platelet aggregation.

- The carbohydrate fraction seems to play an important role, possibly by binding to the fibrinogen receptor on the platelet membrane.

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