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AORTIC ENDOTHELIAL CELLS IN CULTURE SECRETE GLYCOPROTEINS REACTING WITH BLOOD PLATELETS

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The culture medium of bovine aortic endothelial cells contains proteins which inhibit the aggregation of platelets induced by aortic microfibrils but not by type III collagen. From this medium, fibronectin, thrombospondin and a glycoprotein with MW of 128 Kd (GP 128), similar to a glycoprotein described in a microfibrillar extract from bovine aorta were separated by affinity and ion exchange chromatography. GP 128 was further purified by molecular sieve chromatography on SW 3000 column. GP 128 inhibited the aggregation of platelets by microfibrils. This suggests a role of GP 128 in the platelet/subendothelium interaction.

After a lesion of the endothelial cell layer, the adhesion of platelets to certain fibrillar structures of subendothelium initiates haemostasis and thrombosis and also participates to the atherosclerotic process. These fibrillar structures are collagen and microfibrils. Microfibrils are present just under the endothelial cells and within the elastic lamina (1). Histochemical and ultrastructural studies of collagenase treated rabbit aortae submitted to a blood flow showed an adherence of platelets to microfibrils associated with elastin (1). Microfibrils were thereafter extracted from bovine aortic intima as an insoluble extract which, after dispersion of the molecules by sonication, induced an aggregation of platelets (2). On a biochemical aspect, this material was heterogenous, but polyacrylamide gel electrophoresis (PAGE) revealed one single glycoprotein PAS positive band, with an apparent molecular weight of 128 Kd (GP 128). The insolubility of microfibrils is an impediment in the isolation of this glycoprotein. This study demonstrates the presence of GP 128 in culture media of endothelial cells and describes the procedure applied to its separation from fibronectin and thrombospondin, two other glycoproteins synthetized by endothelial

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cells (3,4) and able to react with the platelet membrane (5,6). The ability of GP 128 to interact with platelets was also studied.

MATERIAL AND METHODS

Analysis of the culture media : bovine aortic endothelial cells were isolated and grown according to Jaffe et al (7) and the culture media were pooled in the presence of protease inhibitors phenylmethanesulfonide-fluoride (2 mM), N-ethylmaleimide (5 mM) and EDTA (tetrasodium salt, 25 mM). In some experiments, the medium was supplemented with L 2,3,4,5 ^{3H} proline (10 uCi/ml) (Amersham, france) ; after incubation at 37°C for 24 h, protease inhibitors were added as above. Proteins were then precipitated with ammonium sulfate at 80 % saturation at 4°C and examined by PAGE following Laemmli (8). Radiolabelled macromolecules were also separated on slab gels and detected after protein transfer on nitrocellulose paper (Trans-Blot transfer medium, Biorad USA) by the method of Towbin et al (9), by counting the radioactivity of nitrocellulose strips in a B-counter (SL 4000 Intertechnique). As control, foetal calf serum was treated in the same conditions.

Purification of GP 128 : GP 128 was purified from culture media by a succession of chromatographies on Gelatin-Sepharose, DEAE and molecular sieve columns. Proteins precipitated by 80 % ammonium sulfate were dissolved in 0.05 M, pH 7;5 Tris/HCl buffer , containing 2 mM N-ethylmaleimide and ε-amminocaproic acid (10 mM). All three chromatographies were performed in this buffer. Affinity chromatography on Gelatin-Sepharose was carried out as described by Vuento and Vaheri (10). The flow-through material was analysed by high performance ion exchange chromatography on a TSK 545 DE column eluted stepwise in the above buffer with successive addition of 0.3 M and 2 M NaCl. GP 128 was further purified by high pressure molecular sieve chromatography (SW 3000) ; the homogeneity of the fraction was verified by electrofocusing (LKB ampholine PAG plates pH 3.5 - 9.5). Protein content was determined according to Lowry et al (11) and aminoacid analysis was achieved on acid hydrolysates of the sample in a Beckman 119 CL aminoacid analyzer following Fauconnet and Richemont (12).

Platelet aggregation : Platelet aggregation and inhibition studies were performed as described by Legrand et al(13) using 10 ug aortic MFs and 2.5 ug type III collagen as inducers and incubating the platelets in plasma with 100 µg of each fraction for inhibition studies.

RESULTS

Bovine aortic endothelial cells, in culture, synthetized proteins which were precipitated from the culture medium by 80 % ammonium sulfate. Analysis of these proteins by PAGE demonstrated the presence of several proteins (Figure 1) particularly : fibronectin, thrombospondin*, and also a band with a MW of 128 Kd. This protein stained with PAS and was resistant to collagenase (gels not shown); it thus behaved similarly to the 128 Kd gly-

* Thrombospondin was identified in a culture medium by PAGE followed by blotting using a polyclonal rabbit antibody directed against platelet thrombospondin. (N. Kieffer, A.T. Nurden, Personnal communication).

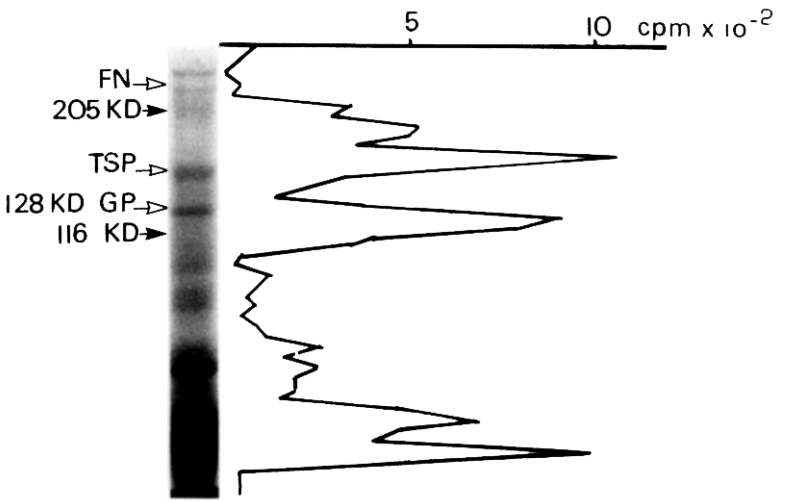


Figure 1 : PAGE of proteins precipitated by 80 % ammonium sulfate

⟶ indicate the migration of fibronectin (FN) thrombospondin (TSP), and the 128 Kd glycoprotein.
⟶ migration of standard proteins : myosin : 205 Kd
B-galactosidase
116 Kd.
The graph corresponds to the radioactivity pattern of ^{3}H proline labelled proteins precipitated from the culture medium.

coprotein already described in a microfibrillar extract from bovine aorta. The absence of this material from foetal calf serum alone must be emphasized (PAGE not shown). Further evidence for its synthesis by endothelial cells was obtained from experiments in which tritiated proline was given to the cells. The analysis of the labelled culture medium by PAGE followed by tranfer on nitrocellulose paper shows (see tracing in Fig. 1) a strong labelling in the migration zone of GP 128. Table 1 shows that proteins precipitated at 80 % ammonium sulfate concentration, which did not induced an aggregation of platelets, inhibited their aggregation by a high dose (10 ug) of microfibrils. The inhibition of microfibrils induced aggregation was total with 80 ug precipitated proteins. On the contrary, they did not affect the aggregation by a small amount of collagen (2.5 µg).

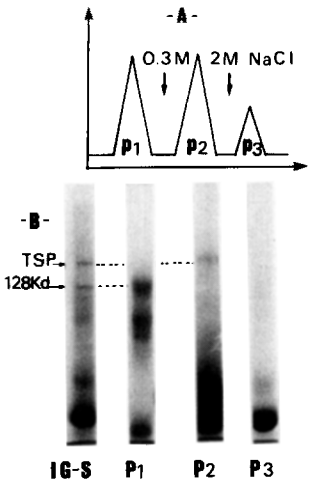
The coexistence of GP 128 with fibronectin and thrombospondin, in the culture medium prompted us to separate these macromolecules. Affinity chromatography on gelatin sepharose first retained fibronectin (not shown). The

TABLE 1
EFFECT OF PROTEINS PRECIPITATED BY 80 % AMMONIUM SULFATE ON MICROFIBRILS AND COLLAGEN INDUCED PLATELET AGGREGATION

	AGGREGATION INDUCED BY 10 µg MICROFIBRILS		AGGREGATION INDUCED BY 2.5 µg COLLAGEN	
	Lag Phase in sec.	Velocity in %	Lag Phase in sec.	Velocity in %
PRP incubated with :				
Saline	60	16.5	102	18
100 µg FCS	60	16.8	102	19
20 µg 80 %	90	7.5	96	17
80 µg 80 %		0	96	17.2

Platelet rich plasma (PRP) was incubated with 20 or 80 % ammonium sulfate precipitate for 3 minutes in an aggregometer and 10 µg of microfibrils or 2.5 µg of collagen were then added. Controls were PRP incubated with 100 ul of saline alone or containing 100 µg foetal calf serum (FCS).

non-retained material (flow through fraction eluted in Tris buffer) contained GP 128 and thrombospondin ; a fibronectin rich fraction was then eluted with 1 M arginine.The non retained material, at a dose of 100 µg, totally inhibited aggregation of platelets by microfibrils whereas fibronectin did not. GP 128 and thrombospondin were then separated from the flow through fractions on a DEAE column eluted by 0.3 M and 2 M NaCl ; this procedure separates neutral from acidic glycoproteins and proteoglycans. As shown on PAGE (Figure 2) GP 128 was not retained on the DEAE column and eluted before addition of NaCl ; thrombospondin was retained and eluted by 0.3 M NaCl. This chromatographic procedure was thus most efficient from their separation. Both GP 128 and thrombospondin rich fractions inhibited to the same extent the aggregation of platelets by microfibrils (Fig. 2). GP 128 was further purified by high pressure chromatography on SW 3000 column : one homogenous peak was obtained which showed one band by isofocusing (Fig. 3), with a pHI of approximately 4.6. Aminoacid analysis of this protein revealed its richness in serine, valine and proline residues and the absence of hydroxyproline and hydroxylysine residues.



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Effect on platelet aggregation by 10 µg aortic MFs

PRP +	Lag phase in sec	Velocity in %
100 µg FCS	90	35
100 µg P1	∞	0
100 µg P2	∞	0
100 µg P3	90	33

Figure 2 : Chromatography on a DEAE column : proteins from the flow-through fraction of gelatin -Sepharose chromatography were injected in a TSK 545 DE column and eluted by increasing stepwise the concentration in NaCl.
A : elution pattern from the DE column
B : PAGE profile of the deposited material (peak 1 Gelatin Sepharose) designated as I-GS, and of the fractions eluted from the DE column (P1, P2, P3).
C : Each fractions (P1, P2, P3, 100 µg) was stirred with platelets rich plasma (PRP) at 37° for 2 minutes before adding 10 µg of microfibrils.
Control is PRP stirred with foetal calf serum (FCS), 100 µg, in the same conditions.

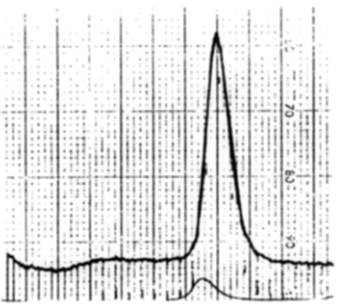
100 ug of purified GP 128 also completely inhibited the aggregation of platelets by microfibrils.

DISCUSSION

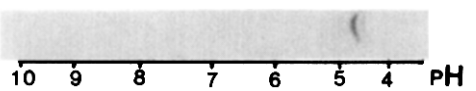
The literature reports on the synthesis of two glycoproteins : fibronectin and thrombospondin by endothelial cells in culture (3,4) and on their reactivity with the platelet membrane (5,6). Endothelial cells also synthetize a soluble 128 Kd glycoprotein (GP 128) which seems similar to an insoluble major constituent of aortic microfibrils (2). In contrast with thrombospondin, GP 128 is not present in serum. Serum thrombospondin can be purified by chromatography on heparin sepharose (14) or hydroxyapatite (4). These techniques were not applied to the separation of tissue thrombospondin from GP 128 because both glycoproteins had similar affinities for these two supports. They were therefore separated by DEAE chromatography. Their inhibitory effect on the aggregation of platelets by aortic microfi-

GP128

MOLECULAR SIEVE CHROMATOGRAPHY ON SW 3000



ELECTROFOCUSING



AMINO ACIDS ANALYSIS

HYP	0	MET	7
ASP	85	ILE	22
THR	83	LEU	82
SER	107	TYR	48
GLU	93	PHE	55
PRO	95	HYL	0
GLY	66	LYS	66
ALA	41	HIS	13
VAL	102	ARG	28
CYS	7		

Figure 3 : Characterization of GP 128
Pic I from the DE column was chromatographed on a molecular sieve (SW 3000) column. The eluted material was analyzed for its amino acid composition and its pHI was determined by isofocalisation, using LKB ampholine PAG plates pH 3.5 - 9.5.

brils suggests that tissue thrombospondin, GP 128 and microfibrils could recognize a commun determinant on the platelet membrane, which is probably different from the determinant(s) involved in the platelet/collagen interaction, because a fraction rich in GP 128 and thrombospondin did not inhibit the aggregation by collagen. An important point is that GP 128 is different from collagen, as shown by the absence of hydroxyproline and hydroxylysine in its amino acid composition and its resistance to collagenase which is mentionned here ; similarly, the insoluble GP 128 described in aortic microfibrils also resisted to collagenase (2) ; GP 128 thus behaves differently from the collagenous GP 128 described by Gibson and Cleary (15) in bovine aortic extract. GP 128 seems more similar to a 130 Kd glycoprotein described by Moczar et al (16) in cultures from intima + media fragments of rabbit aorta.

The data presented in this paper raise different questions concerning the reactivity of GP 128 for platelets : 1°) Is endothelial cell GP 128 a

soluble form of microfibrils GP 128 ? 2°) Does GP 128 aggregate platelets in a particular and still undefined state of polymerization, as it is the case for collagen for which a polymerization of its molecule is a prerequisite for its reactivity with platelets(17).? 3°) Is GP 128 associated with another molecule such as thrombospondin to aggregate platelets ? These questions should be answered in the future.

ACKNOWLEDGEMENT

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