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Interaction of blood platelets with a microfibrillar extract from adult bovine aorta: Requirement for von Willebrand factor

(platelet aggregation/vessel wall/glycoprotein/plasma factor)

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ABSTRACT **Adult bovine aortic tissue was treated with 6 M guanidinium chloride in the presence of proteinase inhibitors to obtain an extract that was essentially devoid of collagenous components and appeared homogeneous by electron microscopy. When this extract was dispersed by sonication it was found to be a very potent inducer of human platelet aggregation. This interaction required the presence of von Willebrand factor and of its receptor (glycoprotein Ib) on platelet membrane. This was demonstrated by the fact that the aggregation of normal blood platelets resuspended in plasmas deficient in von Willebrand factor was significantly diminished as compared to aggregation in control plasma. Moreover, this aggregation was inhibited by a monoclonal antibody, IgG AN51, to platelet glycoprotein Ib. These studies provide direct biochemical evidence for the existence of a thrombogenic constituent of the vessel wall that is noncollagenous and von Willebrand factor-dependent.**

Arterial thrombosis and hemostasis are initiated by the adhesion of blood platelets to certain macromolecules of the subendothelium. A morphological and histochemical study of aortae treated with purified enzymes and exposed to blood flow under the conditions described by Baumgartner and Haudenschild (1) has demonstrated that, in addition to collagen, the microfibrillar component of elastic fibers may represent a second thrombogenic structure in the vessel wall (2). Supporting biochemical evidence has been sought in this study by analysis of extracts of aortic tissue capable of promoting a reaction with blood platelets. An extract of bovine aorta obtained under denaturing conditions known to release "microfibril-related glycoproteins" from elastic fibers of bovine ligamentum nuchae (3, 4) was obtained and partially characterized with respect to its protein and glycoprotein contents and overall amino acid composition. This extract was tested for its ability to induce platelet aggregation in conditions applied to a "microfibrillar extract" of placenta that was found able to aggregate platelets with possible involvement of platelet glycoprotein Ib, as suggested by the role of plasma von Willebrand factor (vWF) (5) and by the inhibitory effect of a monoclonal antibody (IgG AN51) to human platelet glycoprotein Ib. Thus, microfibrillar material present in connective tissues was characterized as able to induce platelet aggregation in a process dependent on the presence of vWF.

MATERIALS AND METHODS

Microfibrillar Material. Adult bovine aortae obtained from Manchester Abbatoirs were freed of adventitial tissue and were finely diced into pieces of 1–2 mm^3. Exhaustive extraction of the tissue with 0.02 M phosphate buffer (pH 7.4) containing 0.05 M NaCl, 0.1 M ε-aminocaproic acid, 2 mM EDTA, 2 mM *N*-ethylmaleimide, 0.05 mM phenylmethylsulfonyl fluoride, and 1% NaN_3 yielded a saline extract. The residue then was extracted twice with 10 vol of 50 mM Tris·HCl buffer (pH 7.4) containing 6 M guanidinium chloride and the above proteinase inhibitors to yield a "G extract." The polypeptide content of this G extract was examined by discontinuous $NaDodSO_4$/polyacrylamide gel electrophoresis (6) by using a 6.5% (wt/vol) separating gel and a 3% (wt/vol) stacking gel. Analyses also were conducted after incubation of G extract for 4 hr at 37°C with 4 units of highly purified bacterial collagenase (collagenase form III, Advance Biofactures, Lynnbrook, NY) per mg of extract in 50 mM Tris·HCl buffer (pH 7.4) containing 5 mM $CaCl_2$, 2 mM *N*-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride. Treatment of G extract with chymotrypsin (Armour Pharmaceutical, U.K.) was carried out in 0.2 M Tris·HCl buffer (pH 7.4) containing 21 mM $CaCl_2$ for 16 hr at 37°C at an enzyme/substrate ratio of 1:400.

Aggregation Studies. Human blood collected by venipuncture, either from healthy volunteers who had not taken any drugs for at least 1 week or from patients with severe vWF deficiency, was anticoagulated with 1 vol of 3.8% (wt/vol) citrate per 9 vol of blood and was centrifuged at 90 × *g* for 20 min at 15°C to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained from PRP by centrifugation at 2,000 × *g* for 20 min at 15°C. In some experiments, normal twice-washed platelets obtained by the method of Patscheke (7) were resuspended in citrated PPP from normal donors or from the patients. The aggregation of platelets was monitored in a Bryston aggregometer at 37°C under continuous stirring at 1,100 rpm with 0.4 ml of platelet suspension. The velocity and intensity of the aggregation were determined by measuring the percentage of aggregation 30 and 120 sec, respectively, after the increase in light transmission (corresponding to the aggregation of the platelets) began to be observed.

The ability of the G extract to promote platelet aggregation was compared with that of type III collagen from calf skin prepared according to Fujii and Kühn (8). The G extract was suspended in water (1 mg/ml) and was dispersed by sonication (10 sec at 4°C at maximal setting with 0.9-cm-diameter probe in MSE Sonicator). Samples in the range of 1.25–20 μg of G extract were added to the platelet suspension (0.4 ml). In control experiments type III collagen was resuspended in water (1 mg/ml) and was treated as described above. In addition, polymerized type III collagen was prepared as follows. Collagen (0.5 mg) was dissolved in 0.1 M Tris·HCl buffer (pH 7.4), kept at 4°C

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Abbreviations: vWF, von Willebrand factor; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

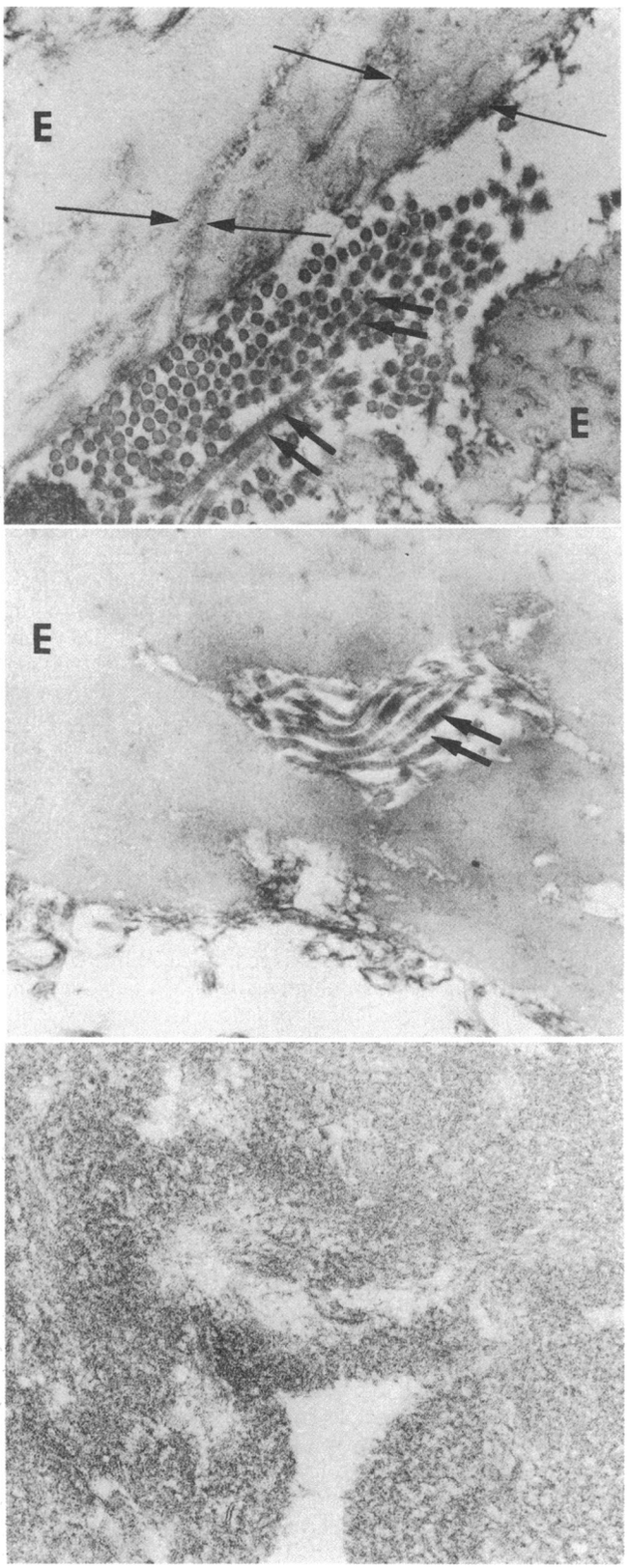

FIG. 1. (*Top*) Nontreated adult bovine aorta. The macromolecular components of the subendothelium visualized are: amorphous elastin (E), collagen fibers (double arrows), microfibrils (single arrows). (×40,000.) (*Middle*) Aorta after the treatment with guanidinium chloride. Collagen fibers (double arrows) are visible. No microfibrils are seen surrounding or in the pockets of elastin (E). (×40,000.) (*Bottom*) Microfibrillar material extracted by guanidinium chloride. (×100,000.)

overnight, and then was polymerized into fibrils by heating at 33°C for 15 min just before use.

Patients. Four patients (B1, B2, P, and Z) presenting with severe von Willebrand disease had bleeding times >15 min. VIII:C, VIII:Ag, and VIII:vWF were measured, respectively, by the cephalin time, the quantitative immunoelectrophoresis described by Laurell (9), and the ristocetin-induced platelet aggregation with either fresh or fixed platelets (10). For each patient these values were found to be below 6%.

Aggregation in the Presence of AN51 Antibody. In this study the purified IgG of monoclonal antibody AN51 to platelet membrane glycoprotein Ib (11) was incubated with PRP under conditions previously described (12). Six micrograms of the sonicated G extract then was added and platelet aggregation was compared to the control without IgG AN51.

RESULTS

Extraction and Characterization of the Microfibrillar Material. The treatment of adult bovine aortic tissue with 6 M guanidinium chloride in the presence of proteinase inhibitors extracted the microfibrillar material surrounding or contained in the pockets of the elastin core (Fig. 1 *Top* and *Middle*) but did not remove the collagen fibers (Fig. 1 *Middle*). This highly insoluble extract (G extract) appeared to be homogeneous under the electron microscope (Fig. 1 *Bottom*). However, gel electrophoretic analysis demonstrated that the G extract was heterogeneous with respect to polypeptide composition (Fig. 2), but staining with periodic acid/Schiff reagent detected only one glycoprotein (apparent M_r 110,000). From this polyacrylamide gel electrophoresis it can be noticed that no polypeptide migrates in the region of interstitial collagen α chains. Accordingly, analyses of the G extract (Table 1) revealed an amino acid

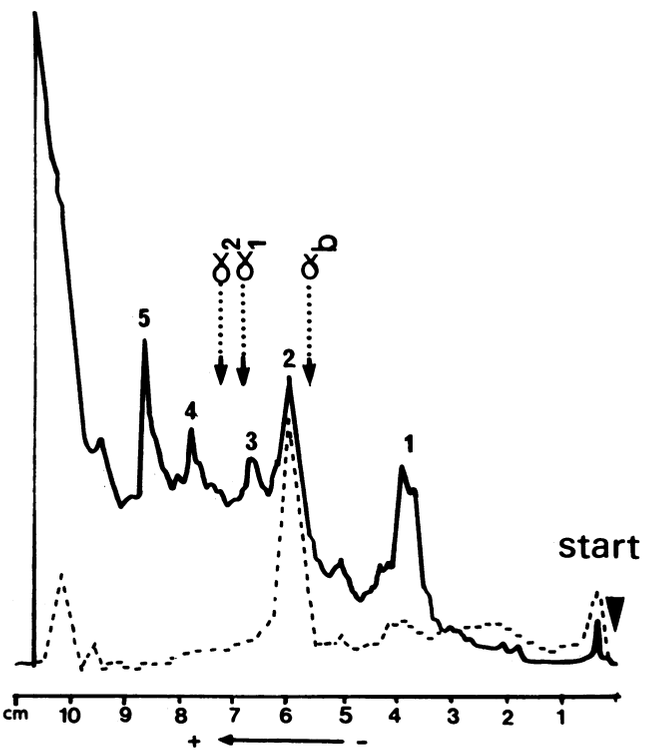

FIG. 2. Electrophoretic analyses of aortic extract. The migration positions of standard collagen chains α1(I), α2(I), and αB(V) that were run concurrently are indicated. Coomassie blue staining (solid line); periodic acid/Schiff staining (broken line); 6.5% separating gel and 3% stacking gel in the presence of 50 mM dithiothreitol.

Table 1. Amino acid analysis of aortic G extract

Amino acid	Residues per 1,000 amino acids
Hyp	7
Asp	81
Thr	60
Ser	66
Glu	137
Pro	60
Gly	97
Ala	80
Cys	12
Val	50
Met	20
Ile	37
Leu	91
Tyr	32
Phe	29
Hyl	Trace
Lys	62
His	19
Arg	59

composition with very low hydroxyproline, hydroxylysine, and glycine contents, as compared to collagen, but a high content of dicarboxylic amino acid residues, demonstrating an overall composition similar to aortic preparations considered to represent "structure glycoproteins" (13). When the G extract was treated with highly purified bacterial collagenase no modification of the electrophoresis profile was observed under conditions in which standard preparations of types I and III collagen were completely digested. In contrast, under conditions in which collagen was not digested by chymotrypsin, the G extract was reduced to only two low molecular weight peptides, as observed by polyacrylamide gel electrophoresis (not shown). A further property that distinguished the G extract from collagenous macromolecules was its marked insolubility in solutions other than dissociative buffers, such as 6 M urea or 6 M guanidinium chloride.

Platelet Aggregation Studies. The ability of the G extract to promote platelet aggregation was examined, but because of its insolubility in mild buffers, the extract was resuspended in water and carefully dispersed by sonication. Under these conditions the G extract was found to be a very potent inducer of platelet aggregation, even when amounts as low as 1.25 μg were used (Table 2). By comparison, type III collagen dispersed in a similar manner was ineffective, even at concentrations 10- to 15-fold higher. However, when type III collagen was first induced to polymerize and to form fibrils, it was found to be effective at concentrations similar to those at which the G extract elicited platelet aggregation, although the G extract always gave rise to a shorter lag phase (Table 2).

Table 2. Comparison of the ability of aortic tissue extracts and type III collagen preparations to promote platelet aggregation in PRP

Preparation	Amount, μg	Aggregation: Lag phase, sec	Aggregation: Velocity, %	Aggregation: Intensity, %
G extract	5	60	47	93.3
	3.5	60	33.3	93.3
	2.5	60	23.1	91.7
	1.25	96	7.2	23
Type III collagen				
Polymerized	2.5	180	34	78
	1.25	180	17.5	55
Sonicated	20	∞	0	0

Table 3. Dependence of platelet aggregation induced by G extract on the presence of vWF

Aggregation condition	Aggregation: Lag phase, sec	Aggregation: Velocity, %	Aggregation: Intensity, %
G extract (5 μg) in			
PRP control	60	47	93.3
PRP patient			
Z	240	4.5	12
B1	∞	0	0
Type III collagen (5 μg) in			
PRP control	80	24.7	83.3
PRP patient			
Z	240	21.2	73.4
B1	240	19.8	74.7
G extract (15 μg) with normal twice-washed platelets resuspended in			
PPP control	74 ± 15*	29.6 ± 2.6*	75.7 ± 6.0*
PPP patient			
P	∞	0	0
B1	60	7.3	24.8
B2	60	6	19.5
Buffer	180	7	25

* Values are shown as mean ± SEM for five experiments.

Platelet Aggregation in the Presence of von Willebrand Plasma. Another important and distinctive feature of the interaction between blood platelets and G extract is the requirement for plasma factors in the aggregation process. Normal washed platelets in the resuspension buffer described by Patscheke (7) can be aggregated by collagen, to the same extent as in plasma, whereas under these conditions no aggregation, or only a weak aggregation, was observed with the G extract (Table 3). This suggested that some component of normal plasma was required to achieve the aggregation. Moreover, the aggregation by the G extract of platelets from patients with severe vWF deficiency was strongly diminished (90–100%), whereas the aggregation induced by type III collagen was not modified, the only difference being a longer lag phase (Table 3). The role of plasma factor was confirmed by the fact that twice-washed normal platelets resuspended in plasma derived from these patients also were abnormally aggregated by the G extract.

Platelet Aggregation in the Presence of AN51 Antibody. AN51 is an antibody directed against the platelet membrane glycoprotein Ib, which is known to be a receptor of vWF (12). When normal platelets were incubated 60 sec with this antibody before the addition of the G extract (Table 4), an inhibition of the aggregation was observed and the degree of inhibition was related to the concentration of antibody. IgG AN51 modified

Table 4. Effect of AN51 antibody on the aggregation of normal platelets in PRP

IgG AN51 final concentration, μg	% of inhibition of control aggregation induced by: G extract, 10 μg/ml	% of inhibition of control aggregation induced by: Type III collagen, 5 μg/ml
0	0	0
1.25	47	0
2.5	75	0
10	100	10

only slightly the aggregation induced by type III collagen at very high concentration (Table 4).

DISCUSSION

Extraction of adult bovine aortic tissue with 6 M guanidinium chloride in the presence of proteinase inhibitors yielded a microfibrillar preparation (G extract) with properties that provide evidence for the occurrence in the vessel wall of a noncollagenous component capable of promoting platelet aggregation. This G extract exhibited biochemical and biological properties different from those observed for collagen. Aortic collagen fibers are very insoluble and were extracted only by pepsinization after reduction and alkylation of the tissue (14). Although the G extract contained very low amounts of hydroxyproline and traces of hydroxylysine, its amino acid composition was quite different from those observed for collagen (Table 1) and presented the characteristics of acidic glycoproteins.

The treatment of the G extract and of type III collagen with collagenase and chymotrypsin confirmed their nonidentity because G extract was resistant to collagenase in conditions in which type III collagen was digested, but the G extract, unlike the collagen, was completely digested by chymotrypsin treatment. Another difference was their solubility because the G extract was insoluble in 0.5 M acetic acid and needed dissociative buffers to achieve solubilization. G extract and collagen also presented differences in their biological activities. Due to its insolubility, G extract must be carefully dispersed by sonication to be a very potent inducer of platelet aggregation. However, collagen must be polymerized into fibers to be active (15) and accordingly, the sonicated type III collagen was inactive towards platelets (Table 2).

An important finding arising from these experiments was the observed role of the vWF in the interaction between blood platelets and the G extract. Thus, the aortic "microfibrillar preparation" was less active or inactive towards twice-washed platelets resuspended in buffer or in plasma from patients with severe vWF deficiency, as compared to resuspension in normal plasma (Table 3). Similar results have been obtained in studies with microfibrils isolated from human placenta (5). This nonreactivity could explain the decreased platelet adhesion in von Willebrand disease. The possibility that vWF may play a role *in vivo* in platelet–matrix interactions also was suggested by the longer lag phase in the aggregation of the platelets from these patients when the inducer was collagen (Table 3). Moreover, platelet aggregation induced by the G extract was inhibited by IgG AN51, a monoclonal antibody to human platelet glycoprotein Ib. The same results have been observed with placental microfibrils and this antibody also inhibited platelet deposition on the collagenase-treated subendothelium of rabbit aorta (12).

The results obtained in this study suggest that the aortic extract contains noncollagenous material that can react with blood platelets in a manner analogous to that reported in the investigations with a microfibrillar protein preparation from the placenta (5). This interaction requires the presence of vWF and confirms that in the vessel wall the microfibrils represent a thrombogenic structure.

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