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Effect of an Aortic Proteoglycan on Platelet Aggregation and Thrombin Time: Plasma Requirement and Active Moieties (39898)[1]

CHUNG-HSIN TS'AO, REUBEN EISENSTEIN, AND BARBARA SCHUMACHER

Department of Pathology, Northwestern University Medical Center, and the Veterans Administration Lakeside Hospital, Chicago, Illinois 60611

Endothelial injury and denudation occur both normally and in many pathologic conditions (1, 2). As a result, the subendothelial region is exposed to circulating blood with resultant activation of platelets and surface-active procoagulants. Thus, a chain reaction is initiated which theoretically should result in the formation and propagation of thrombi. Actually, such a consequence is not indicated by most experimental data. Endothelial denudation alone usually does not result in thrombus formation, but only the adhesion of platelets to the denuded sites (3-5). Although many layers of platelets may clump at a given spot, fibrin strands are rarely present. The extent of fibrin formation and thrombosis may be controlled by factors in blood plasma and platelets. Additional regulators may be present in the vessel wall itself. We describe here studies which demonstrate that a highly purified bovine aortic proteoglycan (PG) affects platelet aggregation and thrombin-induced clotting.

Materials and methods. Reagents. All reagents were analytical grade. Guanidine hydrochloride (GuHCl), cesium chloride (CsCl), and cetylpyridine chloride (CPC) were purchased from Research Plus Laboratories, Denville, New Jersey; soybean trypsin inhibitors (SBTI) and ε-aminocaproic acid (EACA), from Sigma Chemicals, St. Louis, Missouri; and benzamidine hydrochloride, from Eastman Co. Rochester, New York. Trasylol was a generous gift of Dr. G. Schnells, Bayerfabriken, Wuppertal, West Germany. These reagents were used for the PG isolation.

Adenosine diphosphate (disodium salt, ADP), epinephrine (adrenaline-HCl, 1:1000), and human thrombin (Fibrindex) were acquired from Sigma Chemical Co., St. Louis, Missouri; Parke-Davis, Detroit, Michigan; and Ortho Pharmaceutical Co., Raritan, New Jersey, respectively. Collagen was isolated from human skin by the method of Green *et al.* (6). Sodium heparin (Panheparin, 5000 units/ml) was acquired from Abbott Laboratories, North Chicago, Illinois; protamine sulfate, from Upjohn Co., Kalamazoo, Michigan, crystalline papain (16-40 BAEe units/mg), from Sigma, and chondroitinase ABC (minimum 5 units/vial) from Miles Laboratories, Elkhart, Indiana.

Preparation of aortic proteoglycan, platelet-rich and platelet-poor plasmas. The procedure of isolation of aortic proteoglycan (PG) was slightly modified from one previously described (7). Minced inner thirds of freshly slaughtered steer aorta were extracted in 5 vol of 4.0 *M* guanidine hydrochloride (GuHCl) in 0.15 *M* acetate buffer, pH 6.8, for 48 hr at 4°C to which SBTI (10 mg/ml), benzamidine HCl (0.005 *M*), EACA (0.1 *M*), and Trasylol (1 mg/ml) were added. The extract was filtered through glass wool and 0.49 g of CsCl were added per gram of filtrate. It was then centrifuged at 40,000 rpm for 48 hr at 4°C in a Beckman TI 50 rotor. The bottom two-fifths of the gradient was dialyzed against 0.5 M KCl for 24 hr and one-tenth of its volume of 5% CPC was added. The precipitate was washed twice with 0.5 *M* KCl, redissolved in 4.0 *M* GuHCl, and centrifuged at 40,000 rpm for 60 hr at 4°C. The centrifuge tubes were sliced into 10 equal portions or cuts, which were dialyzed separately into 0.15 *M* KCl. Each cut was then precipitated with 10 vol of absolute alcohol. After standing overnight at 4°, the precipitate was dissolved in water and lyophilized. The cuts were designated 1-10 from the top to the bottom of the gradient. Thus, cut 10

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was the densest fraction. Cuts 8 and 9 have been characterized (7) and are highly purified PG, containing dermatan and chondroitin sulfates as their major glycosaminoglycans. Cut 8 was used in the present study.

In some experiments, cut 8 was treated with either papain or chondroitinase. Papain digestion of cut 8 was carried out at 60° overnight in EDTA-cysteine HCl buffer at pH 7.3 (8). Glycosaminoglycans (GAG) were isolated from the digestion mixture by the method of Antonopoulous *et al.* (9). When comparisons were made between PG and GAG, they were on the basis of uronic acid contents measured by the Bitter-Muir method. Chondroitinase digestion was accomplished by incubating 1 mg/ml of cut 8 with 5 units (nominal) of chondroitinase for 30 min at 37°C.

Platelet-rich and platelet-poor plasmas (PRP and PPP) were prepared from citrated whole blood donated by healthy, young volunteers with their consent. Washed platelet suspensions (WPS) were prepared by the method of Rossi (10).

Platelet aggregation and thrombin time studies. Platelet aggregation studies were performed with a Chronolog platelet aggregometer equipped with a recorder (11). An aliquot of 0.02 ml of PG cut 8, dissolved in normal saline, was mixed with 0.33 ml of PRP 60–90 sec prior to the introduction of 0.05 ml of ADP, epinephrine, collagen, or thrombin. The final concentration of aggregating agents varied from experiment to experiment. In general, threshold concentrations of aggregating agents were used to illustrate the effect of PG on platelet aggregation. In other experiments, cut 8 treated with either papain or chondroitinase was used. Appropriate control experiments were done to assess the effect of these two enzymes alone on platelet aggregation. In still other experiments, heparin and/or protamine sulfate, used alone or in combination with cut 8, was mixed with PRP before the addition of aggregating agents.

The effect of PG cut 8 on thrombin time of pooled normal citrated plasma was measured by first mixing plasma with several concentrations of PG. Then 0.2 ml of this mixture was warmed at 37°C for 3 min before the addition of 0.1 ml of thrombin. Two thrombin concentrations were used: One gave a thrombin time of 13–15 sec, the other 55–60 sec for normal plasma. Thrombin time of each sample was determined by a set of two fibrometers (12). In separate experiments 0.5, 1.0, 2.0, or 5.0 units of heparin were incubated with 1 U of chondroitinase or buffer for 30 min at 37°C, and 0.1 ml of each was added to 0.9 ml of pooled normal plasma. Thrombin time was determined.

Results. Details of the isolation procedure of the PG and a preliminary partial characterization have been reported earlier (7). A more detailed characterization is being prepared for publication. PG cut 8 contained 160 μg of uronic acid/mg and heparin contained 2.7 μg of uronic acid/unit. The uronic acid content of GAG isolated from the PG (7) was about 15% greater than of PG, as expected since the PG is 85% GAG. No uronic acid was found in extracted human skin collagen used in the platelet aggregation studies.

Effect of PG on platelet aggregation; requirement of plasma factor. The presence of 50 μg/ml of PG cut 8 in PRP did not affect platelet aggregation induced by ADP, epinephrine and collagen, but inhibited that induced by thrombin. In the latter case, only a small, transient aggregation, which was followed by disaggregation, was observed (Fig. 1). Thrombin (final concentration 0.2 U/ml of reaction mixture) induced aggregation of WPS, and PG cut 8 (50 μg/ml) had no effect on the aggregation. However, in the presence of 5% normal plasma in the reaction mixture, the aggregation of WPS by thrombin was inhibited by cut 8 (Fig. 2).

Comparison of PG and heparin effects; neutralization by protamine sulfate. Heparin also inhibited thrombin-induced platelet aggregation. The extent of inhibition by a given concentration of heparin varied slightly with individual PRP samples. When other conditions such as thrombin concentration remained unchanged, 0.1 U/ml of heparin exerted an inhibiting effect comparable to that of 50 μg/ml of cut 8 (Fig. 3).

Protamine sulfate (PS) neutralized the inhibitory effect of heparin and PG on thrombin-induced platelet aggregation. However, twice as much PS was required to offset the

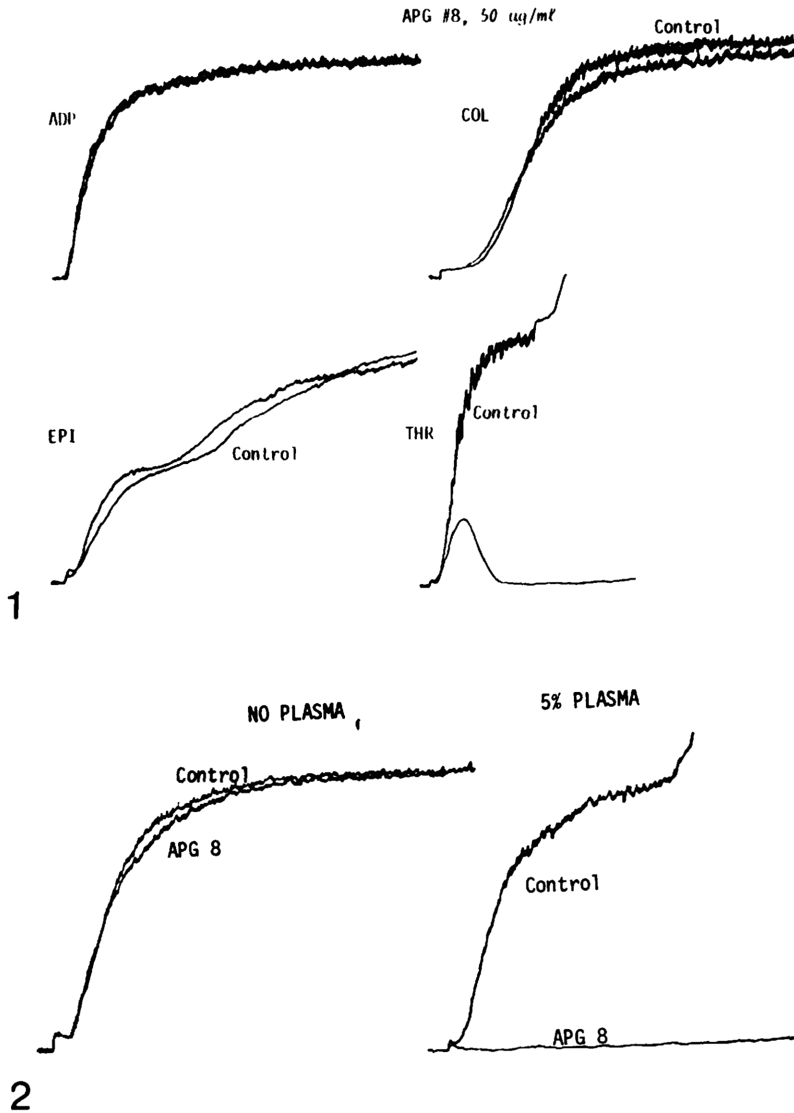

FIG. 1. Light transmission tracings depicting the effect of 50 μg/ml of cut 8 on platelet aggregation induced by adenosine diphosphate (ADP, final concentration 2 μM), epinephrine (2 μM), collagen (1/3200 dilution) and human thrombin (0.2 U/ml). Cut 8 had no effect on platelet aggregation induced by the first three agents, but inhibited thrombin-induced aggregation.

FIG. 2. Thrombin-induced aggregation of washed human platelets illustrating the need for plasma for cut 8 to achieve its inhibitory effect. In the absence of plasma, cut 8 had no effect on aggregation. Cut 8 completely inhibited aggregation in the presence of 5% human plasma. Thrombin concentration 0.2 U/ml.

effect of cut 8 as was required for heparin (Fig. 3).

Effect of papain or chondroitinase digestion on the ability of PG to inhibit thrombin-induced platelet aggregation. Glycosaminoglycans (GAG) isolated from cut 8 by papain digestion had effects on thrombin-induced human platelet aggregation similar to those of the intact PG (Fig. 4). Cut 8 treated with chondroitinase completely lost its inhibitory effect on thrombin-induced aggregation (Fig. 5).

Prolongation of thrombin time by PG. The presence of cut 8 in plasma resulted in a

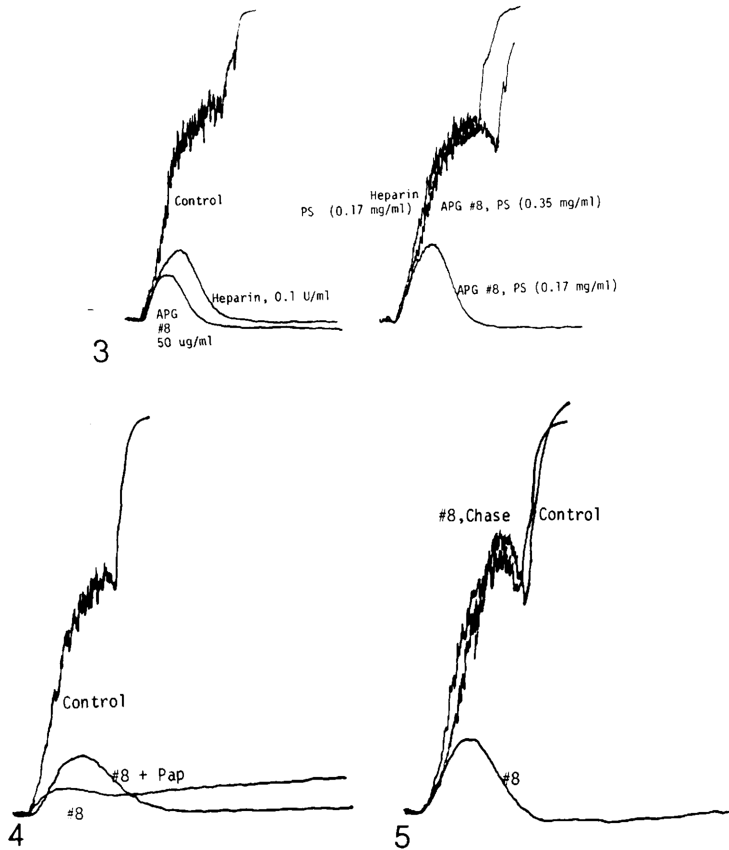

FIG. 3. Comparison of PG and heparin inhibition of thrombin- (0.2 U/ml) induced platelet aggregation and neutralization by protamine sulfate (PS): 0.1 U/ml of heparin exerted inhibition comparable to that of 50 μg/ml of cut 8. However, heparin's inhibition was neutralized by 0.17 mg/ml of PS, but twice as much PS was required to neutralize the inhibition of PG cut 8.

FIG. 4. Effects of glycosaminoglycans (GAG) isolated from cut 8 by papain digestion on thrombin induced platelet aggregation. GAG inhibited thrombin-induced platelet aggregation just as effectively as the intact PG.

FIG. 5. Pretreatment of cut 8 with chondroitinase abolished its ability to inhibit thrombin-induced platelet aggregation.

negligible prolongation of the thrombin time if the concentration of thrombin was adjusted to yield a thrombin time of 13–15 sec for control plasma. However, when the thrombin concentration was reduced to give rise to a 55–60 sec thrombin time for normal plasma, the presence of 10 μg/ml of cut 8 in plasma significantly prolonged the thrombin time (Table I). Cut 8 treated with chondroitinase lost its effect on thrombin time while GAG isolated from cut 8 after papain digestion retained its effect (Table II). Incubation of heparin with chondroitinase did not alter its effects on thrombin time.

Discussion. The anticoagulant activity of vascular GAG is well known, particularly that associated with dermatan sulfate (13–18). These GAG exist in the vascular wall covalently linked to a protein to form a PG. We have demonstrated a mild antithrombic activity of a highly purified PG in which the GAG are largely chondroitin and dermatan sulfates. This activity occurs in both platelet aggregation and the clotting system. Since the antithrombic activity of the PG was not affected after papain hydrolysis, whereas the activity disappeared after chrondroitinase treatment, the entire antithrombic activity of the PG must reside in the GAG. Chondroitinase is a specific enzyme which does not degrade heparin or related molecules such as heparitin sulfate (19). There-

TABLE I. EFFECT OF PG CUT 8 ON THE THROMBIN TIME OF CITRATED NORMAL PLASMA AS DETERMINED WITH TWO CONCENTRATIONS OF HUMAN THROMBIN.

PG #8 μg/ml	Thrombin time, sec
Thrombin concentration 2.70 U/ml	
0	13.9
30	13.4
50	13.9
60	15.4
130	14.9
160	18.7
200	17.9
250	19.3
330	18.4
Thrombin concentration 0.36 U/ml	
0	56.7
6	61.7
10	72.9
15	73.9
33	113.3
60	>180
160	>180

TABLE II. COMPARISON OF EFFECTS OF INTACT PG, ITS ISOLATED GAG (OBTAINED BY PAPAIN DIGESTION) AND ITS PROTEIN (OBTAINED BY CHONDROITINASE DIGESTION) ON THE THROMBIN TIME.

PG #8, μg/ml	Buffer	Chondroitinase	GAG[a]
0	66.8	–	–
16	75.9	64.4	79.1
33	102.7	66.1	128.4

[a] Equilibrated to equal uronic acid concentration with intact PG.

fore, the antithrombic activity of the PG is not due to the presence of heparin or heparitin sulfate.

Here, we showed that a PG, in the natural state in which PG exists in the tissue had no effect on platelet aggregation induced by three physiologic agents, and its antithrombic activity is plasma-dependent. It remains to be investigated whether or not the PG plasma cofactor is identical to the heparin cofactor, antithrombin III. Because the antithrombic activity of PG and its requirement of plasma cofactor are reminiscent of heparin's action, we have attempted to compare PG's action with a commercial heparin preparation. Commercial heparins are not highly purified and vary from batch to batch. With the heparin preparation we used, 0.1 U/ml of heparin exerted inhibitory effects on thrombin-induced platelet aggregation comparable to 50 μg/ml of cut 8. Since 50 μg of PG contained 8 μg of uronic acid, and 0.1 U of heparin contained 0.27 μg, heparin may be viewed as being about 35 times as potent as the PG. However, twice as much protamine sulfate was required to neutralize the effects of cut 8 than was needed to offset heparin's action. This may be a reflection of the charge distribution on the two molecules, or of the recent observation that only a segment of the heparin molecule participates in its antithrombic activity (20).

The aortic PG has been shown to be particularly concentrated in the subendothelial area where it coats collagen (18, 21, 22). It and other arterial PGs are synthesized by at least one cell type, the smooth muscle cell (23). These observations coupled with our findings appear to suggest an admirable engineering design. The arterial wall is constantly under stress and thus likely to suffer from continuous, focal, mild injury. When the injury involves the exposure of subendothelial structures as a result of loss or separation of lining cells, certain elements in the blood, especially platelets and procoagulants, may be activated by connective tissue elements such as collagen. A chain of reactions is thus initiated, reactions which presumably are for the purpose of leading to the repair of the intimal defects. These reactions may also lead to massive platelet clumping and clot formation. So massive a response is not needed if the injury is superficial. Under these circumstances, one only needs to have the injured sites covered by a layer of platelets, so that further interactions between circulating blood and the denuded vessel wall can be prevented and reparative processes can proceed. When a vascular injury involves many layers and the vessel is in danger of bursting, simple platelet clumping may not be sufficient. A solid fibrin clot is now required to stabilize the platelet clump and prevent a catastrophic consequence, even if it may compromise an adequate blood supply to distal organs. A nondiffusible, extracellularly structured macromolecule present in higher concentrations in the intima and in-

ner media than in the outer media and the adventitia, having an antithrombic activity and with little effect on platelet aggregation, might well act as a damper and limit the response of the arterial wall to mild injury. What the biological role of the arrangement of the GAG as part of a larger PG molecule may be is not clarified from these studies.

Summary. A highly purified bovine aortic proteoglycan (PG) prolongs thrombin-induced clotting and inhibits thrombin-induced platelet aggregation, but has no appreciable effect on platelet aggregation induced by ADP, epinephrine, or collagen. Inhibition of thrombin-induced aggregation requires the presence of plasma factors. Glycosaminoglycans (GAG) isolated from the PG contained all the antithrombic activity. Hydrolysis of GAG by treating the PG with chondroitinase abolishes the activity. The antithrombic action of the PG was compared with that of a commercial heparin. Twice as much protamine sulfate was required to neutralize the antithrombic effect of the PG as to neutralize that of heparin. The location of the PG in the arterial wall and its antithrombic activity suggest it plays a role in regulating the response of the circulating blood to vascular injury.

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