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INHIBITION OF PLATELET AGGREGATION BY FIBRONECTIN

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SUMMARY: Fibronectin is a large dimeric glycoprotein found in plasma, on cell surfaces and as a component of the extracellular matrix which has been implicated in a variety of adhesive processes. The role of fibronectin in platelet function has not been clarified. The present investigation demonstrates that an excess of exogenously added fibronectin inhibits platelet aggregation induced by either thrombin or A23187 at a step subsequent to platelet activation and secretion. Similar concentrations of fibrinogen or von Willebrand factor, both of which also bind to the surface of activated platelets, are not inhibitory. These results are consistent with the concept that fibronectin is one of the important mediators of platelet aggregation.

Fibronectin is a large adhesive glycoprotein found in plasma, on cell surfaces and as a component of the extracellular matrix. Several excellent reviews of this important molecule have recently appeared (1-4). Fibronectin has been implicated in a variety of cellular functions including adhesiveness and spreading, motility, control of morphologic phenotype and opsonization. Plasma fibronectin exists as a disulfide-bonded dimer; the cell surface form exists as higher order polymers (5). The diverse functions of fibronectin appear to be mediated by the ability of fibronectin to interact specifically with cell surfaces, proteoglycans and other proteins via several discrete functional domains (4).

While the evidence supporting a role for fibronectin in the biologic processes outlined above as accumulated, the evidence supporting a role for fibronectin in hemostasis has been less well developed. Following the identification of fibronectin as one of the several proteins remaining associated with collagen after collagen-induced platelet aggregation and subsequent lysis of platelets by sonication and detergent treatment, Bensusan and colleagues (6) suggested that fibronectin was the collagen receptor on

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platelets. More definitive studies (7,8) have not supported this proposal. Recent demonstrations that fibronectin is secreted from platelets following activation by thrombin or collagen (9), that fibronectin is not present on the surface of unactivated platelets (7,10,11) but becomes expressed on the surface of activated platelets (10,11) and that platelet activation is accompanied by the development of specific fibronectin binding sites (12) all suggest that fibronectin may play a significant role in the later steps of platelet aggregation.

MATERIALS AND METHODS

Materials - Gelatin and the ionophore A23187 were obtained from Sigma. [^{14}C]serotonin (50mCi/mmol) was purchased from Amersham. Purified human thrombin with a specific activity of 3390 U/mg was generously provided by Dr. Joseph P. Miletich. Sepharose 4B and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia. Fibrinogen (grade L) was obtained from Kabi. Von Willebrand factor was purified from cryoprecipitate as described (13).

Purification of fibronectin - Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose 4B (14). Plasma was made 2mM in PMSF, diluted with an equal volume of 50mM Tris (pH 7.6), 2.5mM EDTA, 0.15M NaCl and applied to a Sepharose 4B column equilibrated with the above buffer to remove material which binds to the Sepharose matrix prior to chromatography on gelatin-Sepharose 4B. Fibronectin was eluted from the gelatin-Sepharose 4B with 4M urea (14).

Platelet aggregation and secretion - Washed platelets were prepared by centrifugation as described earlier (13). For use in the present studies, the platelets were resuspended in 0.14M NaCl, 2.7mM KCl, 12mM $NaHCO_3$, 0.42mM NaH_2PO_4, 0.55mM glucose, 5mM HEPES (pH 7.35), 0.3% bovine serum albumin at a platelet count of 6 x 10^8/ml. Aggregation studies were performed using a Payton dual channel aggregometer. The reaction mixture typically consisted of 150µl of the platelet suspension and 250µl of 0.15M NaCl, 0.05M sodium phosphate (pH 7.4) containing fibronectin or fibronectin domains. After mixing and incubation at 37 degrees for 2 min with stirring at 950 rpm in the reaction cuvette, the sample was made 2mM in $CaCl_2$ and the aggregating agent added. Thrombin was added in a volume of 30µl to give a final concentration of 0.1 U/ml. A23187 dissolved in Me_2SO was added in a volume of 10µl to give a final concentration of 2µM A23187. Serotonin secretion studies were performed as recently described (16) using washed platelets which had been preloaded with [^{14}C]serotonin.

RESULTS AND DISCUSSION

This investigation is based on the underlying assumption that if plasma fibronectin and, by implication, also platelet-secreted fibronectin, are involved in platelet aggregation, the molecule functions to form bridges between platelets by virtue of its divalent structure. When present in a large excess relative to platelet surface components and other platelet-secreted or plasma proteins involved in platelet aggregation one would predict

that fibronectin would become inhibitory in a manner analagous to that in which immune complexes may be soluble under conditions of antibody excess. This condition was achieved by studying platelet aggregation induced by two potent agents, thrombin and the calcium ionophore A23187. Since these agents are each capable of aggregating platelets in the absence of exogenously added proteins such as fibrinogen, it is possible to achieve the desired excess of fibronectin in a simple, well-defined system.

As shown in Fig. 1, under these conditions a progressive inhibition in the extent of platelet aggregation occurs as the concentration of fibronectin is increased. Maximal inhibition is achieved at approximately 500μg/ml of fibronectin. Complete inhibition of aggregation, as reflected by the aggregometer tracings, is not achieved. The extent of inhibition appears to correspond to the loss of the second phase of thrombin-induced aggregation.

It is unlikely that the observed results are attributable to inhibition of thrombin binding, the proteolytic action of thrombin or other early event in

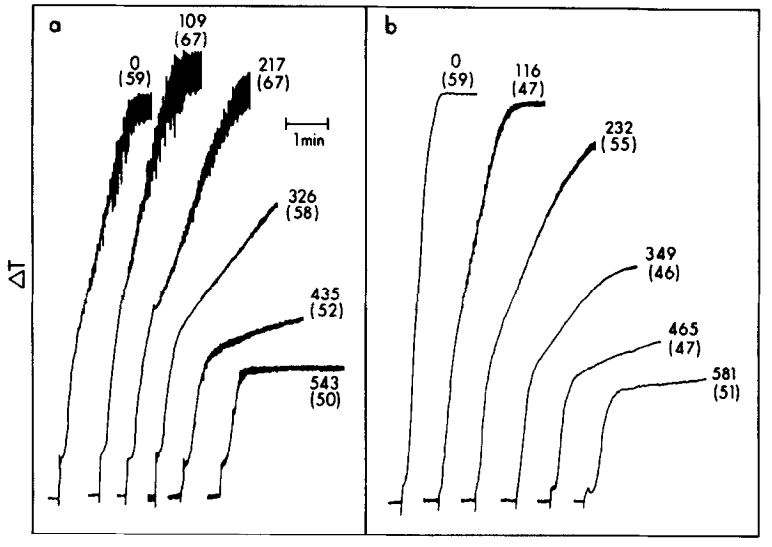

Fig. 1. Inhibition of platelet aggregation by fibronectin. The reaction mixture consisted of 150μl of the washed platelet suspension (6 x 10^8 platelets/ml), and 250μl of 0.05M sodium phosphate (pH 7.4), 0.15M NaCl containing various concentrations of fibronectin. The suspension was made 2mM in $CaCl_2$ and either thrombin (0.1U/ml) or A23187 (2μM) added to initiate aggregation. The uppermost number over each aggregation profile indicates the final concentration (μg/ml) of fibronectin. The number in parentheses indicates the percent of [^{14}C]serotonin secreted from the platelets in response to the stimulus. Aggregation induced by thrombin (a), Aggregation induced by A23187 (b).

the action of thrombin on platelets since A23187-induced aggregation is also effectively inhibited by essentially identical concentrations of fibronectin (Fig. 1). Furthermore, since the secretion of [^{14}C]serotonin induced by both thrombin and A23187 is not markedly inhibited by concentrations of fibronectin which inhibit aggregation (Fig. 1), it is clear that the inhibition of aggregation must occur at a step subsequent to platelet activation and secretion.

Microscopic examination confirmed the inhibition of platelet aggregation by fibronectin. Examination immediately following the aggregation experiment revealed only small platelet aggregates composed of 4-8 platelets. This is consistent with the partial inhibition of aggregation observed using the aggregometer. After prolonged standing even these small aggregates were no longer apparent, although the platelets clearly had undergone the shape change characteristic of activation (17). Thus it appears that fibronectin may play roles in stabilizing small platelet aggregates and in the association of small aggregates which are initially formed into the larger aggregates characteristic of full platelet aggregation.

These findings are not due simply to the presence of a high concentration of a large exogenously added glycoprotein. As shown in Fig. 2, concentrations of fibrinogen or von Willebrand factor similar to those of fibronectin which inhibit platelet aggregation were without effect. Furthermore, the observed inhibition of platelet aggregation by fibronectin is unlikely to arise simply because of steric hindrance at the platelet surface since not only fibronectin (12), but also both fibrinogen (18,19) and von Willebrand factor (20) bind to the surface of activated platelets.

It is interesting to note that the concentration of fibronectin giving maximal inhibition of platelet aggregation in the present investigation is essentially the same concentration at which Plow and Ginsberg (12) observed saturation of the specific fibronectin binding sites on platelets. This observation is consistent with the hypothesis that the inhibition of

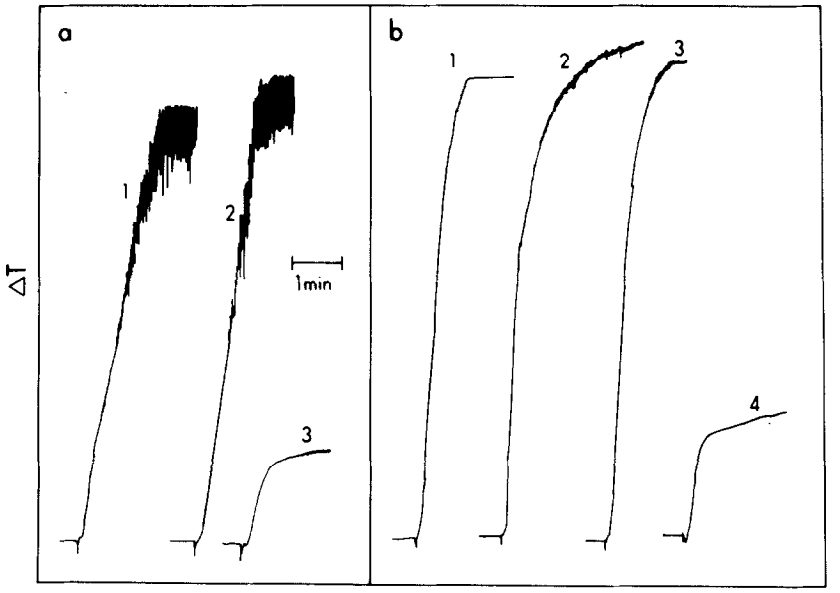

Fig. 2. Specificity of inhibition of platelet aggregation by fibronectin. Experimental conditions were as described in the legend to Fig. 1. Effects on thrombin-induced aggregation are shown in panel a. tracing 1 - aggregation in the absence of exogenously added proteins; tracing 2 - lack of effect of 447μg/ml of von Willebrand factor; tracing 3 - inhibition by 543μg/ml of fibronectin. The effects on A23187 - induced aggregation are shown in panel b. Tracing 1 - aggregation in the absence of exogenous protein; tracing 2 - aggregation in the presence of 581μg/ml fibrinogen; tracing 3 - aggregation in the presence of 479μg/ml von Willebrand factor; tracing 4 - inhibition of aggregation by 581μg/ml fibronectin.

aggregation by high concentrations of fibronectin results from the occupation of each of the fibronectin binding sites by a dimeric fibronectin molecule thus precluding the formation of interplatelet bridges.

The identity of the components with which fibronectin interacts to produce the inhibition of platelet aggregation observed in the present study is not clear. Although fibrinogen would seem a likely candidate, Ginsberg et al (21) have observed the binding of fibronectin to afibrinogenemic platelets and noted that the affinity of fibronectin for fibrin (which is greater than that for fibrinogen) is two orders of magnitude less than the affinity of fibronectin for the platelet. The present contention that fibronectin plays an important role in platelet aggregation is supported by the description of a patient whose plasma was unable to support normal platelet aggregation (22). The abnormality was corrected by the addition of purified fibronectin. We are in agreement with Cohen et al (23) that exogenous fibronectin is not required

for platelet aggregation. Since platelets secrete fibronectin upon activation (9), this is not unexpected. It is probably significant that the proteins now implicated in platelet aggregation, fibrinogen, thrombospondin and fibronectin are all at least divalent. It seems likely that a complex matrix formed by the multiple interactions of these proteins with the platelet surface and with one another gives rise to interactions of sufficient strength to result in the formation of stable platelet aggregates.

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