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Thrombospondin Plays a Role in Platelet–Platelet Recognition during Release-Related Aggregation (41976)

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Abstract. Fixed platelets, bearing covalently bound fibrinogen, participate passively in aggregation of fresh platelets when the aggregation process is release related (G. Agam and A. Livne, Thromb Haemostasis **51**:145–149, 1984). Inhibition of the release by aspirin abolishes the capability of the fresh platelets activated by 10 μM ADP to interact with the fixed platelets. A supernatant fraction from fresh platelets activated by 10 μM ADP (releasate) reconstitutes the interaction. Purified thrombospondin (TSP) replaces the releasate. Moreover, anti-TSP antibodies abolish the reconstituting effect of the releasate. It is concluded that TSP plays a role in the molecular mechanism of platelet–platelet recognition during release-related aggregation.

Macromolecules are recognized as mediators in cell-to-cell attachment (1). For platelet interactions, fibrinogen, thrombospondin (TSP), fibronectin, and von Willebrand factor have been implicated. The role of fibrinogen in platelet aggregation has been established by several studies (2–4). TSP has recently attracted attention as an additional mediator in platelet–platelet interaction during platelet aggregation associated with the release reaction. TSP is an α-granule glycoprotein (5). The endogenous lectin of human platelets was also proven to be an α-granule component (6). TSP has been identified as the endogenous lectin of platelets (7). Its association with the platelet plasma membrane was shown to be Ca^{2+} dependent (8). TSP, released from activated platelets, interacts with fibronectin- or collagen-coated substrata (9). Enzyme-linked immunosorbent assay was used to demonstrate the formation of a complex between purified fibrinogen and TSP (10). It was observed that fibrinogen is the receptor for the endogenous lectin of human platelets (11). Thus, most of the studies which dealt with the role of TSP in the molecular mechanism of aggregation have used stationary systems.

In a previous communication from our laboratory (12) the role of the release reaction in controlling the passive participation of fixed platelets bearing bound fibrinogen (13) was pointed out. It was shown that in contrast with soluble fibrinogen, which binds to the complexed glycoproteins IIb–IIIa regardless of the release reaction, affixed fibrinogen is selectively "recognized" when the fresh platelets have been stimulated to secrete.

The present study utilizes the approach of resolution and reconstitution, namely, a system consisting of fresh platelets at low concentration, supplemented with platelets affixed with fibrinogen, to probe for the role of TSP in platelet aggregation. By this means it is shown that TSP plays a role in the interplatelet interactions during release-related aggregation.

Materials and Methods. Preparation of gel-filtered platelets (GFP), fixed platelets, and acetylsalicylic acid-treated platelets was carried out as previously described (12). The supernatant fraction from activated GFP (releasate) was also obtained according to Agam and Livne (12): GFP (8×10^8–3×10^9/ml) in a Ca^{2+}-, Mg^{2+}-, and albumin-free medium were supplemented with 0.1 mM EDTA and incubated for 10 min at 37°C with ADP under continuous stirring. The platelets were then sedimented by centrifugation for 1 min at 9500 rpm in a Beckman microfuge, the supernatant was collected, kept at 4°C and used within 30 min. The solution derived contained 0.3 mg protein/ml, as determined by the method of Lowry *et al.* (14). Aggregation was quantitated by recording light transmission as described (12).

TSP, prepared in the presence of EDTA (15, 16), and lyophilized in the presence of mannitol, was kindly donated, independently, by Dr. K. Clemetson (Bern) and by Dr.

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L. J. McGregor (Lyon). It was dissolved in water, dialyzed overnight against 10% mannitol in 0.01 *M* Tris–HCl, pH 7.4. The stock solution obtained was 0.5 mg TSP/ml. It was used on the day of completion of dialysis.

Freeze-dried PF_4 was a gift from Dr. J. Dawes (Edinburgh). It was dissolved (0.5 mg/ml) in Tyrode's solution containing 0.26 mg/ml heparin and used on the same day.

Control experiments have shown that neither mannitol nor heparin, at the concentrations introduced when the effect of TSP or PF_4 was studied, had an effect on platelet aggregation. Rabbit anti-human TSP antibodies were prepared (17) by Dr. D. F. Mosher (Madison, Wis.). Control rabbit IgG were obtained from nonimmunized animals. Monoclonal antibodies against TSP (TSP-I-1) and control monoclonal antibodies Fn 18[17] were provided by Dr. M. Ginsberg (La Jolla, Calif.). Protein concentration of all preparations of antibodies was determined by light absorbance (at 1 mg/ml, $A_{280\ nm} = 1.4$).

Results. Modulation of the release reaction during platelet aggregation is possible by changing ADP concentration used for induction, as well as by pretreatment of the cells by aspirin.

Table I shows that aggregation induced by 1 μM ADP (no release) was not augmented by fixed platelets bearing covalently bound fibrinogen (items a, b). Enhancement of aggregation was apparent when 10 μM ADP was used (release takes place, c, d). The importance of the release reaction in the passive participation of the platelets affixed with fibrinogen in aggregation was further supported by the fact that the augmentation of aggregation was abolished by pretreatment of the fresh platelets with aspirin (ASA, e, f). Furthermore, a supernatant fraction from GFP activated by 10 μM ADP ("releasate") reconstituted the participation of the fixed platelets in aggregation (g compared with f). The releasate could be effectively replaced by purified TSP, namely, it enabled the reconstitution of the passive participation of fixed platelets bearing fibrinogen in aggregation of ASA-treated platelets (h compared with f and g). In the absence of fixed platelets TSP also augmented the aggregation of ASA-treated platelets, but to a lesser extent (i). When the release was not hampered, i.e., when untreated GFP were activated by 10 μM ADP, the addition of the same quantity of TSP had no further effect on the augmented aggregation (j compared with d). Purified PF_4,

TABLE I. THE EFFECT OF TSP ON THE PARTICIPATION OF FIXED PLATELETS IN AGGREGATION

GFP	Addition: Fixed platelets bearing fibrinogen	Addition: Solution	ADP (μM)	Aggregation[a] (relative extent)	Enhancement due to addition (% of control)
a. Untreated[a]	−	None	1	7	
b. Untreated	+	None	1	7	0
c. Untreated[b]	−	None	10	17	
d. Untreated	+	None	10	25	40
e. ASA-treated	−	None	10	10	
f. ASA-treated	+	None	10	10	0
g. ASA-treated	+	releasate (57 μg protein)	10	18	80
h. ASA-treated	+	TSP (50 μg)	10	20	100
i. ASA-treated	−	TSP (50 μg)	10	15	50
j. Untreated	+	TSP (50 μg)	10	25	0[c]
k. ASA-treated	+	PF_4 (50 μg)	10	10	0

[a] The assay mixture for the measurement of aggregation in Tyrode medium and in a final volume of 0.45 ml contained 6.6×10^6 GFP or ASA-treated GFP, 100 μg soluble fibrinogen, ADP and TSP as specified, and, where indicated, 6.6×10^6 fixed platelets were added.

[b] With 1 μM ADP no release of [^{14}C]5HT was detected. With 10 μM ADP, 20 ± 10% (range of 10–30%) of incorporated [^{14}C]5HT was released. It was measured as described (22) and modified (23).

[c] Compared with d.

another compound released from platelet α-granules upon activation, when added at a similar molar concentration as TSP, did not substitute for the releasate (k compared with g).

As TSP replaced the releasate and thus appears to play a role in the passive participation of fixed platelets in aggregation, it was expected that anti-TSP antibodies would inhibit aggregation which is release related. This supposition was studied in two systems: (a) ASA-treated GFP supplemented with releasate, and (b) untreated GFP. The effect of the releasate, namely, its ability to permit fixed platelets bearing fibrinogen to participate in aggregation of ASA-treated GFP, was abolished by the addition of rabbit anti-human thrombospondin antibodies (Fig. 1A, trace c compared with b). The antibodies had no effect on the extent of aggregation of ASA-treated platelets (Fig. 1B, trace a superimposed on b). They did not affect aggregation of GFP activated by 1 μM ADP, but decreased the extent of aggregation of GFP induced by 10 μM ADP (Fig. 2). Furthermore, the antibodies abolished the capability

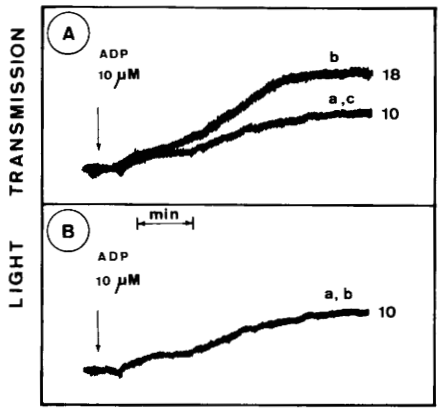

FIG. 1. Participation of fixed platelets bearing fibrinogen in aggregation of ASA-treated GFP: effect of anti-TSP antibodies. The relative extent of aggregation is listed at the right side of the tracing. *Traces:* panel A: (a) ASA-treated GFP (8.8×10^6) + platelets fixed in the presence of 1 mg/ml fibrinogen (6.0×10^6). (b) Same as (a) + 190 μl releasate (added together with ADP). (c) Same as (b) but the releasate was preincubated for 2 min at 23°C with rabbit anti-human TSP antibodies (255 μg protein). Panel B: (a) ASA-treated GFP (8.8×10^6). (b) Same as (a) + rabbit anti-human TSP antibodies (255 μg protein). The values in parentheses are expressed per assay mixture.

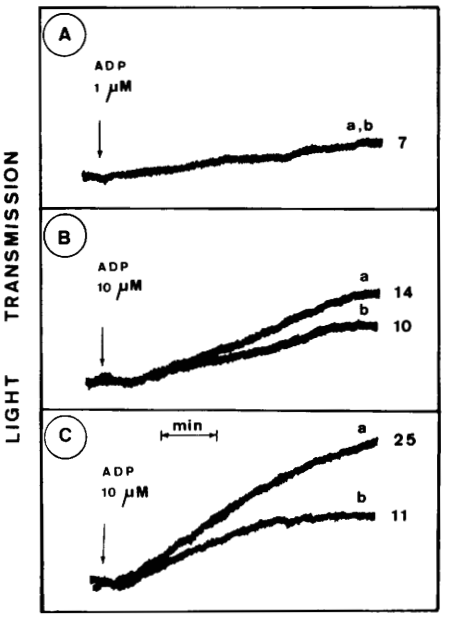

FIG. 2. Effect of anti-TSP antibodies on the aggregation of GFP and on the passive participation of fixed platelets in aggregation of GFP. The relative extent of aggregation is listed at the right side of the tracing. *Traces:* panel A or B: (a) GFP (10^7). (b) GFP (10^7) + rabbit anti-human TSP antibodies (255 μg protein). Panel C: (a) GFP (10^7) + platelets fixed in the presence of 1 mg/ml fibrinogen (6×10^6). (b) Same as (a) + rabbit anti-human TSP antibodies (255 μg protein). The values in parentheses are expressed per assay mixture.

of GFP activated by 10 μM ADP to interact with fixed platelets bearing fibrinogen (Fig. 2C, trace b).

Control experiments to test the specificity of the anti-TSP antibodies showed that IgG from nonimmunized rabbits did not affect ADP-induced aggregation of GFP, or the passive participation of fixed platelets bearing fibrinogen in aggregation.

Monoclonal antibodies against TSP (TSP-I-1) abolished the effect of the releasate similarly to the rabbit anti-human TSP. Another monoclonal antibody, not directed against TSP (Fn 18[17]), was ineffective.

Discussion. In attempts to resolve the molecular mechanism of interplatelet recognition during aggregation an approach of partial resolution and reconstitution of aggregation was utilized in our laboratory (13, 18). This approach and the use of different ADP concentrations to modulate the release reaction

(12) permitted the demonstration of distinct mechanisms of platelet-to-platelet adhesion during aggregation. Several combinations of platelet–platelet bridges formed during aggregation are possible (10, 12, 19): (a) formation of fibrinogen bridge-linking complexes of the glycoproteins IIb–IIIa of adjacent platelets—this probably occurs in aggregation processes which are not accompanied by the release reaction; (b) interaction between released compounds bound to the platelet membrane and fibrinogen; and (c) a combination of (a) and (b) when release takes place. Other possibilities are also feasible.

In the present study the approach of partial resolution and reconstitution of aggregation was used again. It was directed to elucidate whether the thrombospondin participates in the interplatelet recognition that is release related. Two lines of evidence that TSP does function under the conditions employed are presented: (a) TSP purified in the presence of EDTA and added to ASA-treated platelets restored the capability of these platelets to "recognize" fixed platelets bearing covalently bound fibrinogen. In this respect TSP fully replaced the releasate obtained from fresh GFP activated by 10 μM ADP in the presence of EDTA. The use of EDTA in both preparations favors the assumption that the structure of TSP obtained is equal (15). (b) Anti-TSP antibodies, both polyclonal from rabbits and monoclonal from mice, totally abolished the effect of the releasate.

The role of TSP in platelet aggregation was already implicated by different experimental approaches of other laboratories: Leung and Nachman (10) have used purified TSP and showed its complex formation with fibrinogen by ELISA assay. Gartner *et al.* (20) and Nurden *et al.* (21) studied the effect of anti-TSP antibodies on two different activities of activated platelets—the hemagglutination activity and the aggregation capability. From the reports cited, as well as the data presented here, it may be concluded that TSP does play a role in platelet aggregation. It is further concluded that only when platelet aggregation is accompanied by the release reaction, does TSP participate in the interaction between the cells. Interactions between platelets and TSP (8), TSP and fibrinogen (10), as well as platelets and fibrinogen (2, 4) are well established. It is therefore possible that released TSP, bound to the platelet membrane, associates with fibrinogen that is bound to an adjacent platelet. It may also be that TSP serves as a stabilizing molecule for the bridge of fibrinogen that links two platelets.

The modulation theory proposed for cell adhesion (1) asserts that tissues will have only a few cell–cell adhesion molecules (CAMs). Alterations in the temporal expression of these molecules by local surface modulation or by chemical alterations will result in a change in their binding properties. In this respect TSP may be regarded as one of the CAMs which modulate interplatelet interaction during aggregation.

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