Thrombosis and Haemostasis – © F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 59 (3) 504–506 (1988)

Resolution and Reconstitution of Interplatelet Recognition During Aggregation

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Key words

Platelet aggregation – Fibrinogen – Thrombospondin

Summary

In previous studies it was shown that fixed platelets bearing covalently-bound fibrinogen participate passively in release-related aggregation, and that thrombospondin is the released compound which specifically and selectively recognizes the affixed fibrinogen. The present study demonstrates that the phenomenon of passive participation is also obtained with fixed platelets bearing covalently-bound thrombospondin. Moreover, a full resolution and reconstitution of interplatelet recognition during aggregation was obtained with two different systems: (a) fixed platelets bearing affixed fibrinogen were caused to aggregate when stirred and supplemented with soluble thrombospondin; (b) fixed platelets bearing fibrinogen and fixed platelets bearing thrombospondin, each uncapable of undergoing aggregation, aggregated when combined and stirred. It is concluded that fibrinogen and thrombospondin play a major role in the molecular mechanism of interplatelet recognition during aggregation.

Introduction

Preceeding reports from our laboratory have demonstrated that activated releasing platelets recognize fixed platelets bearing covalently bound fibrinogen. The recognition was assayed both as aggregation and binding processes (1–3). Among the glycoproteins released from the activated platelets, thrombospondin was proven to be responsible for the recognition of the affixed fibrinogen (4). These results have raised the possibility that full resolution and reconstitution of interplatelet recognition during release-related aggregation might be achieved with fibrinogen and thrombospondin in their soluble form or attached to platelets. It is envisaged that fibrinogen and thrombospondin function in platelet aggregation as cell-cell adhesion molecules, CAMs, as defined by Edelman (5). The reconstitution afforded by affixed fibrinogen and soluble or affixed thrombospondin, as demonstrated in the present study, indeed supports this supposition.

Materials and Methods

Materials

Fibrinogen (human, 97% clottable) was from Cutter, Berkeley, CA; albumin (bovine) from Hyland, Costa Mesa, CA. The Ca^{2+} ionophore A 23187 was from Calbiochem Behring, La Jolla, CA. Sepharose 2B was from Pharmacia Fine Chemicals, Uppsala, Sweden; formaldehyde (36–38%) from Frutarom, Haifa. Highly purified human thrombin (99.5%) was obtained from Dr. J. W. Fenton II; thrombospondin, prepared in the presence of EDTA (6, 7), and lyophilized in the presence of mannitol, was kindly donated, independently, by Dr. K. Clemetson (Bern) and by Dr. L. J. McGregor (Lyon). It was dissolved in water, dialyzed for 17 h against 10% mannitol in 0.01 M Tris-HCl, pH 7.4. The stock solution obtained was 0.5 mg thrombospondin/ml. It was used on the day of completion of dialysis.

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Preparation of Platelet Suspension

Blood was collected into acid citrate dextrose. Platelet-rich plasma was obtained by centrifugation at 120 × g for 10 min. Platelets were separated by gel filtration as follows: first, the concentration of platelets in platelet-rich plasma was increased 3-fold following centrifugation at 200 × g for 10 min so that the platelets did not pack into a pellet; then 1 ml of concentrated platelet-rich plasma was passed through a Sepharose 2B column (0.8 × 12 cm). The column was equilibrated with Ca^{2+}- and Mg^{2+}-free Tyrode solution (modified, ref. 3) supplemented with 20 mM Hepes-Tris pH 7.4, and 3.6 mg/ml albumin. Platelets were eluted with the same solution.

Preparation of Fixed Platelets

Platelets to be fixed were gel-filtered with columns equilibrated with Ca^{2+}-, Mg^{2+}- and albumin-free Tyrode solution, and eluted with the same solution. Fibrinogen or thrombospondin were added to the suspension of gel-filtered platelets (GFP) at final concentrations of 1.1 and 0.3 μM, respectively. Ca^{2+} was also added to a final concentration of 2.5 mM. Then the suspensions were treated with 2% formaldehyde for 1 h at 23° C, and subsequently washed three times with Ca^{2+}- and Mg^{2+}-free Tyrode solution containing 20 mM Hepes-Tris, pH 7.4.

Assay of Aggregation

Platelets were assayed at low density of about 5×10^7/ml. Aggregation was quantitated by recording light transmission through a stirred platelet suspension in a cuvette of an aggregometer (Chronolog, Model 430, Havertown, PA) at 37° C in a final volume of 0.45 ml. To enable sensitive measurement of the low platelet density, the aggregometer was adjusted to produce a full-scale deflection for platelet count differences of 10^7/ml. The extent of aggregation was defined as the change in light transmission recorded, in relative units, within 5 min after the addition of the inducer. Under these conditions, a linear relationship between platelet concentration and the extent of aggregation was apparent (1). When mixtures of fresh and fixed platelets were assayed, their concentrations were kept equal, unless otherwise stated.

Results

Participation of Fixed Platelets Bearing Covalently Bound Thrombospondin in Aggregation

Formaldehyde-fixed platelets are metabolically inactive, yet they exhibit surface properties. Accordingly, Fig. 1 shows that fixed platelets bearing covalently-bound thrombospondin do not aggregate by themselves in response to thrombin (trace a), but do enhance aggregation of activated fresh platelets (c and d versus b). The enhancement is discernible by the extent and amplitude of the aggregation traces. The fixed platelets bearing thrombospondin were added in concentrations that are close to those of the activated fresh platelets. Under these conditions, the augmentation of aggregation is dose dependent (c versus d).

Reconstitution of Aggregation by Combining Platelets Affixed with Fibrinogen and Platelets Affixed with TSP

Fig. 2 depicts that the recognition between affixed fibrinogen and affixed thrombospondin enables platelet-platelet interaction

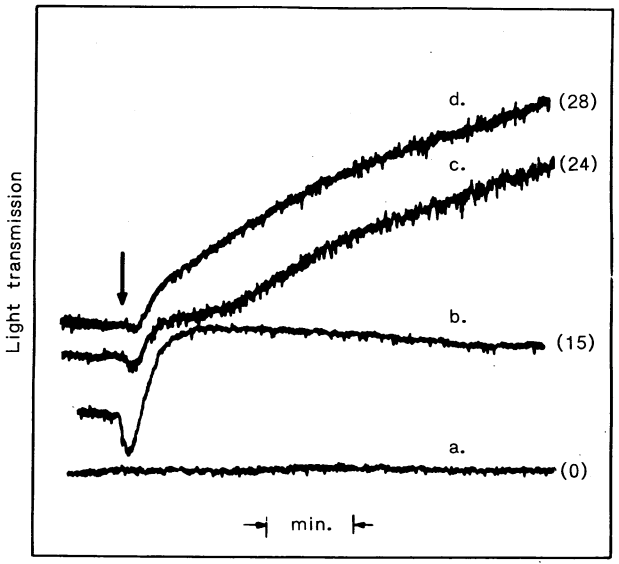

Fig. 1 Fixed platelets bearing covalently bound thrombospondin participate passively in aggregation. The relative extent of aggregation is presented in parentheses. The arrow denotes the addition of thrombin (0.06 u). Traces: a. Fixed platelets bearing thrombospondin (2.0×10^7). b. Fresh platelets (2.2×10^7). c. Fresh platelets (2.2×10^7) + fixed platelets bearing thrombospondin (1.1×10^7). d. Fresh platelets (2.2×10^7) + fixed platelets bearing thrombospondin (2.0×10^7). A representative experiment of three exhibiting an identical pattern

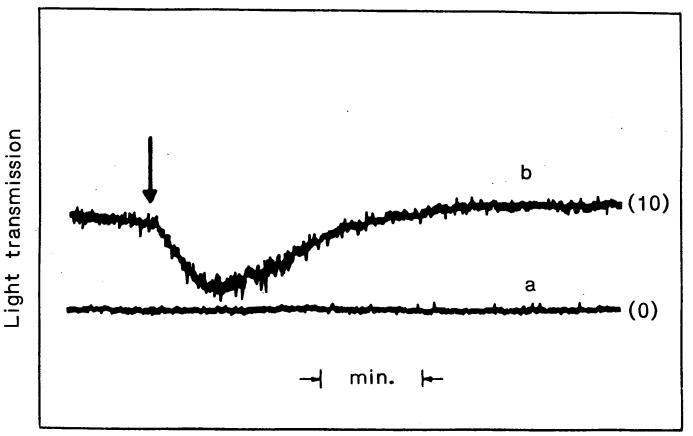

Fig. 3 Reconstitution of aggregation by combining two entities – fixed platelets bearing affixed fibrinogen and soluble thrombospondin. The arrow denotes the beginning of stirring. Traces: a. Fixed platelets bearing covalently bound fibrinogen (2.8×10^7). b. a + soluble thrombospondin (50 μg). A representative experiment of five exhibiting an identical pattern

leading to aggregation. Neither fixed platelets bearing covalently bound fibrinogen, nor fixed platelets bearing covalently bound thrombospondin are capable of undergoing aggregation (traces a, b). Furthermore, fixed platelets bearing thrombospondin, supplemented with soluble fibrinogen, do not aggregate either (trace c). However, combining the two types of fixed platelets – those bearing fibrinogen with those bearing thrombospondin – results in reconstitution of aggregation (trace d). Notably, no inducer was introduced, whereas stirring was indispensible to maintain a constant flow system.

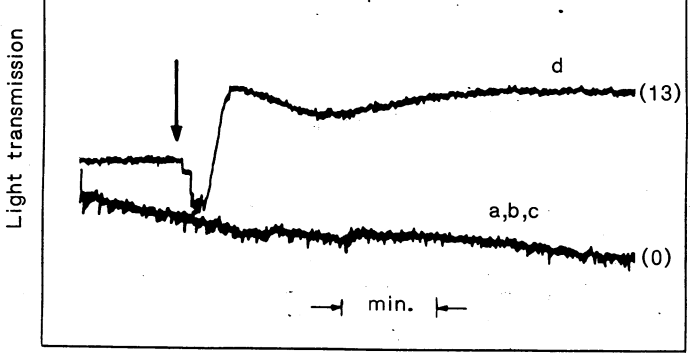

Fig. 2 Reconstitution of aggregation by combining two types of fixed platelets, one bearing covalently bound fibrinogen, the other bearing crosslinked thrombospondin. The arrow denotes the beginning of stirring. Traces: a. Fixed platelets bearing fibrinogen (3.7×10^7). b. Fixed platelets bearing thrombospondin (5.2×10^7). c. b + soluble fibrinogen (1 mg). d. a + b. A representative experiment of five exhibiting an identical pattern

Recognition Between Affixed Fibrinogen and Soluble Thrombospondin Reconstitutes Aggregation

The results presented in Figs. 1 and 2 demonstrate that affixed thrombospondin recognizes affixed fibrinogen (or vice versa), but soluble fibrinogen is not recognized. In contrast, Fig. 3 shows that soluble thrombospondin is recognized by affixed fibrinogen. Namely, as was previously shown (1), fixed platelets bearing covalently bound fibrinogen do not aggregate by themselves (trace a). However, addition of soluble thrombospondin causes aggregation. Aggregation was not only evidenced by the aggregometer tracings; appearance of platelet aggregates was discernible visually in the cuvette. Aggregates were not visible when thrombospondin was not added. Thrombospondin itself did not aggregate either.

In certain traces in Figs. 2 and 3 depicting aggregation a transient decrease in light transmission was apparent, although fresh platelets were absent. This decrease does not reflect shape change that is typical to activated platelets. Rather, it may indicate that the process of recognition proceeds in more than one stage.

Discussion

It is well established that fibrinogen is indispensible for platelet aggregation, its binding to activated platelets being a prerequisite for platelet aggregation (8, 9). Thrombospondin is also recognized as a mediator in platelet-platelet interaction during release-related aggregation (10, 11). Recognition of fibrinogen by thrombospondin has been demonstrated in cell-free systems (12, 13), as well as during platelet aggregation (4, 14).

In the present work two systems of full resolution and reconstitution of interplatelet recognition are demonstrated, both involving fibrinogen and thrombospondin. The first system is comprised of two types of fixed cells – platelets affixed with fibrinogen and platelets affixed with thrombospondin. This result points at a direct linkage that is produced between these two glycoproteins while each of them is attached to platelets, under

conditions of a flow system (in the blood stream) or in a stirred solution (in the aggregometer). The fixation of fibrinogen and thrombospondin is covalent and not physiological. It would not, therefore, depend upon the state of the platelets – resting or activated. However, the product of the fixation – platelets carrying covalently bound fibrinogen or thrombospondin – serves as a model for the interplatelet interaction. Although the molecules in the in vitro system are affixed onto the platelets by formaldehyde, a treatment which probably diminishes flexibility and capability of free coordination changes, an association is being created.

In the second system, recognition, expressed by aggregation, is achieved with soluble thrombospondin and platelets affixed with fibrinogen. With this combination two possible mechanisms of attachment are conceivable: thrombospondin might create a bridge linking two (or more) affixed fibrinogen molecules, or, thrombospondin might serve as a stabilizer of a direct, but unstable, attachment of two fibrinogen molecules. The opposite combination – a mixture of platelets affixed with thrombospondin and soluble fibrinogen – failed to reconstitute aggregation. Thus, it turns out that fibrinogen cannot create a bridge between two (or more) affixed thrombospondin molecules, nor can it stabilize a direct unstable thrombospondin-thrombospondin interaction, if it exists. The inability of soluble fibrinogen to support aggregation of thrombospondin-bearing fixed platelets may be explained by an alternative mechanism: fibrinogen binding to surface-bound thrombospondin occurred, but because fibrinogen was present in excess, no cross-linking and aggregation was observed. Elucidation of this possibility requires further experimentation.

In previous publications we have demonstrated that platelets affixed with fibrinogen are recognized only by releasing activated platelets (1, 3), the released compound responsible for the recognition being thrombospondin (4). In the absence of the release reaction, the molecular mechanism of interplatelet recognition probably involves primarily fibrinogen and its membranal binding sites – the complexed glycoproteins GP IIb–GP IIIa (15–17). It is believed that under these conditions fibrinogen, as a symmetric molecule, bridges between two adjacent platelets. Hence, only soluble, native fibrinogen is recognized by GP IIb–IIIa. On the other hand, the apparently limited flexibility of the affixed fibrinogen, caused by covalent binding onto the platelets, does not interfere with its recognition by thrombospondin, the latter being either in its soluble form or covalently bound to fixed platelets. Alternatively, it might also be suggested that the configuration of the affixed fibrinogen fits the demands for binding to thrombospondin.

Concerning the molecular state of thrombospondin, it was previously suggested by Gartner et al. (18) and Booth et al. (19) that for its haemagglutination activity thrombospondin must become part of a large complex, either by binding to the platelet surface or by becoming polimeric (or "aggregated") in solution. Platelet thrombospondin purified in the presence of calcium was shown to exist in oligomeric state (20). Both calcium- and EDTA-treated platelet thrombospondin have the same haemagglutination activity (21). Therefore, it is conceivable that the EDTA-treated thrombospondin material used in the present work was also polymeric. Thus, for the recognition of affixed fibrinogen the polymeric state of thrombospondin is functional.

In conclusion, this work presents further support that the molecular mechanism of interplatelet interaction during release-related aggregation involves both fibrinogen and thrombospondin.

Acknowledgement

We thank Drs. K. Clemetson and J. L. McGregor for the supply of thrombospondin. We are gratefull to Dr. P. Clezardin for his fruitful information and discussion concerning the thrombospondin state of aggregation.

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Received June 2, 1987 Accepted after revision February 24, 1988