EXPRESSION OF THROMBIN-ENHANCED PLATELET LECTIN ACTIVITY IS CONTROLLED BY SECRETION

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1. Introduction

Recently, we demonstrated that thrombin-activated human platelets express a hemagglutination activity not expressed by non-activated platelets and that this activity mediates the direct platelet-platelet interactions which cause platelet aggregation [1]. Although isolated membranes were found to contain the thrombin-enhanced hemagglutinin [2], the control of its expression was not elucidated. In this study, we report that the expression of the agglutinin is secretion-dependent, caused by secreted materials, and can express itself as both a platelet membrane-bound entity and a soluble agglutinin, and that the expression of the soluble agglutinin is dependent on fibrin formation.

2. Materials and methods

2.1. *Preparation of platelets*

Blood was drawn in ACD (8.6 ml of blood per 1.4 ml of acid citrate which contained 2.5 g of Na_3-citrate·$2H_2O$, 2 g of glucose and 1.5 g of citric acid per 100 ml of H_2O). The platelets were washed as previously described [3].

2.2. *Hemagglutination assays*

The erythrocytes were prepared and the microtiter plate hemagglutination assays were done as previously described [2]. Platelets were at 10^9/ml. The assays using platelets (membrane-bound agglutinin) were done in phosphate buffer saline (0.154 M NaCl, 0.01 M $NaPO_4$) at pH 7.0. The assays using cell-free supernatant fractions were done in TCS (0.154 M NaCl, 0.01 M Tris, 0.002 M $CaCl_2$) at pH 7.4. The washed platelets were suspended in ETS (0.001 M EDTA, 0.01 M Tris, 0.154 M NaCl, 0.2% glucose, pH 7.6) for testing.

3. Results

3.1. *Secretion dependence*

Thrombin-activated platelets were shown to have a greater hemagglutination activity than non-activated washed platelets [1]. As a means of determining if this increased activity was soluble or membrane-bound, activated platelets were removed from suspension by centrifugation and the isolated platelets and supernatant fractions were tested for agglutinin activity. Fig.1 shows that resuspended thrombin-activated platelets had more activity than the unstimulated platelets, but the fraction with the highest specific activity was the cell-free supernatant fraction from the thrombin-activated platelets. Treatment of platelets with prostaglandin E_1(PGE_1), a potent inhibitor of platelet secretion [4], before exposure to thrombin prevented the expression of both platelet bound and the soluble agglutinins (fig.1). Addition of PGE_1 to thrombin-activated platelets and the supernatant fractions from thrombin-activated platelets did not inhibit their agglutinin activities (data not shown). Therefore, since an agglutinin activity appears as a soluble product following platelet activation and since the appearance of all enhanced activity is inhibited by an inhibitor of secretion, it seems that the agglutinin activities of thrombin-activated platelets are derived from secreted materials.

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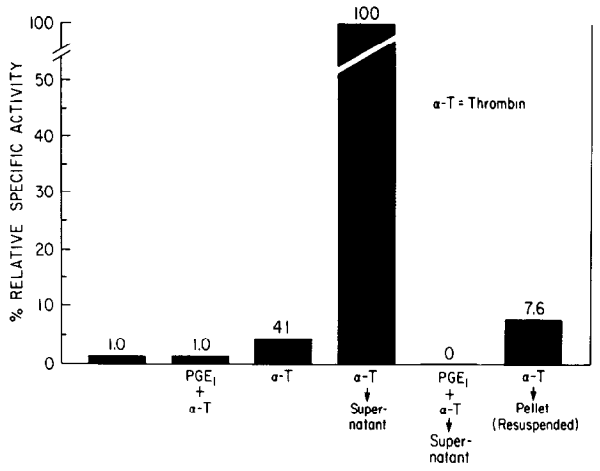

Fig.1. Washed platelets in ETS, untreated or treated as described, were tested for hemagglutination activity. Platelet-free supernatant fractions were prepared as follows: Aliquots of platelet suspensions in ETS were spun for 30 s in a Beckman Microfuge B; the resulting supernatant fractions were spun for another min; 25 μl samples of these supernatant fractions were tested for hemagglutination activity. Platelets were suspended in the original volume of ETS if they were tested following centrifugation. PGE_1 (Upjohn Company) was used at a final concentration of 10 μM.

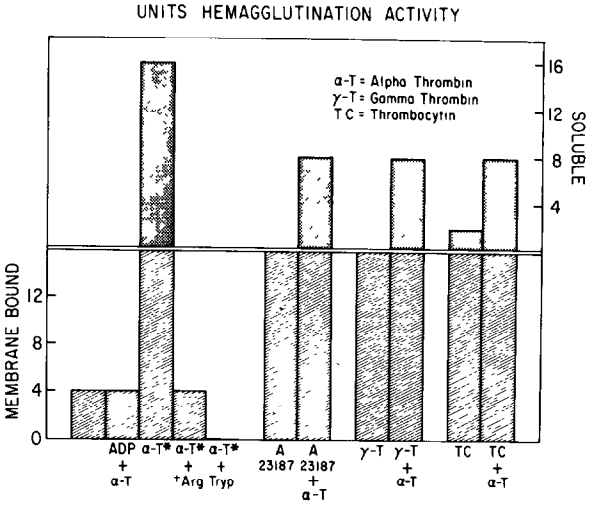

Fig.2. Washed platelets were in ETS unless otherwise stated. The platelets were tested directly or after the treatments described. Washed platelets treated with ADP were in Tyrode buffer. *Washed platelets were exposed to 0.5 U/ml of human thrombin (a generous gift of Dr John Fenton) for 1 min before testing or fractionation. Thrombin-activated platelets and the cell-free supernatant fraction of the activated platelets were treated with 20 μg/ml final concentration of trypsin and incubated at room temperature for 15 min, the reaction was halted by 200 μg/ml (final concentration) of soy bean trypsin inhibitor. Controls were treated with a trypsin preparation previously incubated with a soy bean trypsin inhibitor. Washed platelets were activated with 5 μM ADP, or a 2 μM concentration of A23187, or 5 μg/ml of γ-thrombin (a generous gift of Dr John Fenton), or 5 μg/ml of thrombocytin (a generous gift of Dr Stefan Niewiarowski). The cell-free supernatant fractions were derived from duplicate samples of these preparations and were activated with 0.5 U/ml of thrombin. Cell-free supernatant fractions from non-stimulated platelets occasionally had slight agglutination activity, however, in most cases these fractions had no activity. †All enhanced hemagglutination activities were inhibited by 30 mM arginine, galactosamine, glucosamine, mannosamine, but not by glutamine, galactose, glucose, mannose, and the *N*-acetylated derivatives of the amino sugars at the same concentration.

3.2. *Soluble agglutinin activity*

Washed platelets were activated with three other stimuli which cause platelet secretion to determine if expression of the soluble agglutinin is a unique consequence of thrombin activation. The other stimuli were: (i) γ-thrombin [5], a hydrolytic product of α-thrombin which has limited proteolytic activity; (ii) the Ca^{2+} ionophore A23187 [6]; and (iii) thrombocytin, a serine protease isolated from snake venom [7,8]. The cell-free supernatant fractions derived from platelets activated by γ-thrombin or A23187 had no hemagglutinin activity and the comparable fractions from thrombocytin activated platelets had only slight activity (fig.2). Since all these agents caused secretion, it seems that secretion, per se, is not sufficient for the expression of the soluble agglutinin. Rather, it appears that the secreted materials have to be activated by α-thrombin before they express agglutinin activity.

As a test of this hypothesis, the inactive supernatant fractions described above were treated with α-thrombin and assayed for agglutinin activity. Fig.2 shows that α-thrombin produced agglutinin activity in the previously inactive preparations. PMSF inactivated α-thrombin [9] was without effect and α-thrombin itself did not agglutinate the fixed erythrocytes (data not shown). These results demonstrate that α-thrombin has at least two functions in generating the soluble agglutinin activity: it causes secretion and subsequent activation of the molecules which acquire agglutinin activity.

Since α-thrombin activated the soluble, cell-free secreted materials, but γ-thrombin had no effect and thrombocytin had only a slight effect, it may be that

the differences in the proteolytic specificities of these enzymes account for this behavior. A fundamental functional difference between these enzymes is that α-thrombin can readily cleave the fibrinopeptides A and B from the α and β-chains of fibrinogen, a protein secreted by platelets during activation [10], whereas, γ-thrombin and thrombocytin have very limited ability to catalyze these reactions [5–7]. Therefore, the ability of α-thrombin to activate the secreted materials and thereby generate soluble agglutinin activity may be due to the production of fibrin from secreted fibrinogen.

Fig.3 shows the effect of α-thrombin on the hemagglutination activity of fibrinogen. Fibrinogen at 0.5 mg/ml did not agglutinate the fixed erythrocytes. Addition of ADP, γ-thrombin or thrombocytin to the fibrinogen caused no activation of its hemagglutination activity. In contrast, treatment of fibrinogen with 0.5 U/ml of α-thrombin resulted in activation of the hemagglutination activity. This fibrin-caused agglutination was inhibited by 30 mM arginine, mannosamine, glucosamine, and galactosamine, but not by glutamine, mannose, glucose, galactose, lactose, or the *N*-acetylated derivatives of the amino sugars.

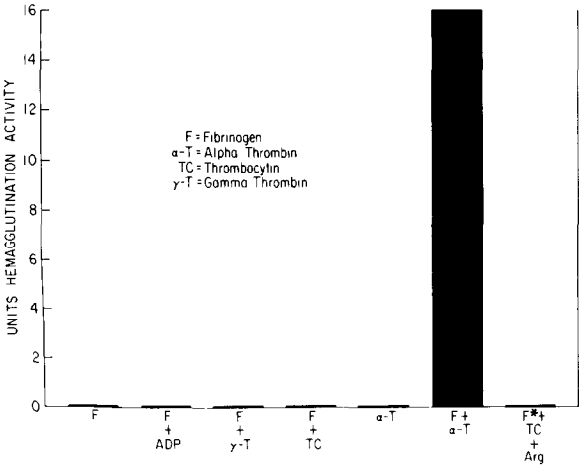

Fig.3. Fibrinogen hemagglutination assays were done in TCS. 25 μl samples of the test substances were evaluated for activity. Fibrinogen (Kabi, Grade L) was at 0.5 mg/ml, ADP at 5 μM, γ-thrombin at 5 μg/ml, thrombocytin at 5 μg/ml and α-thrombin at 0.5 U/ml in the test mixtures. *This fibrin-caused agglutination was inhibited by a final concentration of 30 mM arginine, mannosamine, glucosamine, and galactosamine, but not by glutamine, mannose, glucose, galactose, lactose, or the *N*-acetylated derivatives of the amino sugars at the same concentration.

3.3. *Platelet-bound agglutinin activity*

Fig.2 shows the requirements for the expression of platelet bound enhanced hemagglutination activity. Discoid, washed platelets (the suspensions were birefringent) suspended in Tyrode solution at 37°C were tested for enhanced hemagglutination activity following shape change caused by ADP. Shape change caused by ADP under these conditions does not result in secretion [11]. The non-discoid (shape-changed) platelets lacked both enhanced hemagglutination activities (fig.2). In contrast, washed platelets suspended in ETS (EDTA is required to prevent platelet aggregation) and treated with the Ca^{2+} ionophore A23187, γ-thrombin or thrombocytin had enhanced platelet bound hemagglutination activity. In view of these results and the fact that PGE_1 (fig.1 and [2]) treatment of platelets prevents expression of the platelet-bound thrombin-enhanced hemagglutination activity, it is clear that expression of this activity is not caused simply by either shape change or proteolysis of the platelet surface by thrombin, but is secretion-dependent. In contrast to the soluble agglutinin, its expression is not dependent on the presence of α-thrombin.

4. Discussion

The results presented here demonstrate that the expression of the hemagglutination activity derived from washed platelets is secretion-dependent and can manifest itself in two ways: as platelet-bound enhanced hemagglutination activity and as the soluble agglutination activity of activated cell-free supernatant fractions derived from activated platelets. In both cases, activation of the platelets causes secretion of molecules which mediate agglutination. Platelet-bound enhanced hemagglutination activity is caused by secreted molecules cross-linking activated platelets to fixed erythrocytes. This type of hemagglutination is not dependent on exogeneous protease activity for its expression. In contrast, the soluble agglutinin activity of cell-free supernatant fractions from activated platelets requires activation by an exogeneous protease for its expression. This type of hemagglutination activity is caused by bridging molecules which cross-link the fixed erythrocytes. In both cases, cell-cell interactions are caused by the attachment of secreted molecules to the surfaces of the interacting cells. Both of these agglutinin activities are inhibited by the same

compounds which inhibit platelet aggregation induced by thrombin or ADP and fibrinogen.

The molecules which cause these agglutinin activities have not been identified. However, these activities appear to be dependent on secreted proteins since trypsin inactivates both activities (fig.2 and [1,2]). The agglutinin activities are not caused solely by secreted ADP and fibrinogen because the fixed erythrocytes used in this study cannot be agglutinated by these compounds. However, the results of fibrinogen treatment with α-thrombin vs. γ-thrombin and thrombocytin demonstrate that the expression of the cell-free agglutinin activity is dependent on fibrin formation. This conclusion does not mean, however, that other secreted substances do not participate in mediating the agglutination caused by the activated cell-free supernatant fractions. The results with the platelet-bound enhanced hemagglutinin activity demonstrates that its expression is caused by secreted substances and is not dependent on fibrin formation. Therefore, since ADP and fibrinogen cannot agglutinate the fixed red cells, this platelet-bound activity is caused by secreted substances other than, but not necessarily excluding, ADP and fibrinogen.

Regardless of how the enhanced agglutinin activities were generated, all those activities described here: the soluble (fig.2), the platelet-bound (fig.2) and even that caused by treatment of fibrinogen with α-thrombin (fig.3), were inhibited by the same concentrations of the same compounds which inhibited the hemagglutination activity of isolated platelet membranes [2], and aggregation of platelets induced by thrombin or ADP and fibrinogen [1]. Since all these activities are inhibited by the same compounds, they are probably mediated by a common molecular entity.

These results suggest that secreted materials other than ADP and fibrinogen play a role in platelet aggregation. It is known that washed platelets in Tyrode solution containing fibrinogen aggregate in response to ADP [12]. Furthermore, it is known that damaged red cells release ADP and that plasma contains a high concentration fibrinogen [13,14]. Thus, ADP and fibrinogen mediated aggregation can occur in the absence of thrombin and is not dependent on secreted materials [11]. Therefore, in view of the results presented here, there may be at least two distinct mechanisms for mediating platelet aggregation: one mechanism (exogeneous ADP and fibrinogen) which is dependent on neither thrombin nor platelet-secreted materials, and another mechanism which is dependent on both thrombin and secreted substances.

The control of expression of cell agglutinins or their receptors by secretion is not an exclusive feature of platelets. For example, preliminary studies with the cellular slime molds *Dictyostelium discoideum* and *Polysphondylium pallidum* suggest that the expression of their developmentally controlled receptors for the lectins thought to mediate aggregate formation by cohesive amoebae may also be controlled by a secretion mechanism [15,16]. Likewise, the aggregation of marine sponge cells is mediated by a complex extracellular aggregation factor [17,18] which may be released by secretion. Thus, secretion may be an ancient means of controlling the expression of specific cell agglutinins. These facts raise the possibility that a variety of metazoan cell types may use secretion as a method for controlling the expression of cell agglutinins or their receptors.

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