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Mechanism of Rabbit Platelet Agglutination Induced by Acidic Mucopolysaccharide Extracted from *Stichopus japonicus* Selenka

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Key words

Platelet aggregation – Acidic mucopolysaccharide – Rabbits

Summary

The effects of acidic mucopolysaccharide extracted from sea cucumber (*Stichopus japonicus* Selenka) (SJAMP) on rabbit platelets were studied. Using citrated platelet-rich plasma (PRP), washed platelets, and formaldehyde fixed platelets from 10 New Zealand white rabbits, we investigated the effects of platelet inhibitors and various plasma and its fractions on SJAMP-induced agglutination. It was found that the tracing of platelet agglutination induced by SJAMP showed a single phase without a lag period. The lowest concentration of SJAMP required for the agglutination of rabbit platelets was approximately 2 μg/ml, and the magnitude of agglutination induced by SJAMP was concentration dependent. In 8 out of 10 rabbits, the platelets in PRP were agglutinated by 10 μg/ml of SJAMP. Platelet inhibitors, such as aspirin, indomethacin, apyrase, antimycin, 2-deoxy-D-glucose and EDTA did not inhibit the agglutination induced by SJAMP. Washed rabbit platelets were not agglutinated by SJAMP even though the concentration of SJAMP was raised up to 50 μg/ml. When rabbit plasma, serum, or 50–60% ammonium sulfate saturated plasma fraction was added to the reaction mixture, agglutination of washed platelets by SJAMP was recovered completely. But human plasma or fibrinogen did not have any effect on the reactivity of washed rabbit platelets to SJAMP. From these data we conclude that the SJAMP-induced rabbit platelet agglutination is independent of energy metabolism but requires plasma cofactor(s) other than fibrinogen. The plasma cofactor is present in 50–60% ammonium sulfate saturated plasma fraction.

Introduction

It has been reported that the acidic mucopolysaccharide extracted from sea cucumber (*Stichopus japonicus* Selenka) [SJAMP] causes the clumping of platelets from humans, rabbits, rats, and mice (1). After intravenous injection into rabbits and mice, SJAMP caused a dramatic decrease of platelet count and spontaneous bleeding. SJAMP is a heparin-like substance with a molecular weight ranging from 30,000 to 50,000, containing galactosamine, glucuronic acid, fucose, and sulfate (2). SJAMP possesses an antithrombin-like activity even in the absence of antithrombin III (3). We have described some properties on the mechanism of SJAMP-induced human platelet aggregation (4). In this communication, we demonstrate the effects of platelet storage, platelet inhibitors, various plasmas and their fractions on the agglutination of rabbit platelets by SJAMP, and discuss the different mechanisms of the clumping of rabbit and human platelets induced by SJAMP.

Materials and Methods

Reagents, preparation of platelet-poor plasma and sera, and fractionation of plasma with ammonium sulfate were the same as described elsewhere (4).

Preparation of Platelet-Rich Plasma, Washed Platelet Suspension, and Formaldehyde Fixed Platelets from Rabbits

Blood was drawn from the central aural artery of ten New Zealand white rabbits by a butterfly infusion set. The first 3 to 4 drops were discarded and thereafter blood was collected into a plastic tube containing 3.8% sodium citrate solution. The ratio of blood to 3.8% sodium citrate was 9:1. The citrated blood was centrifuged at 180 g for 10 min at 22° C. The platelet-rich plasma (PRP) was obtained and kept at 22° C until use.

Platelets were washed according to the method of Walsh et al. (5) with some modification. Platelet-rich plasma was transferred to a conical plastic tube. A volume of 35% bovine albumin equal to 1/25 volume of PRP was introduced to the bottom as cushion. After centrifugation at 1600 g for 15 min at 22° C, the supernatant was discarded. The platelets were suspended in calcium-free Tyrode's buffer, pH 6.8, and 1 unit/ml of apyrase. The washing was repeated twice. Finally platelets were suspended in Tyrode's buffer, pH 7.4, with 0.5 mM Ca^{2+} and adjusted to 750 $\times$ 10^9/l.

The formaldehyde-fixed platelets were prepared as described elsewhere (4), according to the method of Macfarlane (6).

Platelet Agglutination/Aggregation Studies

Platelet agglutination was carried out according to the method described by Born (7) using Chrono-log platelet aggregometer (Chrono-log Corporation, Havertown, PA). A 0.4 ml of PRP (containing approximately 200–300,000 platelets/μl) was stirred in a cell at 1200 rpm at 37° C for 3 min then 2 μl of platelet agglutinating/aggregating agent was added. The change of optical density as a result of platelet agglutination/aggregation was recorded.

Study of the Effects of Serum, Plasma and Its Fractions on the Agglutination of Washed Platelets

A mixture of 0.15 ml of washed platelet suspension containing 750 $\times$ 10^9/l platelets, 0.15 ml Tyrode's buffer, pH 7.4, and 0.1 ml of serum, plasma, plasma fraction or human fibrinogen solution in Tyrode's buffer was incubated in a cell at 37° C for 3 min and then 4 μg of SJAMP

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Table 1 The magnitude of maximal agglutination of platelets in PRP from 10 rabbits induced by 10 μg/ml SJAMP or 2.5 μM ADP

Magnitude of maximal agglutination (%)	No. of rabbits SJAMP	ADP
	10	10
0–10	2	0
11–30	0	0
31–50	7	5
>50	1	5

in 2 μl solution was added. The change of optical density as a result of platelet agglutination was recorded.

Study of the Effects of Platelet Inhibitors and Thrombin Inhibitors on the SJAMP-Induced Platelet Agglutination

A mixture of 0.35 ml of PRP and 0.05 ml of inhibitor or Tyrode's buffer was incubated at 37° C for 5 min in a cell in the Chrono-log aggregometer. Then, 2 μl of solution containing 4 μg of SJAMP was added. The percent decrease of optical density resulting from platelet agglutination with or without inhibitor was recorded and compared.

Study of the Effects of SJAMP on Formaldehyde-Fixed Platelets

A mixture of 0.15 ml of formaldehyde-fixed rabbit platelet suspension (750×10^9/l), 0.1 ml of rabbit platelet-poor plasma, and 0.15 ml of Tyrode's buffer, pH 7.4, was incubated in a cell in the Chrono-log aggregometer at 37° C for 5 min. Then, 2 μl of solution containing 4 μg of SJAMP was added. The percent decrease of optical density resulting from platelet agglutination was recorded.

Results

Magnitude and Shape of Rabbit Platelet Agglutination Induced by SJAMP

At the concentration of 10 μg/ml of SJAMP, among the 10 rabbits, there was no agglutination of platelets in PRP in 2, 31–50% agglutination in 7, and >50% agglutination in 1. When the washed platelets from the 2 rabbits whose platelets did not agglutinate were suspended in the plasma from a rabbit whose platelets were agglutinated, agglutination took place. In comparison the aggregation by ADP at the concentration of 2.5 μM was 31–50% in 5 and >50% in 5. At the equal concentration or even if the concentration was raised to 50 μg/ml, heparin from bovine lung or porcine intestinal mucosa failed to induce platelet clumping. With rabbit platelets, there was an immediate agglutinating reaction upon addition of SJAMP. The agglutination tracing was shown to be a single phase and irreversible. The magnitude of agglutination was concentration-dependent (Fig. 1).

Effect of Platelet Storage on SJAMP-Induced Agglutination

The magnitude of platelet agglutination induced by SJAMP in PRP stored at 22° C up to 8 h after drawing of blood did not have any significant change.

Effect of Platelet and Thrombin Inhibitors on SJAMP-Induced Platelet Agglutination

Aspirin (1 mM) and indomethacin (8 mM) (inhibitors of cyclooxygenase), apyrase (15 units/ml) (which removes ADP), antimycin (1.4 μM), and 2-deoxy-D-glucose (8 mM) (energy metabolic inhibitors) and N-ethylmaleimide (50 μM) (a sulfhydryl-group blocking agent) had no inhibitory effect on the SJAMP-induced rabbit platelet agglutination. Neither did EDTA at 4 mM (a Ca^{2+} chelating agent) have a significant effect on agglutination induced by SJAMP. The thrombin inhibitors (1 μM PPACK, 10 units/ml heparin or 2 units/ml hirudin) did not have any effect on the SJAMP-induced agglutination.

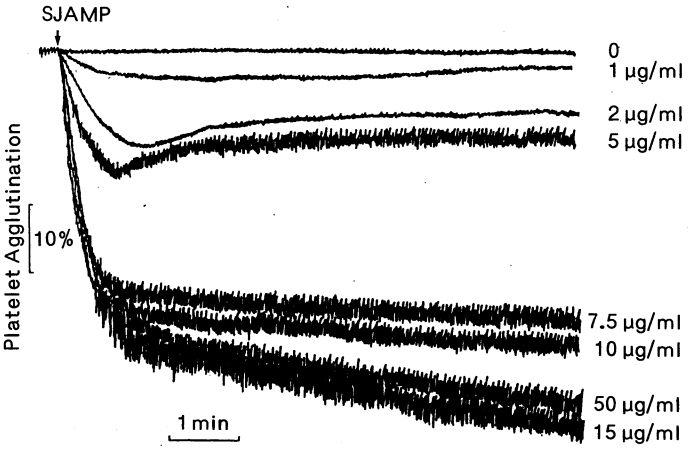

Fig. 1 The magnitude of rabbit platelet agglutination is dependent on the concentration of SJAMP using platelets from one of the responsive rabbits

Effects of Sera, Plasmas, Plasma Fractions and Fibrinogen on the Agglutination of Washed Rabbit Platelets by SJAMP

Washed rabbit platelets alone were not agglutinated by SJAMP. The platelet agglutination was restored completely upon addition of rabbit plasma, serum, or 50–60% saturated ammonium sulfate plasma fraction (Fig. 2). The 0–50% or >60% saturated ammonium sulfate plasma fraction did not have any effect. The minimal concentration of rabbit plasma required in the platelet agglutination mixture was approximately 12%. The human plasma, human serum, and human fibrinogen failed to recover the rabbit platelet agglutinating activity of SJAMP.

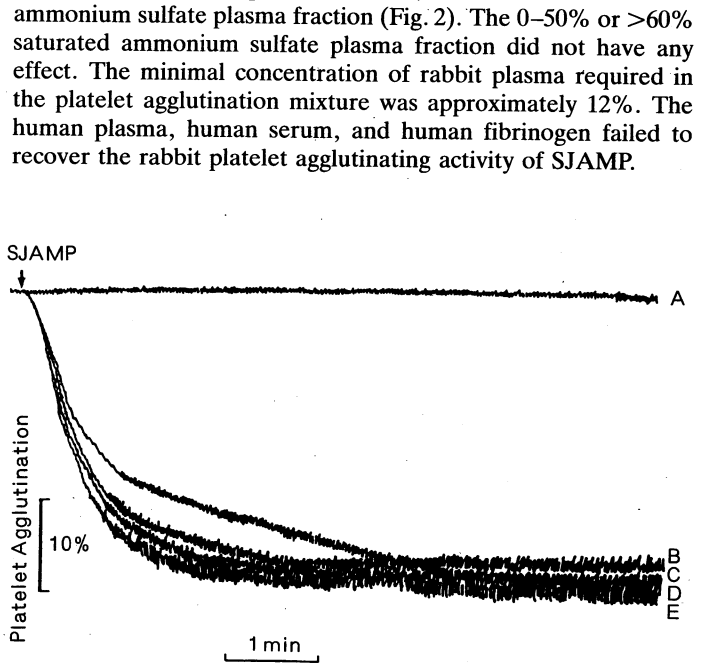

Fig. 2 Requirement of a rabbit plasma factor(s) in the agglutination of rabbit platelets by SJAMP. A mixture of 0.15 ml of washed rabbit platelet suspension containing 750×10^9/l platelets, 0.15 ml of Tyrode's buffer, pH 7.4, and 0.1 ml of buffer (A), rabbit plasma (B), rabbit serum (C), or rabbit 50 to 60% ammonium sulfate saturated plasma fraction (D) was incubated at 37° for 3 min before addition of 4 μg SJAMP. E represents the agglutination of rabbit platelets in the platelet-rich plasma by the same concentration of SJAMP for comparison. (Representative of 3 experiments)

Effect of SJAMP of Formaldehyde-Fixed Platelets

The formaldehyde-fixed platelets mixed with rabbit plasma were agglutinated by SJAMP at the concentration of 10 μg/ml; the magnitude of agglutination was slightly lower than that of fresh platelets.

Discussion

The acidic mucopolysaccharide isolated from sea cucumber (*Stichopus japonicus* Selenka) caused a variable magnitude of agglutination of rabbit platelets in 8 of 10 rabbits at the final concentration of 10 μg/ml. Platelets from 2 of them have no reaction to SJAMP even though the concentration of SJAMP was raised to 50 μg/ml. When these platelets were resuspended in the solution containing PRP, from a rabbit whose platelets in PRP were agglutinated by SJAMP, the agglutination occurred. This implies that these 2 rabbits have a defect in the plasma factor required for the SJAMP-induced agglutination of rabbit platelets or possess an inhibitory substance against the agglutination.

For comparison, the effect of heparin from bovine lung and procine intestinal mucosa on rabbit platelets was studied. Heparin was found not to induce the clumping of rabbit platelets in PRP at the concentration of 10 μg/ml or 50 μg/ml. It is different from the in vivo study in rabbits reported by Eika and Godal (8), in which thrombocytopenia was observed. We have also shown (3) that infusion of SJAMP to rabbits at the dose of 3 mg/kg body weight caused serious thrombocytopenia, which was similar to the heparin studies described by other workers (8–12).

It has been reported that endotoxin, a lipopolysaccharide, from varied bacteria caused rabbit platelet aggregation (13–16). Endotoxin-induced rabbit platelet aggregation occurred in two phases and was accompanied by secretion of platelet granule contents. The second phase of aggregation was abolished by N-ethylmaleimide, PGE_1, and methyl xanthines, and partially inhibited by aspirin (17). Therefore, the mechanism of endotoxin-induced aggregation is different from that of SJAMP-induced platelet agglutination.

In contrast to human platelets, the clumping of rabbit platelets induced by SJAMP was not inhibited by aspirin, indomethacin, apyrase, antimycin, and 2-deoxy-D-glucose. The formalin-fixed platelets were also clumped by SJAMP. Therefore, the clumping of rabbit platelets by SJAMP is an agglutination instead of aggregation because it does not require the release of ADP, thromboxane A_2 formation and energy metabolism.

The agglutination of rabbit platelets by SJAMP requires a plasma factor because washed platelets were not agglutinated by SJAMP unless rabbit plasma, serum, or 50–60% ammonium sulfate saturated plasma fraction was added. Human plasma or fibrinogen could not restore the aggregation of rabbit platelets. Therefore, the plasma factor(s) required for the SJAMP-induced agglutination of rabbit platelets is different from that of human platelets, in which human fibrinogen is required (4). Mason and Read (10) reported that species differences in the reactivity of platelets from twelve mammals to various agglutinating or aggregating agents, but did not mention that an agonist-induced aggregation/agglutination of platelets from two species can be caused by entirely different mechanisms. In this report, we demonstrated that the mechanism of platelet aggregation/agglutination by SJAMP is different between human and rabbit platelets. Therefore, the mechanism of platelet aggregation/agglutination by an agonist cannot be extrapolated from one species to another.

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