SUPPRESSION OF THE ABNORMALLY STRONG AGGREGATION OF WASHED PLATELETS FROM BOVINE WITH SERUM ALBUMIN

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1. Introduction

During the course of investigations of platelets with functions such as adhesion, aggregation, retraction and transport of metal ions across the membrane, many investigators have tried to give platelets intrinsic functions in an artificial medium in place of plasma. No ADP-induced aggregation of washed platelets from pig [1] or from human [2,3] was reported in an artificial medium; washed platelets from pig suspended in buffered saline do not cause ADP-induced aggregation and those from human in buffered saline or in tyrode solution give rise to slight aggregation only with a great amount of ADP. However, the addition of bovine serum albumin and apyrase to tyrode solution containing platelets from human caused aggregation by ADP and fibrinogen from human, and maintained the shape of platelets in disc form [4]. The necessity of serum albumin to keep the functions of platelets was pointed out [5,6]; the existence of serum albumin in an artificial medium containing platelets prevents a leakage of nucleotides and serotonin from platelets, and suppresses the loss of lactate dehydrogenase and the oxidation of glucose in platelets.

Plasma cofactors participated in ADP-induced aggregation of unwashed platelets in Tris–ACD from bovine [7,10]. In the present study, an abnormally strong aggregation and disaggregation of washed platelets was observed by a simultaneous addition of serum albumin and ADP plus Ca^{2+}. This abnormally strong aggregation was suppressed by incubation of washed platelets in an artificial medium with serum albumin. This phenomenon was discussed in relation to stabilization of washed platelets with serum albumin and also to the effect of plasma cofactors on ADP-induced aggregation of unwashed platelets.

2. Materials and methods

Platelet-rich plasma was obtained from bovine blood anticoagulated by acid–citrate dextrose as in [8] and centrifuged at 500 × *g* for 5 min to obtain platelet pellet. The platelet pellet was suspended in Tris–ACD buffer at pH 7.35 (unwashed platelets in Tris–ACD). The Tris–ACD buffer is a mixture of 5 vol. 115 mM NaCl, 15 mM KCl, 25 mM Tris (2-amino-2(hydroxy-methyl)-1,3-propanediol) and 5 mM glucose, and 1 vol. ACD solution [9]. To prepare washed platelets, unwashed platelet in Tris–ACD was centrifuged at 500 × *g* for 5 min and washed with Tris–ACD. This procedure was repeated 2 times and then washed platelet pellet was suspended in Tris–ACD (washed platelets in Tris–ACD). Unwashed platelet pellet was also suspended in plasma (unwashed platelets in plasma). These three platelet suspensions (unwashed platelets in plasma, unwashed platelets in Tris–ACD and washed platelets in Tris–ACD) were used in the present experiments. The concentration of platelets in each suspension was 3–5 × 10^5 cells/ml. Aggregation of platelets was recorded spectrophotometrically as follows [7]: 50 μl 0.5 mM ADP containing 1.5 M $CaCl_2$ was added to 5 ml platelet suspension in a cylindrical photocell, preincubated for 10 min at 37°C. ΔA_{600} of sample suspension was recorded with time at 37°C under stirring at 1000 rev/min using a Simadzu recording spectrophotometer SV-50. Upon the addition of ADP, the A_{600} increases for a few

seconds and then decreases sharply with time to reach the lowest level of absorbance (see curve A, fig.1). After that the absorbance gradually increases with time to reach the original absorbance level. This absorbance change reflects the aggregation and disaggregation of platelets induced by ADP. The difference, ΔA_{max}, between the highest and the lowest absorbance values is taken as the aggregability of platelets. Crystalline bovine serum albumin was purchased from Kokusan Kagaku Co. Alkaline denatured serum albumin was obtained by incubating serum albumin at pH 13.1 for 2 h at room temperature. The plasma cofactors participating in ADP-induced aggregation of unwashed platelets in Tris–ACD from bovine were obtained as in [7,10].

3. Results and discussion

Figure 1 shows profiles of a normal aggregation (curve A) and an abnormally strong aggregation (curves C, D) of platelets. An addition of 5 μM ADP plus 15 mM Ca^{2+} to unwashed platelets in plasma (80 mg protein/ml) caused the normal aggregation

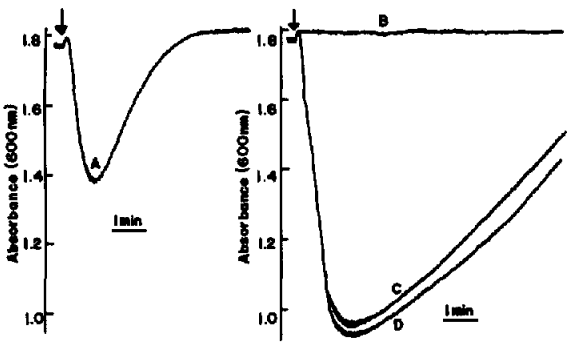


Fig.1. Aggregation and disaggregation of washed and unwashed platelets from bovine. Curve A: unwashed platelets in plasma, normal aggregation induced by ADP plus Ca^{2+}. Curve B: washed platelets in Tris–ACD, no aggregation with ADP plus Ca^{2+}. Curve C: washed platelets in Tris–ACD, abnormally strong aggregation induced by simultaneous addition of diluted plasma (3.5 mg protein/ml) and ADP plus Ca^{2+}. Curve D: washed platelets in Tris–ACD, abnormally strong aggregation induced by simultaneous addition of serum albumin (1.0 mg/ml) and ADP plus Ca^{2+}. At points indicated by arrows, ADP and others were added. The concentration of ADP and Ca^{2+} in a reaction mixture were 5 μM and 15 mM, respectively. Platelet concentration: 3.9×10^8 cells/ml.

and disaggregation with an aggregability of ΔA_{max} 0.42 (curve A). However, adding the same reagents to washed platelets in Tris–ACD did not cause any aggregation and disaggregation at all (curve B). A simultaneous addition of diluted plasma (3.5 mg protein/ml) and 5 μM ADP plus 15 mM Ca^{2+} to washed platelets in Tris–ACD did cause the abnormally strong aggregation with aggregability of ΔA_{max} 0.85 (curve C). Similarly, the abnormally strong aggregation was observed for washed platelets in Tris–ACD by simultaneous addition of serum albumin (1.0 mg/ml) and 5 μM ADP plus 15 mM Ca^{2+} (curve D). The difference in profiles of ADP-induced aggregation between unwashed platelets in plasma and washed platelets in Tris–ACD may be due to the change of physiological state of platelets, probably because of the removal of plasma components such as serum albumin from the surface of platelets. The abnormal aggregability of washed platelets in Tris–ACD with diluted plasma (3.5 mg protein/ml) or with serum albumin (1.0 mg/ml) is 2 times as great (0.85/0.42) as the normal aggregability of unwashed platelets in plasma.

In order to see the effect of serum albumin (0–1.0 mg/ml) on the aggregability of washed platelets in Tris–ACD or of unwashed platelets in plasma, the aggregability, ΔA_{max}, of the each platelet suspension induced by simultaneous addition of serum albumin (0–1.0 mg/ml) and ADP (0 μM, 2.5 μM, 5 μM and 10 μM) plus 15 mM Ca^{2+} was measured. The results are shown in fig.2, in which the left panel shows the plot of ΔA_{max} against serum albumin concentration, when washed platelets in Tris–ACD were used. The right panel represents the same plot when unwashed platelets in plasma were used. In the case of washed platelets in Tris–ACD, the value of ΔA_{max} increased markedly by increasing serum albumin concentration and reached a constant level of ΔA_{max} 1.25 (curve A) at 10 μM ADP, ΔA_{max} 0.83 at 5 μM (curve B) and ΔA_{max} 0.34 at 2.5 μM ADP (curve C). On the other hand, in the case of unwashed platelets in plasma, the ΔA_{max} did not depend upon the concentration of serum albumin and the values of ΔA_{max} at 10 μM, 5 μM and 2.5 μM ADP were 0.61 (curve A), 0.44 (curve B) and 0.27 (curve C), respectively, which are 1/2 ΔA_{max} obtained for washed platelets in Tris–ACD. No aggregation took place in the absence of ADP (curve D in each panel). This indicates that normal aggregability of unwashed platelets in plasma depends

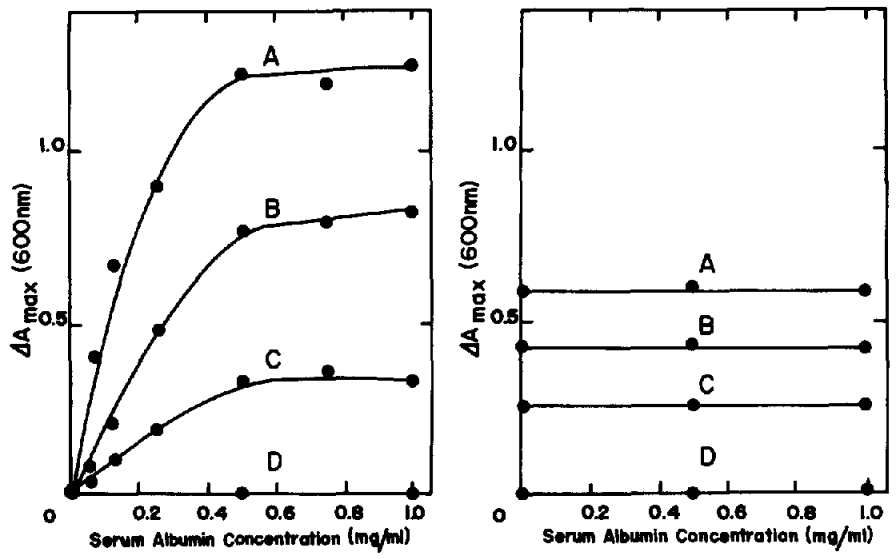


Fig.2. Plots of aggregability, ΔA_{max}, of washed platelets in Tris–ACD or of unwashed platelets in Tris–ACD against serum albumin concentration. Left panel: to washed platelets in Tris–ACD was simultaneously added serum albumin (0–1.0 mg/ml) and ADP plus Ca^{2+}. Curves A–D: 10 μM, 5 μM, 2.5 μM and 0 μM ADP, respectively. Right panel: to unwashed platelets in plasma was simultaneously added serum albumin (0–1.0 mg/ml) and ADP plus Ca^{2+}. Curves A–D: 10 μM, 5 μM, 2.5 μM and 0 μM ADP, respectively. Ca^{2+} concentration: 15 mM. Platelet concentration: 3.7×10^8 cells/ml.

upon only ADP concentration. However, the abnormally strong aggregation of washed platelets in Tris–ACD depends upon the concentrations of serum albumin and ADP.

Figure 3 represents the effect of preincubation of washed platelets in Tris–ACD with serum albumin (higher panel). During the preincubation, the abnormally strong aggregation phenomenon of washed platelets in Tris–ACD disappeared rapidly. At points indicated by arrows, 5 μM ADP plus 15 mM Ca^{2+} was

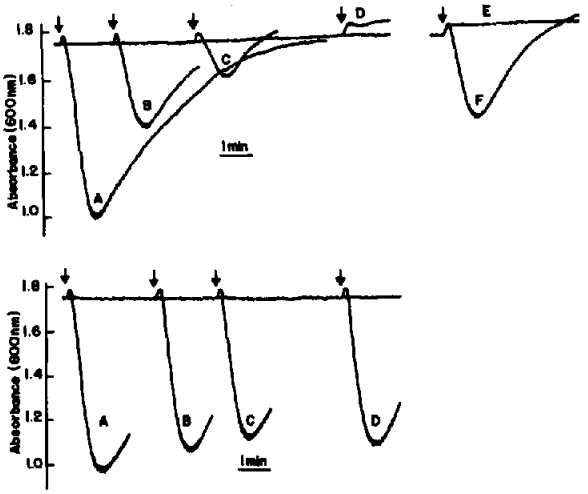


Fig.3. Effect of preincubation of washed platelets in Tris–ACD in the presence (higher panel) and the absence (lower panel) of serum albumin on ADP-induced aggregation. Higher panel, curves A–D: at points indicated by arrows, ADP plus Ca^{2+} was added to washed platelets in Tris–ACD preincubated with serum albumin (1.0 mg/ml for 0 min, 2 min, 5 min and 10 min, respectively. Curve E: addition of ADP plus Ca^{2+} to washed platelets in Tris–ACD preincubated with serum albumin (1.0 mg/ml) for 20 min. Curve F: addition of ADP plus Ca^{2+} to washed platelets in in Tris–ACD preincubated with serum albumin and cofactors (cofactor A in homogeneous state with mol. wt 117 000, 0.18 mg/ml; and a partially purified cofactor B with mol. wt ~280 000, 3.7 mg/ml). Lower panel, curves A–D: at points indicated by arrows, serum albumin (1.0 mg/ml) and ADP plus Ca^{2+} was added to washed platelets in Tris–ACD preincubated for 0 min, 3 min, 5 min and 10 min, respectively. The concentrations of ADP and Ca^{2+} in a reaction mixture were 5 μM and 15 mM, respectively. Platelet concentration: 4.2×10^8 cells/ml.

added to washed platelets in Tris–ACD preincubated with serum albumin (1.0 mg/ml) for 0 min (curve A), 2 min (curve B), 5 min (curve C) and 10 min (curve D). The value of ΔA_{max} was decreased markedly by prolonging the incubation time, and after 20 min incubation no aggregation took place by ADP plus Ca^{2+} (curve E). On the other hand, the abnormally strong aggregability was not reduced by the preincubation in the absence of serum albumin, which is shown in the lower panel. The simultaneous addition of serum albumin (1.0 mg/ml) and 5 μM ADP plus 15 mM Ca^{2+} to washed platelets in Tris–ACD preincubated for 0 min (curve A), 3 min (curve B), 5 min (curve C) and 10 min (curve D) in the absence of serum albumin caused the abnormally strong aggregation with ΔA_{max} 0.78–0.81. These results indicate that the abnormal aggregability of washed platelets in Tris–ACD is suppressed by serum albumin. These washed platelets preincubated with serum albumin have the normal aggregability which could be induced by the addition of plasma cofactors and 5 μM ADP plus Ca^{2+} (curve E, higher panel). Its value of ΔA_{max} 0.40 is closely in agreement with the aggregability, ΔA_{max} 0.42, obtained for unwashed platelets in plasma (curve A, fig.1).

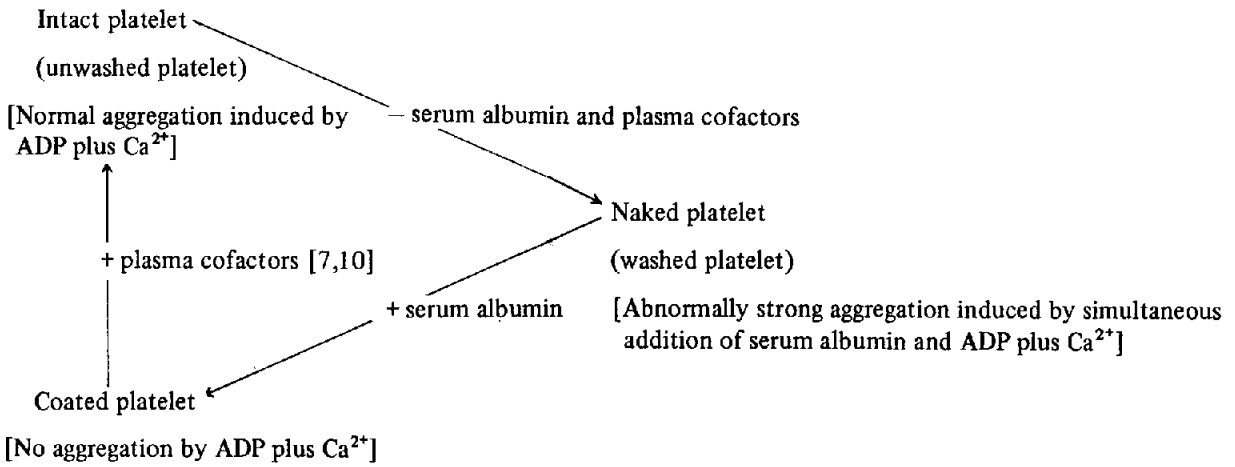
Table 1 shows the effect of various proteins (native and denatured serum albumin and ovoalbumin) on suppression of the ADP-induced abnormal aggregation. A simultaneous addition of serum albumin and 5 μM ADP plus 15 mM Ca^{2+} to washed platelets in Tris–ACDD caused the abnormally strong aggregation with ΔA_{max} 0.85. However, an addition of denatured serum albumin or ovoalbumin in place of serum albumin did not cause any aggregation at all. After the incubation of washed platelets in Tris–ACD with serum albumin for 20 min, the ADP-induced abnormal aggregability was almost completely lost (ΔA_{max} 0.02). A simultaneous addition of serum albumin (1.0 mg/ml) and ADP plus Ca^{2+} to washed platelets in Tris–ACD preincubated with alkaline-denatured serum albumin (1.0 mg/ml) or with ovoalbumin (2.0 mg/ml) led to the abnormally strong aggregation with ΔA_{max} 0.87 or ΔA_{max} 0.85, respectively. This indicates that only native serum albumin is highly effective to suppress the abnormally strong aggregation of washed platelets.

Three plasma cofactors from bovine blood, which participate essentially in ADP-induced aggregation of bovine platelets, were found [7,10]. One of the cofactors, with mol. wt 117 000, is highly effective in ADP-induced aggregation of unwashed platelets in Tris–ACD. Judging from the results obtained [7,10] and in the present studies, a scheme may be proposed as follows:

Table 1
Suppresion of abnormally strong aggregation of washed platelets in Tris–ACD with native serum albumin

[A] (mg/ml)	Aggregability (ΔA_{max})	
	Simultaneous addition of [A] and ADP plus Ca^{2+} (%)	Simultaneous addition of ADP plus Ca^{2+} and serum albumin (1 mg/ml) after preincubation with [A] for 20 min (%)
Serum albumin (1.0 mg/ml)	0.85 (100)	0.02 (2.4)
Alkaline denatured serum albumin (1.0 mg/ml)	0.03 (3.5)	0.87 (102.4)
Ovoalbumin (2.0 mg/ml)	0.00 (0)	0.85 (100)

Each value is the average value obtained by 3 experiments



When intact platelet was washed with Tris–ACD, plasma components such as serum albumin and plasma cofactors may be removed from the surface of platelet to form naked platelet (washed platelet), with the abnormally strong aggregability. Binding of naked platelet with serum albumin leads to coated platelet and further binding of plasma cofactors with coated platelet leads to intact platelet with the normal aggregability.

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