Effect of Human Platelets on the Amidolytic Activity of Human Factor XII Activated by Kaolin

Yutaka Komiyama, Hiroshi Egawa, Hideki Kuriki and Kenjiro Murata

Department of Clinico-laboratory Medicine, Kansai Medical University, Moriguchi, Osaka, Japan

Key words: Factor XII, Blood coagulation, Blood platelets, Serotonin

Summary

We have established an assay system for the amidolytic activity of purified human factor XII which is activated only by the negatively charged substance, kaolin, and examined the effect of washed platelets, ADP and serotonin on the amidolytic activity of kaolin-activated factor XII. Kaolin-treated platelets and serotonin of a physiological content in human platelet were found to promote the amidolytic activity of kaolin-activated factor XII, but ADP of a physiological content in human platelet had no such effect. The promoting effect of kaolin-treated platelets and serotonin were inhibited by the addition of methysergide. Furthermore, it seems that serotonin affects the amidolytic activity of already activated factor XII by negatively charged substance.

Introduction

Intrinsic blood coagulation is initiated by the activation of factor XII, and platelets play an important role in the mechanism of blood coagulation. Recently, Walsh reported that washed human platelets treated with adenosine diphosphate (ADP) promote the proteolytic activation of human factor XII in the presence of plasma prekallikrein and that the cleavage of factor XII was kallikrein-dependent. But Vicic reported that no evidence could be adduced for the activation of factor XII, and Scully reported the inhibition of contact activation by platelet factor.

In this communication, we reported the promoting effect of washed platelets and serotonin on the contact activation of purified human factor XII in the absence of plasma prekallikrein.

Materials and Methods

Preparation of purified human factor XII

Human factor XII was purified from human ACD-blood according to the method of Griffin et al. The biological activity of factor XII was determined by kaolin-activated partial thromboplastin time, during which the factor XII preparation restored the clotting capacity of factor

Supplement to J. Kansai Med. Univ., Vol. 36, Dec, 1984
We confirmed that this factor XII-deficient plasma contained a normal level of factors V and VIII on the basis of its restorative capacity of factor V-deficient plasma and factor VIII-deficient plasma (Dade Diagnostics, Inc.), and fibrinogen, using the thrombin time method. The contamination of plasma prekallikrein in purified human factor XII was examined by kaolin-activated partial thromboplastin time, during which purified human factor XII restored the clotting capacity of prekallikrein-deficient plasma (George King Biomedical Inc.), and it was found that this purified human factor XII contained no plasma prekallikrein activity. The specific activity of purified factor XII is 46.5–51.0 unit/mg protein and the homogeneity of purified factor XII was confirmed by 7.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The protein content was determined by the method of Lowry et al., and the ultraviolet absorbance at 280 nm. The glassware used in the present paper was siliconized by silicon KF-96 or KC-88 (Shin-etsu Kagaku). Purified factor XII was stored in an aliquot at –80°C as a solution of 0.1 M sodium acetate buffer (pH 5.3) containing 0.25 M NaCl, 1 mM benzamidine, 50 mg/l of polybrene and 0.02% NaN₃, and dialyzed against 0.05 M Tris–HCl buffer (pH 7.8) containing 0.117 M NaCl (reaction buffer) for 2 hr before use.

**Amidolytic activity of Kaolin-activated factor XII**

All reagents in this assay system were poured into a reaction container in an ice-cold bath at 20 sec intervals. The synthetic chromogenic substrate, S-2302 (AB Kabi Diagnostics), was dissolved in the reaction buffer and 0.3 mg/0.2 ml of S-2302 was used in the assay system. Kaolin (Fisher Scientific Co., acid washed) was homogenized before use and poured into the reaction container with stirring as a suspension of the reaction buffer (0.5 ml). One ml of washed human platelets, ADP or serotonin was then added to the reaction container. Methysergide was as a solution of the reaction buffer (0.5 ml), if required. Factor XII was employed one unit/0.5 ml diluted with the reaction buffer in this assay system. After factor XII solution was added, the reaction volume was brought to 3 ml by the addition of the reaction buffer, and the system was then incubated. A plastic tube (16.5×100 mm) was used as the reaction container. The incubation temperature was maintained at 37°C in a water bath, and during incubation, the tubes were shaken vigorously. After incubation, the reaction was terminated by the addition of 50% acetic acid (0.3 ml). The reaction mixture was then centrifuged at 1500×g for 10 min and the optical density of the supernatant fraction was measured at 405 nm. Unless otherwise noted, all samples were assayed in duplicate and the data were indicated by mean values. A control experiment was carried out using a mixture of S-2302, kaolin and factor XII. Blank experiments were performed for factor XII, platelet suspension, serotonin and methysergide.
Preparation of washed human platelets

Washed human platelets were prepared from fresh human citrated blood, according to the method of Walsh\textsuperscript{12).} A gradient system of bovine serum albumin (Sigma Chemical Co., fraction V) was formed in a plastic tube by thawing at 37°C and gentle agitation with a siliconized glass stirring rod. A Sepharose 2B column was prepared using a fresh Ca-free Tyrode’s solution (pH 7.3) containing 0.1% bovine serum albumin (elution buffer). The flow rate of this column was 20 ml/hr. ADP aggregation of these washed platelets was observed using a platelet aggregation tracer (NKK Ltd., PAT-2A). The washed platelets were counted by phase contrast microscopy or platelet counter (Coulter Ltd., Thrombocounter C), and diluted with elution buffer.

Time course of dextran sulfate-activated factor XII amidolytic activity

Dextran sulfate (Sigma Chemical Co., M.W., 500,000) was dissolved in the reaction buffer and one \( \mu g/0.5\) ml of dextran sulfate was used in the assay system. The reaction container was a plastic cuvette (Kartell Co., 10 mm). The time course of the dextran sulfate-activated factor XII amidolytic activity was followed by a spectrophotometer and recorder (Hitachi Ltd., 100-60, 200-0510 and 056). The concentration of S-2302, factor XII and serotonin was similar to the reaction system of Kaolin-activated factor XII amidolytic activity. The reaction temperature was maintained at 37°C, unless otherwise noted.

Reagents

Unless otherwise noted, all reagents were of special or highest grade. Methysergide bimaleate was kindly provided by Sandoz Ltd. ADP was purchased from P-L Biomedicals. Polybrene and benzamidine hydrochloride were purchased from Aldrich Chemical Co. Crystallized and lyophilized bovine serum albumin in an elution buffer was purchased from Sigma Chemical Co. Column packing materials were purchased from Pharmacia Fine Chemicals. Thrombin was purchased from Mochida Pharm. Reagents for the kaolin-activated partial thromboplastin time and prothrombin time were purchased from Travenol Laboratories, Inc. Other reagents were purchased from Wako Pure Chemicals.

Results

Reaction time

As shown in Fig. 1, the reaction almost terminated after 60 min in the presence of 1 mg/0.5 ml kaolin suspension, and within 60 min, factor XII showed only slight amidolytic activity in the absence of kaolin suspension. Therefore, the reaction time of 60 min was used.
Dose of kaolin

 Amidolytic activity of factor XII was detected by the addition of at least 0.04 mg kaolin/0.5 ml, and increased with the dose of kaolin to 1 mg kaolin/0.5 ml, as shown in Fig. 2. But this amidolytic activity fell gradually by the addition of more kaolin. Consequently the dose of kaolin of 0.1 mg/0.5 ml in the assay of amidolytic activity of factor XII was adopted.

Effect of washed platelets

 In order to examine the damage to the platelets by the washing procedure, we examined the platelet aggregation of the washed platelets (6×10⁷ platelets/0.1 ml), and confirmed the aggregation using 10 μM ADP. Fig. 3 shows the effect of these washed platelets on the amidolytic activity of kaolin-activated factor XII. We found that the washed platelets promoted the amidolytic activity of factor XII in the presence of 0.1 mg kaolin/0.5 ml. This reaction was promoted most by 1.25×10⁸ platelets/ml, but this effect fell gradually by the addition of
Fig. 3 Effect of washed platelets on the amidolytic activity of kaolin-activated factor XII

*: significant difference with control experiment (mean±s.e., p<0.05, n=4)

more platelets. Fig. 4 shows the effect of the supernatant fraction of centrifuged at 1000 X g for one min of the washed platelets (1.3 X 10^8 platelets/1.4 ml) and kaolin suspension (0.15 mg/0.1 ml) preincubated at 37°C. The washed platelets showed promoting activity in the supernatant fraction soon after preincubation, as shown in Fig. 4.
Effect of ADP and serotonin

As shown in Fig. 5, the effect of ADP on the amidolytic activity of the kaolin-activated factor XII was not confirmed by the addition of 0.004 to 0.4 μmoles/ml of ADP. Although 2 μmoles/ml of ADP caused the increase of optical density at 405 nm, this dose of ADP

**Fig. 5** Effect of ADP on the amidolytic activity of kaolin-activated factor XII

****: significant difference with control experiment (mean±s.e., p<0.02, n=4)

**Fig. 6** Effect of serotonin on the amidolytic activity of kaolin-activated factor XII

****: significant difference with control experiment (mean±s.e., p<0.02, n=4)

*: significant difference with control experiment (mean±s.e., p<0.05, n=4)
was more than the physiological dose of ADP calculated from the platelet count and the content of ADP in the human platelets. Next we examined the effect of serotonin on the amidolytic activity of the kaolin-activated factor XII. Serotonin was dissolved in water (pH 5.6) just before the amidolytic activity assay. As shown in Fig. 6, serotonin had significantly promoting activity when added in a quantity of 0.1 µg/ml. This amount of serotonin did not exceed the physiological content of serotonin calculated from platelet count and the content of serotonin in human platelets.

**Effect of methysergide on the promoting activity of serotonin and washed human platelets**

Table 1 shows the effect of methysergide on the promoting activity of serotonin and washed platelets. The promoting activity of serotonin was inhibited in the presence of methysergide (10-fold the weight of serotonin) as well as that of washed platelets.

<table>
<thead>
<tr>
<th>line</th>
<th>factor XII (1.0 unit)</th>
<th>kaolin (0.1 mg)</th>
<th>platelet (1.25x10^8)</th>
<th>serotonin (0.1 µg)</th>
<th>methysergide (1.0 µg)</th>
<th>relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>100.0±2.1</td>
</tr>
<tr>
<td>2</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>113.9±4.0**</td>
</tr>
<tr>
<td>3</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>98.0±2.0</td>
</tr>
<tr>
<td>4</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>100.0±4.5</td>
</tr>
<tr>
<td>5</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>116.4±4.3*</td>
</tr>
<tr>
<td>6</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>101.3±4.6</td>
</tr>
<tr>
<td>7</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>98.5±3.6</td>
</tr>
</tbody>
</table>

1) Amidolytic activity of control experiment (line 1 and line 4) is referred as to 100 (mean±s.e.).

** : significant difference with control experiment (line 1, p<0.02, n=4)

* : significant difference with control experiment (line 4, p<0.05, n=4)

**Time course of dextran sulfate-activated factor XII amidolytic activity**

In order to characterize the reaction between serotonin and factor XII, we observed the time dependent variation of optical density at 405 nm using the soluble negatively charged substance, dextran sulfate, instead of kaolin. As shown in Table 2, the promoting effect of serotonin on the dextran sulfate-activated factor XII amidolytic activity was dependent on time when serotonin was added to the reaction system. The addition of 10 min following the reaction's
Table 2  Effect of serotonin on the amidolytic activity of dextran sulfate-activated factor XII

<table>
<thead>
<tr>
<th>line</th>
<th>time following commencement of incubation</th>
<th>( \Delta ) OD at 405 nm (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>10 min</td>
</tr>
<tr>
<td>1</td>
<td>factor XII (^2)</td>
<td>0.017</td>
</tr>
<tr>
<td>2</td>
<td>factor XII, serotonin (^3)</td>
<td>0.020</td>
</tr>
<tr>
<td>3</td>
<td>factor XII, serotonin (^4) (preincubation)</td>
<td>0.020</td>
</tr>
<tr>
<td>4</td>
<td>factor XII (^2) serotonin (^5)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

1) These samples were assayed in duplicate and the data are indicated by mean values.
2) factor XII: 1.0 unit/ml
3) factor XII, serotonin: Factor XII (1.0 unit/ml) and serotonin (0.1 \( \mu g/ml \)) was poured into reaction container at the same time.
4) factor XII, serotonin: Factor XII (1.5 unit/1.5ml) and serotonin (0.15\( \mu g/1.5ml \)) were preincubated at 37°C for 10 min in another plastic tube and 2.0ml of the mixture was poured into the reaction container.
5) serotonin: Serotonin (0.1 \( \mu g/0.05ml \)) was poured into reaction mixture whose volume was already 2.95ml through the addition of the reaction buffer at 0 min.

commencement to the reaction system (line 4) resulted in the greatest amidolytic activity of the dextran sulfate-activated factor XII.

Discussion

We investigated the contribution of washed human platelets to the contact activation mechanism of human factor XII. The reason for employing S-2302 was its superiority as a synthetic chromogenic substrate of the factor XII fragment reported by Hojima et al. \(^{15}\). By the assay system of kaolin-activated factor XII amidolytic activity, we found that washed platelets could promote amidolytic activity. Nevertheless, kaolin activated not only factor XII but also the washed platelets, thus, this promoting reaction did not derive simply from washed platelets but from kaolin-treated platelets. To determine the cause of this reaction, we examined the shift of promoting activity to see if it derived from a precipitate or a supernatant fraction on the basis of our findings, it was confirmed that the promoting activity resulted from a supernatant fraction.

Many substances are released from platelets. In this communication, we examined ADP and serotonin which are the most typical substances released from platelets. ADP is also a stimulating substance of platelets\(^{7,16}\), but in our assay system, ADP did not promote amidolytic activity by the addition of a physiological dose. Although 2 \( \mu mols/ml \) of ADP caused the increase of optical density at 405 nm, in view of the amount of the dose, we consider that
ADP play only a small role in our assay system. The other substance which we investigated, serotonin, is said to induce a local vascular contraction and exposure of the basement membrane by the contraction of endothelial cells in the vascular wall\(^{17-20}\). We found a significant effect of serotonin in a physiological dose in promoting the amidolytic activity of the kaolin-activated factor XII. Neutralization of this activation was affected through the addition of a serotonin antagonist, methysergide, whose dose weight was 10 times that of serotonin. Furthermore, the promoting activity of kaolin-treated platelets was also neutralized by methysergide. These observations suggested that serotonin plays an important role in the promoting reaction by kaolin-treated platelets. The observation of the time course of the dextran sulfate-activated factor XII suggests that serotonin affects the amidolytic activity of already activated factor XII by dextran sulfate which is negatively charged substance. Walsh has reported the existence of factor XI-like activity in washed human platelets\(^7\) \(^{11}\). But in our assay system, the promoting activity of kaolin-treated platelets was substituted by serotonin and neutralyzed by the addition of methysergide. Therefore, we consider that the direct participation of factor XI–like activity in washed platelets is only a small role in our assay system.

References


