Factor XIII is Not Involved in Human Platelet-Collagen Interaction

Morio Aihara, Yoshihiko Sawada, Hideki Takami, Katsutoshi Kariya, Ikuo Kudo, Asano Kimura and Yutaka Yoshida

The First Department of Internal Medicine, Hirosaki University School of Medicine, Hirosaki 036

AIHARA, M., SAWADA, Y., TAKAMI, H., KARIYA, K., KUDO, I., KIMURA, A. and YOSHIDA, Y. Factor XIII is Not Involved in Human Platelet-Collagen Interaction. Tohoku J. Exp. Med., 1989, 159 (1), 37-44 —— A role of factor XIII (FXIII) on the interaction of human platelets with collagen was investigated using either formaldehyde fixed-washed platelets (FWP) or nonfixed platelets. The adhesion of FWP to bovine type I collagen was measured by using either an aggregometer or a collagen immobilized glass beads column. The interaction of non-fixed human platelets with collagen was measured with in vitro bleeding time (Thrombostat-4,000), which was performed by passing citrated whole blood through the filter covered with rat type I collagen under the constant shear stress. FWP adhesion to the collagen immobilized column (1,300 μg collagen) was not changed by the addition of commercial FXIII preparation (Fibrogammin); the adhesion was 42.7% in the presence of 1% human serum albumin, 42-43% in the presence of 1-2 U/ml of FXIII. The addition of rabbit antibody to FXIII to normal FWP did not change the degree of adhesion; 42.3% (1:100 anti-FXIII) and 46.1% (normal rabbit serum). Furthermore, platelets from the patient with congenital FXIII deficiency normally aggregated by bovine collagen and the adhesion of the patient FWP to the collagen was similar to that of normal FWP. Prolongation of partial thromboplastin time and the changes of thromboelastograph of normal plasma were observed after mixing with the collagen, and factor VIII, FXIII and von Willebrand factor were adsorbed by the collagen. The amount of FXIII in normal human plasma bound to collagen was 17, 23 and 54% at the concentration of the collagen 250, 500 and 1,000 μg/ml, respectively. The binding of plasma ristocetin cofactor was not different between normal control and the patient with FXIII deficiency. These data suggest that FXIII is not involved in human platelet interaction with the type I collagen, while FXIII in normal human plasma binds to the collagen. —— platelet; collagen; factor XIII and factor XIII deficiency

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Correspondence to: Morio Aihara, M.D., the First Department of Internal Medicine, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036, Japan.
Platelet adhesion to vascular subendothelium is an initial important reaction in normal hemostasis and platelets immediately spread and aggregate after the adhesion. Collagen is one of the important components among the vascular subendothelium and several reports on the platelet membrane receptors which are involved in platelet-collagen interaction have been described in recent years (Shadle et al. 1984; Tsunehisa et al. 1984; Chiang et al. 1987; Kotite and Cunningham 1986; Nieuwenhuis et al. 1986; Sugiyama et al. 1987). Saito et al. (1986) have reported that bovine factor XIII (FXIII) may be a platelet receptor for collagen. We have previously described that the adhesion of human fixed washed platelets (FWP) to the fibrillar collagen is affected by plasma adhesive proteins such as von Willebrand factor or fibronectin (Aihara et al. 1986) and platelet membrane glycoprotein Ib has a partial role in this interaction (Aihara et al. 1988).

In this article, we describe a role of FXIII on the interaction of human platelets with collagen. Our data suggest that FXIII is not involved in the interaction of human platelet with collagen although FXIII in normal human plasma binds to fibrillar collagen. Furthermore, the binding of plasma ristocetin cofactor (RCo) to collagen was not different between normal and FXIII deficiency.

**MATERIALS AND METHODS**

**Materials.** Bovine serum albumin (BSA; crystallized and lyophilized) was obtained from Sigma Chemical Co., St. Louis, MO. Polyclonal antibodies to the coagulation factors were obtained as follows: anti-FXIII a subunit, anti-factor IX (FIX) and anti-fibrinogen were from Berhringwerke AG, Marburg, W. Germany; anti-factor V, Dako Japan Co., Kyoto. Agarose used for electroimmunoassay was Seakem ME (FMC Marine Colloids Division, Rockland, ME, USA) or agarose C (Pharmacia Fine Chemicals, Tokyo). Purified FXIII preparation was purchased from Hoechst Co. and the dried material was dissolved in 0.05 M cacodylate buffer containing 0.1 M NaCl, pH 7.3 and used by no further purification.

**Patient with FXIII deficiency.** The patient was a 27-year-old woman who had repeated episodes of intramuscular bleeding since her childhood. Family history was negative and an inheritance was obscure. Prothrombin time (PT), activated partial thromboplastin time (aPTT), plasma fibrinogen and von Willebrand factor were all within normal range. Plasma level of a subunit of FXIII antigen measured by Laurell electroimmunoassay was less than 6% of normal pooled plasma. Bleeding time measured by the method of Duke was 1.5 min. The platelet aggregation test was normal; 91% (1 μg/ml collagen), 93% (2 μg/ml collagen), 89% (3 μM ADP) and 84% (5 μg/ml epinephrine). She was diagnosed as having FXIII deficiency and her massive bleeding tendency has been treated by the infusion of either normal blood or commercial FXIII preparation. When the patient platelets were washed in 0.05 M Tris-0.15 M NaCl (TBS) containing 5 mM ethylene diamine tetra acetic acid (EDTA), 5 mM N-ethylmaleimide and 10 μM Leupeptin (washing buffer), and were solubilized by 1% Triton X-100 in the washing buffer, the amount of FXIII antigen in 10⁷ platelets was also less than 6%.

**Coagulation tests of normal human plasma after mixing with collagen.** Blood samples were collected with informed consent from healthy volunteers into 1/10 volume of 3.8% sodium citrate and the plasma was obtained by the centrifugation at 1,000 × g for 15 min. Plasma samples of 0.3 ml were mixed with 0.3 ml of several concentrations of the collagen
in distilled water and incubated for 10 min at 37°C, and the supernatant materials isolated by the centrifugation at 1,000 x g for 5–10 min were tested for coagulation test. PT was tested by using a rabbit lung and brain thromboplastin (Simplastin; Organoteknika Co., Durham, NC, USA), and aPTT was measured by standard techniques using Platelin plus activator (Organoteknika Co., Morris Plains, NJ, USA). One-stage assays were performed for the measurement of each coagulation factor using commercial plasmas deficient in the corresponding factor. Measurement of FXIII was performed by Laurell electroimmunoassay using commercial antibody against human FXIII. Plasma thromboelastograms were measured using Hellige-Thrombelastograph by a modification of the method of Hartert (1948), and coagulation was initiated by the addition of 0.05 ml of 0.36% CaCl₂. The parameters of reaction time (r-value) which represents the time (sec) from the beginning of the tracing to the point on the tracing with the width of 2 mm and maximum amplitude (ma-value) were measured.

**FWP preparation.** Platelets were isolated from the citrated blood by the centrifugation at 160 x g for 10 min at 26°C, and washed with the buffer. FWP were prepared by fixing with 1.8% formaldehyde as previously described (Cooper et al. 1975). For the adhesion experiments, FWP were suspended in 0.05 M cacodylate-HCl, 0.1 M NaCl, pH 7.3 (Cacodylate buffer salt solution; CBS).

**Collagen and collagen coated glass beads.** Dispersed bovine flexor collagen in 50% methanol/1% cyanacetic acid was obtained from Ethicon Inc., Somerville, NJ, USA (a gift from Dr. R.L. Kronenthal). The stock solution of collagen was prepared by suspending the original material in distilled water by rocking overnight at 4°C. To prepare collagen coated glass beads, 5 ml of the collagen in distilled water (250–2,000,ug/ml) was added by 1.0 ml of 0.1 M ammonium bicarbonate in a plastic box. Fifteen grams of glass beads (0.3–0.4 mm in diameter, Eiken, Tokyo) were then added to the collagen solution and the mixture was vacuum dried overnight at room temperature (Aihara et al. 1986).

**Quantitation of the adhesion of FWP to collagen.** Adhesion of FWP to collagen was tested by two different methods, using either an aggregometer (Payton) or a collagen immobilized column as previously described (Aihara et al. 1984a, b). In brief, two tenth milliliters of FWP (initial concentration 8 x 10⁸/ml in CBS) was mixed with 0.2 ml of test materials in an aggregometer cuvette. The mixture was then added by 0.1 ml collagen (1,000 μg/ml in distilled water) and stirred at 600 rpm, 37°C.

The adhesion of FWP on collagen coated glass beads column was measured by passing FWP solution (platelet concentration, 4 x 10⁸/ml), which were mixed with either the buffer (CBS containing 2% BSA) or purified FXIII, through the collagen column at the constant flow speed. Each 0.5 ml fraction was collected and platelet count was measured by an electroparticle counter (Thrombocounter-C, Coulter, FL, USA). The degree of adhesion was calculated from the difference between the count of non adherent FWP in 4 factions (2 ml) and that of initial FWP solution, and expressed as percent of the initial FWP.

**In vitro bleeding time.** The interaction of human platelets with collagen was also investigated by the method of in vitro bleeding time (Thrombostat 4,000, Von Der Goltz, Seeon-Chisnagau, FRG) (Kratzer and Born 1985). The collagen used in this experiment was rat tail collagen, type I. One milliliter of normal citrated whole blood was mixed with 10 μl of either rabbit anti-FXIII or normal rabbit serum and the mixtures were incubated for 5 min at 37°C. The parameters measured in this experiment were in vitro bleeding time and bleeding volume. As abnormal control, normal human blood was mixed with 1/100 volume of monoclonal antibody against vWF (CLB-RAg 35; a gift from Dr. van Mourik, Amsterdam, The Netherlands), which inhibits the binding of vWF to collagen, and incubated in the similar manner.

**RESULTS**

**Binding of coagulation factors in normal plasma to collagen.** When normal
plasma was incubated with bovine collagen and the supernatant materials were assayed for routine coagulation test, aPTT was prolonged to 77.4 sec (final concentration of collagen, 100 μg/ml), 73.9 sec (500 μg/ml) and 72.9 sec (250 μg/ml), while aPTT of control normal plasma which was diluted 1 : 2 in saline was 60.1 sec. PT, however, was not changed by the addition of the collagen to normal plasma; PT 13.5 sec (collagen 1000 μg/ml) and 13.6 sec (in the absence of collagen). As shown in Table 1, factor VIII (FXIII) and FIX were the factors which bound to the collagen among the intrinsic coagulation factors.

When plasma coagulation was measured by thromboelastogram, both the prolongation of r-value and the decrease of ma-value of the supernatant plasmas were observed; the value of r-value and ma-value of the supernatant plasma after mixing with collagen (final 1,000 μg/ml) were 67 and 2.8 mm, while the values of control sample (1 : 2 diluted plasma with 0.15 M NaCl) were 25 and 8.1 mm (Fig. 1). The binding of FXIII antigen in plasma to collagen was dose dependent and 17, 23 and 54% of FXIII antigen bound to 250, 500 and 1000 μg/ml collagen, respectively.

**FWP adhesion to collagen.** FWP adhere to bovine type I collagen without the platelet aggregation in the absence of any plasma factor in our system. The addition of commercial FXIII to the test system did not change adhesion. The degree of adhesion which was measured by collagen column were 42% and 43% in the presence of 1 and 2 U/ml of commercial FXIII, while the adhesion in the absence of FXIII was 42%. In addition, the adhesion of FWP which were added by 1 : 100 rabbit antibody to FXIII was 42.3 ± 1.4% and was not different from that of non-immune rabbit serum, 46.1 ± 1.3%.

**In vitro bleeding time.** As shown in Fig. 2, the pattern of the in vitro bleeding time was not changed by the addition of rabbit antibody to FXIII to normal human citrated blood. The in vitro bleeding time of the plasma incubat-

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<th>Table 1. Residual functional activities of the coagulation factors in the supernatant plasma after mixing with collagen</th>
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<tr>
<td>Final concentration of bovine collagen (μg/ml)</td>
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<tr>
<td>Fitzgerald factor</td>
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<td>Flectcher factor</td>
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<td>Factor XII</td>
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<td>VIII</td>
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<td>Fibrinogen</td>
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Each activity of the coagulation factors in the supernatants was expressed as a percent of normal human plasma which was diluted 1 : 2 with distilled water. Values are means of duplicate examinations.
Fig. 1. Plasma thromboelastogram.
Normal human plasma was mixed with equal amount of collagen (2,000 μg/ml), and the supernatant material was tested for the thromboelastogram (upper). Normal human plasma was diluted 1:2 with 0.15 M NaCl and was tested as a control (lower).

Fig. 2. In vitro bleeding time.
One milliliter of normal human citrated blood was mixed with 10 μl of either normal rabbit serum (a), anti-FXIII antibody (b), or anti-vWF (c). The in vitro bleeding time was measured after the incubation for 5 min at 37°C.
ed with anti-FXIII antibody was 119 sec and the bleeding volume was 290 μl, which value was not significantly different from that of normal control (317 ± 93.8 μl, n = 19). The in vitro bleeding time of the blood mixed with 1/100 of monoclonal anti-vWF was abnormal and similar to that of the patient with von Willebrand disease (Table 2).

In vitro studies of the patient with FXIII deficiency. When the patient’s platelets were fixed and the adhesion to collagen was measured by an aggregometer method, he adhesion was not different from normal control; the

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<th>Normal range</th>
<th>Anti-FXIII</th>
<th>Anti-vWF</th>
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<tr>
<td>Bleeding time (min)</td>
<td>180.0 ± 12.0*</td>
<td>119</td>
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<td>Bleeding volume (μm)</td>
<td>317.7 ± 93.8</td>
<td>290</td>
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Normal human citrated blood, 1 ml, was added by 10 μl of the antibodies and incubated for 5 min at 37°C. *value from Kratzer and Born (1985).

Fig. 3. Adhesion of FWP to collagen.

FWP were obtained from normal human volunteer and the patient with FXIII deficiency. FWP adhesion to collagen was measured using an aggregometer. The final concentration of the collagen was (a) 200 μg/ml, (b) 100 μg/ml, and (c) 50 μg/ml.

<table>
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<tr>
<th>Final concentration of bovine collagen (μg/ml)</th>
<th>Normal plasma</th>
<th>FXIII def. plasma</th>
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<tbody>
<tr>
<td>600</td>
<td>&lt;6%</td>
<td>&lt;6%</td>
</tr>
<tr>
<td>400</td>
<td>11%</td>
<td>12%</td>
</tr>
<tr>
<td>200</td>
<td>26%</td>
<td>16%</td>
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<tr>
<td>100</td>
<td>34%</td>
<td>22%</td>
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The plasma of the patient with FXIII deficiency was diluted 1:1.7 with TBS containing 5% BSA because of the amount of RCo in neat plasma was 1.7 U/ml. The collagen used was different lot from that used in Table 1. Single determination.
adhesion of the patient's FWP was 73.1% (collagen 200 μg/ml), 47.5% (100 μg/ml) and 17.6% (50 μg/ml), while that of normal FWP was 70.6% (200 μg/ml), 42.4% (100 μg/ml) and 17.6% (50 μg/ml) (Fig. 3). Using the patient's citrated plasma, the binding of plasma RCo to collagen was measured by batch method. The residual amount of RCo in the supernatant plasma which was incubated with collagen was not significantly different between the patient and normal control (Table 3).

**DISCUSSION**

Several platelet membrane proteins have been reported as the receptor for the collagen which is a major component in the vessel wall (Shadle et al. 1984; Nieuwenhuis et al. 1986; Chiang et al. 1987; Sugiyama et al. 1987). Platelet FXIII was recently described as collagen receptor for bovine platelet-collagen aggregation interaction (Saito et al. 1986). In our experiments with human platelets, the adhesion was not changed by the addition of either commercial FXIII preparation or the addition of polyclonal antibody against FXIII to FWP, suggesting that FXIII is not involved in human platelet-collagen interaction. Although platelet membrane fluidity may be changed by the fixation of human platelets and this may modify an influence of exogenous FXIII on platelet-collagen interaction, this possibility can be contradicted by our several results. First, in vitro bleeding time by which the interaction of non-fixed human platelets with collagen could be evaluated was not changed by the addition of the antibody to FXIII to the normal blood. Second, the collagen induced platelet aggregation was normal in our patient with congenital FXIII deficiency as described by Ozsoylu and Hicsonmez (1976). Third, the adhesion of the patient's FWP to collagen was not different from that of normal FWP. Thus, it is suggested that the initial platelet-collagen interaction in human is not mediated by FXIII.

Although dose dependent binding of FXIII antigen in human plasma to the collagen was observed, the binding of RCo in the patients with congenital FXIII deficiency to the bovine collagen was not different from normal human control in our batch experiments. The decrease of ma-value in plasma Thrombelastograph after the incubation of plasma with collagen was likely due to the binding of FXIII to the collagen, because the level of fibrinogen in the supernatant material was not changed after mixing of normal plasma with collagen. The decrease of the functional activity of FIX in our experiments may be artificial result of FVIII deficiency because FIX antigen which was measured by immunological method was not changed after mixing with collagen. As previously reported (Aihara et al. 1984b), the binding of FVIII coagulant activity was not complete even at the concentration of the collagen which removed most of vWF: Ag from human plasma.

In conclusion, human plasma FXIII binds to collagen but is not involved in the initial interaction of human platelets with collagen.
Acknowledgments

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References