Contributions of Human Platelets to the Proteolytic Activation of Blood Coagulation Factors XII and XI

By Peter N. Walsh and John H. Griffin

Previous studies have suggested that human platelets can promote the activation of factor XI by two different mechanisms, one requiring factor XII and ADP-treated platelets and the other requiring collagen-treated platelets in the apparent absence of factor XII. To investigate these hypotheses, isolated platelets were tested for their capacity to promote the activation and cleavage of purified factors XII and XI in various mixtures of purified factor XII, kallikrein, high molecular weight (HMW) kininogen, and factor XI. The activation of factor XII or factor XI was tested in clotting assays using plasmas deficient in factor XI. The activation of factor XII or factor XI was tested in clotting assays using plasmas deficient in factor XI, XII, or VII and the limited proteolytic cleavage of 125I-labeled factor XII or XI was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence of reducing agents. That ADP- or collagen-treated platelets can promote the proteolytic activation of factor XII in mixtures containing kallikrein and HMW kininogen was shown by: (1) the proteolytic cleavage of factor XII; (2) the development of factor XII coagulant activity; and (3) the proteolytic cleavage of 125I-factor XI. Platelets treated with collagen or thrombin were shown by both coagulant assays and by cleavage studies to participate in the absence of platelets or other surfaces; and (4) the localization of cleavage products of factor XI in platelet pellets. Experiments with celite or with unstimulated platelets present showed a linear relationship between factor-XII coagulant activity and proteolysis of factor XII. However, with activated platelets present the coagulant activity was two to fourfold greater than that observed for an equivalent amount of factor XII cleaved with celite present. These studies demonstrate that platelets can promote the proteolytic activation of factor XII by kallikrein and of factor XI by both factor-XII dependent and factor-XII-independent mechanisms.

The sequence of reactions required to initiate the intrinsic mechanism of blood coagulation involves a complex interaction between negatively-charged surfaces and at least four plasma proteins: factor XII (Hageman factor), prekallikrein (Fletcher factor), high molecular weight (HMW) kininogen (Fitzgerald, Williams or Flaujeac factor) and factor XI (plasma thromboplastin antecedent). During contact activation in vitro, factor XII is absorbed to negatively charged surfaces such as kaolin or glass where it undergoes a conformational change as evidenced by studies employing circular dichroism and fluorescence spectroscopy. Factor XII is thereby rendered more susceptible to proteolytic cleavage by plasma kallikrein as well as other proteases. The zymogen form of factor XII is converted either by conformational alteration alone or by the resultant limited proteolysis to various molecular species with enzymatic activity capable of effecting the cleavage and activation of both prekallikrein and factor XI, processes that also can occur on negatively charged surfaces.

Previously evidence was presented that platelets have a role in contact activation of intrinsic coagulation. It was shown that ADP-treated platelets could shorten clotting times of noncontacted plasma in the presence of factor XII but not in its absence. It was further shown that collagen-treated platelets could accelerate coagulation reactions occurring prior to the activation of factor X in the apparent absence of factor XII provided either plasma factor XI or platelet factor-XI-like activity was present. These observations suggested that platelets could promote contact activation by two apparently separate and distinct mechanisms, one of which requires factor XII and the other of which is independent of factor XII.

We have examined the effect of washed human platelets on the activation and proteolytic cleavage of purified human factor XII and factor XI and here present evidence that platelets stimulated by ADP, collagen or thrombin promote proteolytic activation of factor XII by plasma kallikrein, and of factor XI by...
mechanisms both dependent upon and independent of factor XII.

**MATERIALS AND METHODS**

**Preparation of Washed Platelet Suspensions**

Nine volumes of blood from apparently healthy donors not on medication were collected by clean venipuncture with a 19-gauge needle and catheter directly into 1 volume of 3.8% w/v trisodium citrate using plastic containers and equipment throughout. Platelet-rich plasma was obtained and platelets were washed by albumin density gradient centrifugation in calcium-free Tyrode's solution pH 6.5 by a modification of a previously described method. After two separations into albumin density gradients the platelet suspension (3-4 ml) containing 1.0-1.5 x 10^8 platelets/ml in calcium-free Tyrode's solution pH 6.5 was applied to a Sepharose 2B column (1.5 x 30 cm) that had been equilibrated with calcium-free Tyrode's solution pH 7.3 containing 1 g/liter of crystallized bovine serum albumin (Sigma Chemical Company, St. Louis, Mo.). The platelets were gel-filtered according to the method of Tangen et al. the following composition: NaCl, 0.138 M; KCl, 0.0027 M; NaH₂PO₄·2H₂O, 0.000417 M; MgCl₂·6H₂O, 0.00205 M; NaHCO₃, 0.0119 M; dextrose, 0.0056 M; crystallized bovine serum albumin, 1.0 g/liter. Platelets were counted by phase contrast microscopy. The mean platelet recovery from platelet-rich plasma after 2 albumin density gradient centrifugations was 84% and the mean platelet recovery of washed platelets after gel-filtration was 65% for a total platelet recovery from platelet-rich plasma after both procedures of 55%. The time required from venipuncture to final collection of washed and gel-filtered platelets ranged from 2 to 2.5 hr. Washed platelet suspensions contained procoagulant activities of factors V and XI but no detectable procoagulant activities of factors II, VII, VIII, IX, X, or XII. Washed platelets from patients with hereditary factor XII deficiency gave results similar to washed normal platelets in factor XII clotting assays. Experiments with 125I-labeled bovine serum albumin and alternatively with 125I-labeled factor XI demonstrated that contamination of platelet suspensions with plasma proteins amounted to less than 0.06%.

**Purified Proteins**

Factor XII was isolated from pooled normal human plasma as previously described and was more than 95% homogeneous when examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The specific clotting activity of the purified protein was 82 U/mg protein where 1 U is defined as the amount of clotting activity in 1 ml of pooled normal human plasma. The human kallikrein preparation was obtained in activated form from a preparation designed for the isolation of prekallikrein employing chromatography on QAE-, DEAE- and SP-Sephadex, and on concanavalin A-Sepharose. The protein was more than 95% homogeneous on SDS-PAGE and had a specific clotting activity of 30 U/mg. Kallikrein migrated on unreduced gels with a mol wt of 85,000, and on reduced gels exhibited the characteristic heavy and light chains of kallikrein. It contained no detectable plasminogen, plasmin, factor XI, or β₂ glycoprotein I and less than 5% IgG. Kallikrein was assayed with the chromogenic substrate, benzoyl-pro-phe-arg-p-nitroanilide (Chromozym PK, Pentapharm, Basel) as previously described.

HMW kininogen was isolated from normal human plasma as previously described. The purified protein containing two polypeptide chains linked by disulfide bonds, was more than 95% homogeneous on SDS-PAGE and had a specific clotting activity of 12.5 U/mg of protein. Factor XI was isolated from pooled normal human plasma as previously described and was more than 95% homogeneous when examined by SDS-PAGE. The specific clotting activity of the purified protein was 250 U/mg protein. Factor XI was radiolabeled with 125I by the chloramine T method to 33 μCi/μg and retained its clotting activity during the radiolabeling procedure.

**Clotting Assays**

Incubation mixtures (100–125 μl) containing purified proteins and washed platelet suspensions as indicated in Results were with modification as stated here and suspended at concentrations of 5–7 x 10⁷ platelets/ml in calcium-free Tyrode's solution with assayed for coagulant activity in plasmas deficient in factor XII, factor XI, factor IX, or factor VII. To the incubation mixture in siliconized glass tubes (10 x 75 mm) at 37°C was added 100 μl of substrate plasma diluted 1 in 2 in 0.01 M tris-buffered saline (0.14 M NaCl), pH 7.4 (TBS) and 100 μl of cephalin (rabbit brain cephalin, Sigma Chemical Company, St. Louis, Mo.) added at a concentration determined to be optimal for clotting of dilutions of normal plasma in each assay. To the mixture was added 100 μl of 0.05 M CaCl₂ and the clotting time was measured at 37°C by visual inspection of tubes at regular 5-sec intervals. In experiments in which percentage factor XII, coagulant activity developing in incubation mixtures was calculated, a standard curve was constructed by plotting the logarithm of clotting times in factor-XII-deficient plasma of dilutions of normal pooled plasma obtained in the presence of kaolin versus the logarithm of factor XII concentration.

**Polyacrylamide Gel Electrophoresis**

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) was carried out on 7.5% gels 8 cm long from apparently healthy donors not on modification as stated here and suspended at concentrations of 5–7 x 10⁷ platelets/ml in calcium-free Tyrode's solution with assayed for coagulant activity in plasmas deficient in factor XII, factor XI, factor IX, or factor VII. To the incubation mixture in siliconized glass tubes (10 x 75 mm) at 37°C was added 100 μl of substrate plasma diluted 1 in 2 in 0.01 M tris-buffered saline (0.14 M NaCl), pH 7.4 (TBS) and 100 μl of cephalin (rabbit brain cephalin, Sigma Chemical Company, St. Louis, Mo.) added at a concentration determined to be optimal for clotting of dilutions of normal plasma in each assay. To the mixture was added 100 μl of 0.05 M CaCl₂ and the clotting time was measured at 37°C by visual inspection of tubes at regular 5-sec intervals. In experiments in which percentage factor XII, coagulant activity developing in incubation mixtures was calculated, a standard curve was constructed by plotting the logarithm of clotting times in factor-XII-deficient plasma of dilutions of normal pooled plasma obtained in the presence of kaolin versus the logarithm of factor XII concentration.

**Polyacrylamide Gel Electrophoresis**

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) was carried out on 7.5% gels 8 cm long in 4.5 x 100 mm glass tubes according to the method of Weber and Osborn. To the incubation mixtures (100–125 μl) containing washed platelet suspensions and purified proteins was added 50 μl of a solution containing 10% SDS, 1% β-mercaptoethanol, 8 M urea, and 0.01 M EDTA. The mixture was boiled for 10 min, bromophenol blue added as a tracking dye and the sample applied in glycerol to the gel. Following electrophoresis the gels were sliced into 1.2 mm sections and counted for radioactivity in a Model 1195 Searle Analytic Gamma Counter. When 125I-labeled factor XII or factor XI was analyzed on reduced SDS-polyacrylamide gels without the addition of other reactants, a large peak of radioactivity was observed corresponding to the native molecule with a mol wt of 80,000, also demonstrated on stained gels.
activated radioactivity appeared in two polypeptide fragments with mol wt of 52,000 and 28,000; and in the case of factor XI mol wt of 48,000 and 32,000. The percentage of factor XII or factor XI cleaved was calculated as the relative amount of radiolabel appearing in the two smaller fragments divided by the amount of radioactivity appearing in all three peaks. When the native molecules were analyzed in control samples without other reagents, less than 5% of the total amount of radioactivity appeared in the two smaller peaks and this background percentage was subtracted from total percent cleavage in experimental samples to give net percent cleavage of $^{125}$I-factor XII or factor XI.

**Reagents Used in Experiments**

Purified collagen from human skin was the generous gift of Dr. George Wilner, Washington University, St. Louis and was prepared by him by the method of Nishihara as described by Steven and Jackson. The collagen preparation was homogenized at 4°C in a no-clearance Teflon pestle. Adenosine diphosphate (ADP) (Sigma Chemical Company, St. Louis, Mo.) was stored as a 10 mM stock solution in TBS and diluted appropriately before use. Celite was obtained from J.T. Baker Chemical Co., Phillipsburg, N.J. Indomethacin (Sigma) was dissolved in 0.1 M Na$_2$CO$_3$ at a concentration of 0.1 M and diluted to 0.5 mM with TBS for storage at 4°C until use. The human α-thrombin preparation was kindly provided by Dr. John W. Fenton, II, New York State Department of Health, Albany, N.Y.

**RESULTS**

**Effects of Platelets on Proteolytic Cleavage of Factors XII and XI**

**Factor XII.** Since the conversion of factor XII from zymogen to active protease is accompanied by fragmentation of the 80,000 mol wt molecule to heavy and light chains of 50,000 and 30,000 mol wt respectively, attempts were initially made to determine whether the limited proteolytic cleavage of factor XII by kallikrein was enhanced in the presence of platelets. Washed collagen-treated platelets were incubated with HMW kininogen, $^{125}$I-labeled factor XII and kallikrein and the extent of cleavage of factor XII was assessed by SDS-PAGE. A gel profile from one of ten similar experiments is shown in Fig. 1 in which 16% cleavage (range 14%–32% in 10 experiments) of $^{125}$I-labeled factor XII occurred in the presence of collagen-treated platelets, HMW kininogen and kallikrein compared with 5.5% cleavage (range 2.8%–7.2%) in a similar incubation mixture in which platelets were omitted.

Electrophoretic studies were carried out under unreduced and reduced conditions simultaneously in order to ascertain the nature of the factor-XII cleavage. When factor XII is cleaved internal to intramolecular disulfide bonds (α-factor XII) it migrates as a single 80,000 mol wt protein on unreduced gels and as two polypeptide chains of 50,000 and 30,000 mol wt on reduced gels. When cleavage occurs outside these disulfide bonds (β-factor XII), the protein migrates as two polypeptide chains on both reduced and unreduced gels. A comparative analysis of reduced and unreduced gels showed that in the presence of either platelets or celite, the percentage of factor XII cleaved internally (i.e., α-factor XII) compared with the total amount cleaved ranged between 54% and 62%.

**Factor XI.** The conversion of the zymogen, factor XI, to an active serine protease involves the limited proteolytic cleavage of the native molecule (mol wt = 160,000 without reduction, 80,000 in the presence of reducing agents) to yield two polypeptide chains of mol wt = 48,000 and 32,000 that are held together by disulfide bonds. To determine whether the platelet-dependent proteolytic cleavage of factor XII by kallikrein, observed in Fig. 1, was associated with the appearance of enzymatic activity, a series of experiments was carried out in which washed platelet suspensions or Tyrode’s solution were added to collagen, ADP or buffer and incubated with factor XII, HMW kininogen, kallikrein and $^{125}$I-labeled factor XI. Radiochromatograms of $^{125}$I-labeled factor XI on reduced SDS polyacrylamide gels from a representative experiment are shown in Fig. 2. When platelets were absent and kallikrein, factor XII, HMW kinin-
ROLE OF PLATELETS IN CONTACT ACTIVATION

Fig. 2. SDS polyacrylamide gel electrophoresis of \(^{125}\text{I}\)-factor XI in mixtures of factor XII, HMW kininogen, and kallikrein in presence and absence of collagen-treated or ADP-treated platelets. A suspension (75 \(\mu\)l) of platelets (5.58 \(\times\) 10\(^9\)/ml) in calcium-free Tyrode's solution, pH 7.3, containing bovine serum albumin (1 mg/ml) or buffer was placed in a 10 \(\times\) 75 mm siliconized glass tube with 5 \(\mu\)l of either collagen (300 \(\mu\)g/ml), ADP (50 \(\mu\)M), celite (1.57 mg/ml) or buffer. To the mixture was added 5 \(\mu\)l of each of the following reactants: factor XII (0.36 \(\mu\)g), HMW kininogen (0.54 \(\mu\)g), kallikrein (0.5 \(\mu\)g), and \(^{125}\text{I}\)-Factor XI (0.15 \(\mu\)g). After a 120-min incubation at 37\(^\circ\)C, 30 \(\mu\)l of a solution containing 10% SDS, 1% \(\beta\)-mercaptoethanol, 8 M urea and 0.01 M EDTA was added. The sample was boiled for 10 min, electrophoresed on 7.5% polyacrylamide and analyzed as described in Materials and Methods. Open circles represent results with platelets absent; filled circles, platelets present. (A) Collagen; (B) celite (platelets absent); (C) ADP; (D) buffer.

Open circles represent results with platelets absent; filled circles, platelets present. (A) Collagen; (B) celite (platelets absent); (C) ADP; (D) buffer.

Table 1. Clotting Times of Factor-XII-Deficient Plasma and Cleavage of \(^{125}\text{I}\)-Factor XII in Presence and Absence of Platelets, HMW Kininogen, Kallikrein, and Factor XII

<table>
<thead>
<tr>
<th>Incubation Mixtures</th>
<th>Clotting Times (sec)</th>
<th>Amount (ng) of (^{125}\text{I}) F XII Cleaved</th>
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<td>Platelets Present</td>
<td>Platelets Absent</td>
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<td>12</td>
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One hundred microliters of washed platelets (5.31 \(\times\) 10\(^9\)/ml) in calcium-free Tyrode's solution pH 7.3 with bovine serum albumin (1 mg/ml) or buffer was incubated in a 10 \(\times\) 75 mm siliconized glass tube for 1 min at 37\(^\circ\)C with 2 \(\mu\)l (0.54 \(\mu\)g) of high molecular weight kininogen (or TBS) and 5 \(\mu\)l (0.2 \(\mu\)g) of a mixture of cold and \(^{125}\text{I}\)-labeled factor XII. To this mixture was added 5 \(\mu\)l of collagen (250 \(\mu\)g/ml), ADP (50 \(\mu\)M), celite (1.35 mg/ml) or TBS pH 7.3 for a further 5-min incubation. 5 microliters of kallikrein (0.5 \(\mu\)g) was then added and the mixture was incubated for 20 min at 37\(^\circ\)C after which 200 \(\mu\)l of a mixture of deficient plasma and rabbit brain cephalin and 100 \(\mu\)l of 50 mM CaCl\(_2\) were added and clotting times were determined. Similar samples were examined in parallel by SDS-PAGE.
sample with platelets absent (Fig. 2B). Enhanced platelet-dependent cleavage of $^{125}$I-labeled factor XI was also observed in ADP-treated and buffer-treated samples but to a lesser extent than that in the collagen-treated sample. It can be concluded from the experiments presented in Figs. 1 and 2 that platelets promote the proteolytic cleavage of factor XII (presumably) by kallikrein and that this fragmentation of the factor XII molecule is associated with enzymatic activation as measured by factor XI cleavage.

Effects of Platelets on Coagulant Activities

To gain additional information about whether the proteolytic cleavage of factor XII or factor XI is associated with activation of these molecules, the coagulant activities of purified proteins were tested in plasmas deficient in factors XII, XI, IX and VII.

Effects of platelets in factor-XII-deficient plasma. In seven similar experiments, washed platelets were incubated with collagen or ADP, HMW kininogen, kallikrein, and factor XII after which clotting times were determined in factor-XII-deficient plasma (Table 1). Although generally similar results were obtained on each occasion, the variability in clotting times from experiment to experiment due to the use of different platelet preparations, different concentrations of reactants, and different experimental conditions precluded meaningful averaging of results. Therefore in this and subsequent experiments representative experiments are presented. When mixtures containing platelets and collagen, ADP or buffer were compared with similar mixtures not containing platelets (lines 1–3), shortened clotting times were observed which were most marked with collagen-treated platelets, less with ADP-treated platelets and least with buffer-treated platelets. When celite replaced platelets in the incubation, clotting times were even shorter (line 4). The coagulant activity developing in the presence of collagen-treated or ADP-treated platelets or in the presence of celite was entirely dependent on the presence of kallikrein (lines 9–12). In contrast, the exclusion of HMW kininogen greatly diminished the coagulant activity developing in mixtures containing celite (lines 4 and 8), but only slightly prolonged clotting times in the presence of platelets (lines 1–3 and 5–7). These data are consistent with a platelet-dependent, kallikrein-dependent factor-XII activation that is enhanced by treatment of platelets with collagen or ADP.

Effects of platelets in factor-XI-deficient plasma. To examine the effects of factor XI$^\alpha$ on the coagulant activity of factor XI in the presence and absence of platelets, washed platelets or calcium-free Tyrode’s solution were incubated with collagen, buffer or kaolin, and factor XI$^\alpha$, HMW kininogen, and factor XI were added to the incubation mixture (Table 2). Clotting times of the incubation mixture were then determined in factor-XI-deficient plasma. When mixtures containing platelets and collagen or buffer were compared with similar mixtures not containing platelets (Table 2, lines 1 and 2), marked shortening of clotting times was observed, indicating that the presence of platelets was associated with the generation of a coagulant activity that corrected the clotting time defect of factor-XI-deficient plasma. In the absence of platelets, kaolin shortened the clotting time of factor-XI-deficient plasma from 163 sec (line 1) to 120 sec (line 7). In comparison collagen-treated platelets in the absence of kaolin shortened the clotting time to 102 sec (line 1). Collagen had no effect in the absence of platelets (lines 1 and 2) whereas a significant shortening of clotting time occurred in the presence of collagen and platelets (line 1 and 2). This experiment suggests that collagen-treated or untreated platelets can promote the activation of factor XI by factor XII$^\alpha$ in the presence of HMW kininogen and that platelets may have an additional advantage over kaolin by enhancing the rate of some subsequent coagulation reaction.

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Clotting Times (sec)</th>
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<tbody>
<tr>
<td></td>
<td>Platelets Present</td>
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<tr>
<td>Collagen or Kaolin</td>
<td>Factor XI$^\alpha$</td>
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<td>Line 1</td>
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<td>Line 2</td>
<td>Buffer + + +</td>
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<td>Line 3</td>
<td>Collagen - + +</td>
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<td>Line 4</td>
<td>Buffer - + +</td>
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<tr>
<td>Line 5</td>
<td>Collagen + - -</td>
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<tr>
<td>Line 6</td>
<td>Buffer + - -</td>
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<td>Line 7</td>
<td>Kaolin + + +</td>
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<td>Line 8</td>
<td>Kaolin + - -</td>
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</table>

A suspension (100 μl) of platelets (4.75 x 10⁹/ml) in calcium-free Tyrode’s solution, pH 7.3, containing bovine serum albumin (1 mg/ml), prepared as described (see Materials and Methods) or buffer was incubated in a 10 x 75 mm siliconized glass tube for 30 sec at 37°C with 5 μl of purified collagen (250 μg/ml) or TBS or kaolin. To this mixture was added 15 μl of factor XI$^\alpha$ (0.95 μg), 5 μl of a mixture of HMW kininogen (0.26 μg), and factor XI (0.12 μg) or TBS. After a further 2-min incubation, 200 μl of a mixture of factor-XI deficient plasma and rabbit brain cephalin and 100 μl of 50 mM CaCl₂ were added and clotting times were determined.

*The numbers in parentheses represent mU of factor XI$^\alpha$ activity derived from the clotting times and a standard curve of normal pooled plasma assayed in factor-XI-deficient plasma in the presence of kaolin as described in Materials and Methods. They are presented for reference only and should not be interpreted literally as amount of factor XI$^\alpha$ generated, since as pointed out in Results and Discussion, platelets enhance the apparent activity of factor XI$^\alpha$. From www.bloodjournal.org by guest on November 9, 2017. For personal use only.
Effects of platelets in factor VII- and -IX-deficient plasmas. To explore the possibility that the advantage of collagen-treated platelets over kaolin in the experiment described above was a result of the acceleration of some subsequent coagulation reaction and to determine the specificity of the effect observed in factor-XI-deficient plasma, experiments similar to those described above were carried out with incubation mixtures containing factor XIIa, HMW kininogen, factor XI, platelets, or Tyrode's solution, and either collagen or buffer or kaolin tested for their coagulant activity in plasmas deficient in either factor XI, IX, or VII. It was reasoned that if the coagulant advantage of platelets was operative in the extrinsic system of clotting, it would not be observed if factor-VII-deficient plasma were used to test it. Very similar clotting times were observed whether factor-XI-deficient or factor-VII-deficient plasma was used (Table 3). Thus, the coagulant advantage of platelets would appear to reflect exclusively their role in the intrinsic clotting mechanism and subsequent activation of prothrombin. In contrast to the results with either factor VII- or factor-XI-deficient plasma, when these studies were carried out using factor-IX-deficient substrate plasma the clotting times were uniformly prolonged indicating that an activity was not generated in the incubation mixture which bypassed factor-IX activation. Therefore, these results suggest that the coagulant activity observed in factor-XI-deficient plasma involved the activation of factor XI itself. Also an additional enhancement of subsequent reactions that are dependent on the presence of factor IX may have been involved.

Effects of HMW kininogen, factor XIIa and factor XI. To examine the dependency of the platelet-related activation of factor XI upon factor XIIa, HMW kininogen and factor XI, platelets, or Tyrode's solution were incubated with factors XIIa, HMW kininogen, or factor XI in the presence of collagen or buffer. When collagen-stimulated platelets were present and factor XIIa was excluded from the incubation mixture (Table 2, line 3 and Table 3, line 5), a marked prolongation of clotting time occurred compared with the results in the presence of factor XIIa (Table 2, line 1 and Table 3, line 1). A similar prolongation of clotting time was observed when collagen-stimulated platelets were tested in the absence of HMW kininogen and factor XI (Table 2, line 5) compared with the results of the complete system (Table 2, line 1). Thus, the activation of factor XI as judged by its coagulant activity in the presence of collagen-treated platelets would appear to be dependent upon the presence of factor XIIa, HMW kininogen, and factor XI.

### Relationship of Coagulant Activities and Proteolytic Cleavage

An important question concerns the relationship between coagulation assays and determination of proteolytic cleavage as measures of zymogen activation in the presence of platelets. The relationship between coagulant activity, measured in factor-XII-deficient plasma, and cleavage of factor XII, measured by SDS-PAGE, was examined in mixtures containing either celite or washed platelets, treated with collagen, ADP, or buffer and incubated for 1-40 min with HMW kininogen, kallikrein, and 125I-labeled factor XII (Fig. 3). It is evident that a linear relationship exists between coagulant activity and cleavage of factor XII during incubation either with unstimulated platelets or with celite. In contrast, in the presence of collagen-treated or ADP-treated platelets, the coagulant activity measured in factor-XII-deficient plasma greatly exceeded that observed for an equivalent

### Table 3. Clotting Times of Factor-XI, -IX and -VII Deficient Plasmas in Presence and Absence of Collagen-Treated Platelets, Factor XII, High Molecular Weight Kininogen, and Factor XI

<table>
<thead>
<tr>
<th>Incubation Mixtures</th>
<th>Clotting Times (sec) in Substrate Plasma</th>
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<tbody>
<tr>
<td>Line</td>
<td>Platelets</td>
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A suspension (100 µl) of washed platelets (5.81 x 10⁸/ml) or buffer was incubated with the specified reactants as indicated in Table 1. Clotting times were then determined in substrate plasmas deficient in factors XI, IX, and VII as detailed in Table 1.

*When purified factor IX was added to the incubation mixture, the clotting time of factor-IX deficient plasma was 133 sec.
amount of factor XII cleaved in the presence of unstimulated platelets or celite. Furthermore, the apparent coagulant activation of factor XII in mixtures containing collagen-treated or ADP-treated platelets occurred within the first few minutes of incubation and increased only slightly thereafter whereas cleavage of the molecules occurred progressively over a 40-min time course.

To examine the relationship between coagulant activity and cleavage of factor XI by factor XII, in the presence of HMW kininogen and platelets or celite, the experiment shown in Table 4 was done. After a relatively short (10-min) incubation, clotting times of factor-XI-deficient plasma were much shorter in the presence of collagen-treated platelets (line 1) than with celite (line 5), whereas a greater quantity of factor XI was cleaved in the presence of celite (line 5) than with collagen-treated platelets (line 1). It is apparent from these observations that the activation of factors XII and XI in the presence of stimulated platelets results in a considerable enhancement of their apparent coagulant activities. Thus an equivalent or lesser amount of factor XII or factor XI cleaved in the presence of platelets or celite gives rise to greatly enhanced coagulant activity in the presence of stimulated platelets. Possible explanations of these observations are discussed below.

**Requirements for Proteolytic Activation of Factor XII**

To examine the requirements for the proteolytic activation of factor XII, HMW kininogen, kallikrein, and \(^{125}\)I-labeled factor XII were incubated in the presence or the absence of either celite or collagen-treated or ADP-treated platelets (Table 1). Factor-XII cleavage was platelet or celite dependent and was

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**Table 4. Relationship Between Coagulant Activity and Cleavage of Factor XI in Presence of Platelets or Celite**

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Clotting Times (sec)</th>
<th>(^{125})I F XI Cleaved (ng)</th>
</tr>
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<tbody>
<tr>
<td>Line 1</td>
<td>Platelets</td>
<td>Collagen</td>
</tr>
<tr>
<td>Line 2</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Line 3</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Line 4</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Line 5</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

A suspension (125 \(\mu\)l) of washed platelets (5.74 \(\times\) \(10^{4}\)/ml) or Tyrode’s solution was incubated in a 10 \(\times\) 75 mm siliconized glass tube with 5 \(\mu\)l of purified collagen (250 \(\mu\)g/ml), celite (1.35 mg/ml), or TBS. To this mixture was added 5 \(\mu\)l of factor XII (0.95 \(\mu\)g), 5 \(\mu\)l of a mixture of HMW kininogen (0.26 \(\mu\)g), and cold \(^{125}\)I-labeled factor XI (0.12 \(\mu\)g) or TBS. After a further 10-min incubation, clotting times were determined in factor-XI deficient plasma as described in the footnote to Table 2 or samples were analyzed by SDS-PAGE as described in Materials and Methods.
greater in the presence of celite (line 4) than with platelets (lines 1–3). The amount of factor XII cleaved in the presence of platelets was enhanced by collagen (line 1), by ADP (line 2) and by HMW kininogen (lines 1–3 and 5–7). Factor-XII cleavage with either platelets or celite was entirely kallikrein-dependent (lines 1–4 and 9–12).

The nonsteroidal anti-inflammatory drug indomethacin is known to inhibit the secretion of stored contents of platelet granules by inhibiting the cyclooxygenase enzyme that catalyzes the conversion of arachidonic acid to prostaglandin endoperoxide. To determine whether inhibition of platelet responses to ADP or collagen could influence the platelet-dependent cleavage of factor XII by kallikrein, the effect of 20 µM indomethacin on the cleavage of 125I-labeled factor XII was determined. Indomethacin inhibited the cleavage of factor XII by kallikrein 66% in the presence of collagen-treated platelets and 69% in the presence of ADP-treated platelets, and less than 4% in the presence of buffer-treated platelets. In a parallel experiment the release of [3H]-5-hydroxytryptamine from collagen-treated platelets was 80% inhibited by 20 µM indomethacin. In contrast, in control experiments done in the absence of platelets, indomethacin had no effect on the amount of factor XII cleaved by kallikrein in the presence of celite.

**Requirements for Proteolytic Activation of Factor XI**

A series of experiments was carried out to determine to what extent the platelet-dependent proteolytic cleavage of factor XI was dependent upon the presence of HMW kininogen, factor XII, factor XIIa, and kallikrein. In the experiment shown in Table 5, either collagen-treated platelets, collagen alone, or celite alone was incubated with various mixtures of HMW kininogen, factor XII or factor XIIa, kallikrein, and 125I-labeled factor XI. When platelets were absent and collagen was incubated with various mixtures of the purified proteins, only minimal cleavage (< 2%) of factor XI was observed. Cleavage of factor XI was increased 13-fold in the presence of collagen-treated platelets and 23-fold with celite present in the absence of platelets (line 1). Generally similar results were obtained when factor XII replaced factor XII and kallikrein in the incubation mixture (line 5). When HMW kininogen was excluded from an incubation mixture containing factor XII, kallikrein and factor XI (line 2) the amount of factor XI cleaved was decreased 34% in the presence of collagen-treated platelets and 46% in the presence of celite alone. When factor XII was excluded from the incubation mixture containing HMW kininogen, kallikrein and factor XI (line 3) insignificant amounts of factor XI were cleaved in the sample containing celite without platelets. Thus the celite-dependent cleavage of factor XI strictly required factor XII. In contrast, 54% of the amount of factor XI cleaved in the presence of collagen-treated platelets, HMW kininogen, kallikrein and factor XII was cleaved in a similar incubation mixture to which factor XII was not added (line 3). Finally when kallikrein was excluded from the incubation mixtures, insignificant cleavage of factor XI occurred in any of the incubation mixtures. It can be concluded from this experiment that the platelet-dependent proteolytic activation of factor XI in mixtures containing factor XII, HMW kininogen and/or kallikrein required kallikrein and was enhanced two to three-fold by factor XII, thus demonstrating that significant proteolytic activation of factor XI occurred in the absence of added factor XII, provided platelets were present.

**Association of Factor-XI Cleavage Products With Platelets**

It was important to determine whether the cleavage products of factor XI were associated with platelets. Therefore, mixtures containing HMW kininogen, kallikrein, and 125I-labeled factor XI, incubated with or without factor XII or platelets, were centrifuged and the supernatant and pellet were analyzed separately by SDS-PAGE (Table 6). Insignificant cleavage of factor XI was observed in the absence of platelets, whereas with platelets present the cleavage

**Table 5. Cleavage of 125I-Factor XI in Presence and Absence of Platelets, HMW Kininogen, Factor XII, Factor XIIa, and Kallikrein**

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Amount (ng) 125I F XI Cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets + Collagen</td>
<td>Buffer + Collagen</td>
</tr>
<tr>
<td>1</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
</tr>
</tbody>
</table>

To 75 µl of platelet suspension (6.77 x 10⁷/ml) or calcium-free Tyrode’s solution, pH 7.3, containing bovine serum albumin (1 mg/ml) was added 5 µl of each of the reactant indicated or TBS buffer. The amount of factor XII added was 0.36 µg. Otherwise the amount of each reactant added and experimental conditions are as detailed in the legend to Fig. 2.
products appeared in the pellet, not in the platelet-free supernatant. Treatment of platelets with thrombin or collagen was associated with a 150%–230% increase in the number of factor-XI molecules cleaved either in the presence of absence of added factor XII. The total number of factor XI molecules sedimenting with the platelets was similarly increased by treatment of platelets with thrombin or collagen, as was the number of molecules cleaved per platelet (Table 7), either in the presence of absence of added factor XII.

**DISCUSSION**

Previously published investigations, utilizing platelet and plasma samples obtained from patients with congenital deficiencies of plasma coagulation proteins, presented evidence that platelets treated with ADP or collagen have a role in the interaction and activation of factors XII and X.

In the present study we have shown that platelets could promote the initiation of intrinsic coagulation in the apparent absence of factor XII provided either plasma factor XI or platelet factor-XI-like activity was present. Observations by some investigators have failed to confirm a possible role of platelets in contact activation. However, one of these studies examined the effect of platelets on factor-XII activation in the presence of HMW kininogen and prekallikrein, not examined in the present study, whereas another concluded that collagen-treated platelets do not activate factor IX directly, a conclusion also made in previous studies from our laboratory. The purpose of the experiments reported here was to evaluate further the contribution of platelets to contact activation using purified coagulation factors and isolated platelets.

In the present study we have shown that platelets can promote the proteolytic activation of factor XII by kallikrein. At least two lines of evidence support this conclusion. First, proteolytic cleavage of factor XII occurred in the presence of kallikrein, HMW kininogen, and either platelets or an artificial negatively charged surface such as celite (Fig. 1 and Table 1). Second, the development of factor XII, coagulant activity in mixtures containing factor XII, kallikrein, HMW kininogen, and either platelets or celite provides evidence that fragmentation of the factor XII molecule was associated with the appearance of coagulant activity (Table 1). Possible further evidence of factor-XII activation is provided by the proteolytic cleavage of factor XI observed when activated platelets or celite were incubated with factor XII, kallikrein, HMW kininogen, and factor XI (Fig. 2 and Table 5), although the factor XI cleavage observed in the presence of platelets could also be factor XII independent as discussed below.

It has been suggested that the mechanism by which negatively charged surfaces promote the activation of factor XII involves a conformational change in its...
native structure,89 making it much more susceptible to proteolytic activation by plasma proteases such as kallikrein.10 To define the mode of action of platelets in the activation and cleavage of factor XII, it was important to determine whether the platelet contribution was dependent upon or independent of the presence of kallikrein. The data presented here support the conclusion that the platelet contribution to factor-XII proteolytic activation and cleavage is kallikrein-dependent. Therefore, we do not conclude that platelets directly activate factor XII, but rather that platelets can provide a surface, perhaps similar to that provided by such negatively charged substances as kaolin or glass, which renders factor XII more susceptible to proteolytic activation by plasma kallikrein.

The evidence presented here that platelets can also promote the proteolytic activation of factor XI is as follows. The coagulant activity in factor-XI-deficient plasma of mixtures of purified factor XII, HMW kininogen, and factor XI was greatly enhanced in the presence of collagen-treated platelets or mildly enhanced by untreated platelets whereas collagen had no effect in the absence of platelets (Table 2). The development of this coagulant activity was (1) dependent upon the presence of factor XII, HMW kininogen and factor XI (Table 2); (2) independent of the extrinsic system of blood coagulation (Table 3); and (3) not observed in the absence of factor IX (Table 3). Therefore, the observed coagulant activity involves intrinsic coagulation reactions occurring prior to the activation of factor IX. The hypothesis that the observed coagulant activity results from a platelet-dependent proteolytic activation of factor XI was borne out by the demonstration that cleavage of 125I-labeled factor XI occurred in incubation mixtures containing factor XII, HMW kininogen, and kallikrein in the presence of collagen-treated, ADP-treated or untreated platelets (Fig. 2 and Table 5).

When the effects of HMW kininogen, kallikrein, and factor XII on the proteolytic cleavage of 125I-labeled factor XI were examined in the presence and absence of collagen-treated platelets (Table 5), several important observations were made. First, in the absence of platelets collagen had no discernible effect on factor-XI cleavage in the presence of kallikrein, HMW kininogen, and factor XII. When either a suspension of collagen-treated platelets or celite was present, significant amounts of factor XI were cleaved in mixtures containing either kallikrein, HMW kininogen, and factor XII (Table 5, line 1) or HMW kininogen and factor XII (Table 5, line 5). HMW kininogen is known to form a complex with prekallikrein and also with factor X and to enhance the proteolytic activation of factor XI by factor XII.116 Thus it was not surprising to observe that when HMW kininogen was excluded from the incubation mixtures containing either platelets and collagen or celite (Table 5, line 2), a lesser extent of cleavage of factor XI was observed than in its presence (Table 5, line 1). Kallikrein is known to cleave and activate factor XII,37 and was required for factor-XI cleavage in mixtures containing HMW kininogen and factor XII (Table 5, lines 1 and 4). Factor XII, apparently activated by kallikrein, was also required for factor-XI cleavage in the presence of celite (Table 5, line 3). In contrast when collagen-treated platelets were substituted for celite, significant factor-XI cleavage occurred in mixtures containing HMW kininogen and kallikrein in the absence of factor XII. This result confirms the hypothesis that factor-XI activation in the presence of collagen-stimulated platelets can proceed in the absence of added factor XII. This suggests that platelets may possess a factor-XII-like activity that can substitute for added purified plasma factor XII. Observations presented previously indicate that platelets have factor-XI-like coagulant activity35 and factor-XI antigen,36 whereas only trace factor-XII-like activity can be detected.1835 We conclude that platelets can promote the proteolytic activation of factor XI in the absence of added factor XII provided kallikrein is present.

The mechanism by which collagen-treated platelets can participate in the proteolytic activation of factor XI is conjectural. The most likely explanation is that platelets provide a negatively charged surface upon which coagulation factors can be assembled and proteolytically activated. A similar mechanism has been proposed for the orientation and proteolytic cleavage of factor XI by factor XII, in the presence of HMW kininogen on a negatively charged surface such as celite, kaolin, or glass.1227 However, as pointed out above, collagen-treated or thrombin-treated platelets would appear to provide more than simply a surface such as that provided by kaolin or celite since they effect the proteolytic activation of factor XI in the absence of added factor XII and since they contribute to the development of significantly more coagulant activity in mixtures of purified contact factors than does either kaolin or celite. Another possible mechanism by which platelets might promote the proteolytic activation of coagulation zymogens is by the secretion or stimulus-specific activation of platelet proteolytic enzymes, although none of the proteases described in platelets3739 is known to recognize coagulation proteins as substrates.

Several lines of evidence indicate that the zymogen activations observed are platelet-related. First activation of platelets by ADP, collagen, or thrombin
enhanced both coagulant activity and proteolytic cleavage of factor XII and of factor XI. Secondly, inhibition of platelet responses by indomethacin was associated with inhibition of platelet-dependent cleavage of factor XII. Finally, the cleavage products of factor XI generated either in the presence or in the absence of factor XII were localized in the platelet-rich pellet and were not present in the platelet-poor supernatant of centrifuged samples. The experiments presented in Tables 6 and 7 suggest that both factor XI and factor XI<sub>a</sub> are bound to platelets; however, further studies will be required to determine whether or not this binding is specific, saturable, and reversible and to define the kinetic and thermodynamic constants for the binding of factor XI and factor XI<sub>a</sub>. The observation that activated platelets bind approximately 500–800 molecules of factor XI<sub>a</sub> per platelet seems reasonable in light of the known binding capacities of platelets for factor X<sub>a</sub> and thrombin<sup>41</sup>.

Some of the experiments reported here were designed to examine the correlation between the coagulant activity and the proteolytic cleavage of factor XII or factor XI observed in the presence of either platelets or artificial surfaces. When mixtures containing kallikrein, HMW kininogen, and factor XII were analyzed, a linear relationship between factor XII coagulant activity and cleavage of factor XII was observed in the presence of either unstimulated platelets or celite (Fig. 3). In contrast, with collagen-treated or ADP-treated platelets present, the coagulant activity was two to ten-fold greater than that observed for an equivalent amount of factor XII cleaved with celite present. Therefore, platelets incubated with collagen or with ADP would appear to enhance the coagulant activity of factor XII<sub>a</sub>. A similar effect was observed in mixtures of factor XII<sub>a</sub>, HMW kininogen and factor XI, which produced a clotting time of 117 sec in factor-XI deficient plasma with 47% cleavage of factor XI<sub>a</sub> in the presence of celite, compared with a clotting time of 97 sec and 9% cleavage of factor XI in a similar mixture with celite absent and collagen-treated platelets present (Table 4). It is inferred from this observation that collagen-treated platelets have a coagulant advantage over a combination of celite and phospholipids in some reaction subsequent to factor-XI activation. Possible sites of action of collagen-treated platelets are factor-X or prothrombin activation in which available evidence indicates platelets are involved.<sup>40–45</sup> Consistent with this possibility is our recent observation of a 5–50-fold enhancement of the factor-XI activity, measured in an activated partial thromboplastin time, of a mixture of isolated platelets or platelet membranes and purified factor XI or factor XI<sub>a</sub>, compared with the sum of the factor-XI activity of either separately.<sup>46</sup> Potentiation of the coagulant activity of contact factors by activated platelets may serve to promote localization of initial coagulation reactions and subsequent biologic amplification within platelet plugs.

Any consideration of the physiologic relevance of these studies, which utilized relatively artificial incubation conditions, is extremely complex. In most of the experiments the ratios of reactants were similar to the ratios of these coagulation proteins in plasma. The incubation times required to detect significant proteolysis of factors XII and XI were quite long, but coagulant activity reflecting activation of these zymogens developed within 1 min, which is consistent with a variety of interpretations. One of these is that the activation of only a very few molecules of factor XII or factor XI is sufficient to trigger intrinsic coagulation that is further amplified on the platelet surface. Another possible interpretation is that proteolysis is not essential for activation. However, the present studies were not designed to address this extremely difficult and controversial question.

The physiologic significance of the observations presented here may relate to the mechanisms by which normal hemostasis and coagulation can be initiated and sustained in vivo. It has been suggested that the events of primary hemostasis, i.e., platelet adhesion, aggregation and secretion, and blood coagulation are linked<sup>47,48</sup> As platelets aggregate to form a hematic plug, they provide a surface for assembling, activating, and binding coagulation proteins in a sequential manner beginning with the initiation of clotting by proteolytic activation of "contact factors" on platelets and culminating in the local explosive generation of large amounts of thrombin and fibrin. The protection by platelets of activated clotting factors from inactivation by natural inhibitors in plasma may further localize the clotting process to a protective nidus provided by aggregating platelets.<sup>54,55</sup> As shown here platelets may provide and in vivo mechanism by which intrinsic coagulation can be triggered in the apparent absence of plasma factor XII thus providing a possible explanation for the absence of a hemostatic defect in patients with factor-XII deficiency or Hageman trait.<sup>50</sup> Our data do not explain the absence of a hemostatic defect in patients with prekallikrein deficiency. The possible pathophysiologic significance of the part played by platelets in promoting coagulation reactions, particularly those early events related to contact activation, is further supported by observations indicating deficient platelet coagulant activities in patients with hemostatic deficiency states such as
ROLE OF PLATELETS IN CONTACT ACTIVATION

thrombasthenia\textsuperscript{51} and the hereditary giant platelet syndrome of Bernard and Soulier,\textsuperscript{52} and hyperactive platelet coagulant activities in patients with certain thrombocytopenic disorders such as venous thrombembolism\textsuperscript{53} and transient cerebral ischemia.\textsuperscript{54}

REFERENCES


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Contributions of human platelets to the proteolytic activation of blood coagulation factors XII and XI

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