Evidence for Tissue Factor-Dependent Activation of the Classic Extrinsic Coagulation Mechanism in Blood Obtained From Bleeding Time Wounds

By Harvey J. Weiss and Bruce Lages

The activation of platelets and the coagulation mechanism was studied by collecting blood from a standard bleeding time incision at 30-second intervals and measuring the plasma concentrations of fibrinopeptide A (FPA), platelet factor 4 (PF₄), and thromboxane B₂ (TxB₂). FPA was observed in the first samples (30 to 60 seconds) obtained, increased progressively until cessation of bleeding, and was markedly diminished after heparin administration, thus indicating that thrombin formation occurs early in incisional blood. PF₄ increased monotonically throughout blood sampling, whereas the major increase in TxB₂ appeared near the cessation of bleeding. The initial increase in FPA content occurred normally in patients with deficiencies of either factor IX or VIII, was markedly diminished in patients with factor X or V deficiency, and was delayed in patients with factor VII deficiency. These studies suggest that tissue factor activation of the classic (activation of factor X) extrinsic coagulation mechanism occurs as an early event during the arrest of bleeding from bleeding time incisions. The relation of the aforementioned to platelet activation is less clear because there was no consistent correlation between decreased FPA formation and impaired PF₄ secretion or TxB₂ production. In fact, the latter were normal in some subjects with the most impaired FPA formation, which suggests that both collagen and thrombin, perhaps synergistically, may contribute to platelet activation during the primary arrest of bleeding.

MATERIALS AND METHODS

Bleeding Time and Collection of Incision Blood for Sequential Measurement of Blood Loss and Radioimmunoassays

After compressing the upper arm with a sphygmomanometer cuff to a pressure of 40 mm Hg, two longitudinal incisions (perpendicular to the antecubital crease) were made on the volar surface of the forearm by using a Simplate II bleeding time device (Organon-Teknika Corp, Durham, NC). At 30-second intervals, blood from each of the two wounds was collected into heparinized microhematocrit capillary tubes (Fisher Scientific Co, Pittsburgh) of known diameter. The incision and blood collection were performed by the same operator throughout the study. The height of the column of blood was immediately measured, and from this the blood volume was determined. The blood was then gently expressed into microcentrifuge tubes containing either 120 µL phosphate-saline buffer (0.025 mol/L NaH₂PO₄, 0.025 mol/L, Na₂HPO₄, 0.15 mol/L NaCl), pH 6.8, containing 12 µmol/L indomethacin and 5 mmol/L EDTA. The major increase in TxB₂ occurred as an early event during the arrest of bleeding from bleeding time incisions.
phylline for the measurement of PF₄ and FPA levels in blood from the distal wound. The diluted wound blood samples were centrifuged at 12,000 g for two minutes at room temperature, and 100 μL of the supernatants was removed. Aliquots of these supernatants were processed for FPA radioimmunoassay (see the next section) or stored frozen at -80°C before assay of the other substances. As a control on the assay procedures for determining TxB₂, PF₄, and FPA levels, several drops of unanticoagulated venous blood were taken directly from the butterfly infusion set tubing onto a piece of parafilm and then drawn up into capillary tubes, measured, and processed as described for the wound blood samples. The results on these samples are indicated as control values. All studies were performed under a protocol approved by the Institutional Review Board of the St Luke’s-Roosevelt Institute for Health Sciences.

**FPA**

Radioimmunoassay of FPA was performed by a modification of the method of Nossel et al. Briefly, samples were incubated (10.5, vol/vol) for ten minutes at room temperature with a suspension of 80 mg/mL bentonite (Sigma Chemical Co, St Louis) in Tris-buffered saline (0.1 mol/L NaCl, 0.05 mmol/L Tris), pH 8.9, containing 0.1% chicken ovalbumin (Sigma). The mixture was then centrifuged at 4,800 g for ten minutes at 4°C and the supernatant plasma was removed and refrozen until radioimmunoassayed for FPA. Adsorbed plasmas were diluted in the same buffer, incubated with anti-FPA serum at 4°C for 18 hours, and after the addition of 125I-desaminotyrosyl-FPA, incubated for an additional two hours. A charcoal suspension was added and the assay mixtures centrifuged at 4°C. Decanted supernatants were counted on a Beckman Gamma 4000 counter, and values were determined from a curve obtained by testing serial dilutions of FPA standards. FPA antiserum, FPA standards, and desaminotyrosyl-FPA were obtained from IMCO Corp, Stockholm; desaminotyrosyl-FPA was labeled with 125I by the chloramine-T method.

**PF₄**

PF₄ levels were measured by radioimmunoassay using the assay kit from Abbott Laboratories, Diagnostics Division (Chicago). 125I-labeled PF₄ was used as a tracer, and the separation of bound and free fractions was achieved by ammonium sulfate precipitation. The sensitivity of our assay procedure expressed as nanograms per milliliter of PF₄ giving 10% displacement of tracer, was 3.5 ± 1.0 (SD) ng/mL, and the interassay coefficient of variation was 11.0%.

**TxB₂**

TxB₂ levels were measured by radioimmunoassay using TxB₂ standards obtained from the Upjohn Co (Kalamazoo, MI), 3H-TxB₂ tracer obtained from NEN Products (Boston), and a sensitive and specific antibody obtained from Dr J.B. Smith, Temple University, Philadelphia, PA. This assay had a sensitivity of 0.0072 ± 0.0041 pmol TxB₂ (TxB₂ was required for 10% displacement of the tracer) and interassay and intraassay coefficients of variation of 12.8% and 11.0%, respectively. The cross-reactivities of this antibody with other prostaglandins (PG), including 6-keto-PGF₁α, were all less than 0.05%.

**Coagulation Assays**

Coagulation factors were assayed by one-stage methods using as substrates plasmas from patients with severe congenital deficiencies of these factors or, in the case of factor V, artificially depleted plasma.

**Subjects With Coagulation Defects**

Ten patients, aged 24 to 47, had severe congenital deficiencies of one coagulation factor (VIII, IX, V, VII, or X), as indicated in Table 1, and had not had a transfusion for at least 2 months before the study. The four patients with factor VIII deficiency and the one patient with factor IX deficiency required periodic transfusions for...
episodes of clinical bleeding. Two of the factor VIII–deficient patients had recently ingested either ibuprofen (VIII-1) or indomethacin (VIII-4) and were found to have impaired TxB2 production (see Results). A third patient (VIII-3) had no detectable TxB2 in his bleeding samples but had not been specifically questioned about drug ingestion. Patient VIII-1 was retested after abstaining from all medication for at least 1 week. The patients with factor V and X deficiency had moderate bleeding symptoms occasionally requiring factor replacement. Among the three patients with factor VII deficiency, all of whom had symptoms of a moderate bleeding disorder, two (patients VII-1 and VII-2) were reported previously to have factor VII levels <5% (patients 12 and 16, respectively), and factor VII antigen levels obtained recently on all three patients (VII-1, 2, 3) by using an enzyme-linked immunosorbent assay method have been found to be 1%, 50%, and 1.7% of normal, respectively (Dr Douglas A. Triplett, Muncie, IN, personal communication). Two normal subjects (aged 24 and 28) were studied before and 15 minutes after intravenous administration of 5,000 units of heparin (Liquaemin sodium, Organon, West Orange, NJ).

Normal Subjects

Controls for the study were normal hospital personnel (n = 13), aged 25 to 50, who denied recent ingestion of drugs known to affect platelet function or hemostasis. Bleeding time and volume studies were performed on 13 subjects (five males, eight females) and radioimmunoassay studies on nine subjects (four males, five females).

RESULTS

Normal Subjects

Average sequential values (±SEM) for blood volume, FPA, PF4, and TxB2 obtained in normal subjects are shown in Fig 1. Because of the variable bleeding times (3.0 to 8.0 minutes), the average values for times greater than 3.0 minutes were obtained from a progressively lesser percentage of the total number of subjects studied, and the last two points on the assay curves are the average of two values (for which no SEM are shown).

The volume of blood increased during the first two minutes and then declined until cessation of bleeding. Both FPA and PF4 were consistently detectable in the earliest blood samples. The average FPA values in the 1-, 2-, and 3-minute samples were 203, 547, and 1,107 ng/mL (control values, 3.9 ± 3.1 [SD]), and the average concentration during the first three minutes was 491 ng/mL (Table 2), thus reflecting an increase in thrombin concentration. More direct confirmation for prothrombin activation was obtained by using a specific radioimmunoassay25,26 for the factor Xa–mediated prothrombin fragment F1+2, kindly performed by Drs Kenneth Bauer and Robert Rosenberg, Boston. In one normal subject, F1+2 was found to increase progressively over the control value (1.0 nmol/L) to a value of 30.2 nmol/L in the final sample (FPA concentration, 1,784 ng/mL) obtained before cessation of bleeding at 3.5 minutes. The average PF4 values in the first three minutes were 246, 456, and 811 ng/mL (control values, 29.6 ± 20.2 [SD]), and the average concentration was 404 ng/mL (Table 2). Visual inspection of the curves (Fig 1) suggests a linear increase for PF4 and an exponential increase for FPA throughout the period of sampling. In most normal subjects, TxB2 was undetectable during the first minute, after which TxB2 increased linearly in the midportion of the bleeding time curve and then rapidly near the cessation of bleeding.

Coagulation Defects

To study further the mechanisms that might account for the early burst of thrombin activity and its possible relation to platelet activation (increasing PF4 and TxB2), we studied patients with well-characterized coagulation defects. In addition to the curves in Figs 2 to 4 that show the sequential values for FPA, PF4, and TxB2 in patients and controls (gray area), we have also tabulated their average concentration during the first three minutes of bleeding and for the entire bleeding period (Table 2).

FPA. The initial increases in FPA content in all factor VIII–deficient patients were within the range of normal values during the first three minutes (Fig 2A), and this was true whether or not TxB2 was detectable in the incisional blood. In addition, the average plasma concentration (689 ng/mL) was similar to that observed in controls (491 ng/
In two subjects (VIII-2 and VIII-4), the bleeding time was at the upper limit of normal, and the FPA values either declined (patient VIII-4) or did not rise as rapidly (patient VIII-2) from three minutes to the termination of bleeding. FPA production was normal in the patient with factor IX deficiency.

As seen in Fig 2B, FPA concentrations in patients with factor V or X deficiency were strikingly decreased throughout the entire period of bleeding (Fig 2B), and the average concentrations (11 and 13 ng/mL, respectively) during the first three minutes of bleeding were approximately 2% of the normal value (Table 2). FPA generation was also abnormal in the three patients with factor VII deficiency but less strikingly so (Fig 2C). Each of the six values obtained during the entire period of bleeding (Fig 2C), as were the average concentrations of 21, 82, and 89 ng/mL (normal range, 202 to 825; Table 2). In all three subjects, FPA values increased rapidly in the terminal portion of the bleeding time and approached or achieved normal values. Administration of heparin (5,000 units) to two normal subjects markedly inhibited FPA production as expected (Fig 2D and Table 2).

**PF₄**  The findings for sequential PF₄ values are shown in Fig 3. PF₄ generation was normal in factor VIII deficiency, except for a moderately delayed production in one subject (VIII-4), and was normal in the patient with factor IX deficiency. The initial rate of PF₄ production was normal in factor X deficiency (Fig 3B and Table 2) and remained, for the most part, near the lower limit of normal values. In contrast, PF₄ generation was somewhat decreased in the patient with factor V deficiency (Fig 3B and Table 2), but the abnormality was less pronounced than was the impairment of FPA production. (The total releasable PF₄ determined in serum after clotting venous blood with thrombin, was the same in this patient as in normal subjects [data not shown].) Heparin reduced PF₄ production (to the same extent as factor V deficiency) in one normal subject (H-1), but PF₄ values in the other subject (H-2) remained within the normal range. PF₄ production was normal in all three patients with factor VII deficiency.

**TxB₂**  Results are shown in Fig 4 and Table 2. As seen in Fig 4A, TxB₂ production was decreased in three factor VIII-deficient patients, two of whom (VIII-1 and VIII-4) were known to have recently ingested nonsteroidal antiinflammatory agents. It was normal (or somewhat increased) when repeated in patient VIII-1 (after he had abstained from these drugs), patient VIII-2, and the patient with factor IX deficiency. TxB₂ production was also normal in the patients with factor V and factor X deficiency (Fig 4B) and in two of the three patients (VII-2 and VII-3) with factor VII deficiency (Fig 4C). In the one factor VII-deficient patient (VII-1) with a prolonged bleeding time, TxB₂ production was normal during the first 8.5 minutes but decreased in the later samples (9 to 12.5 minutes). In normal subjects receiving heparin, the initial rate of TxB₂ production was normal, but in subject H-2 the curve flattened at values that were generally somewhat decreased during the remainder of bleeding.

**Blood Loss After Bleeding Time Incisions**  In patients with factor VIII deficiency, the blood loss (volume)-v-time curve and the bleeding time were essentially

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### Table 2. Average Blood Concentrations and Bleeding Time Values

<table>
<thead>
<tr>
<th>Substances Assayed</th>
<th>Normal Subjects</th>
<th>VII</th>
<th>Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V X 1 2 3 Mean</td>
<td>1 2</td>
<td>VIII IX</td>
</tr>
<tr>
<td><strong>A. First 3 min</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPA (ng/mL)</td>
<td>491±86 (202-825)</td>
<td>11 13</td>
<td>21 82 89 64±22</td>
</tr>
<tr>
<td>PF₄ (ng/mL)</td>
<td>404±77 (220-773)</td>
<td>160 254</td>
<td>241 400 315 318±46</td>
</tr>
<tr>
<td>TxB₂ (pmol/mL)</td>
<td>4±2 (1-16)</td>
<td>5 6</td>
<td>1 6 6 4±2</td>
</tr>
<tr>
<td><strong>B. Entire period</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPA (ng/mL)</td>
<td>1,617±347 (727-4,221)</td>
<td>40 110</td>
<td>1,437 198 277 637±400</td>
</tr>
<tr>
<td>PF₄ (ng/mL)</td>
<td>827±122 (504-1,409)</td>
<td>399 417</td>
<td>1,096 539 492 791±181</td>
</tr>
<tr>
<td>TxB₂ (pmol/mL)</td>
<td>13±3 (4-27)</td>
<td>44 17</td>
<td>10 10 15 12±2</td>
</tr>
<tr>
<td><strong>Hemostasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleeding time (min)</td>
<td>6.0±0.5 (3-8.0)</td>
<td>15 7</td>
<td>12.5 4 7.5 8±2</td>
</tr>
<tr>
<td>Total blood loss (μL)</td>
<td>99±11 (17-181)</td>
<td>337 59</td>
<td>261 37 83 127±68</td>
</tr>
</tbody>
</table>

Values are the average values for the first six (1/2-minute) samples (A), or for all the samples (B) until cessation of bleeding. For groups where three or more subjects were studied (normal, factor VII, and factor VIII), SEM (±) and the range (in parentheses) of values are also shown.
COAGULATION MECHANISMS IN BLEEDING WOUNDS

Fig 2. FPA concentration in bleeding time blood in subjects with coagulation defects. (A) Factor IX (O) deficiency (n = 1) and factor VIII (O) deficiency (n = 4). Factor VIII-deficient patients in whom no TxB2 was detectable (TxB2-negative, see Fig 4) are distinguished (dashed lines) from those with measurable TxB2 (TxB2-positive, solid lines). Patient VIII-1 was studied under both conditions. (B) Factor V (O) and X (O) deficiency. (C) Factor VII deficiency (n = 3). (D) Two normal subjects (H-1 and H-2) 15 minutes after receiving 5.000 units of heparin. Subjects are identified by number in Table 1. Values obtained in normal subjects were within the area shown in gray.

normal except for a slight prolongation of the bleeding time to nine minutes in patients VIII-2 and VIII-4 (who had ingested indomethacin) and an increase in volume in patient VIII-2. The bleeding time and volume were normal in factor IX deficiency. These results are summarized in Table 2. As seen in Fig 5 and Table 2, the volume-v-time curves and total blood loss were strikingly abnormal in factor V but not factor X deficiency. These were abnormal in only one (VII-1) of the factor VII-deficient subjects (Fig 5).

DISCUSSION

The method described herein for sequentially measuring FPA, PF4, and TxB2 levels in blood emerging from bleeding time incisions was developed to study the activation of both platelets and coagulation during the primary arrest of bleeding. The early appearance of FPA in normal subjects and its suppression by heparin strongly suggest that both thrombin and fibrin production occur within 30 to 60 seconds of the incision and are consistent with observations from histological examination of bleeding time wounds and with the results of a recently published study independently demonstrating an early increase in FPA levels of the same order of magnitude as observed herein. In addition, in one normal subject studied, the F1+2 fragment of prothrombin increased progressively in bleeding time samples, thereby providing more direct evidence for prothrombin activation in incisional blood.

To determine the mechanism that might be operative in generating thrombin in bleeding time incisions, we studied patients with severe congenital deficiencies of coagulation factors. The normal generation of FPA that we observed in all patients with factor VIII deficiency during the first three minutes of bleeding is consistent with recent observations that fibrin can be observed at the periphery of the bleeding time wounds three minutes after an incision in patients with hemophilia. The additional findings that FPA generation is normal in factor IX deficiency, delayed in factor VII deficiency, and markedly abnormal in patients deficient in either factor V or X is consistent with a mechanism in which factor Xa and, subsequently, thrombin are generated initially by direct tissue factor VII activation of factor X (classic extrinsic system) and not through either the intrinsic pathway or the alternate tissue factor pathway involving factor VIII and Factor IX. The results and conclusions of other studies suggest that the type, depth, and location of an incisional wound may strongly influence the coagulation
were obtained from a different incision from that used for FPA and with coagulation disorders. Same legend as in Fig 2. Blood samples for performing bleeding time tests. For example, factor VIII but not factor IX. Thus, it is highly likely that activation of coagulation is influenced by the shear rate (which exceeds 1,000 s⁻¹ in arterioles and is probably less than 20 s⁻¹ in the wound, Dr V.T. Turitto, personal communication) and by the composition of the tissue to which the blood is exposed. Finally, it must be emphasized that our conclusions concerning thrombin formation apply particularly to the earliest (first three minutes) events in hemostasis where normal FPA values were observed in all hemophilic patients. The reason for the wider variation observed at later time periods, perhaps related to the prolongation of the bleeding time in a subset of these patients, requires further study.

The role of thrombin in the early activation of platelets and the permanent arrest of bleeding also requires further study. The increase in TxB₂ content that we and others observed and the rapid appearance of both FPA and PF₄ (similar to that recently reported for β-thromboglobulin) suggest that platelet activation/secretion could be, in part, thrombin mediated. That this may not be the sole mechanism, however, is suggested by the results obtained in subjects with coagulation defects. First, although the most severe defects in FPA production were observed after heparin administration and in the patients with factor V and factor X deficiency, PF₄ values were either normal (heparin subject 2), marginally reduced (factor X), or reduced to a lesser extent than FPA (factor V and heparin patient 1). In addition, TxB₂ production was normal in the patients with factor V and X deficiency. One possible explanation for these findings is that the formation of FPA from fibrinogen requires a higher concentration of thrombin than is required for TxB₂ production or the secretion of platelet α granule substances such as PF₄. This appears unlikely, however, because in previous in vitro studies this was never observed. The conclusions of Kaplan et al could be relevant to our findings: in areas where collagen is exposed, platelet release by collagen might be expected to precede evidence of fibrin formation. Thus, our findings and the evidence from previous in vitro studies suggest that thrombin and collagen may provide dual mechanisms for activating platelets during the early arrest of bleeding.

Finally, the technique reported here for studying the activation of platelets and the coagulation system in vivo after bleeding time wounds might be useful in detecting abnormalities of hemostasis that are not possible by current in vitro techniques. For example, abnormalities in tissue factor or in tissue factor expression might be detected by this method.

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