

Ex Vivo Coagulation Test on Tissue Factor-Expressing Cells with a Calibrated Automated Thrombogram

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ABSTRACT

A calibrated automated thrombogram is not affected by the turbidity of platelet and cell preparations because the measurement is based on fluorescence. To examine conditions that mimic the physiological state, we investigated thrombograms that show thrombin generation on tissue factor (TF)-bearing cells. An increase in the number of J82 cells did not affect the endogenous thrombin potential (ETP) of normal plasma, although the lag time (LT), the peak height, and the time to peak (ttPeak) did depend on cell concentration. When 5 parameters of coagulation factor-deficient plasmas were plotted on a radar graph, the thrombogram pattern of factor XI (FXI)-deficient plasma became slightly reduced. The thrombogram did not improve when washed normal platelets or washed normal platelets with adenosine diphosphate (ADP) were added. FVII-depleted plasma, FVIII-deficient plasma, and FIX-deficient plasma showed remarkably reduced peak heights, ttPeaks, and times to the end of thrombin generation (start tails). The thrombogram of FVII-depleted plasma was characterized by a remarkably prolonged LT, unlike the patterns of FVIII- or FIX-deficient plasma and FXI-depleted plasma. The ETP of FVIII- and FIX-deficient plasma, but not FVII-depleted plasma, improved significantly upon addition of washed normal platelets or washed normal platelets with ADP. The thrombograms of coagulation factor-deficient plasma containing TF-bearing cells differed from those for recombinant TF and phospholipid in the liquid phase. We suggest that thrombograms using TF-bearing cells can be a useful *ex vivo* test, because this experimental model may be analogous to most coagulation processes *in vivo*.

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KEY WORDS: *Ex vivo* test • Thrombin generation
• Tissue factor-bearing cell •
Coagulation factor-deficient plasma •
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INTRODUCTION

Blood coagulation tests have generally been performed by determining both the activated partial thromboplastin time to test intrinsic-pathway function according to the waterfall-cascade sequence and the prothrombin time to test extrinsic-pathway function. Although these coagulation tests are useful, they do not always accurately reflect clinical symptoms because they are carried out under nonphysiological conditions [1-2]. It has been thought that factor VII (FVII) and activated FVII (FVIIa) bind *in vivo* to tissue factor (TF) derived from TF-bearing cells on damaged endothelium and that this complex activates FX and FIX to generate abundant thrombin via amplification of the coagulation cascade [3,4].

The calibrated automated thrombogram (CAT), which measures time-dependent thrombin generation with trace tissue thromboplastin without defibrination treatment, was developed to assess coagulation mechanisms relevant to abnormal hemostasis [5]. The CAT is not influenced by turbidity because it measures fluorescence from a thrombin-cleaved substrate [6]. Although the CAT has been reported to be useful for assessing a thrombotic tendency [7-9], there are no reports of its use as an *ex vivo* test to assess blood coagulation defects in assays using TF-bearing cells.

To examine thrombin generation under conditions that mimic the physiological states of patients with coagulation defects, we carried out CAT assays and generated thrombograms to analyze thrombin generation by normal plasma, FXI-depleted plasma, FIX-deficient plasma, FX-deficient plasma, FVIII-deficient plasma, and FVII-depleted plasma by using cells from the bladder carcinoma cell line J82,

which expresses TF at high density [10]. We also carried out CAT assays with washed normal platelets, with and without added adenosine diphosphate (ADP).

MATERIALS AND METHODS

Samples of peripheral venous blood were drawn from 20 healthy volunteers who had not taken any drugs for at least a week and were collected into a one-tenth volume of a 3.2% trisodium citrate solution (wt/vol). Plasma was obtained by immediate centrifugation of the samples at room temperature for 15 minutes at 3000 rpm; the individual plasma fractions were combined to make a pool of normal plasma. FVII-depleted plasma was prepared from the normal pooled plasma by immunoaffinity chromatography with insolubilized antihuman FVII antibody [11], and FXI-depleted plasma was prepared by immunoaffinity chromatography with insolubilized monoclonal antihuman FXI antibody (kindly provided by Dr. Yutaka Komiyama, Department of Clinical Sciences and Laboratory Medicine, Kansai Medical University, Osaka, Japan). FX-deficient plasma, FIX-deficient plasma, and FVIII-deficient plasma were purchased from Sysmex (Kobe, Japan).

The bladder carcinoma cell line J82 was kindly provided by Prof. Katsusuke Naito, Department of Urology, Graduate School of Medicine, Yamaguchi University, Ube, Japan. The cells were cultured at 37°C in a 5%-CO₂ atmosphere in Dulbecco Modified Eagle Medium supplemented with fetal bovine serum (10% by volume), 2 mmol/L L-glutamine, 10 mmol/L HEPES (pH 7.2), 100 units/mL penicillin G, and 100 µg/mL streptomycin. For CAT, J82 cells were cultured overnight in medium-containing wells of 96-well microplates at cell concentrations of 1, 10, 100, and 1000

cells/well. We washed the cells twice with 0.01 mol/L Tris-HCl buffer containing 0.15 mol/L NaCl (TBS) and measured thrombin generation by adding 80 µL of plasma, 20 µL of HEPES buffer, and 20 µL of 2 mmol/L solution of a fluorogenic substrate (Z-GGR-AMC; Calbiochem, Darmstadt, Germany) containing 2 mmol/L CaCl₂ to the washed cells in the 96-well microplates and then monitoring the fluorescence with a fluorometer (Fluoroskan Ascent; Thermo Fisher Scientific, Waltham, MA, USA). Corn trypsin inhibitor (final concentration, 60 µg/mL; Calbiochem) was added to the plasma samples to eliminate contact factor activation. As a control, we used 20 µL of Thrombin Calibrator (Synapse, Maastricht, the Netherlands) instead of HEPES buffer. After measuring the fluorescence, we calculated 5 thrombin-generation parameters with Thrombinoscope software (Synapse): endogenous thrombin potential (ETP), peak height, lag time (LT), time to peak (ttPeak), and time to the end of thrombin generation (start tail) (Figure 1). In addition, to measure the effect of platelets on thrombin generation, we measured thrombograms by adding 80 µL of plasma sample and either 10 µL of washed platelets (final concentration, 5 × 10⁴/µL) plus 10 µL HEPES buffer or 10 µL washed platelets plus 10 µL ADP (final concentration, 0.2 µmol/L; Sigma-Aldrich, St.

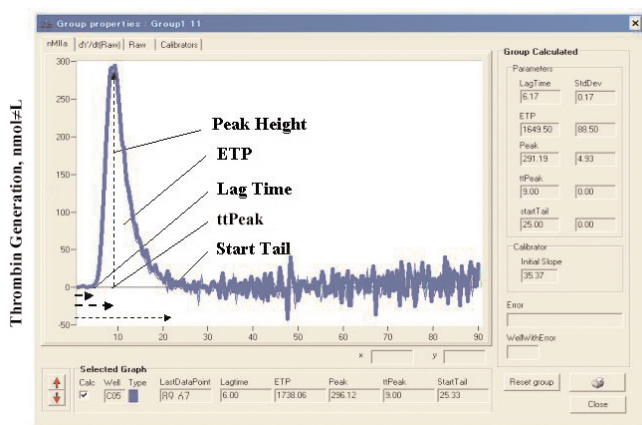


FIGURE 1. Pattern of thrombin generation by calibrated automated thrombography. ETP indicates endogenous thrombin potential; ttPeak, time to peak; start tail, time to the end of thrombin generation.

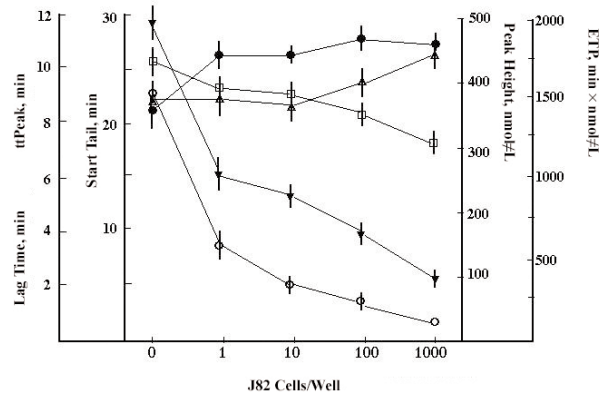


FIGURE 2. Parameters of thrombin generation at different concentrations of J82 cells. J82 cells were added to medium-containing wells of a 96-well microplate at concentrations of 0, 1, 10, 100, and 1000 cells/well, and the cells were cultured overnight. Cells were washed twice with 0.01 mol/L Tris-HCl buffer containing 0.15 mol/L NaCl; 80 µL plasma, 20 µL HEPES buffer, and 20 µL 2 mmol/L fluorogenic substrate containing 2 mmol/L CaCl₂ were then added to the microplate wells. Thrombin generation was measured with a fluorometer. Indicated are the lag time (○), endogenous thrombin potential (ETP) (●), peak height (Δ), time to peak (ttPeak) (▼), and time to the end of thrombin generation (start tail) (□).

Parameters of Thrombin Generation Using Normal Plasma and Coagulation Factor-Deficient Plasmas*

| | Normal Plasma | FXI-Depleted Plasma | FIX-Deficient Plasma | FVIII-Deficient Plasma | FVII-Depleted Plasma |
|---------------------|---------------|---------------------|----------------------|------------------------|----------------------|
| Lag time, min | 1.28 ± 0.282 | 1.61 ± 0.332 | 1.94 ± 0.456† | 1.28 ± 0.23 | 8.26 ± 2.313† |
| ETP, min × nmol/L | 1411 ± 325 | 1432 ± 372 | 924 ± 203† | 1108 ± 266 | 1175 ± 352 |
| Peak height, nmol/L | 346 ± 107 | 264 ± 76.6 | 61 ± 20† | 73 ± 24.8† | 74 ± 22.9† |
| ttPeak, min | 3.27 ± 0.69 | 4.27 ± 0.85 | 8.6 ± 2.56† | 7.27 ± 2.25† | 17.91 ± 7.16† |
| Start tail, min | 17 ± 4.76 | 19 ± 6.27 | 60 ± 21† | 59 ± 19.5† | 41 ± 13.94† |

*Data are presented as the mean ± SD. FXI indicates factor XI; ETP, endogenous thrombin potential; ttPeak, time to peak; start tail, time to the end of thrombin generation.

†P < .05, Welch t test for comparison of results for normal plasma with results for coagulation factor-deficient plasmas.

Louis, MO, USA) instead of the HEPES buffer. We isolated normal platelets from platelet-rich plasma collected from healthy volunteers via gel filtration on a Sepharose 4B column (2.5 × 40 cm) at a flow rate of 1.2 mL/min with HEPES/Tyrode buffer (126 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, 0.38 mmol/L NaH₂PO₄, 5.6 mmol/L dextrose, 6.2 mmol/L sodium HEPES, pH 6.5). Two different experiments were performed via the double test for CAT; results are presented as mean values. Values for measurement parameters of normal plasma and coagulation-deficient plasma were compared with the Welch t test.

To quantitate thrombomodulin (TM) in J82 cells, we

washed 1000 cells twice with TBS buffer, lysed the cells with lysis buffer (0.1% Igepal [vol/vol], 0.1 mol/L benzamidine, 10 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L Tris-HCl, pH 8.0), centrifuged the cell preparation at 15,000 rpm to remove cell debris, and then measured TM in the cell lysates by enzyme-linked immunosorbent assay (TM Panasera; Fujirebio, Tokyo, Japan). In addition, J82 cells were grown on cover slips (Lab-Tek II Chamber Slide System; Nunc, Naperville, IL, USA), fixed with 4% formaldehyde (vol/vol) in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4, for 20 minutes at room temperature, washed twice with PBS, and incubated with antihuman TM immunoglobulin G (IgG) (diluted 1:100 in PBS) for 2 hours at room temperature. The cells were washed twice with PBS and then incubated with alkaline phosphatase-labeled antirabbit IgG (diluted 1:50; Dako, Carpinteria, CA, USA) and levamisole (to inhibit endogenous alkaline phosphatase) in PBS for 1 hour at room temperature. The cells were washed twice with PBS, incubated with the Dako New Fuchsin Substrate System, and examined under a microscope. The antihuman TM rabbit IgG was kindly provided by Prof. Ikuro Maruyama, Graduate School of Medicine, Kagoshima University, Kagoshima, Japan.

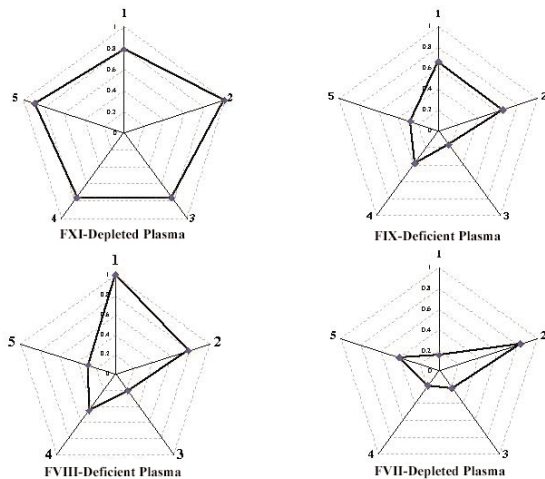


FIGURE 3. Thrombograms showing thrombin generation of factor XI (FXI)-, FIX-, FX-, FVIII-, and FVII-deficient plasmas in the presence of tissue factor-bearing cells for inducing coagulation. Parameter values were normalized to those for normal plasma; the ratios of the results relative to those of normal plasma are presented as radar graphs. Lag time, 1; endogenous thrombin potential (ETP), 2; peak height, 3; time to peak (ttPeak), 4; time to the end of thrombin generation (start tail), 5.

RESULTS

In normal plasma, J82 cells shortened the LT and the ttPeak in a concentration-dependent manner but did not reduce the ETP and the peak height. The LT and ttPeak were remarkably prolonged without the cells (Figure 2). The J82 cells did not hydrolyze the fluorogenic substrate directly in the absence of plasma, even when the Ca²⁺ ion was present (data not shown).

We measured thrombin generation of normal plasma and coagulation factor-deficient plasma after overnight culture of J82 cells adjusted to a concentration of 100 cells/well. FX-deficient plasma generated less thrombin, and the thrombogram was inaccurate. The LT of FVII-depleted

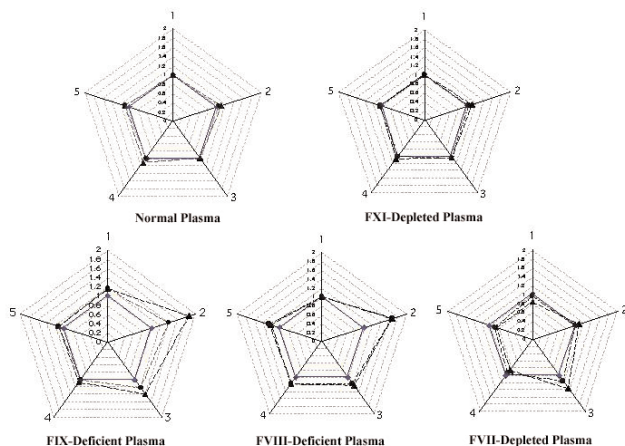


FIGURE 4. The influence of normal washed platelets and of normal washed platelets plus adenosine diphosphate (ADP) on thrombograms of factor XI (FXI)-depleted plasma, FIX-deficient plasma, FVIII-deficient plasma, and FVII-depleted plasma in the presence of tissue factor-bearing cells for inducing coagulation. The ratios of values for the 5 thrombogram parameters to those for normal plasma are shown in the radar graph. The lag time, the time to peak (ttPeak), and the time to the end of thrombin generation (start tail) were not remarkably different for the coagulation factor-deficient plasmas in the presence of platelets with or without ADP, compared with normal plasma. The endogenous thrombin potential (ETP) and the peak height clearly distinguished the coagulation factor-deficient plasmas in the presence of platelets, with or without ADP, from normal plasma. Lag time, 1; ETP, 2; peak height, 3; time to peak (ttPeak), 4; time to the end of thrombin generation (start tail), 5. Indicated are results with normal washed platelets (●) and with normal washed platelets plus ADP (▲).

plasma was remarkably prolonged compared with normal plasma and other coagulation factor-deficient plasmas. The ETP of FIX-deficient plasma apparently decreased compared with normal plasma. The peak heights of FIX-deficient plasma, FVIII-deficient plasma, and FVII-depleted plasma were significantly reduced compared with normal plasma. Both the ttPeak and the start tail of FIX-deficient plasma, FVIII-deficient plasma, and FVII-depleted plasma were apparently prolonged compared with normal plasma (Table). When the values of the measurement parameters for these results were normalized to those for normal plasma (ie, expressed as a ratio to the values for normal plasma) and represented as radar graphs, the values for 4 of the parameters of FXI-depleted plasma demonstrated slight reductions, but the ETP value was unchanged. Both FVIII-deficient plasma and FIX-deficient plasma showed similar patterns in that the peak height, the ttPeak, and the start tail were remarkably reduced. On the other hand, the LT, peak height, ttPeak, and start tail values were remarkably reduced

for FVII-depleted plasma (Figure 3).

Next, we added washed normal platelets or washed normal platelets with ADP and measured the thrombogram in microplate wells in the presence of washed J82 cells. Thrombograms of normal plasma and washed normal platelets (both with and without added ADP) hardly changed compared with assays carried out with only J82 cells. Addition of washed platelets or washed platelets plus ADP to FXI-depleted plasma also had little influence on any of the parameter values. The ETP values of FIX-deficient plasma and FVIII-deficient plasma apparently increased when platelets were added, with or without ADP. The peak heights of FVII-depleted plasma, FIX-deficient plasma, and FVIII-deficient plasma increased in the presence of platelets with or without ADP, compared with the peak heights of only J82 cells (Figure 4).

The TM reading in J82 cells was $700 \text{ mg}/10^6$ cells by enzyme-linked immunosorbent assay. Immunohistochemical techniques for detecting TM in J82 cells indicated that TM was present diffusely throughout the cytoplasm.

DISCUSSION

Homeostasis *in vivo* requires TF-bearing cells and activated platelets at the sites of vascular injury; however, for reasons of convenience, both the activated partial thromboplastin time and the prothrombin time have traditionally been evaluated in liquid-phase reactions under nonphysiological conditions. Such tests cannot take into account physiologically relevant anticoagulants, such as TM on the endothelium surface, which inhibits the thrombin generated during the coagulation process. Hemker et al reported that the addition of activated protein C and TM to plasma inhibited the generation of thrombin with the CAT [5]. TM was demonstrated in human urinary bladder cells and in the BOY cell line, which was generated from transitional carcinoma cells of the urinary bladder [12]. We also confirmed the existence of TM in J82 cells, which are carcinoma cells of the urinary bladder, by both enzyme immunoassay and immunohistochemical staining. J82 is considered a useful model for *ex vivo* coagulation testing because it mimics the physiological coagulation system by assembling a functional prothrombinase complex on the cell surface. The interactions between FVII and J82 cells have been studied extensively by Kisiel's group [13-15]. J82 cells express TF on >90% of the cells without any stimulation [16]. Although some tumor cells are known to directly convert prothrombin to thrombin or FX to FXa [17], J82 cells have been shown to convert FX to FXa via the FVII/FVIIa-TF complex pathway [14,15].

FX-deficient plasma did not generate thrombin in the presence of J82 cells, although a trace peak height was visible. It was not clear whether FX-deficient plasma lacked FX completely, so this nominal peak may have been an artifact.

Increasing the concentration of J82 cells had little effect on the ETP, although the LT depended on the concentration of the cells. When 5 parameters of coagulation factor-deficient plasma were presented on radar graphs, all of the parameters of FXI-depleted plasma except ETP were slightly reduced compared with normal plasma. We also demonstrated that thrombin generation by FXI-depleted plasma was not amplified in the presence of either platelets or activated platelets. FXI binds specifically to high-affinity sites on leucine-rich repeats of glycoprotein 1b (GP1b) located on the surface of stimulated platelets, and FXI is activated by thrombin binding to an anionic region on GP1b [18]. The radar graphs for FVII-depleted plasma, FVIII-deficient plasma, and FIX-deficient plasma showed similar patterns except for the LT. The thrombogram of FVII-depleted plasma had a remarkably prolonged LT. Neither the LT nor the ETP of FVII-depleted plasma was improved by the addition of platelets or activated platelets. Barnett et al reported that a patient with FVII-deficient plasma (<1% of the normal level) had an ETP and peak height that were significantly decreased and a slightly delayed ttPeak. Platelet-rich plasma from this patient significantly increased the ETP and peak height and prolonged the ttPeak [19]. Giansily-Blaizot et al reported that FVII-depleted plasma treated by defibrination did not generate thrombin, but the ETP and peak height of plasma with 1% FVII were 32% and 28% of normal control values, respectively [20]. We thought that this result reflected the difference between coagulation in the liquid phase and that on TF-bearing cells. Chantarangkul et al have suggested that ETP measurement with low levels of TF may be helpful for assessing thrombin generation in hemophilia patients [8]. Dargaud et al reported that the ETP for severe hemophilia A (<1%) and severe hemophilia B (<1%) was 3% to 49% of normal controls when measurements were made with 4 μ mol/L phosphatidylserine and 1 pmol/L TF [21]. The ETP, peak height, and ttPeak values for plasma from hemophilia patients were reported to apparently decrease, but the LT was within the normal range [22]. Our results with TF-bearing cells were approximately similar to previous results obtained with thrombography. The thrombograms for FVIII- and FIX-deficient plasmas showed that platelets or activated platelets amplified the ETP remarkably in these plasmas. The ETP in the thrombogram for FIX-deficient plasma increased more in the presence of washed platelets plus ADP than in the presence of washed platelets without ADP. Siegemund et al reported that the difference between hemophilia A and B in thrombin generation might be attributed to the role of platelets in the assembly of the tenase complex on the platelet surface [23]. Dargaud et al reported that thrombin generation was retarded in the presence of intact platelets because unactivated platelets do not undergo phospholipid scrambling, which leads to surface exposure of phosphatidylserine [22]. We supposed that trace levels of FVIII

and FIX, which might have been present in the deficient plasmas, increased the ETP in the presence of activated platelets, because FVIIIa and FIXa can amplify coagulation *in vivo* on activated platelets. Thrombograms for FVIII-depleted plasma were not affected by the presence of activated or unactivated platelets, probably because FVIIIa at low levels does not act at the surface of the activated platelet.

The results obtained for thrombograms for coagulation factor-deficient plasmas with TF-bearing cells were somewhat different from results obtained with recombinant TF and phospholipid in the liquid phase. We suggest that thrombograms using TF-bearing cells can be a useful *ex vivo* test, because this experimental model may be analogous to most coagulation processes *in vivo*.

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