Plasma Tissue Factor Plus Activated Peripheral Mononuclear Cells Activate Factors VII and X in Cardiac Surgical Wounds

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OBJECTIVES
The purpose of this study was to test the hypothesis that activated monocytes with soluble plasma tissue factor (pTF) activate factors VII and X to generate thrombin.

BACKGROUND
Despite heparin, thrombin is progressively generated during cardiac surgery with cardiopulmonary bypass (CPB), produces intravascular fibrin and fibrinolysis, and causes serious thromboembolic and nonsurgical bleeding complications. Thrombin is primarily produced in the surgical wound, but mechanisms are unclear.

METHODS
In 13 patients, interactions of mononuclear cells, platelets, pTF, and pTF fractions to activate factors VII and X were evaluated in pre-bypass, perfusate, and pericardial wound blood before and during CPB.

RESULTS
Monocytes are activated in wound, but not in pre-bypass or perfusate plasma (monocyte chemotactic protein-1 (MCP-1) is elevated compared to other locations). Wound pTF is substantially elevated compared to other locations (3.64 ± 0.45 pmoles/l vs. 0.71 ± 0.65 pmoles/l and 1.31 ± 1.4 pmoles/l). Supernatant wound pTF contains 81.7% of TF antigen; wound microparticle pTF contains 18.3%. Wound monocytes and all C5a-stimulated monocytes (but not activated platelets) completely convert factor VII to factor VIIa with wound pTF. Activated monocytes more efficiently activate factor X with wound supernatant TF/factor VII(VIIa) complex than with wound microparticle TF/factor VII(VIIa). The correlation coefficient (r) between wound thrombin generation (F1.2) and wound pTF concentration is 0.944 (p = 0.0004).

CONCLUSIONS
During cardiac surgery with CPB, wound monocytes plus wound pTF or wound microparticle-free supernatant pTF preferentially accelerate activation of factor VII and factor X. This system represents a novel mechanism for thrombin generation via the TF coagulation pathway.

During cardiac surgery using cardiopulmonary bypass (CPB), thrombin is progressively generated to produce a consumptive coagulopathy that is responsible for the thromboembolic and nonsurgical bleeding complications associated with these operations (1–5). The majority of thrombin is produced in the pericardial wound (3,6), but the timing and amounts of thrombin produced are not fully explained by cell-bound tissue factor (TF) expression (6–10).

Soluble plasma tissue factor (pTF) is an undefined mixture of procoagulant microparticles (0.1 to 1 μm) and possibly procoagulant protein fragments of TF (6,9,11). Within 15 min of starting CPB, Philippou et al. (6) found increased concentrations of soluble plasma tissue factor (pTF) and evidence of factor VII (FVII) activation and thrombin formation in the pericardial wound. Earlier, Nieuwland et al. (9) demonstrated elevated concentrations of procoagulant microparticles, primarily derived from platelets and erythrocytes, in pericardial wound blood during cardiac surgery; later studies confirmed the procoagulant activity of microparticles (10).

The roles of blood cells, microparticles, and pTF in thrombin generation in the pericardial wound is not clear. Chung et al. (8) observed more monocyte procoagulant activity, FVII activation, and thrombin generation in the wound than in the circulating perfusate. Shibamiya et al. (12) found more neutrophils and monocytes positive for TF antigen in pericardial blood than in the perfusate.

Monocytes synthesize TF, but peak concentrations occur 12 h after in vitro endotoxin stimulation, and only a third of peak is reached within 2 h (13). Therefore, TF synthesis does not explain the rapid increase in monocyte procoagulant activity in the pericardial wound (8). This study tests the hypothesis that cardiac surgery with CPB-activated wound monocytes that have not synthesized TF provides a preferred phospholipid platform for increased concentra-
tions of pTF that activate fVII and factor X (fX) to initiate thrombin generation by the extrinsic coagulation pathway.

The results show that wound monocytes combined with the supernatant fraction of wound pTF more rapidly and completely activate fVII and fX than does the microparticle fraction. In this system activated monocytes, as compared to activated platelets, more completely convert fVII to fVIIa.

METHODS, MATERIALS, AND PATIENTS

Sample sources and processing. Thirteen patients (three women) who required cardiac surgery with cardiopulmonary bypass consented to donate 120 ml of blood/perfusate during operation after approval by the Institutional Review Board (protocol #703385). The perfusion circuit included a centrifugal pump and Maxima oxygenator (Medtronic Cardiopulmonary, Anaheim, California) and was primed with 2L Normosol-R, (Abbott Laboratories, North Chicago, Illinois). Activated clotting times were maintained >400 s with bovine lung heparin. Pericardial blood (mean hematocrit 20 ± 1.8) was aspirated from the wound, washed (Haemonetics Inc., Braintree, Massachusetts), and reinfused as packed red cells.

The mean age of the patients was 65.1 ± 15.4 years (median 60 years, range 34 to 82 years); two were insulin-dependent diabetics and three were smokers. Operations were first-time myocardial revascularization (n = 5), single or double valve repair or replacement (n = 3), valve replacement with myocardial revascularization (n = 2), aortic replacement graft (n = 2), and reoperative myocardial revascularization (n = 1). Cardiopulmonary bypass lasted 152 ± 67 min. Blood samples were obtained from three locations: the arterial catheter after heparin, before CPB (pre-bypass), and simultaneously from the pericardial wound (pericardial) and perfusion circuit (perfusate) 30 to 45 min after starting CPB. Additional blood samples were obtained from aspirin-abstaining volunteer donors.

Cells. Citrated (3.8%, 9:1 vol) blood samples (20 to 40 ml) were centrifuged at 1,500×g for 20 min at 23 °C to separate cells and plasma, which was then frozen at −70 °C. Platelets were isolated from blood by Mustard’s method (14). Resuspended cellular pellets (25 nmol/l HEPES in Hank’s Buffer Salt Solution (1:1 vol/vol) were layered onto a Histopaque-1077 gradient (Sigma Chemical, St. Louis, Missouri) to isolate viable (by trypan blue exclusion test) mononuclear cells (15).

Donated platelets were activated by the thrombin activation peptide derived from Par 1, SFLRN-NH2 (Bachem Inc., King of Prussia, Pennsylvania), after a dose/response trial, which showed aggregation within 10 min with 50 μmoles/l and none at 45 min with 20 or 10 μmoles/l. Mononuclear cells isolated from blood samples and donated mononuclear cells were activated by incubation with recombinant human complement C5a (70 μg/ml [Sigma Chemical Co.] incubated at 37 °C for 60 min in HBSS buffer).

Tissue factor (TF). An Imubind Tissue Factor enzyme-linked immunosorbent assay (ELISA) kit (American Diagnostica Inc., Greenwich, Connecticut) was calibrated by standard TF antigen (American Diagnostica Inc.). Enzyme-linked immunosorbent assay was used to measure TF antigen in all forms of TF, including TF/fVII(fVIIa) complex; soluble plasma tissue factor (sTF) taken from each of three locations (pre-bypass, perfusate, and pericardium), and fractions of pTF: microparticle-bound pTF (mpTF) and pTF in supernatant plasma (spTF). Recombinant TF was used as a standard; the curves of recombinant TF and pTF were parallel.

Fractionation of soluble plasma tissue factor (pTF). Circulating microparticles present in plasma were isolated as described previously (16). Briefly, 10 aliquots of 250 μl of plasma in 1-ml tubes were centrifuged for 30 min at 17,570 g in a SORVALL superspeed RC 2-B automatic refrigerated centrifuge (DuPont, Wilmington, Delaware) at 23 °C. The microparticle pellet was recovered and washed once with phosphate-buffered saline. The pellet was dissolved with 500 μl of buffer (10 mM HEPES, 5 mM KCl, 1 mM MgCl2, and 136 mM NaCl; pH 7.4). Microparticles derived from 5.0 ml of plasma were analyzed. Tissue factor antigen bound to microparticles (mpTF) and spTF were measured by ELISA.

Wound pTF (but not pre-bypass or perfusate plasma) fractionation was further evaluated. One milliliter of wound pTF was diluted with buffer (1:1 vol) (10 mM HEPES, 5 mM KCl, 1 mM MgCl2, and 136 mM NaCl; pH 7.4) and centrifuged at 260,000 g for 4 h (17). A microparticle pellet and three distinct particle-free fractions were separated (17). The particle-free fractions were a floating lipid layer; a middle clear aqueous fraction; and a lower pigmented stratum, which had the highest concentration of TF antigen. Microparticle pellets were washed once with phosphate-buffered saline and dissolved in 500 μl of buffer.

Measurements of factors VIIa and Xa. Purified plasma fVII (lot CP2052U, Cortex Biochem Inc., San Leandro, California), of which >98% was in a single band on SDS-polyacrylamide gel electrophoresis, was used. Factor VIIa (fVIIa) was measured by chromogenic assay (Spectrozyme fVIIa; American Diagnostica Inc.). Specificity for fVII was verified using recombinant fVII (gift of Dr. Rodney Camire); recombinant TF (10 nmol/l) did not affect the assay (0.01 to 10 nmol/l fVIIa). The assay was
free of fXa contamination as tested using fXa substrate, S-2765 Chromogenix, (Diapharma Group Inc., West Chester, Ohio). Below 1.0 nmole/l, fXa does not cleave fVIIa substrate.

Factor X activation by microparticle pTF or supernatant pTF with either unstimulated or C5a-stimulated mononuclear cells (2 × 10^6/ml) from each of the three sample locations was assessed. Cells were incubated with fVII (10 nmol/l), CaCl_2 (5 mmol/l), and supernatant pTF (2.0 ml) or microparticle pTF (2.0 ml) from pre-bypass, pericardial wound, or perfusate samples for 10 min at 37°C. Factor X (100 nmol/l, American Diagnostica Inc.) was added and incubated for another 10 min at 37°C. An aliquot of 100 µl from the mixture was added to S2765 Xa substrate (5 mmol/l) and incubated at 37°C for 20 min; fXa activity was detected at absorbance 405 nm. As control, cells alone or C5a-stimulated cells without pTF were used.

**Western blotting.** Western blotting was performed as described (18). After measuring protein concentrations in supernatant pTF and microparticle pTF samples from each location, plasma was subjected to SDS-PAGE, and the separated proteins were transferred onto nitrocellulose membranes. Each gel well contained 20 µg of protein as measured by Comassie blue protein assay. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20, then incubated with rabbit anti-human TF immunoglobulin G (IgG) (American Diagnostica Inc.) or rabbit anti-human fVIIa IgG (Abcam, Inc., Cambridge, Massachusetts), followed by alkaline phosphatase-conjugated rabbit IgG secondary antibodies (Abcam, Inc.), and visualized with phosphatase substrate, BCIP/NBT (KPL, Gaithersburg, Maryland) (n = 6). One blot was stained with an antibody to alternatively spliced human TF (17).

Digitized Western blot band images were quantified with Quantity One Software 4.5.2 (Bio-rad Laboratories, Hercules, California) using the volume rectangular tool and analyzed for volume density (n = 3). The volume density was defined as the total sum of intensity (inverted range 0 to 255 for each pixel) of all the pixels in the volume divided by the area of the volume and is reported as intensity/ mm^2. A background volume density was subtracted from each band.

**Thrombin generation.** Prothrombin fragment F1.2 and thrombin-antithrombin complex (TAT) were measured to compared with pTF concentrations in wound and perfusate plasma using kits (Dade Behring Inc., Newark, Delaware).

**Mechanistic studies.** pTF was measured in plasma from pre-bypass, perfusate, and wound samples by ELISA. Plasma human monocyte chemotactic protein-1 was measured by human MCP-1 immunoassay Quantikine assay kit (R & D Systems, Minneapolis, Minnesota).

Plasma taken from each sample location (2.0 ml) was mixed with unstimulated or C5a-stimulated sample mononuclear cells (2 × 10^6/ml) in various combinations and then incubated for 10 min at 37°C with fVII (10 nmol/l) and calcium (5 mmol/l). For control purposes a similar volume of buffer was used instead of plasma or cells.

Stimulated and unstimulated donor mononuclear cells (2 × 10^6/ml) were also incubated with 1.46 pmol/l wound pTF or fractions of wound pTF for 10 min at 37°C to determine the effects of monocyte activation on fVII activation at equivalent TF concentrations. Factor VIIa was measured after fVII (10 nmol/l) and CaCl_2 (5 mmol/l) were added and incubated 10 min longer.

Donated platelets (2 × 10^6/ml) were preincubated with...
SFLLRN-NH2 (20 μmoles/l) for 10 min at 37°C and then incubated with wound pTF for 10 min at 37°C. Factor VII (10 nmoles/l) and CaCl$_2$ (5 mmoles/l) were added and incubated 10 min longer, after which fVIIa was measured.

**Statistical analysis.** Data are expressed as mean ± SEM. Comparisons between serial samples taken from different groups are made using one-way ANOVA. Comparisons for various combinations of different plasma and monocyte samples from patients are made by paired $t$ tests. Correlation coefficients are calculated by ANOVA (StatView version 4.51, Abacus Concepts Inc., Berkeley, California).

### RESULTS

Concentrations of pTF and MCP-1 in blood samples obtained from each sampling location are shown in Figure 1A. Note that the highest concentrations of pTF (3.64 ± 0.45 pmoles/l) and MCP-1 (29.5 ± 2.1 pmoles/l) are in wound plasma (n = 7). Respective mean concentrations were 0.71 ± 0.65 and 2.8 ± 1.2 pmoles/l in pre-bypass plasma and 1.31 ± 0.47 and 3.3 ± 1.4 pmoles/l in perfusate (n = 7).

Wound pTF with unstimulated wound monocytes and C5a-stimulated monocytes from each sample location convert all available fVII (10 nmoles/l) to fVIIa (Fig. 1B). Unstimulated pre-bypass and perfusate monocytes fail to do so. Interestingly, after adding fVII (10 nmoles/l) and CaCl$_2$ (5 mmoles/l), wound monocytes (2 × 10$^6$/ml) with pre-bypass plasma generated 2.02 ± 2.82 nmoles/l fVIIa; thus, wound monocytes without increased pTF are weakly procoagulant (n = 5, data not shown).

Resting and stimulated (20 nmoles/l SFLLRN-NH2) platelets with wound pTF generated 2.01 ± 0.02 and 2.57 ± 0.11 nmoles/l fVIIa, respectively (data not shown, n = 3).

When wound pTF (250 μl) was fractionated for 30 min at 17,570 g (16), supernatant pTF contained 3.58 ± 1.05 (81.7%) pmoles/l TF antigen and microparticle pTF contained 0.80 ± 0.23 (18.3%) pmoles/l (n = 4) (Fig. 2A). When the same wound pTF was centrifuged at 260,000 g for 4 h (17), TF antigen in the lower, pigmented supernatant fraction of diluted wound pTF (2 ml) was 6.98 ± 0.51 pmoles/l (87.5%) and was 1.00 ± 0.12 pmoles/l (12.5%) in the microparticle pellet (n = 4) (Fig. 2A).

Equal concentrations of TF antigen (1.46 pmoles/l) taken from wound pTF, wound microparticle pTF or wound supernatant pTF were combined with donor unstimulated or C5a-stimulated mononuclear cells (2 × 10$^6$/ml) (n = 3). After adding fVII (10 nmoles/l) and CaCl$_2$ (5 mmoles/l), fVIIa was measured (Fig. 2B). Compared with microparticle pTF, wound pTF and supernatant pTF strongly catalyzed fVIIa production with unstimulated and stimulated mononuclear cells; however, only part of the available fVII (10 nmoles/l) was activated. Mononuclear cell stimulation modestly increased Factor VIIa generation with all TF preparations.

**Figure 3** (left panel) shows the molecular weight distribution of TF antigen in fractionated samples of pTF taken from each location in a representative patient. Wound supernatant pTF is markedly increased compared with pre-bypass or perfusate supernatant pTF and with all microparticle pTF samples. All TF proteins migrate at Mr = 47.5 kDa except recombinant TF (Mr = 33 kDa). An antibody to alternatively spliced human TF, which cross-reacted with 47 kDa standard TF antigen (American Diagnostica Inc.), showed the presence of a 31 kDa band (blot not shown).

**Figure 3** (right panel) shows the molecular weight distri-
bution of fVIIa (fVII) antigen for the same plasma samples shown in the left panel. Note that most of the fVII/VIIa complex in wound plasma is associated with microparticle pTF.

Figure 4 shows the amount of fX converted to fXa by the two fractions of pTF from each sample location. The fractions, supernatant pTF and microparticle pTF, were centrifuged 30 min at 17,570 g (16) after combination with donor unstimulated or C5a-stimulated monocytes (2 × 10⁶/ml). Although wound microparticles contain high concentrations of fVII/VIIa, activated mononuclear cells with wound supernatant pTF more readily activate fX (p = 0.013; n = 3). The same plasma centrifuged at 260,000 g provided identical results (data not shown).

The correlation (r) between wound pTF concentrations and prothrombin fragment F1.2 in wound plasma is 0.944 (n = 7) (Fig. 5A). The coefficient of determination (R²) for perfusate samples is 0.0136. Figure 5B shows the correlation between wound pTF concentrations and TAT (r = 0.98). For the perfusate sample R² is 0.126.

**DISCUSSION**

This study supports our hypothesis and establishes a central role for wound pTF and activated mononuclear cells in the generation of thrombin in the pericardial wound during cardiac surgery using CPB. Activated monocytes with wound pTF convert all available fVII to fVIIa, wound pTF concentrations are sufficient for generation of fVIIa, wound mononuclear cells are activated, wound monocytes alone weakly activate fVII, a strong correlation exists between wound pTF levels and thrombin formation, and wound supernatant pTF contains significantly more pTF antigen than wound microparticle pTF. Although wound microparticle pTF binds fVIIa (fVII) with high affinity, wound supernatant pTF/fVII complexes have a larger role in fX activation. These observations explain the paradox of rapid monocyte procoagulant activity in the wound (8) and delayed monocyte procoagulant activity in the perfusion circuit (19); where few monocytes are activated, pTF concentrations are low and monocyte synthesis of TF has not yet occurred (20).

Previous studies demonstrated increased concentrations of pTF in the pericardial wound of cardiac surgical patients requiring CPB (6,10). Our studies confirm this observation and predominant thrombin generation in the pericardial wound (3,6,8–10). Sturk-Maquelin et al. (10) demonstrated a role for procoagulant microparticles with “exposed” TF in wound thrombin generation and found a median of 77% (range 20% to 78%) of wound non–cell-bound TF is associated with microparticles. Our study shows only 12% to 18% of wound pTF is associated with wound microparticles. This discrepancy may reflect differences in method of preparation or sampling time. Moreover, we find that with mononuclear cells, wound microparticle pTF is less efficient in activation of fVII. In addition, although microparticles bind more fVIIa (fVIIa), wound microparticle pTF/fVIIa complex activates less fX than mononuclear cells with wound supernatant pTF/fVIIa. These observations and the demonstration of fVII catalytic activity by microparticle-free wound supernatant pTF associated with mononuclear cells are novel; others (21–23) have demonstrated transfer of microparticle TF to monocytes, neutrophils, platelets, and aggregates of these components, but not transfer of microparticle-free supernatant TF.

The mechanisms of pTF activation of fVII are unknown, and many variables may be involved. The molecular weight and concentration of TF antigen may be important; recom
binant TF (kDa 33) is less efficient than wound pTF and wound supernatant pTF (kDa 47) (data not shown). With stimulated mononuclear cells, 1.46 pmoles/l wound pTF and wound supernatant pTF failed to convert all available fVII to fVIIa. Furthermore, wound microparticle TF (kDa 47) in combination with stimulated mononuclear cells does not efficiently catalyze fVII activation. Microparticle TF may not associate with mononuclear cells to bind fVII or may impair association of microparticle-bound TF with mononuclear cells; in either event, activation of fVII is diminished as compared with supernatant pTF. The larger mononuclear cell may spatially orient microparticle-free supernatant pTF better for fVII capture and assembly of supernatant pTF/fVII(fVIIa) complex, as compared to free microparticles or platelet-bound TF. In addition, activated mononuclear cells express the CD11b receptor that binds fX (24); thus, mononuclear cells may also facilitate assembly of the supernatant pTF/fVII(fVIIa)/fXa complex for cleavage of prothrombin. The possible importance of pTF concentration, pTF molecular weight, phospholipid platform for pTF, cell activation, spatial orientation, and substrate binding is consistent with the observation that in diabetic patients platelet microparticles exposing TF are not procoagulant (25).

This study does not address the composition or sources of wound pTF, which may or may not be unique to cardiac surgery with CPB. Increased concentrations of circulating pTF occur in a variety of diseases associated with intravascular coagulation, including unstable angina and myocardial infarction, diabetes, sepsis, and some malignancies (11,21,26,27). However, pTF may not be the same between diseases. Presumably all procoagulant microparticles or microparticle-free protein TF fragments contain the 219-amino-acid extracellular domain that complexes with fVII (17,28). If microparticle-bound TF and microparticle-free protein fragments in pTF differ between procoagulant diseases (26), the abilities of these molecules to capture fVII also may differ. The present study raises the possibility that circulating protein fragments of pTF and peripheral mononuclear cells activated by complement, cytokines, or other agonists may generate thrombin in procoagulant diseases that have an inflammatory component.

Bogdanov et al. (17) described recombinant alternatively spliced human TF, which has procoagulant activity and which retains the extracellular domain of cellular TF joined to a novel terminus not incorporated into a phospholipid layer. Ruf et al. (29) demonstrated that recombinant TF protein, TF1-219, which lacks transmembrane and intracellular domains, retains enhanced catalytic activity when complexed with fVIIa but does not form a stable relationship with a phospholipid surface (29). Partial activation of fVII by recombinant TF (Morrissey) with activated mononuclear cells is consistent with this observation. A secure transmembrane anchor for the extracellular domain of TF may be necessary for efficient capture of fVII. Nevertheless, our data suggest that mononuclear cells also may provide the best platform for assembly of truncated forms of pTF and fVII complexes.

Cellular TF is present in all exposed tissues in the cardiac surgical wound except pericardium (7,8). Thus, the proportionate contributions of cellular TF, wound pTF associated with activated peripheral mononuclear cells, and non–cell-bound TF exposed on microparticles, platelets, leukocytes, and platelet-leukocyte aggregates to generate thrombin in heparinized pericardial wound blood are unknown. When wound blood is washed, perfusate monocytes and low pTF concentrations weakly activate fVII. Thus, washing or discarding pericardial wound blood reduces circulating thrombin.

Generation of thrombin during CPB produces a consumptive coagulopathy that causes most of the nonsurgical
bleeding problems and thromboembolic complications associated with cardiac surgery and all applications of extracorporeal circulation (30,31). Despite elevated wound concentrations of TF pathway inhibitor (6), thrombin is produced progressively during CPB (6,8,10). Pericardial blood cannot always be washed or discarded during complex cardiac operations; therefore, a means to suppress thrombin generation in the pericardial wound remains an important and worthy goal. The present study indicates that wound pTF and activated mononuclear cells are major targets for this effort.

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