

# Simultaneous Expression of Tissue Factor and Tissue Factor Pathway Inhibitor by Human Monocytes. A Potential Mechanism for Localized Control of Blood Coagulation

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## Summary

Cells of monocytic lineage can initiate extravascular fibrin deposition via expression of blood coagulation mediators. This report is about experiments on three mechanisms with the potential to modulate monocyte-initiated coagulation. Monocyte procoagulant activity was examined as a function of lipid cofactor, protein cofactor, and specific inhibitor expression during short-term culture *in vitro*. Lipid cofactor activity was measured as the initial rate of factor X activation by intrinsic-pathway components, the assembly of which depends on this cofactor. Lipid cofactor activity levels changed by <30% during 48-h culture. Protein cofactor, *i.e.*, tissue factor (TF) antigen was measured by enzyme immunoassay. It increased from 461 pg/ml to a maximum value of 3,550 pg/ml at 24 h and remained at 70% of this value. Specific TF activity, measured as factor VII-dependent factor X activation rate, decreased from 54 to 18 nM FXa/min between 24 and 48 h. TF activity did not correlate well with either lipid cofactor or TF protein levels. In contrast, the decrease in TF activity coincided in time with maximal expression of tissue factor pathway inhibitor (TFPI) mRNA, which was determined using reverse transcriptase polymerase chain reaction (RT-PCR), and with maximal TFPI protein levels measured by immunoassay. The number of mRNA copies coding for TFPI and TF in freshly isolated blood monocytes were 46 and 20 copies/cell, respectively. These values increased to 220 and 63 copies/cell during short-term cell culture in the presence of endotoxin. Results demonstrate concomitant expression by monocytes of genes coding for both the essential protein cofactor and the specific inhibitor of the extrinsic coagulation pathway. Together with functional and antigenic analyses, they also imply that the initiation of blood clotting by extravascular monocyte/macrophages can be modulated locally by TFPI independently of plasma sources of the inhibitor.

Hemostatic balance is maintained by localized crosscatalytic and inhibitory reactions connecting the intrinsic, extrinsic, and common coagulation pathways (1–4). These reactions are ultimately dependent on tissue factor (TF)<sup>1</sup>, an integral membrane protein selectively expressed by procoagulant cells. TF procoagulant activity is associated with monocytes/macrophages and perturbed endothelial cells, but not with polymorphonuclear leukocytes, lymphocytes, or platelets (4–6). TF functions as an essential cofactor for factor

VIIa in the extrinsic pathway–protease complex and is therefore indispensable for rapid proteolytic activation of coagulation factors IX and X. In addition, TF facilitates the activation of factor VII by factor Xa and is required for efficient inhibition of TF/VIIa complexes by antithrombin III (2, 7, 8). Thus, identification of mechanisms that regulate TF expression and function on procoagulant membranes is a necessary step in understanding how hemostatic balance is maintained *in vivo*.

Available information suggests that among the TF-pathway inhibitors described to date, antithrombin III (7) and lipoprotein-associated coagulation inhibitor (9–11) are likely the most important regulators *in vivo*. This latter inhibitor is also referred to as extrinsic pathway inhibitor (EPI) and more recently as tissue factor pathway inhibitor (TFPI) (12–15). TFPI is primarily associated with lipoproteins, and is found in human plasma at a concentration of ~3 nM. In sharp contrast with antithrombin III, TFPI does not inhibit thrombin

<sup>1</sup> Abbreviations used in this paper: EIA, enzyme immunoassay; LPSR, low protein serum replacement; Mo-MLV-RT, Moloney murine leukemia virus RT; RT, reverse transcriptase; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

Parts of this study have been published in abstract form (1993. *FASEB [Fed. Am. Soc. Exp. Biol.] J.* 7:A116).

and thus, is considered a more selective regulator of the extrinsic pathway (9, 10). Structurally, TFPI is related to the inhibitor family of the pancreatic trypsin inhibitor (kunitz-type) of protein inhibitors of proteinases (9, 10). It has been recently shown that TFPI binds TF, factor VII, and factor Xa on cell membranes *in vitro* (16). Functionally, TFPI inhibits the activation of factors IX and X by TF-factor VIIa complexes and the amidolytic activity of factor VIIa and Xa toward small synthetic substrates. Plasma concentration of TFPI is at least 10-fold lower than its dissociation constant with factor Xa (10), and of the same order as its dissociation constant with TF/factor VII on cell membranes (16). These observations suggest that TFPI must be generated close to its proposed site of action on procoagulant surfaces (9, 10). However, *de novo* expression of TFPI by nontransformed procoagulant cells other than endothelial cells remains to be demonstrated unambiguously.

Monocytes and macrophages are characteristically present in neoplastic, inflammatory, and atherosclerotic lesions. Expression of TF activity by infiltrating monocyte/macrophages is believed to be an important factor in the thromboembolic manifestation often associated with these pathological processes (17–24). Monocytes also express the lipid cofactor necessary for efficient assembly of intrinsic and common pathway coagulation proteases. The physicochemical characteristics of membrane lipids have been shown to influence the functional activity of TF (25). The present studies examine kinetics of monocyte procoagulant expression during short-term culture *in vitro* in the presence of endotoxin. Three hypothetical mechanisms of TF control amenable to experimental testing are investigated: namely, modulation of TF activity by changes in lipid membrane cofactor, regulation of activity via changes in levels of TF protein, and regulation via *de novo* synthesis of specific TF inhibitor.

## Materials and Methods

**Monocyte Isolation and Culture.** Human mononuclear cells were obtained by fractionation of 200–500 ml heparinized or citrated blood by differential sedimentation and centrifugation in density gradients (26, 27). Mononuclear cell populations were enriched in monocytes by centrifugation through hypertonic Percoll gradients, 45% Percoll in NaCl at 9.3 mg/ml. Contaminating platelets were removed by repeated washes with sterile physiologic saline solution. Cells in the purified population were 85–95% monocytes and 5–15% lymphocytes by morphologic criteria (Wright's stain and nonspecific esterase stains) (4). Cells were >95% viable as determined by trypan blue dye exclusion and contained <1 platelet/10 nucleated cells. For culture, these monocyte-enriched populations were resuspended at  $4\text{--}5 \times 10^6/\text{ml}$  in serum-free medium M199 supplemented with 2% low protein serum replacement (LPSR; Sigma Chemical Co., St. Louis, MO), containing 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 2  $\mu\text{g}/\text{ml}$  of bacterial LPS (*Escherichia coli* Serotype 0127:B8; Sigma Chemical Co.). In selected experiments, cells were also incubated in the absence of LPS-LPSR stimulus. Cultures were maintained up to 48 h in polypropylene culture tubes in a slanted position at 37°C, and in an atmosphere of 95% air, 5% CO<sub>2</sub>. Cell viability decreased by <7% during the observation interval and the range of total cell counts remained within  $4.47 \pm 1.4\%$ , of initial counts in replicated cultures.

**Identification of mRNAs Coding for TFPI and TF.** Steady-state levels of TF and TFPI mRNA were measured by PCR amplification of cDNAs transcribed from RNA using reverse transcriptase (RT) and oligo dT primers. Amplified DNA was measured fluorometrically and the PCR efficiency was determined by analysis of amplification kinetics on each primer-template set. The rationale for this approach is based on the fact that the final amount of DNA generated by PCR after each cycle is determined not only by the number of copies initially present but also by the overall experimental efficiency. These two factors can be related by the formula:  $N = N_0(1 + E)^n$  where N is the final amount of DNA generated, N<sub>0</sub> is the amount initially present and E is the overall amplification efficiency. The term E includes the combined effect of hybridization efficiency, enzyme decay, and other factors on the deviation of reaction kinetics from the optimal theoretical,  $N = N_0 2^n$ . The observed efficiency, E<sub>obs</sub>, was derived from the slope of linear plots of log DNA concentration vs. cycle number. E<sub>obs</sub> was corrected for decay of Taq DNA polymerase activity during reaction time in order to estimate the initial efficiency, E<sub>i</sub>. Decay constant of Taq polymerase was calculated assuming a half-life of 40 min at 95°C. The overall efficiency E was approximated as the arithmetic average of initial and observed efficiencies such that  $E = (E_i + E_{obs})/2$ .

Total RNA was isolated from monocytes by a variation of the guanidinium thiocyanate method (28) using RNeasy reagent from Cinna Scientific, Inc. (Friendswood, TX). The RNA was washed with ethanol, resuspended in H<sub>2</sub>O, and quantified spectrophotometrically by measuring absorbance at 260 nm. Preparations of RNA used in these studies had 260/280 optical density ratios of 1.8 and  $0.6 \pm 0.5 \mu\text{g}$  of total RNA was recovered from 10<sup>6</sup> cells. Purified RNA samples were stored in ethanol at –20°C. The mRNA in 0.5  $\mu\text{g}$  total RNA was transcribed to cDNA in reaction mixtures containing 2.1 U/ml of Moloney murine leukemia virus reverse transcriptase (Mo-MLV-RT), 2.1  $\mu\text{M}$  oligo dT primers, 4 mM MgCl<sub>2</sub>, 0.8  $\mu\text{M}$  each of deoxynucleoside triphosphates, and 0.8 U/ $\mu\text{l}$  of RNase inhibitor, in 10 mM Tris-HCl buffer, pH 8.3, with 40 mM KCl. Reactions were carried at 42°C for 1 h at which time Mo-MLV-RT was inactivated by heating at 99°C for 5 min. A known number of copies of control RNA template transcribed from plasmid pAW109 containing IL-1 $\alpha$  sequences was incubated under conditions identical to those used for monocyte RNA. This control was used to confirm the linearity of the RT reaction and to validate the method for determination of efficiency by PCR amplification kinetics. In some experiments reactions included an excess of mRNA (0.25  $\mu\text{g}$  of rabbit  $\beta$ -globin mRNA; Clontech, Palo Alto, CA) to insure that reaction conditions allowed for transcription of all mRNA in RT mixture. Mixtures with either no RT or RNA were also cycled to control for the possibility of amplifying traces of genomic DNA that may be present in the total RNA preparations and to detect possible contamination of reagents, respectively.

Samples, 10  $\mu\text{l}$  each, from each RT mixture were adjusted to 3 mM of MgCl<sub>2</sub> and amplified for 35 cycles with Taq-polymerase, 2.5  $\mu\text{g}/\text{ml}$ , using 0.15  $\mu\text{M}$  each of oligonucleotide primers. Each set of primers frame a 528 and 513 base pair sequence starting at nucleotides 217 and 292 from the 5' end of the published cDNA translated sequences of TF and TFPI respectively (29, 30). Amplifications were carried in a thermocycler, 2 min at 95°C for one cycle, and 1 min at 95°C and 1 min at 60°C for 35 cycles. All reagents used for RT-PCR were obtained from Perkin-Elmer Cetus (Norwalk, CT).

To measure amplification kinetics, reaction mixtures were rapidly sampled during the melting step of cycles 20–35, and the concentration of DNA in each sample was determined fluorometrically.

cally with Hoechst dye 33258 (31). Standard curves were constructed with calf thymus DNA and the sensitivity of the assay was adjusted to give a linear signal with concentrations ranging from 2 to 100  $\mu\text{g}$  DNA/ml. The purity of the PCR products was analyzed in representative experiments by agarose gel (1.2% low melting point; Clontech) electrophoresis using ethidium bromide to identify the size of DNA bands. The specificity of the amplification reaction was further demonstrated by subcloning and sequencing of randomly chosen amplified transcripts using PCR II vector (Invitrogen, San Diego, CA) and Sequenase 2, sequencing kit (United States Biochemical Corp., Cleveland, OH).

**Detection of TF and TFPI Protein by Immunoassay.** TF antigen on monocytes was measured in cell lysates by enzyme immunoassay (EIA) using Immunobind Tissue Factor kit from American Diagnostica, Inc. (Greenwich, CT). In this assay the TF antigen in cell extracts and supernatants is captured specifically with monoclonal antibody that recognizes intact TF (32). Standard curves were constructed using a recombinant 47-kD TF protein. TFPI antigen was measured using EIA Immunobind TFPI Kit from American Diagnostica with minor modifications. The instructions in the kit prospectus were followed except that reagent volumes were 50  $\mu\text{l}$  instead of the 100  $\mu\text{l}$  recommended and the chromogenic reaction was extended to 90 min instead of 30 min. Standard curves were constructed using a recombinant 43-kD TFPI protein.

**Measurement of Procoagulant Activity.** Procoagulant activity was quantified using a one-stage clotting assay with pooled human plasma (George King, Bio-Medical, Inc., Overland Park, KS) as described previously (33). 60  $\mu\text{l}$  of the sample were mixed to 60  $\mu\text{l}$  of 0.025 M  $\text{CaCl}_2$  and 60  $\mu\text{l}$  of citrated plasma. Clotting times were measured using a fibrometer (Becton Dickinson & Co., Mountain View, CA) and converted to arbitrary TF units using logarithmic plots of clotting times vs. dilution of a standard TF solution prepared with crude human brain homogenate. By definition, 1,000 mU/ml of procoagulant is the concentration of TF giving a coagulation time of 50 s in this test.

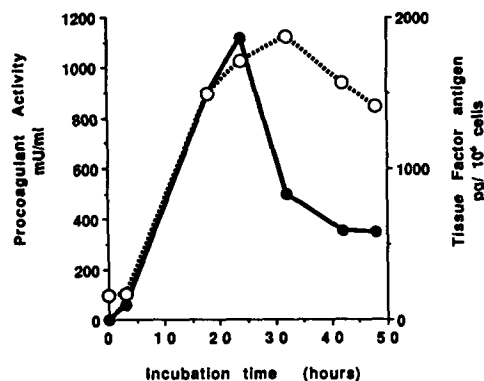
**Measurements of Factor X Activation Rates on Monocytes.** Activation of factor X via the extrinsic and intrinsic pathway protease complex assembled on monocytes was measured as described previously (4, 6). Briefly, reaction mixtures contained  $2.6 \times 10^6$ /ml monocytes, 5 mM  $\text{CaCl}_2$ , 5 nM factor VIIa in 50 mM Tris buffer, 0.15 M NaCl, and 0.5 mg/ml ovalbumin, pH 7.2–7.4. Reactions were initiated with factor X at 70 nM. 40  $\mu\text{l}$  samples were withdrawn at 30 s intervals and mixed with 40  $\mu\text{l}$  of 0.2 M EDTA in 50 mM Tris, 0.8 N NaCl, pH 8.3, in microplate wells. Chromogenic substrate either S-2222 (*N*-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride) or S-2765 (*N*- $\alpha$ -Benzoyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide-dihydrochloride), both from Kabi Vitrum (Stockholm, Sweden) was added (0.32 and 0.12 mg/ml, respectively) and initial rates of *p*-nitrophenyl generation were measured using a Vmax-microplate reader (Molecular Devices Corp., Menlo Park, CA). Activation of factor X via intrinsic pathway was measured in reaction mixtures constructed as above except that factor VIIa was replaced by 0.8 nM factor IXa 0.8 nM and 1 nM factor VIII. Factor VIII was activated to factor VIIIa with 0.5 nM thrombin added 30 s before initiation of factor X activation reaction (4).

**Coagulation Factors.** Purified human coagulation factors X, IXa, and IX with specific activities of 125, 200, and 125–200 U/mg of protein, respectively, were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Human factor Xa was prepared by activation of factor X with Russell Viper Venom as previously described (4). Recombinant factor VIIa was kindly donated by Dr. Ulla Hedner (Novo Nordisk A/S, Copenhagen, Denmark).

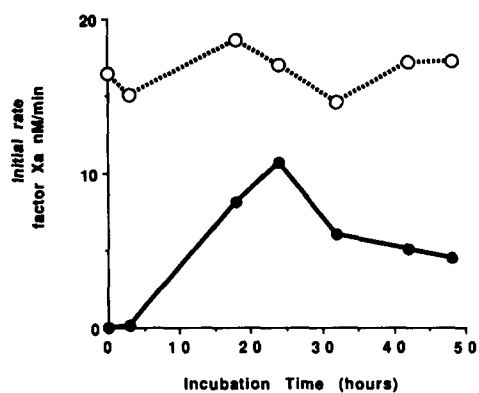
This recombinant product is homogeneous as determined by high-performance liquid chromatography in the supplier's laboratory and has activity of  $\approx 2180$  U/ml as determined by clotting test with monodeficient human plasma. The factors IX, X, and IXa used are electrophoretically homogenous as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis and functionally pure in coagulation tests with monodeficient human plasma as substrate. Immunopurified human factor VIII with specific activity greater than 3,000 U/mg of protein was obtained from Armour Pharmaceutical Co. (Kankakee, IL).

## Results

**Monocyte Procoagulant Activity during Short-term Culture In Vitro** Mononuclear phagocytes isolated from human blood were incubated at  $4\text{--}5 \times 10^6$ /ml for 0, 3, 20 and 48 h. Cells were suspended in medium M-199 with 2% LPSR and 2  $\mu\text{g}$ /ml of bacterial LPS. Incubation under these conditions resulted in morphologic and functional changes that are comparable to those observed in monocyte/macrophages at extravascular, inflammatory, and atherosclerotic sites (18, 21, 22, 33). These changes include increases in size and procoagulant activity and decreases in nucleus to cytoplasmic ratios. Total levels of procoagulant activity in the monocyte suspension, measured as the shortening of plasma clotting times, were maximal between 18–24 h and decreased to 75% of maximum by 48 h (Fig. 1). The rate of procoagulant activity accumulation was estimated to be maximal between 6 and 15 h of culture at a value of 39.8 mU/ml/h. After 15 h the average rate of procoagulant output decreased rapidly and was only 1.0 mU/ml/h from 15 to 22 h. The rapid changes in procoagulant activity generation in the absence of cell death suggest specific modulation of this activity during maturation.



**Figure 1.** Procoagulant activity and TF antigen expressed by monocytes during culture. Procoagulant activity (closed circles) was measured as the shortening of plasma clotting time upon mixing with the cell suspension. Activity is expressed in milliunits (mU) and was quantified using a reference curve constructed with human brain homogenate as a TF standard. In this assay, 1 U is the concentration of TF that shortened the clotting time of recalcified plasma to 50 s. TF antigen (open circles) was measured by enzyme immunoassay using a monoclonal antibody that recognizes intact TF. TF antigen was quantified using a reference curve constructed with human recombinant TF as a standard. Data are from one of six similar experiments. Mean and SE of normalized values from additional experiments are in Table 1.



**Figure 2.** Specific TF activity and lipid cofactor activity expressed by monocytes during culture. TF activity (closed circles) was measured in the presence of purified factors VIIa (5 nM) and X (120 nM) as the initial rate of factor Xa generation in Tris buffer, 0.15 M NaCl, 4 mM CaCl<sub>2</sub>, pH 7.3. Concentration of factor VIIa used was saturating and initial rates reflect concentration of functional TF expressed by cells. Lipid cofactor activity (open circles) was measured independently of TF by substituting factor VIIa with intrinsic protease components, factor IXa (0.8 nM) and VIIIa (1 nM). These concentrations yield half maximal rates of Xa generation. Rates reflect number of functional assembly sites expressed on the monocyte membrane. Data were obtained from one experiment with cells from the same donor used to obtain data in Fig. 1. Means and SE from additional experiments are in Table 1.

tion. To determine if the decrease in procoagulant activity is associated with specific changes in TF activity, initial rates of factor Xa generation were measured in reaction mixtures assembled with purified human coagulation factors VIIa and X. Concentration of factor VIIa in these experiments was 5 nM, i.e., similar to plasma concentration and saturating for membrane TF. This insures that observed rates reflect TF concentration rather than possible changes in the dissociation/association rates of TF/VIIa interaction. Initial rates of factor X activation increased with time of culture and were

maximal between 15 and 24 h but decreased to  $\approx 30\%$  of maximum by 48 h (Fig. 2 and Table 1).

**Monocyte Lipid Cofactor Function during Short-term Culture.** The observed changes in TF activity could be, in theory, secondary to changes in the membrane lipid cofactor activity necessary for functional assembly of coagulation protease complexes (1, 2, 25). Lipid cofactor activity was measured as the ability of monocytes to provide membrane sites for efficient assembly of the intrinsic factor X activating protease. This protease complex, like the extrinsic pathway protease TF/VIIa, is composed of a protein cofactor, factor VIIIa, and an enzyme component, factor IXa. However in contrast with TF, factor VIIIa is not an integral membrane protein but binds reversibly to lipid surfaces with an apparent  $K_d$  of  $\approx 10^{-9}$  M (6, 34). This characteristic was exploited to measure lipid cofactor activity independently of changes in the protein cofactor, i.e., TF. The intrinsic pathway protease was assembled on monocytes cultured for 0–48 h using fixed and limiting concentrations of factor IXa and VIIIa (Fig. 2, dotted line, and Table 1). Rates of factor X activation by this protease complex did not decrease significantly during monocyte maturation; instead, a modest increase in activity was observed during culture. This result indicates that the decrease in specific (i.e., factor VII dependent) TF activity observed (Fig. 2, solid line, and Table 1) is not likely due to concomitant changes in protease assembly sites expressed during monocyte maturation in culture.

**TF Antigen Levels in Monocytes during Short-Term Culture.** Levels of TF protein antigen in monocytes were measured by enzyme immunoassay at 0, 3, 24 and 48 h, after initiation of culture in the presence of stimulus. Results of these measurements (Fig. 1, and Table 1) show that TF antigen was present in freshly isolated monocytes at 178 pg/mg of total cell protein, increased to a maximum value of 1407 pg/mg (7.9-fold) by 24 h, and stayed at  $>70\%$  of maximal values for up to 48 h. In contrast, specific TF activity measured

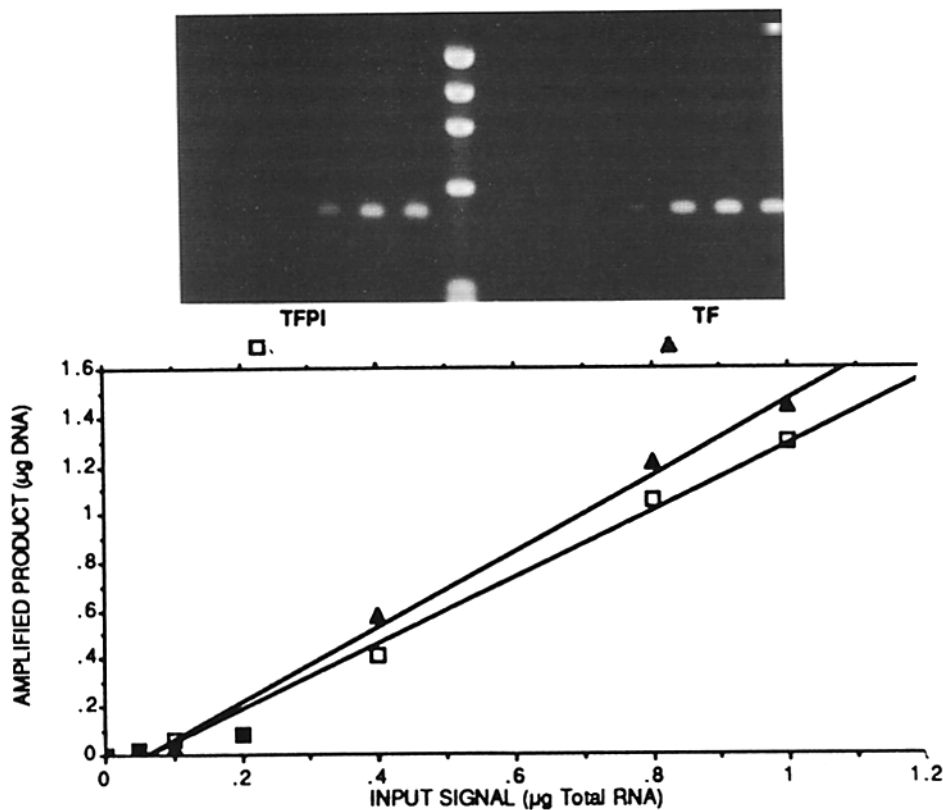
**Table 1.** TF Activity, Lipid Cofactor Activity, and TF Antigen Expressed during Monocyte Culture

Culture time	TF activity*	Lipid cofactor activity	TF antigen†	TFPI Antigen
<i>h</i>				
0	1.5 ± 0.7	74 ± 6	13 ± 2	45 ± 9
3	8.5 ± 3.2	80 ± 4	22 ± 7	51 ± 6
24	100 ± 0.0	95 ± 3	100 ± 20	72 ± 8
48	34.3 ± 4.5 ( <i>n</i> = 6) <sup>§</sup>	96 ± 3 ( <i>n</i> = 6)	73 ± 20 ( <i>n</i> = 5)	98 ± 2 ( <i>n</i> = 5)

\* TF and lipid cofactor activity were measured as initial rate of factor X activation in reaction mixtures with  $2.0 \times 10^6$  cell/ml, and either factor VIIa (5 nM) or factors IXa/VIIIa (0.8 and 1 nM). Rate values are normalized as percent of maximal rate measured during culture. Maximal rates were  $9.0 \pm 1.0$  and  $18 \pm 0.2$  nM factor Xa/min for TF and lipid cofactor activity, respectively. Lipid cofactor activity, TF antigen, and TFPI antigen levels were maximal at 24–48 h of culture. TF activity was maximal at 24 h of culture.

† TF and TFPI antigens, were quantified by EIA. Maximal value for tissue factor was 1.4 ng/mg of cell protein. Maximal value for tissue factor pathway inhibitor was 0.57 ng/mg of cell protein.

§ Data are mean ± SE from *n* different monocyte cultures, each with cells from a different donor.

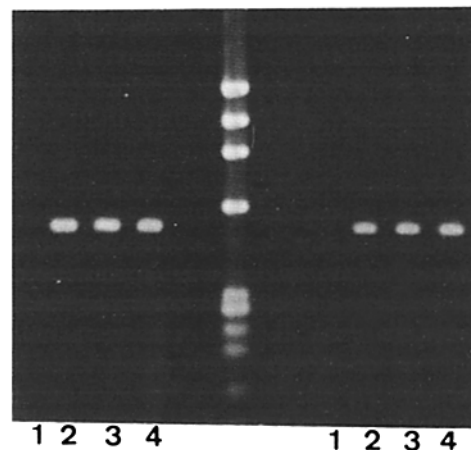


**Figure 3.** Signal to product relationship for TFPI and TF mRNA in RT-PCR assay. Total RNA (0, 0.05, 0.2, 0.4, 0.8, and 1 µg) isolated from monocytes after 3 h in culture was reverse transcribed and amplified as indicated in Table 2. (Top) Results obtained using ethidium bromide and agarose gel electrophoresis; (Bottom) results from Hoechst dye 33258 and fluorometry for DNA quantitation. Note the increased sensitivity and precision obtained with Hoechst dye method. Within the range of mRNA levels measured, input RNA signal was directly related to amplified cDNA.

kinetically was maximal at 24 h with a rate of  $9.1 \pm 1$  nM factor Xa/min (Fig. 2, and Table 1) and decreased to 30% of this maximum by 48 h. In the absence of stimulus, TF protein levels were five-, three-, and fourfold above those in freshly isolated monocytes at 3, 20, and 48 h of culture. TF activity measured kinetically in these cultures was maximal at 48 h, however, rate was only 1.5 nM factor Xa/min. The observed discrepancy between TF activity, lipid cofactor, and TF protein antigen levels suggest modulating of activity by specific TF pathway inhibitor.

**Expression of TFPI and TF mRNA by Monocytes during Short-Term Culture.** The possibility that human monocytes can regulate procoagulant activity via synthesis and expression of specific TF pathway inhibitor was explored by analysis of mRNA. Relative levels of mRNA coding for TFPI and TF expressed during monocyte culture were determined in total RNA preparations isolated from monocytes after 0, 3, 24, and 48 h of culture. RT and oligo dT primers were used to generate cDNA from poly A mRNA in total RNA preparations. Two identical aliquots of the RT reaction mixture were used as the template source to amplify specific cDNA sequences with synthetic oligonucleotide primers complementary to either TFPI or TF sequences. The PCR products yielded sharp single bands of the expected size when analyzed by gel electrophoresis and ethidium bromide staining (Figs. 3 and 4). The DNA sequences of amplified products were shown to have >99% homology with human TF and TFPI sequences stored in GenBank. Amounts of PCR product generated were proportional to initial amounts of total RNA

included in the RT mixture (Fig. 3, bottom). The number of mRNA copies/cell for each gene was estimated from the average amplification efficiency determined independently for each primer template set by kinetic measurement of



**Figure 4.** Time course of amplified TF and TFPI cDNA. Detection using ethidium bromide and agarose gel electrophoresis. Total RNA, 0.5 µg isolated from monocytes was reverse transcribed and amplified as indicated in Table 2. Amplified products and standard DNA fragments (*Hae* III digest of phage RF  $\Phi$ x174) were electrophoresed in 1.2% agarose gel and stained with ethidium bromide. Amplified TFPI cDNA and amplified TF cDNA are to the left and to the right of the standards, respectively. For each cDNA, bands 1, 2, 3, and 4 were from mRNA samples isolated at 0, 3, 24, and 48 h of culture. The band from 0 time samples was not visible for TF cDNA and only faintly visible for TFPI cDNA.

**Table 2.** Levels of TF and TFPI mRNA Expressed by Monocytes during Culture.

Monocyte* Maturation time	TF		TFPI	
	DNA <sup>†</sup> μg	mRNA <sup>‡</sup> copies/cell	DNA μg	mRNA copies/cell
<i>h</i>				
0	0.27 ± 0.07 (7)	22.0	0.35 ± 0.11 (7)	46.2
3	0.63 ± 0.16 (7)	50.9	0.65 ± 0.16 (7)	83.6
24	0.52 ± 0.16 (7)	42.5	1.03 ± 0.21 (7)	133.3
48	0.53 ± 0.04 (4)	42.6	1.70 ± 0.64 (3)	220.0

\* Total RNA 0.5 μg isolated from monocytes after the indicated culture intervals was used to generate cDNA from mRNA using 1 U/μl Mo-MLV-RT. Identical aliquots of the RT reaction mixture were amplified by PCR using sense and antisense primers, 0.15 μM each complementary to either TF or TFPI cDNA sequences. Taq polymerase was added at 2.5 U/100 μl; deoxynucleoside triphosphates at 1 mM each and MgCl<sub>2</sub> at 2 mM. The reaction was carried in 10 mM Tris HCl buffer, pH 8.3, for 35 cycles, each cycle at 95°C for 1 min and at 60°C for 1 min.

† The amplified DNA was measured fluorometrically using Hoechst dye 33258 and calf thymus DNA as a standard. No DNA was detected in cycled reaction mixtures that did not include either RT or RNA.

‡ The number of copies/monocyte was estimated from the amounts, *N*, of DNA generated by PCR using the formula,  $N = N_0(1 + E)^n$ , where *N*<sub>0</sub> is the initial number of copies of cDNA, *E* is the average efficiency and *n* is the number of cycles. The average efficiency was calculated from the observed efficiency *E*<sub>obs</sub> and the initial efficiency, *E*<sub>i</sub> i.e.,  $E = \frac{1}{2}(E_i + E_{obs})$ . The initial efficiency was calculated from *E*<sub>obs</sub> using the relationship  $E_{obs} = E_i e^{-\lambda t}$ , where *t* is the time in minutes and λ is the decay constant for Taq polymerase with half-life of 40 min at 95°C. The observed efficiency *E*<sub>obs</sub> was determined from the slope of semilogarithmic plots of *N* vs. cycle number. The *E* was 0.351 ± 0.016 and 0.368 ± 0.027 for TFPI and TF, respectively. To demonstrate the reliability of *E* estimates, the *E* of control pAW109 RNA was determined directly, using known number of initial mRNA copies ranging from 0.15 to 1.25 × 10<sup>4</sup> and resulting in amplified DNA ranging from 0.13 to 5.4 × 10<sup>12</sup> copies. The average efficiency was 0.75 ± 0.06, which is within experimental error of the value 0.715 calculated from the kinetic measurement for the control template-primer set. Thus, the decrease in efficiency during amplification time can be accounted for by the loss in activity of the enzyme. These later results also demonstrate that neither the primers nor the dNTP were limiting during the reaction, and that the kinetic approach followed provides reliable estimates of average efficiency and of the number of mRNA copies. Data include results from the number of different donors indicated in parentheses.

amplification reactions (Table 2). The kinetic method used to estimate mRNA yielded the expected known number of copies of control synthetic RNA template. The value of amplification efficiency of control template calculated directly from the observed DNA amounts and the formula  $N = N_0(1 + E)^n$  was within experimental error of values obtained using the kinetic measurement corrected for Taq polymerase decay.

Freshly isolated monocytes expressed both TF and TFPI messages, but whereas the levels of TF message reached a maximum after 3 h of culture, levels of TFPI message continued to increase for at least 48 h. Thus, the increase in TFPI mRNA coincided in time with the specific decreases in TF activity measured using functional assays with purified components.

To further examine the relationship between TFPI and TF expression, TFPI protein was measured in cell extracts (1% Triton X-100) and culture supernatants by EIA using monospecific monoclonal and polyclonal IgG as capture and detection antibodies respectively. Results from these experiments show that TFPI antigen was detected in freshly isolated monocytes at 250 pg/mg of total cell protein and at a maximum value of 570 pg/mg by 48 h of culture with LPS (Table 2). In the absence of stimulus TFPI antigen levels were 2.7-, 2.4- and 2.3-fold above those in freshly isolated monocytes at 3, 20, and 48 h. The molar ratio TF/TFPI varied during culture and was 0.7, 1.0, 3.4, and 1.7 at 0, 3, 24, and 48 h in stimulated cultures and 0.25, 0.45, 0.32, and 0.28 in unstimulated cultures respectively. The TF activity

calculated as mole factor Xa generated per mole of TF was better correlated with TF/TFPI ratios than with TF protein levels. At the concentrations of factors VII and X used in these studies, TF activity in stimulated cultures was 6 × 10<sup>2</sup>/s, 2 × 10<sup>3</sup>/s, 11 × 10<sup>4</sup>/s and 2.6 × 10<sup>3</sup>/s at 0, 3, 24, and 48 h, respectively. In contrast, TF protein levels were 4.5, 7.5, 35.0, and 25.5 pmole/mg cell protein.

## Discussion

The studies described here demonstrate the simultaneous expression of TF and TF mRNA by human blood monocytes. Steady-state levels of both TF and TFPI mRNA changed during cell culture, but the kinetic profile for each of the two mRNA species was different. The highest ratios of TFPI to TF mRNA coincided in time with low levels of TF activity measured specifically using purified factors VIIa and X.

Demonstration of specific mRNA is essential to differentiate de novo endogenous expression from endocytosis and subsequent secretion of ingested protein. It has been reported that lipoprotein internalized by monocytes/macrophages can be recycled later to the plasma membrane and released into the culture medium (35). Since plasma TFPI is largely associated with lipoproteins, it is possible that a fraction of the TFPI demonstrated by immunological methods represents endocytosed protein. Similarly, functional inhibitor measurements are not a good parameter of TFPI gene expression, since net TF activity is always detected. Absolute levels of TF and TFPI mRNA were estimated using a quantitative

RT-PCR approach based on direct efficiency determination from amplification kinetics. Using this method, low copy numbers of both messages were detected on freshly isolated nonstimulated monocytes.

In contrast to TF activity, lipid cofactor activity increased slightly, as reflected in the ability of monocytes to provide sites for efficient assembly of the intrinsic pathway protease components. Thus, the decrease in procoagulant activity was specific for the extrinsic pathway components.

The biological importance of TFPI in the regulation of blood coagulation *in vivo* has not yet been established. However, very suggestive evidence for a role of TFPI in the control of hemostasis has been obtained in sublethal disseminated intravascular coagulation (DIC) models. Depletion of TFPI in rabbits treated with anti-TFPI antibodies rendered the animals susceptible to endotoxin administered intravenously (36). Low doses of TF induced DIC in TFPI-depleted rabbits but had no demonstrable effect on intact animals (37). From these studies, it has been proposed that the inhibitor is important in maintaining low basal levels of TF activity that may be expressed under physiological conditions (9, 36, 37). The results presented here are also consistent with this hypothesis. Neither procoagulant nor inhibitory activity were expressed at detectable levels in freshly isolated monocytes. However, mRNA and protein were consistently identified. It is possible that the low levels of functional TF expressed by monocytes are balanced by concomitantly generated TFPI. The simultaneous generation of TF and TFPI and the for-

mation of inactive TF/TFPI complexes may serve to limit the excessive accumulation of functional TF at inflammatory sites, and to check functional TF expression on circulating and margined monocytes.

Endothelial cells, megakaryocytes, and several continuous cell lines including monoblastoid U-937 cells, have been shown to express TFPI mRNA. However, RNA isolated from diverse human tissues and cells, including liver and bone marrow-derived cells, did not hybridize with TFPI probes in Northern blots (14). To our knowledge, there are only two additional reports (published as abstracts) on the subject of monocyte/macrophage TFPI mRNA expression (38, 39). One of the reports indicates that human monocytes in adherent cultures express TFPI mRNA upon stimulation with endotoxin (38). In contrast, the other report indicates that expression of TFPI does not increase upon endotoxin stimulation in adherent cultures (39). The apparent contradiction between these two previous studies both using Northern blot analysis of mRNA, may reflect differences in monocyte isolation and culture protocols and in the sensitivity and efficiencies of the hybridization protocols.

The RT-PCR technique used here demonstrated that freshly isolated monocytes express TFPI and that its expression is inducible concomitantly with TF during short-term culture *in vitro* in the presence of endotoxin. Regulation of monocyte TF activity at neoplastic, inflammatory, and atherosclerotic sites can thus be mediated by TFPI generated locally independently of intravascular sources.

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## References

1. Nemerson, Y. 1992. The tissue factor pathway of blood coagulation. *Semin. Hematol.* 29:170.
2. Mann, K.G., M.E. Nesheim, W.R. Church, P. Haley, and S. Krishnaswamy. 1990. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood.* 76:1.
3. Jesty, J. 1991. Interaction of feedback control and product inhibition in the activation of factor X by factors IXa and VIII. *Haemostasis.* 21:208.
4. McGee, M., and L. Li. 1991. Functional difference between intrinsic and extrinsic coagulation pathways - kinetics of factor X activation on human monocytes and alveolar macrophages. *J. Biol. Chem.* 266:8079.
5. Nawroth, P.P., D.A. Handley, C.T. Esmon, and D.M. Stern. 1986. Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. *Proc. Natl. Acad. Sci. USA.* 83:3460.
6. McGee, M.P., L.C. Li, and M. Hensler. 1992. Functional assembly of intrinsic coagulation proteases on monocytes and platelets. Comparison between cofactor activity induced by thrombin and factor Xa. *J. Exp. Med.* 176:27.
7. Lawson, J.H., S. Butenas, N. Ribarik, and K.G. Mann. 1993. Complex-dependent inhibition of factor VIIa by antithrombin III and Heparin. *J. Biol. Chem.* 268:767.
8. Rao, L.V.M., S.I. Rapaport, and A.D. Hoan. 1993. Binding of factor VIIa to tissue factor permits rapid antithrombin III/Heparin inhibition of factor VIIa. *Blood.* 81:2600.
9. Rapaport, S.I. 1991. The extrinsic pathway inhibitor: a regulator of tissue factor-dependent blood coagulation. *Thromb Haemostasis.* 66:6.
10. Broze, G.J., T.J. Girard, and W.F. Novorty. 1991. Lipoprotein-

- associated coagulation inhibitor. *Curr. Studies Hematol. Blood Transfus.* 58:22.
11. Carson, S.D., W.D. Haire, G.J. Broze, Jr., W.F. Novorty, S.J. Pirruccello, and M.J. Duggan. 1991. Lipoprotein associated coagulation inhibitor, factor VII, antithrombin III and monocyte tissue factor following surgery. *Thromb Haemostasis.* 66:534.
  12. Rao, V.M., and S.I. Rapaport. 1981. Studies of a mechanism inhibiting the initiation of the extrinsic pathway of coagulation. *Blood.* 69:643.
  13. Novorty, W.F., T.J. Girard, J.P. Miletich, and G.J. Broze. 1989. Purification and characterization of the lipoprotein-associated coagulation inhibitor from human plasma. *J. Biol. Chem.* 264:18832.
  14. Bajaj, M., M.N. Kuppuswamy, H. Saito, and S. Spritzer. 1990. Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor: evidence that endothelium is the principal site of its synthesis. *Proc. Natl. Acad. Sci. USA.* 87:8869.
  15. Nordfang, O., S.E. Bjorn, S. Valentin, L.S. Nielsen, P. Wildgoose, T.C. Beck, and U. Hedner. 1991. The C-terminus of tissue factor pathway inhibitor is essential to its anticoagulant activity. *Biochemistry.* 30:10371.
  16. Callander, N., V.M. Rao, O. Nordfang, P. Sandset, B. Warn-Cramer, and S. Rapaport. 1992. Mechanisms of binding of recombinant extrinsic inhibitor (rEPI) to cultured cell surfaces that rEPI can bind and inhibit factor VIIa-tissue factor complexes in the absence of factor Xa. Evidence. *J. Biol. Chem.* 267:876.
  17. Sandset, M., U. Abildgaard, and M.L. Larsen. 1988. Heparin induces release of extrinsic coagulation pathway inhibitor (EPI) *Thromb Res.* 50:803.
  18. Gerrity, R.G. 1981. The role of the monocyte in atherosclerosis II migration of foam cells from atherosclerotic lesions. *Am. J. Pathol.* 103:119.
  19. Lando, P.A., and T.S. Edgington. 1985. An innate host response to the neoplastic cell: syngeneic rat tumor cells can elicit a rapid de novo lymphoid procoagulant response. *J. Immunol.* 135:3587.
  20. Rickles, F.R., and R.L. Edwards. 1983. Activation of blood coagulation in cancer: Trousseau's sign revisited. *Blood.* 62:14.
  21. Cole, E.H., J. Schulman, M. Urowitz, E. Keystone, C. Williams, and G.A. Levy. 1985. Monocyte procoagulant activity in glomerulonephritis associated with systemic lupus erythematosus. *J. Clin. Invest.* 75:861.
  22. Wilcox, J.H., K.M. Smith, S.M. Schwartz, and D. Gordon. 1989. Localization of tissue factor in the normal vessel wall and in atherosclerotic plaques. *Proc. Natl. Acad. Sci. USA.* 86:2839.
  23. Edwards, R., and F. Rickles. 1992. The role of leukocytes in the activation of blood coagulation. *Semin. Hematol.* 29:202.
  24. Weiss, H.J., V.T. Turitto, H.R. Baumgartner, Y. Nemerson, and T. Hoffman. 1989. Evidence for the presence of tissue factor activity on subendothelium. *Blood.* 73:968.
  25. Conkling, P.R., K.L. Patton, Y.A. Hannun, C.S. Greenberg, and J.B. Weinberg. 1989. Sphingosine inhibits monocyte tissue factor-initiated coagulation by altering factor VII binding. *J. Biol. Chem.* 264:18440.
  26. O'Flaherty, J.T., D.L. Kreutzer, and P.A. Ward. 1978. Chemotactic factor influences on the aggregation swelling and foreign surface adhesiveness of human leukocytes. *Am. J. Pathol.* 90:537.
  27. Bauldry, S.A., C.E. McCall, S.L. Cousart, and D.A. Bass. 1991. Tumor necrosis factor- $\alpha$  on priming of phospholipase A<sub>2</sub> activation in human neutrophils. An alternative mechanism of priming. *J. Immunol.* 146:1277.
  28. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
  29. Morrissey, J.H., H. Fakhrai, and T.S. Edgington. 1987. Molecular cloning of the cDNA for tissue factor, the cellular receptor for the initiation of the coagulation protease cascade. *Cell.* 50:129.
  30. Wun, T.C., K.K. Kretzmer, T.J. Girard, J.P. Miletich, and G.J. Broze. 1988. Cloning and characterization of a cDNA coding for the lipoprotein associated coagulation inhibitor shows that it consist of three tandem kunitz-type inhibitor domains. *J. Biol. Chem.* 263:6001.
  31. Brunk, C., K. Jones, and T. James. 1979. Assay for nanogram quantities of DNA in cellular homogenates. *Anal. Biochem.* 92:497.
  32. Carson, S.D., S.E. Ross, R. Bach, and A. Guba. 1987. An inhibitory monoclonal antibody against human tissue factor. *Blood.* 70:490.
  33. Rothberger, H., M.P. McGee, and T.K. Lee. 1984. Tissue factor activity. A marker of alveolar macrophages maturation in rabbits. Effects of granulomatous pneumonitis. *J. Clin. Invest.* 73:1524.
  34. Nesheim, M.E., D.D. Pittman, J.H. Wang, D. Slonosky, and A.R. Giles. 1988. The binding of <sup>35</sup>S-labeled recombinant factor VIII to activated and inactivated platelets. *J. Biol. Chem.* 263:16467.
  35. Robenek, H., and N.J. Severs. 1992. Endocytosis of lipoproteins and cholesterol homeostasis. In *Cell Interaction in Atherosclerosis*. H. Robenek and N.J. Severs, editors. CRC Press, Boca Raton, FL. 288-292.
  36. Sandset, P.M., B.J. Warn-Cramer, S.L. Maki, and S.I. Rapaport. 1991. Immunodepletion of extrinsic pathway inhibitor sensitizes rabbits to endotoxin-induced intravascular coagulation and the generalized Shwartzman reaction. *Blood.* 78:1496.
  37. Sandset, P.M., B.J. Warn-Cramer, L.V. Rao, S.L. Maki, and S.I. Rapaport. 1991. Depletion of extrinsic pathway inhibitor (EPI) sensitizes rabbits to disseminated intravascular coagulation induced with tissue factor: evidence supporting a physiologic role for EPI by a natural anticoagulant. *Proc. Natl. Acad. Sci. USA.* 88:708.
  38. Ameri, A., S. Hyers, S. Tricom, M.N. Kuppuswamy, and P. Bajaj. 1991. Transcriptional expression of tissue factor pathway inhibitor (TFPI) in alveolar macrophages of patients with adult respiratory distress syndrome (ARDS). *Blood.* 78:107a (Abstr.).
  39. van der Logt, C.P., H.P. Reitsma, and R.M. Bertina. 1991. LACI transcription in human monocytes is not stimulated by agonists that induce tissue factor expression. *Thromb Haemostasis.* 65:702a (Abstr.).