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ORIGINAL ARTICLE

Human polymorphonuclear leukocytes produce and express functional tissue factor upon stimulation[1]

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Summary. *Background:* Blood-borne tissue factor (TF) plays a crucial role in thrombogenesis. *Aim:* To study whether polymorphonuclear leukocytes (PMN) are a source of TF. *Methods and Results:* Human PMN were carefully separated from other blood cells and stimulated for 3 min with purified P-selectin or the chemotactic peptide formyl-MetLeu-Phe (fMLP): they expressed both TF procoagulant activity, as identified by specific TF MoAb and inactivated factor VIIa blockade; and TF:Ag (four to six times), as shown by flow-cytometry and immunocytochemistry. About 40% of permeabilized PMN, both resting and stimulated, contained TF:Ag, indicating that stimulation only modifies the location of TF:Ag within PMN. By real time-polymerase chain reaction (RT-PCR), a very low amount of TF mRNA was detectable in resting PMN, but a 3- to 5-fold increase was observed after 1-h stimulation with P-selectin or fMLP, respectively. *Conclusions:* These findings suggest that TF is not constitutively expressed in peripheral PMN, but can be up-regulated and produced upon stimulation and specific gene transcription, as for instance during contact with activated platelets or endothelium. The stored TF is rapidly expressed *in vitro* as a functional molecule on the surface of activated PMN. The availability of PMN TF supports the relevance of inflammatory cells and their interaction with platelets for fibrin deposition and thrombus formation.

Keywords: inflammation, platelet P-selectin, polymorphonuclear leukocytes, thrombosis, tissue factor.

Introduction

Tissue factor (TF) is the primary cellular initiator of the blood coagulation cascade. After vascular injury, the TF-FVIIa complex triggers a number of protease reactions that lead to fibrin formation and deposition. This mechanism contributes to intravascular thrombosis associated with atherosclerosis, cancer and other chronic diseases [1,2]. Inflammation is a common substrate of these various diseases and plays a crucial role in thrombogenesis [3].

While the classical view of TF was that it is expressed locally within a vascular lesion, more recent studies suggest that an additional source of TF, known as blood-borne TF, may also contribute to thrombosis [4]. One controversial issue is the form of blood-borne TF. Presently, it is accepted that monocytes [5] and platelets [6–8] may express TF and that TF-positive microparticles may favor incorporation of monocyte-derived TF into a thrombus by a P-selectin-mediated mechanism [9–11]. As polymorphonuclear leukocytes (PMN) are the most important and abundant circulating cells involved in the inflammatory process and are implicated in the pathogenesis of tissue injury [12], a direct contribution of PMN to TF production could be expected. However, whether PMN are able to produce and/or express TF is still controversial and matter of debate [13–16].

In previous studies, using an experimental model, where whole blood was stimulated for 24 h with phorbol myristate acetate (PMA) (an unphysiological agonist) no presence and/or production of TF was observed in PMN [15]. On the contrary, *in vivo* experiments, in an animal model of inflammation, described fibrin precipitation on PMN surface and the presence of mRNA codifying for TF on the PMN migrated into septic tissues [14], thus suggesting that PMN are able not only to express functional TF, but also to produce it. However, whether TF was endogenously

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synthesized or engulfed by PMN could not be established [15].

In the context of this debate and of our interest in the role of platelet-PMN interaction in thrombosis [17], we evaluated the capacity of PMN to express and synthesize TF after stimulation with physiological agonists, such as P-selectin. The results obtained extend to PMN the concept of 'P-selectin, tissue factor, coagulation triad', recently proposed on the basis of studies with monocytes [18].

Materials and methods

Preparation of PMN leukocytes

Venous blood was collected, after informed consent was obtained, from healthy volunteers, who denied having received any medication for at least 2 weeks. Nine parts of blood were mixed with one part of 3.8% trisodium citrate. Platelet-rich plasma was prepared by centrifugation of whole blood at $180 \times \boldsymbol{g}$ for 20 min and removed. PMN, isolated from the remaining blood by Dextran sedimentation followed by Ficoll-Hypaque gradient and hypotonic lysis of erythrocytes, were washed and resuspended in HEPES-Tyrode buffer (pH 7.4) containing 1 mM $CaCl_2$. All procedures for PMN separation [19] were performed at 4 °C.

The purified PMN suspensions were checked by flow cytometry to exclude the presence of monocytes [CD14+ cells: 0.02% ± 0.02% (means ± SEM; $n = 25$) in the PMN suspension] and platelets (CD61+ cells: virtually 0) as contaminants in the CD45+ PMN populations.

The PMN suspensions for real time polymerase chain reaction (RT-PCR) were prepared by magnetic immunoselection of CD14+ cells (MACS® MicroBeads kit, Miltenyi Biotec, Bologna, Italy) and/or a second Ficoll-Hypaque gradient. This procedure showed similar CD14+ cell contamination as compared with the standard procedure (0.012% ± 0.013%, $n = 5$).

TF activity

The procoagulant properties of PMN were evaluated by two different methods. (i) Diluted aPTT test: 100 μL of PMN suspension, containing 150×10^4 PMN, were stimulated with the peptide formyl-MetLeuPhe (fMLP) or P-selectin for 2 min at 37 °C, then 100 μL of pooled normal human plasma was added and further incubated for 1 min. Finally 100 μL of aPTT reagent (SynthASil, IL, USA) was added and the coagulation time recorded. The final concentration of PMN in this case was 5×10^6 mL^{-1}. (ii) Recalcification time assay: 100 μL of PMN suspension, containing 450×10^4 PMN, were stimulated with fMLP or P-selectin for 1 min at 37 °C, then 100 μL of pooled normal human plasma was added and further incubated for 1 min. Finally 100 μL of 25 mM $CaCl_2$ solution was added and the coagulation time recorded. The final concentration of PMN in this case was 15×10^6 mL^{-1}.

Neutralization tests

The TF-dependent procoagulant activity was also characterized using a FVII-deficient plasma (American Diagnostica, Foster City, CA, USA). Annexin V (placental anticoagulant protein-I, vascular anticoagulant-alpha, from Sigma, Milan, Italy) was used for its potent anticoagulant properties *in vitro* that are based on its high affinity for anionic phospholipids and its capacity to displace coagulation factors from phospholipid surfaces [20]. In a recalcification time system, annexin V was added to the PMN suspension prior to activation. The final concentration of annexin V used was 1.3 mM, which was found to completely block the shortening of the recalcification time by adding 150 000 platelets μL^{-1} to the plasma (this shortening being similar to that obtained with stimulated PMN).

The HTF-1 (Pharmingen, Becton Dickinson, Milan, Italy), a specific blocking MoAb against TF, or irrelevant IgG1 (isotypic control, Pharmingen) were added to the cell suspension immediately before the clotting assay at a final concentration of 5 μg mL^{-1}.

For competitive inhibition, site-inactivated FVIIa (iVIIa) was obtained by treatment of FVIIa (Novoseven®, Novo Nordisk, Bagsvaerd, Denmark) as previously described [21]. Ten micrograms per milliliter of iVIIa were added to the cell suspension immediately before the clotting assay.

Tissue factor antigen (TF:Ag) determination

Monoclonal antibodies (MoAb) against CD14 (clone RMO52, fluorochrome PE) against CD45 (clone J33, fluorochrome PC5), against the platelet glycoprotein IIIa (CD61, clone SZ21, fluorochrome FITC), its isotypic controls and fixing solution (IO Test 3) were from Immunotech (Instrumentation Laboratory, Milan, Italy). MoAb against TF (clone HTF-1) and it isotypic control were from Becton Dickinson (Milan, Italy). MoAb against myeloperoxidase (MPO), its isotypic control and Fix and Perm Cell Permeabilization Kit were from Caltag (Valter Occhiena, Turin, Italy).

All MoAb were used at saturating concentrations. These concentrations were experimentally determined and usually corresponded to the concentrations suggested by the manufacturers.

Sample preparation Polymorphonuclear leukocytes (5×10^6 PMN mL^{-1}), resuspended in Hepes Tyrode buffer with $CaCl_2$ 1 mM were placed in an aggregometer cuvette (under constant stirring, at 37 °C) and a saturating concentration of HTF-1 (a MoAb anti-TF), or the same amount of isotypic control were added. After 1 min, samples were stimulated with 0.5 μM fMLP, or 5 μg mL^{-1} P-selectin, or 0.5 μM PMA, or vehicle (basal value). The concentrations of P-selectin (5 μg mL^{-1}) and fMLP (0.5 μM) were those that in preliminary experiments induced submaximal TF expression, without inducing cell damage. The reaction was stopped by addition of a fixing solution and placed at 4 °C until flow cytometry or immunocytochemical characterization.

Flow cytometry This was performed in a Coulter Epics XL (Instrumentation Laboratory, Milan, Italy). The fluorescence channels were set at logarithmic gain. Morphological distribution of PMN was defined using forward light scatter (FSC) vs. side-angle light scatter (SSC) intensity dot-plot representation. PMN cluster was defined considering the characteristics of FSC vs. SSC within the CD45+ population. Ten thousand CD45+ PMN sized events were analyzed and counted by triggering on a preset threshold of PC5 fluorescence of the PMN marker CD45 [22]. Consequently, TF:Ag expression was the percentage of positively TF-labeled PMN among the 10 000 CD45+ PMN.

For detection of intracellular TF:Ag, PMN were fixed and permeabilized using the Fix and Perm Cell Permeabilization Kit (according to the kit indications) and incubated with a MoAb against MPO, as positive control of permeabilization, or MoAb HTF-1 or its isotype control, all FITC-conjugated. Cells were washed and analyzed for expression of TF:Ag.

Immunocytochemistry The PMN samples treated with HTF-1 as primary MoAb and fixed were then washed three times and resuspended in phosphate-buffered saline. One hundred microliters of cell suspension (5×10^6 PMN mL^{-1}) were spun on a slide using a Shandon centrifuge. Immunocytochemical staining was performed using a labelled streptavidin biotin peroxidase method (LSABPx). Endogenous peroxidase was blocked with 3% hydrogen peroxide and methanol for 15 min. After washing with phosphate-buffered saline, visualization was performed with the Dako LSAB 2 Kit peroxidase, which contains labeled streptavidin biotin reacting with primary mouse antibody and diaminobenzidine; the cell preparations were incubated in a wet chamber, at room temperature, for 1 h. Cells were briefly counterstained with hematoxylin, dehydrated, cleared and cover-slipped. As a negative control, cells were processed in the same manner as described above, except that the isotype Ab was added instead of HTF-1 MoAb.

Determination of PMN apoptosis by flow cytometry

Hypodiploid nuclei as marker of apoptosis [23,24] were identified by means of DNA histograms at flow cytometry. PMN suspensions were washed, pelleted, and fixed in cold 70% ethanol. After 30 min incubation at 4 °C, cells were washed and resuspended in 500 μL of 20 mg mL^{-1} propidium iodide solution (Sigma). After 30 min incubation, cells were analyzed by flow cytometry and 10 000 events (CD45+ cells) were counted. Propidium iodide fluorescence of individual nuclei was recorded.

RNA extraction and RT-PCR

The PMN and monocyte total RNA were isolated by the guanidinium isothiocyanate procedure as previously described [7]. Five hundred nanograms of total RNA was reverse transcribed using 20 U Stratascript Reverse Transcriptase (Stratagene, La Jolla, CA, USA) and 0.5 μg random primer together with manufacturer's buffer, 500 nM dNTP and 40 U RNase inhibitor. Incubation was carried out at 42 °C for 60 min. cDNA (25 ng) was incubated in 25 μL IQ Supermix containing TF or 18S primers (100 and 50 nM, respectively) [7] and the fluorescent dye SYBRGreen (Bio-Rad Laboratories, Milan, Italy). Primers for TF (for: 5′-tgatgtggataaaggagaaaac-tactgt; rev: 5′-tctaccgggctgtctgtactctt, selected on exon 5, which is missing in the alternatively spliced TF isoform) and 18S (for: 5′-cggctaccacatccaaggaa; rev: 5′-cctgtattgttatttttcgtcactacct) were designed using Primer Express (PE Applied Biosystems, Monza, Italy). RT-PCR was carried out in two steps (denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s, 50 cycles) on iCycler Optical System (Bio-Rad Laboratories) as previously described [7].

Amplification of ribosomal 18S was used to correct for fluctuations in input RNA levels and efficiencies of reactions. Specificity of amplified products was monitored by performing melting curves at the end of each amplification; melting curves were acquired by stepwise increase of the temperature from 55 to 95 °C. All the amplicons generated a single peak, thus reflecting the specificity of the primers. Relative quantitation of TF mRNA in each sample was obtained by normalizing TF Ct (cycle threshold) values with 18S Ct values (Δ_{Ct}).

Statistical analysis

Data of TF antigen and activity are reported as means and SEM; statistical analysis was performed by ANOVA and Dunnett's test. The differences between relative gene expression in stimulated and control samples (arbitrarily set to 1) were considered statistically significant if the ranges did not overlap the normalized value of 1 (*t*-test; $P = 0.05$).

Results

Procoagulant activity of PMN

Tables 1 and 2 report that both in the diluted activated partial thromboplastin time (aPTT) and recalcification time tests PMN were able to shorten the plasma coagulation time upon P-selectin or fMLP stimulation, resting cells being unable to modify the clotting time.

In both tests, treatment of PMN with the MoAb HTF-1 or iVIIa before P-selectin or fMLP stimulation, completely prevented the procoagulant activity expressed on stimulated PMN. This indicates that TF expressed on the PMN surface contributes to the procoagulant effect of stimulated PMN.

In order to rule out the contribution of PMN phospholipids to accelerate the plasma coagulation time after stimulation, annexin V was used for its high affinity for anionic phospholipids and its capacity to displace coagulation factors from phospholipid surfaces. Table 2 shows that annexin V did not modify the procoagulant activity of stimulated PMN in the recalcification time test.

Table 1 Activated partial thromboplastin time of cell-free citrated human plasma added to different polymorphonuclear leukocyte (PMN) samples: the procoagulant activity of activated PMN is tissue factor-dependent

Sample	Clotting time (s)
Buffer (control)	64 ± 3
PMN	61 ± 3
PMN + P-selectin	37 ± 1*
PMN + fMLP	25 ± 2*
PMN + HTF-1 + P-selectin	54 ± 6
PMN + iVIIa + P-selectin	65 ± 3
PMN + HTF-1 + fMLP	68 ± 4
PMN + iVIIa + fMLP	70 ± 5

Mean ± SEM, $n = 6$; $^*P < 0.001$ vs. unstimulated PMN by ANOVA and Dunnett's test. PMN were pre-incubated with vehicle, or P-selectin (5 μg mL^{-1}), or fMLP (0.5 μM) at 37 °C for 2 min before addition of citrated cell-free plasma and of an activated partial thromboplastin time reagent (1 min later). Inactivated factor VIIa (iVIIa) or a MoAb anti-TF (HTF-1) were added to PMN immediately before clotting assay. In the test system used, the control clotting time (64 ± 3 s) was shortened to 38–22 s by 6.25–25 μL of recombinant TF (Recombiplastin, IL). fMLP, formyl-MetLeuPhe.

Table 2 Recalcification time of cell-free citrated human plasma added to different polymorphonuclear leukocyte (PMN) samples: the procoagulant activity of activated PMN is tissue factor-dependent and not due to leukocyte phospholipid expression

Sample	Clotting time (s)
Buffer (control)	132 ± 4
PMN	125 ± 4
PMN + P-selectin	81 ± 5*
PMN + fMLP	71 ± 7*
PMN + Annexin V + P-selectin	92 ± 2*
PMN + Annexin V + fMLP	78 ± 10*
PMN + HTF-1 + P-selectin	124 ± 5
PMN + iVIIa + P-selectin	121 ± 3
PMN + HTF-1 + fMLP	124 ± 3
PMN + iVIIa + fMLP	115 ± 4

Means ± SEM, $n = 3$; $^*P < 0.001$ vs. unstimulated PMN by ANOVA and Dunnett's test. PMN were pre-incubated with vehicle, or P-selectin (5 μg mL^{-1}), or fMLP (0.5 μM) at 37 °C for 2 min before addition of citrated cell-free plasma and of $CaCl_2$ (1 min later). Annexin V, inactivated factor VIIa (iVIIa) or a MoAb anti-TF (HTF-1) were added to PMN immediately before clotting assay. In the test system used, the control clotting time (132 ± 3 s) was shortened to 78–65 s by 3.12–6.25 μL of recombinant TF (Recombiplastin, IL). fMLP, formyl-MetLeuPhe.

In neither test, did MoAb HTF-1, isotypic control, or iVIIa in the absence of PMN, by themselves modify the coagulation time; similarly, the addition of the isotypic control MoAb did not alter the procoagulant activity of PMN (not shown).

Determination of TF antigen

Flow cytometry The presence of TF:Ag was evaluated in resting and 3-min stimulated PMN suspensions by flow cytometry. Figure 1 shows that the percentage of PMN expressing TF in unstimulated samples was very low (3.6% ± 0.4%, mean ± SEM, $n = 25$). P-selectin (12.3% ± 2.8%, $n = 15$) or fMLP (19.3% ± 4.5%, $n = 25$) induced a statistically significant increase in TF:Ag expression. On the contrary, no increase in the basal TF:Ag expression was observed (4.1% ± 0.5%, $n = 8$) in experiments in which PMN were stimulated with PMA.

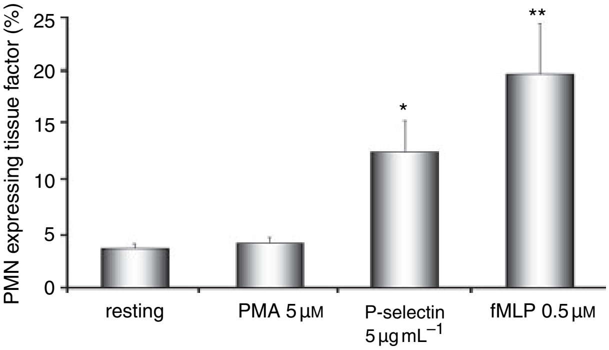

Fig. 1. Determination of tissue factor (TF) expression on polymorphonuclear leukocyte (PMN) by flow cytometry. TF expression on PMN, resting, or activated by 5 μM PMA, 5 μg mL^{-1} purified P-selectin or 0.5 μM formyl-MetLeuPhe (fMLP) for 3 min was evaluated by flow cytometry. Data are reported as the percentage of PMN expressing TF. $^*P < 0.05$ and $^{**}P < 0.01$ vs. resting PMN by one-way ANOVA and Dunnett's test. Mean and SEM, $n = 8$–25.

TF expression is not due to PMN apoptosis As fMLP might stimulate (or potentiate) PMN apoptosis [23], experiments ($n = 6$) were performed to rule out the possibility that the expression of TF on PMN surface was the result of the apoptotic process. The percentage of hypodiploid cells was 2.8% ± 0.3% and 1.4% ± 0.4% in fMLP and P-selectin stimulated PMN, respectively. Also if the proportion of hypodiploid cells was double after fMLP treatment, as compared with resting (1.2% ± 0.3%) or P-selectin (1.4% ± 0.4%) stimulated PMN, this is unlikely to explain the significant difference in TF expression after stimulation with fMLP in respect to P-selectin. In these particular experiments, TF expression was 13.0% ± 2.7% and 20.9% ± 3.7% after P-selectin and fMLP, respectively and CD14+ cells contamination was <0.005%.

Immunocytochemistry The results of immunocytochemistry largely supported the flow cytometric determinations. Figure 2A shows TF expression on resting (unstimulated) cells and shows that only the few PMN with morphological aspect of eosinophils (bilobulated nuclei) express TF positivity. After stimulation with either P-selectin (Fig. 2B) or fMLP (Fig. 2C) a larger number of PMN expressed TF. This expression appeared to be confined to the plasma membrane (Fig. 2C inset), even if it is difficult to differentiate, by optical microscopy, cytoplasm from membrane staining in whole cell preparations obtained by centrifugation.

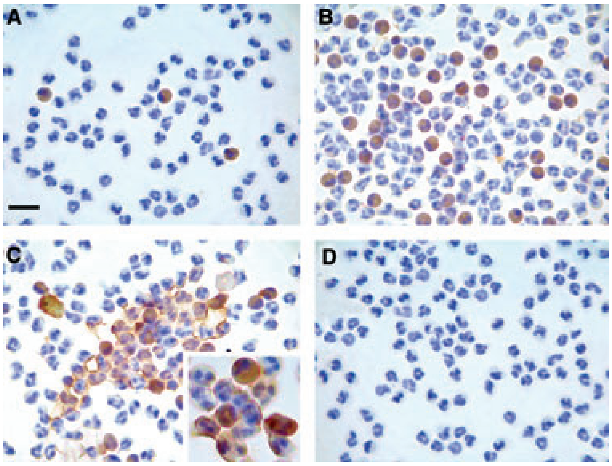

Fig. 2. Determination of tissue factor (TF) expression on polymorphonuclear leukocyte (PMN) by immunocytochemistry. TF expression in untreated (A), P-selectin- (B) and formyl-MetLeuPhe (fMLP)-treated PMN (C). (A) In untreated PMN positivity for TF is observed only in scattered PMN, eosinophil type. (B) After P-selectin stimulation several PMN stain positive for TF. (C) fMLP stimulation induces TF expression in a larger number of PMN with a certain tendency to cell aggregation and membrane staining segregation (inset). (D) No signal is observed when the isotype IgG1 was used in the staining procedure. Calibration bar is 20 μm in A–D and 10 μm in C inset. For further details, see Materials and methods.

Determination of TF mRNA

mRNA The RT-PCR experiments showed that resting PMN contain a very low, but still detectable, amount of TF (Fig. 3A). Stimulation of PMN for 1 h with fMLP or P-selectin induced a 5- and 3-fold increase of the mRNA, respectively. The rise in TF mRNA levels was already detectable 30 min after P-selectin stimulation (1.6-fold increase compared with resting PMN). In order to rule out the contribution of monocyte contamination to PMN TF mRNA expression, specific experiments were performed. Indeed, when definite amount of monocytes (0.02–0.12%) were added to a fixed amount of PMN (5×10^6 mL^{-1}), either resting or stimulated with fMLP, no significant increase in TF mRNA was observed (Fig. 3C). Moreover, TF mRNA expression in monocytes (2.5×10^6 mL^{-1}) was induced as expected by lipopolysaccharide (LPS; 1 μg mL^{-1}, for 60 min), but was not affected by fMLP (1 μM, for 60 min; Fig. 3B).

TF expression after 3 min activation is due to intracellular localization As PMN appear to express functional TF in only 3 min after challenge with fMLP or P-selectin, while at least 30 min were required to induce the appearance of TF mRNA, we considered the possibility that TF expressed upon agonist activation may already be present within PMN. In order to clarify this point, permeabilization experiments were performed. The results, reported in Fig. 4, show that 39.0 ± 2.5% ($n = 4$) of resting PMN contain TF:Ag. A similar percentage of TF:Ag expression was observed in permeabilized samples after stimulation (31% ± 3.2%; 31% ± 0.7% and 36% ± 1.4% of PMN positive for TF:Ag after PMA, P-selectin and fMLP, respectively), indicating that stimulation only modifies the location of PMN TF:Ag. On the other hand, the percentage of PMN expressing TF on their surface in respect to total content (permeabilized cells expressing TF) was 14.5% ± 3.6%, 23.4% ± 5.9%, 34.6% ± 8.7% and 51.7% ± 13% in resting, PMA-, P-selectin- and fMLP-stimulated PMN, respectively.

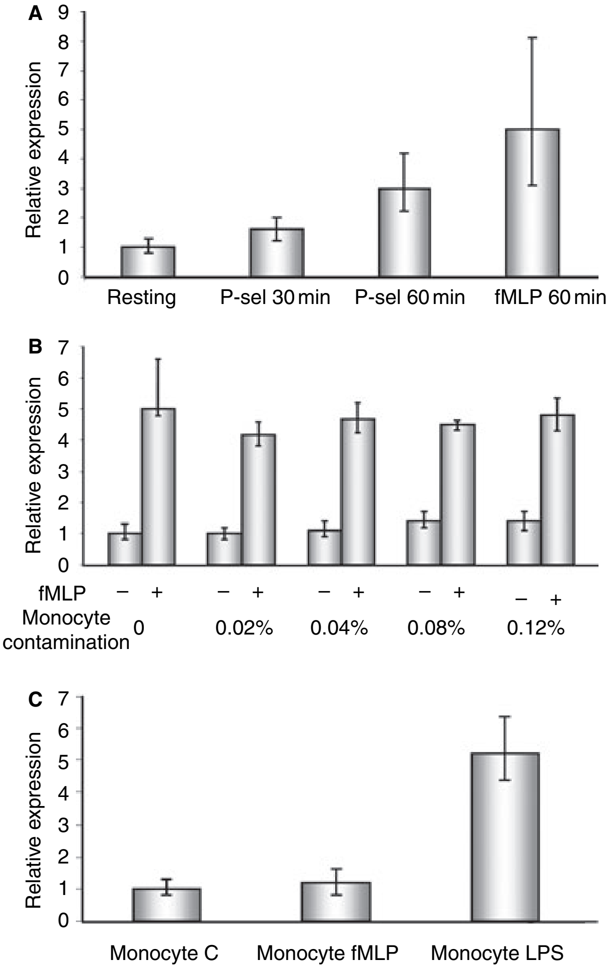

Fig. 3. Determination of TF gene transcription on PMN by real time-polymerase chain reaction. Histograms represent the relative expression calculated as 2-*ΔΔ*Ct, including the range [2-(*Δ*ÄCt ± SEM)]. Gene expression levels in stimulated samples vs. control (arbitrarily set to 1) are significantly different if the range does not overlap the normalized value of 1 (*t*-test; $P = 0.05$). Measurements were carried out in triplicate for each sample with RNA preparations from at least three independent experiments. (A) TF mRNA expression in resting and stimulated PMN. (B) Effect of addition of increasing amount of monocytes (isolated from the same blood donor) on TF mRNA expression in resting and fMLP-stimulated PMN. (C) TF mRNA expression in monocytes stimulated for 1 h with vehicle (C), fMLP (1 μM), or LPS (1 μg mL^{-1}).

Discussion

This study shows that human PMN, carefully separated from other blood cells, are able to express both TF activity and antigen upon stimulation with physiological inflammatory

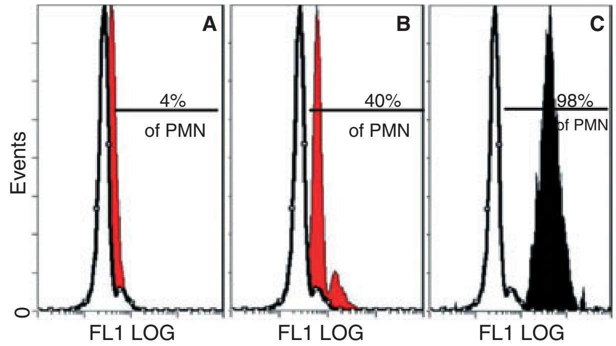

Fig. 4. Intracellular localization of tissue factor. (A) percentage of PMN expressing TF (non-permeabilized cells). (B) total content of TF in PMN (permeabilized cells). (C) Total content of MPO (control of permeabilization). Washed PMN were fixed and/or permeabilized using the Fix and Perm Kit in the presence of MoAb against myeloperoxidase (MPO), as positive control of permeabilization and characterization of PMN, or the MoAb HTF-1 or the isotype control, all FITC-conjugated. Control with isotypic Ab (white histograms); PMN labeled with MoAb against TF (red histograms) or myeloperoxidase (black histogram). Representative of four different experiments.

stimuli, such as P-selectin and fMLP. Both inducers are also able to stimulate TF mRNA synthesis in PMN. These data constitute a further link between inflammation and coagulation and underline the crucial role of adhesion molecule-mediated cell–cell interaction in thrombogenesis [17].

The primary role of TF in blood coagulation and thrombus propagation has been recognized for many years. TF is expressed on the surface of various cells such as monocytes, macrophages, platelets and endothelial cells in response to various stimuli [7,25–27].

Blood-borne TF [4] was observed in a perfusion model of human whole blood flowing over thrombogenic surfaces. In those studies, blood was shown to contain circulating TF-bearing microparticles derived from leukocytes, which could be transferred to a growing platelet-rich thrombus. It was also proposed that intravascular TF may be important in the initiation and propagation of blood coagulation.

It is currently accepted that circulating monocytes are the major cell type that respond to inflammatory stimuli by developing TF coagulant activity [26,27]. In contrast, whether other leukocyte lineages such as PMN express any TF-like activity still is a matter of debate [13–16].

Pioneering studies had already suggested the generation of TF procoagulant activity by PMN after 6 h incubation of citrated human whole blood with LPS. This activity, reduced by the protein synthesis inhibitor cycloheximide, was lacking in blood from a patient with agranulocytosis but increased over a number of days, as the patient's neutrophil count recovered [28]. However, transfer of TF from other blood cells or contamination of PMN samples with monocytes was an alternative explanation [15].

In a model of disseminated intravascular coagulation in rabbits and monkeys, PMN accumulated in liver sinusoids were reported to express TF [28–31]; the same was true for PMN infiltrating skin sites, showing an Arthus reaction [29]. In two studies, mRNA coding for TF was observed by *in situ* hybridization; in both cases the PMN migrated into tissues with an activated endothelium, expressing P-selectin [31,32].

Expression of both TF coagulant activity and antigen on activated PMN surface

In our *in vitro* study on isolated human PMN, we found that immunoreactive TF – as determined by both flow cytometry and immunocytochemistry – is located inside circulating PMN (about 40% of total cell number, as shown in Fig. 4). This finding agrees with a previous observation of TF inside PMN, using immunogold electron microscopy [9]. In resting conditions PMN expressing TF on their surface (non-permeabilized) were about 15% of the permeabilized cells expressing TF (total content).

A very short (3-min) stimulation by either P-selectin or fMLP, but not by PMA, induces translocation of TF onto the cell surface in 10–20% of total PMN (Figs 1 and 2). Failure of PMA to stimulate TF expression on PMN surface is in agreement with previous observations [33]. After stimulation with P-selectin and fMLP, the proportion of PMN expressing TF on their surface (non-permeabilized) rose to 35% and 52%, respectively, of the permeabilized cells expressing TF (total content).

The TF expressed on activated PMN surface is functionally active, as shown by its procoagulant activity (Tables 1 and 2). The procoagulant activity of PMN was completely neutralized by inactivated FVIIa or by a TF-blocking MoAb. The results of experiments performed using annexin V ruled out a major influence of phospholipids (which are able to accelerate the coagulation cascade) [20] expressed on PMN after activation [15].

Possible origin of TF expressed on PMN surface

The origin of TF transported by PMN is unclear. Upon stimulation, PMN rapidly express TF procoagulant activity, which is already present within the cells. In this respect, PMN would behave like monocytes: indeed, we evidenced stored TF in permeabilized PMN, a finding in agreement with a recent report showing TF in both resting and LPS-stimulated monocytes [5].

The observation that less than half of peripheral PMN show the presence of TF *in vitro*, and only a relatively lower number of them express TF upon activation, is difficult to explain. One can suggest that *in vivo* older and younger cells differ in their capacity to react to stimuli and/or to synthesize endogenous TF. This interpretation would favor the hypothesis that TF is not constitutively present in peripheral PMN, but only produced upon stimulation and specific gene transcription, as for instance during random contact with activated platelets or endothelium.

We could not establish, however, whether PMN would also be able to acquire ('engulf') extracellular TF antigen [15]. In the latter case, platelet- or monocyte-derived microvescicles might be the source of TF in PMN leukocytes [9,13]. Due to the remarkable phagocytic characteristics of PMN, it is unlikely, however, that 'engulfed' proteins/antigens would not rapidly be destroyed by digestive proteases that are abundant in the phagocytic vacuoles [12]. The possibility that TF on activated PMN would be the expression of a previously incorporated extra-PMN TF seems unlikely, also considering that PMN are unable to express the 'incorporated' antigens as such, but rather express antigen-derived peptides [12].

TF gene transcription in activated PMN

The RT-PCR assay indeed showed the presence of a significant amount of TF-mRNA in PMN after 1 h incubation with P-selectin (or fMLP). The TF primers used in our study were designed on exon 5, which is missing in the alternatively spliced TF (asHTF) isoform. We are therefore unable to confirm in PMN a previous observation showing that undifferentiated precursor HL-60 cells are able to express the asHTF isoform after stimulation [34].

The most important finding of the present study is that P-selectin, an adhesive molecule expressed on activated circulating platelets and/or on platelet-derived microparticles [35], can induce, by distinct kinetics, both TF expression and production following platelet-PMN conjugates.

Conclusions

Although we do not know yet whether blood-borne PMN TF might contribute to thrombotic phenomena *in vivo*, probably in the area of stasis [11], our observations constitute a possible link between inflammation and blood coagulation and add still another mechanism by which P-selectin, and by inference blood platelets, can contribute to the procoagulant activity of white blood cells [36–38].

Further studies will evaluate the signalling pathway(s) by which PMN PSGL-1 engagement by platelet P-selectin induces TF expression and generation, and whether other receptors on PMN or counter-receptors on platelets could act in synergy with P-selectin/PSGL-1 system to produce and express TF.

The possible generation of TF-positive microparticles from PMN also remains to be investigated. As platelet-PMN interaction is a well established phenomenon [17,18,39,40] and platelets too, when activated, are able to express and produce TF [7,41], it would be interesting to know whether and how TF from either cell type contributes to the blood-borne TF pool and, if so, in which physio-pathological or clinical situations such a contribution would occur.

Whatever the answers to these (and other) questions will be, it appears that a new avenue may be developed on blood cell contribution to inflammation, hemostasis, thrombosis and related diseases [42,43].

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