Role of Platelet P-Selectin and CD40 Ligand in the Induction of Monocytic Tissue Factor Expression

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Abstract—Activated platelets can express CD40 ligand (CD40L) and trigger inflammatory response and tissue factor (TF) expression in endothelial cells through interaction with CD40. This pathway is also important for T cell–induced monocyte and endothelial cell procoagulant activity. We have studied the potential role of the CD40–CD40L pathway in platelet-induced TF expression in a monocytic cell line and in whole-blood monocytes. In vitamin D₃–differentiated U-937 cells, thrombin-stimulated platelets increased TF expression as measured by mRNA quantification, flow cytometry, and procoagulant activity. Maximum antigen expression occurred after 2 hours. Neutralizing anti–P-selectin antibody yielded a 50% suppression of procoagulant activity, whereas antibody to CD40L had no effect. In thrombin receptor activator–stimulated citrated blood, monocytes were up to 77% TF-positive, with peak expression after only 15 minutes. However, no TF mRNA was detectable at that time. Anti–P-selectin antibody reduced TF by 50%, whereas antibody to CD40L gave a 17% reduction. Thus, we conclude that P-selectin exposed on activated platelets induces the expression of TF in both U-937 cells and whole-blood monocytes but by different mechanisms. Platelet CD40L does not display any significant effect on U-937 cells but may be of some importance on whole-blood monocytes. This suggests a possible functional difference between U-937 and monocyte CD40. Another important finding in this study is the rapid appearance of surface TF on monocytes without detectable mRNA formation. This indicates that TF may be stored intracellularly in these cells and can be exposed on the surface independent of de novo protein synthesis.

Key Words: tissue factor ■ P-selectin ■ CD40 ligand ■ monocytes ■ U-937 cells

Tissue factor (TF), a 47-kDa transmembrane glycoprotein, is the cellular receptor for coagulation factor VII/VIIa. TF is the main initiator of the coagulation cascade and also plays important roles in angiogenesis and tumor metastasis. Although not normally expressed by cells within the circulation, TF gene transcription can be induced in both monocytes and endothelial cells. TF induction in monocytes can occur by direct contact with other cells. A number of studies have shown that engagement and cross-linking of the counter-receptors for several adhesion molecules, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin, and P-selectin (CD62P), will result in transcription of the TF gene and subsequent surface expression of the protein. Such aberrant TF expression is known to be associated with thrombotic complications like disseminated intravascular coagulation and atherosclerosis, and it is therefore of great importance to further study the regulation of monocytic TF.

CD40 is a 50-kDa membrane-bound glycoprotein that is expressed primarily by B lymphocytes but also by a variety of other cell types: dendritic cells, monocytes, and endothelial cells, among others. Its ligand, CD40 ligand (CD40L, CD154, a member of the tumor necrosis factor gene superfamily) is expressed predominantly on activated CD4+ T-helper cells, but in recent years, it has been shown to exist in its functional form on smooth muscle cells, macrophages, activated basophils, and, most recently, activated platelets as well. For several years, CD40–CD40L–mediated signals were thought to be of importance primarily in contact-dependent T-cell/B-cell cross talk. More recently, however, the involvement of CD40–CD40L interactions in inflammatory processes such as atherosclerosis has received great attention, because it is becoming increasingly clear that this receptor–ligand couple has a variety of functions in the body. T lymphocytes have been reported to induce monocyte and endothelial cell adhesion molecules, TF, and cytokine secretion in vitro by CD40–CD40L ligation. Moreover, recent studies have shown that activated platelets trigger inflammatory response and procoagulant activity (PCA) in endothelial cells via the CD40–CD40L pathway. Because platelets are also known to induce and potentiate monocyte TF expression in a P-selectin–dependent manner, we have studied the possible importance of CD40–CD40L interactions in activated platelet–induced TF expression in human mononuclear cells. We have used 2 systems: coculture of the vitamin D₃–differentiated monocytic cell line U-937, which has a
monocyte-like phenotype, with purified human platelets, and stimulation of platelets in anticoagulated whole blood. We show that activated platelets cause TF expression by different mechanisms in U-937 cells compared with whole-blood monocytes. Platelet CD40L seems to be of some importance in whole blood but not in the cell line. We also demonstrate rapid TF surface expression in monocytes, independent of mRNA formation.

Methods

Culture and Differentiation of U-937 Cells

The human monoblastic cell line U-937 (U-937-1) was maintained and differentiated as previously described. This resulted in monocyte-like, growth-arrested, CD14-positive cells with very low basal TF expression. We also examined the cells for CD40 expression and found them to be >90% positive as measured by flow cytometry.

Preparation of Platelets and Coculture With U-937 Cells

Platelet-rich plasma was prepared by centrifugation from citrated whole blood (Vacutainer, Becton Dickinson) freshly drawn from healthy volunteers. Plasma (1 mL) was passed over a 150×10 mm Sephacryl S-1000 column (Amersham Pharmacia Biotech) packed according to the manufacturer’s instructions. The column was equilibrated in Tyrode’s/HEPES buffer (in mmol/L: NaCl 138, KCl 2.9, MgCl2 1, NaH2PO4 0.5, glucose 1, and HEPES 20, and 0.3% BSA, pH 7.4), and the platelet fraction was also collected in this buffer. The degree of platelet activation was assessed by flow cytometry before and after stimulation with 1 U/mL thrombin (Enzyme Research Laboratories). For coculture experiments, platelets were also activated with 1 U/mL thrombin, with or without preincubation with neutralizing anti–P-selectin antibody, anti-CD40L antibody (9E1, R&D Systems and M90, Genzyme Diagnostics; 5 μg/mL each), or isotype control (11711.11, R&D Systems) for 20 minutes. The U-937 cells were incubated accordingly with blocking anti-CD40 antibody (M3, Genzyme).

For the coculture experiments, U-937 cells were placed in a 24-well plate (Nunc) at 5×105 cells/well, and 10 μL of platelet suspension was added to each well. Buffer from the column fraction just before the platelets was used as control. The cells were then incubated in 5% CO2 at 37°C.

Isolation of Lymphocytes and Coculture With U-937 Cells

Mononuclear cells were isolated from fresh, heparinized blood (Venoject) by centrifugation for 40 minutes at 400g on a Ficoll-Paque density gradient (Amersham Pharmacia Biotech). Lymphocytes were then further purified by adherence of the monocytes to cell culture-grade plastic and subsequent harvesting and washing of the lymphocytes. The final cell preparation contained 94% to 97% lymphocytes as determined by staining with Türk’s solution.

U-937 cells (5×105 per well) and lymphocytes (1×106 per well) (24-well plates) were then cocultured in RPMI 1640/7.5% FBS in 5% CO2 at 37°C in the presence or absence of neutralizing or control antibodies.

TF mRNA Quantification by Real-Time PCR

The total RNA from 3×106 U-937 cells was isolated with Trizol reagent (Life Technologies) and treated as previously described. For whole-blood total RNA, a QIAamp RNA Blood Mini Kit (Qiagen) was used. Reverse transcription and real-time polymerase chain reaction (PCR) were carried out as described earlier with the TaqMan real-time PCR assay and an ABI PRISM 7700 Sequence Detection System (Perkin Elmer Applied Biosystems).

Flow Cytometry on Cultured Cells

After U-937–platelet coculture, the surface antigen expression was analyzed by immunofluorescence. The cells were washed with PBS+0.1% BSA, incubated for 30 minutes on ice with primary antibody (TF9-9C3, American Diagnostica, or isotype control, Dakopatts, 2.5 μg/mL), washed again twice, incubated for another 30 minutes on ice with 10 μg/mL of FITC-conjugated rabbit anti-mouse IgG (Dakopatts), and washed twice before analysis. For evaluation of the platelet–U-937 complexes and their degree of activation, the cells were stained with FITC- or phycoerythrin-labeled antibodies against glycoprotein (GP) IIb/IIIa, CD40L (Dakopatts), or P-selectin (SeroSpect, dilution 1:40 from stock). Mean channel fluorescence intensity (MFI) and percentage of positive cells were determined for each sample by setting, on the fluorescence scale, the positivity gate on the uppermost 2% of the cells stained with isotype control antibody. Analyses were performed with a Coulter Epics XL flow cytometer (Coulter Electronics). The instrument was calibrated daily with Flow-Check beads (Coulter Immunology).

Measurement of U-937 PCA

The PCA of cell surface TF was determined in a previously described 2-stage amidolytic assay.

Whole-Blood Stimulation and Flow Cytometry

Blood from healthy volunteers was drawn into Vacutainer tubes containing 3.2% sodium citrate with a 21-gauge needle and no or very little stasis. Aliquots of blood (0.3- to 0.6-mL) were placed into sterile polystyrene tubes (Falcon, Becton Dickinson) and incubated for 15 minutes in 5% CO2 at 37°C with 300 ng/mL of the GP IIb/IIIa inhibitor fradafiban (to prevent platelet aggregation; kindly supplied by Boehringer Ingelheim Pharma, Biberach, Germany) and, in some cases, 5 μg/mL of neutralizing antibodies to P-selectin, CD40, or CD40L (M90 or TRAP1, Immunotech, alone or in combination) or control antibody (11711.11 or 24822.111 from R&D Systems). After this time, thrombin receptor activator (TRA; SFLLRNPNNDKYEFP, Sigma Chemical Co) was added to some tubes at a final concentration of 20 μmol/L, and the blood was further incubated for up to 2 hours. The tubes were gently rotated every 30 minutes to prevent complete sedimentation of the cells. In some experiments, lipopolysaccharide (LPS) at a final concentration of 5 ng/mL was also used as a comparative stimulus.

At the end of the incubation, the blood was stained with FITC- or phycoerythrin-conjugated monoclonal antibodies for GP IIb/IIIa, P-selectin, CD40L, and TF (4508CJ, American Diagnostica), with CD14 antibody (Dakopatts) used as a selective marker for monocytes. The procedure for TF staining was described previously. For platelet-monocyte complexes and their content of P-selectin and CD40L, the blood was very gently mixed with the buffer and conjugated antibodies (1 μL anti-CD14 and 3 μL anti–GP IIb/IIIa/anti-P-selectin/anti-CD40L/isotype control; all stock solutions were 100 μg/mL) and incubated for 15 minutes at room temperature. The red cells were then lysed and the other cells fixed without prior washing by use of Coulter Whole Blood Lysing Reagents.

In the following flow cytometry analysis, monocytes were identified by gating on the CD14-positive cells, and the positivity for the different markers was determined in this population.

Endotoxin Contamination

All reagents were screened for endotoxin contamination with CoaT-est (Chromogenix, Haemochrom Diagnostica). For whole blood, final LPS concentrations were always <10 pg/mL. For the cell line, slightly higher values (<100 pg/mL) were accepted, because these cells do not respond to LPS at these concentrations and because a buffer control was always included in the experiments.

Statistical Analysis

Data were analyzed with Statistica for Windows software (StatSoft). A Student’s t test for dependent samples was used to determine statistical significance between different data sets. Results are expressed as mean±SD unless stated otherwise. Values of P<0.05 were considered statistically significant.

Results

Platelet/U-937 Coculture

U-937 cells bound large amounts of resting platelets, as indicated by the high MFI value for GP IIb/IIIa. With
thrombin-stimulated platelets, however, these complexes were up to 96% positive for P-selectin and 67% for CD40L (Figure 1a).

Platelet Effect on TF Antigen and mRNA Levels in U-937 Cells

Vitamin D$_3$–differentiated U-937 cells were incubated with thrombin-stimulated platelets or buffer for up to 6 hours. TF antigen was analyzed by flow cytometry. Antigen levels were measurable already after 30 minutes of incubation (Figure 2a) and peaked after 2 hours with 54±17% TF-positive cells, after which they started to decrease.

Activated platelets induced TF mRNA expression, which peaked at 30 minutes (Figure 2b) with an up to 12-fold increase compared with fresh cells, then declined to reach background levels again after 4 hours. Background values of TF mRNA, ie, incubation with buffer or resting platelets, were in general low but with a slight increase peaking at 2 hours.

Platelet Influence on PCA in U-937 Cells

U-937 cells were incubated with buffer or resting or thrombin-stimulated platelets, with or without preincubation with neutralizing antibodies. Activated platelets generated a 6-fold increase in PCA compared with background (buffer) (Figure 3). Anti-P-selectin antibody suppressed TF activity by 47±7% (P<0.001), whereas anti-CD40 or anti-CD40L had no effect. Blocking both P-selectin and CD40L simultaneously did not result in further inhibition compared with P-selectin alone.

Lymphocyte/U-937 Coculture

To clarify whether vitamin D$_3$–differentiated U-937 cells were at all able to express TF in response to CD40 ligation, coculture experiments with isolated human blood lymphocytes were performed. This would cause the T lymphocytes to become allogenically activated in the same way as in a mixed lymphocyte reaction.

Lymphocytes were isolated as described and incubated together or separately with U-937 cells for 18 hours in the presence or absence of blocking antibodies. Coculture resulted in a 10-fold increase in PCA compared with U-937 cells alone and 5-fold compared with lymphocytes alone (Figure 4). This induction could be almost completely blocked by anti-CD40L antibody (P<0.001), whereas antibody to P-selectin had no significant effect. Surprisingly, anti-CD40 antibody had no inhibitory effect on its own. Contaminating monocytes were not responsible for the TF activity measured, because a mixed lymphocyte reaction between the different donors did not yield a PCA that differed from that of lymphocytes alone (data not shown).

Platelet Activation in Whole Blood

Citrated whole blood was stimulated with TRA and incubated for up to 2 hours. Complexes formed between monocytes and platelets were then analyzed by flow cytometry (monocyte
positivity for GP IIb/IIIa), and their contents of P-selectin and CD40L were assessed. In unstimulated blood, only a small number of monocytes stained positive for the platelet marker. In TRA-activated blood, however, a great number of large complexes were formed (monocyte MFI for GP IIb/IIIa, 200) that were 100% positive for P-selectin and 45% to 85% positive for CD40L (Figure 1b). The results were similar at 15 minutes and at 2 hours.

Kinetics of Platelet-Induced Monocyte TF Antigen in Whole Blood
Citrated blood was incubated with or without TRA or LPS. Samples were removed for antibody staining after 15 minutes, 30 minutes, 1 hour, and 2 hours. Whereas LPS stimulation resulted in a gradually increasing TF expression over time, TRA gave a rapid exposure of TF antigen at 15 minutes, after which the levels declined (Figure 5a). The peak levels of TF with TRA stimulation were typically between 30% and 40% TF-positive monocytes (mean 41 ± 6), although ranging from 17% to 77%. The degree of decline varied between individuals, with mean TF levels of 23 ± 12% at 2 hours.

Platelet Induction of TF mRNA in Whole Blood
Aliquots of blood were incubated with or without TRA for 15 minutes to 2 hours. LPS was used as a positive control. Total RNA was isolated and TF mRNA quantified. Flow cytometry analyses of TF antigen were made on the same samples. Two experiments were performed, which were in complete accordace with each other.

At 15 minutes, no TF mRNA was detectable in any of the samples (Figure 5b), even though the monocytes expressed plenty of TF antigen in the TRA-stimulated blood (35% and 76% TF-positive cells, respectively; data not shown). After 1 hour’s incubation, some mRNA induction by TRA could be seen, albeit much weaker than that by LPS. At 2 hours, the levels remained the same or declined slightly. Antigen levels followed the same kinetics as seen earlier.

Blocking Experiments in Whole Blood
Aliquots of blood were preincubated with neutralizing antibodies or isotype control, then stimulated with TRA and further incubated for 15 minutes or 2 hours. At 15 minutes, anti-P-selectin caused a significant decrease in TF antigen in all cases (Figure 6), with a mean inhibition of 51 ± 16%
Anti-CD40L had a negligible effect in 2 of the 6 experiments and gave 20% to 30% suppression in the other 4. Overall, the inhibition was significant at \( P < 0.02 \). The 2 antibody clones against CD40L gave comparable results, which were therefore treated as 1 group. Oddly, anti-CD40 antibody caused very little or no TF decrease. Anti–P-selectin and anti-CD40L together did not result in further inhibition compared with anti–P-selectin alone. At 2 hours, the results did not differ from those at 15 minutes (data not shown).

Discussion

The understanding of the importance of CD40 signaling in other events than the humoral immune response is continuously growing, especially in the field of atherosclerosis. It has been demonstrated by several groups that CD40L activates atheroma-associated cells by the induction of adhesion molecules, cytokines, matrix metalloproteinases, and TF, all thought to be involved in the atherogenic process. In a recent publication, Mach et al showed reduction of atherosclerosis in mice by administration of anti-CD40L antibody. Platelets, known to activate leukocytes by P-selectin tethering, together did not result in further inhibition compared with anti–P-selectin alone. At 2 hours, the results did not differ from those at 15 minutes (data not shown).

That activated platelets express CD40L and also that they can induce TF in a P-selectin–dependent manner in both systems. As to the importance of CD40 ligation and signal transduction, there seems to be a difference between the vitamin \( \Delta_2 \)-differentiated U-937 cells and whole-blood monocytes. In the cell culture experiments, no significant effect on TF activity could be discerned when a blocking antibody to CD40L was added. In whole blood, however, a small and varying yet significant inhibition was observed at the antigen level. The U-937 cells did, however, respond to CD40 ligation when brought on by activated T lymphocytes, and this effect could be blocked with the appropriate antibody. It should be mentioned that this induction was measured after 18 hours’ incubation; at 4 hours, no PCA had yet been developed (data not shown). However, extending the incubation time did not improve the induction of PCA in platelet–U-937 cocultures (data not shown). We interpret these results as indicating that there seems to be a discrepancy between CD40 signaling in vitamin \( \Delta_2 \)-differentiated U-937 cells and human monocytes in a whole-blood environment that reduces the effect of platelet CD40L in the cell line system. This would not be surprising, because the pattern of TF induction in vitamin \( \Delta_2 \)-differentiated U-937 cells differs slightly from that of isolated human monocytes. Possibly, costimulatory factors from lymphocytes that are not present in the platelet preparation are needed for induction in the cell line.

The importance of CD40 ligation for platelet-induced monocyte TF in blood is difficult to estimate, because the addition of P-selectin and CD40L blocking antibodies together did not result in further inhibition compared with P-selectin alone. It appears that P-selectin is required for TF induction. Possibly, platelet CD40L enhances P-selectin–induced TF, although it does not cause TF expression on its own. One cannot exclude the possibility, however, that platelet CD40L may be of importance in vivo, where cell aggregates that are formed on or near an atherosclerotic plaque are in closer proximity to each other than they are in whole blood. Factors such as shear stress, locally secreted...
cytokines, and engagement of other adhesion molecules may influence the responsiveness of monocytes to CD40 ligation.

Our results regarding TF upregulation by platelet CD40L in monocytic cells are in contrast to observations previously made on endothelial cell models. This only underlines the existing differences in TF induction between different cell types.

The kinetics of TF expression induced by platelets differed between our 2 experimental systems. The cell line responded as could be expected for a gene-level induction, with gradually rising levels of both mRNA and antigen over time. In whole blood, however, large amounts of TF antigen appeared on the monocyte cell surface after only 15 minutes’ incubation with TRA, after which the levels decreased, indicating shedding or internalization. Interestingly, no TF mRNA could be detected at the earliest time point of antigen detection. Early TF antigen induction by platelets in whole blood was observed by Amirkhosravi et al., who also used 15-minute incubations for their collagen stimulation experiments. Similar observations have been made by other workers. We now show, for the first time, the TF mRNA kinetics for platelet-induced monocytic TF. The complete lack of mRNA formation at the time of antigen maximum in whole blood indicates that TF has been exposed on the cell surface independent of de novo protein synthesis, which has previously been considered a requirement for monocyte TF expression.

Our results correlate well with those of Giesen et al., who recently demonstrated TF-containing microvesicles as well as TF-positive monocytes and neutrophils in blood from healthy individuals and hypothesized that leukocytes are the main source of this blood-borne TF. The same group has previously shown 3 cellular pools of encrypted TF in smooth muscle cells, finding that raises the possibility that other cell types can have a similar organization. In forthcoming studies, we will further penetrate the possible existence of intracellularly stored TF in monocytes.

The facts that TRA stimulation of whole blood resulted in TF mRNA formation after 1 hour of incubation and that antibodies to P-selectin and CD40L had the same inhibitory effects at 2 hours as at 15 minutes suggest a biphasic response by monocytes to activated platelets. Possibly the TF first exposed on the surface is shed, but protein brought on by the P-selectin antibody. We cannot exclude the possibility that other cell types can have a similar organization. In forthcoming studies, we will further penetrate the possible existence of intracellularly stored TF in monocytes.

In conclusion, we show that activated platelets cause TF expression by different kinetics in U-937 cells compared with whole-blood monocytes. CD40L seems to be of some importance in whole blood. We also demonstrate a rapid, seemingly novel mechanism of TF surface exposure in blood monocytes, independent of mRNA formation. The idea of an in vivo crosstalk between monocytes, endothelial cells, and activated platelets, expressing both P-selectin and CD40L, raises the possibility of this interaction being a key event in the initiation of plaque formation and progression, as well as in the initiation and propagation of thrombus formation at the plaque surface.

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