Tissue Factor Gene Expression Analysis in Circulating Monocytes*

Jens Müller Jutta Maria Rox Bernd Pötzsch

Institute of Experimental Haematology and Transfusion Medicine, University of Bonn, Germany

Key Words

Tissue factor · Gene expression · Monocytes ·
Real-time PCR

Summary

Background: There is growing evidence that an increase in stimulated and tissue factor(TF)-expressing monocytes drive the coagulation system to a hypercoagulable state. In order to further investigate this phenomenon, methods are needed that allow sensitive, reliable and specific quantification of TF-expressing monocytes. **Materials and Methods:** Two procedures of one-step multiplex real-time RT-PCR were developed that allow absolute quantification of TF, CD14 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA transcripts. In order to minimize preanalytical changes of the mRNA profile, a blood sampling system which is based on integrated RNA stabilization was used. **Results:** An in vitro lipopolysaccharide (LPS) model was used for initial assay evaluation. LPS-induced TF gene expression patterns were similar to previously described findings. With the use of the whole blood RNA stabilization system, the marker transcripts were preserved for up to 24 h at room temperature and for up to 5 days at 4 °C. Monocytic TF transcription analysis in different patient groups identified significant higher levels in thrombophilic patients than in a representative control population. In all patients and control probands analyzed we found significantly higher levels of TF transcripts in carriers of the G allele of the TF-603A/G promotor polymorphism. **Conclusion:** The newly developed RT-PCR allows sensitive, reliable and specific quantification of TF transcripts in whole blood samples.

Schlüsselwörter

Tissue Factor · Genexpression · Monozyten ·
Echtzeit-PCR

Zusammenfassung

Hintergrund: Eine Vielzahl von Daten spricht dafür, dass stimulierte, Tissue-Factor(TF)-exprimierende Monozyten prokoagulatorisch aktiv sind. Für weiterführende Untersuchungen zu dieser Thematik bedarf es standardisierter Methoden, die eine sensitive, zuverlässige und spezifische Quantifizierung von TF-exprimierenden Monozyten ermöglichen. **Material und Methoden:** Zwei Echtzeit-RT-PCR-Verfahren wurden zur parallelen, absoluten Quantifizierung von TF-, CD14- und Glyceraldehyd-3-Phosphat-Dehydrogenase(GAPDH)-Transkripten etabliert. Zur Konservierung des zellulären RNA-Profiles ex vivo wurde ein hierzu zur Verfügung stehendes Blutentnahmesystem verwendet. **Ergebnisse:** Die in einem zur Evaluierung der Methodik eingesetzten Lipopolysaccharid-Modell beobachtete monozytäre TF-Transkription entsprach den in der Literatur beschriebenen Charakteristiken. Unter Verwendung des beschriebenen Blutentnahmesystems konnte eine Stabilisierung der untersuchten Transkripte für bis zu 24 h bei Raumtemperatur und für bis zu 5 Tage bei 4 °C erreicht werden. Im Vergleich zu einer Kontrollgruppe wurden erhöhte Konzentrationen monozytärer TF-mRNA in thrombophilen Patienten nachgewiesen. Eine generell gesteigerte TF-Transkription konnte bei Trägern des G-Allels bezüglich des TF-603A/G-Promotor-Polymorphismus festgestellt werden. **Schlussfolgerung:** Die beschriebenen Methoden erlauben eine sensitive, zuverlässige und spezifische Quantifizierung der monozytären TF-Transkription in Vollblutproben.

*Dedicated to Prof. Dr. Peter Hanfland, Bonn, on the occasion of his 65th birthday.

Introduction

Tissue factor (TF) is the primary initiator of the coagulation cascade *in vivo*. It is expressed on the surface of nearly all extravascular tissues. After vessel injury, extravascular TF comes into contact with coagulation factor VII (FVII) that circulates in plasma in its active form, activated FVII (FVIIa). The resulting FVIIa/TF complex activates FX and FIX, leading to thrombin generation and subsequent formation of a fibrin clot [1].

This traditional view of coagulation has been challenged by the demonstration of intravascular TF that participates in the coagulation reaction. Among the blood cells, monocytes have been shown to be a major source of intravascular TF [2, 3]. The active role of monocytic TF in activation of the coagulation cascade is convincingly demonstrated in lipopolysaccharide (LPS)-induced disseminated intravascular coagulation. In addition, enhanced levels of monocytic TF have been reported in various diseases that are associated with increased basal levels of activation of the coagulation system such as solid cancer and hematopoietic tumors [4–7]. These data demonstrate that increased levels of TF expression in circulating monocytes drive the hemostatic system to a procoagulant state. Therefore, quantification of TF expression in circulating monocytes might be a helpful diagnostic tool to characterize a hypercoagulable state.

Detection of TF-expressing monocytes (TF+ monocytes) is hampered by the fact that TF+ monocytes are rapidly cleared from the circulation by adhesion to the endothelial cell lining [8]. This makes determination of monocytic TF expression unreliable if performed on the protein level by e.g. FACS analysis or measurement of TF activity. Analysis of TF expression on the transcriptional level has been shown to be more sensitive in identifying monocytes that become procoagulant [9].

Nowadays, reverse transcription followed by real-time PCR is the method of choice for quantification of specific mRNA transcripts [10–12]. Generally, the advent of the homogenous real-time PCR format in 1992 greatly improved the reliability and speed of nucleic acid detection [13]. In contrast to heterogeneous assay formats, real-time PCR is fast and combines high sensitivity with a broad dynamic range. Due to the closed-tube system, false-positive results from carryover of PCR products are very unlikely [14]. Furthermore, the use of fluorescence-labeled probes offers the possibility to apply multiplex reactions in order to detect both the target and a possible reference template in a single amplification reaction [15–17]. Because of these advantages we used the real-time PCR format to develop a diagnostic PCR that allows quantification of TF-mRNA in whole blood samples.

Accurate quantification of mRNA in living cells including blood cells is hampered by the degradation of gene transcripts or unintended *ex vivo* gene expression caused by sample handling or uncontrolled activation of coagulation [18, 19]. This is especially a problem when working with monocytes since

monocytes are highly reactive and TF expression can be induced by contact with foreign surfaces and extended blood storage [20]. Thus, the mRNA profile must be immediately stabilized *in vitro* to enable reliable determination of monocytic TF gene expression by quantitative detection of TF mRNA transcripts [21]. To minimize changes of the mRNA profile *ex vivo*, a blood sampling system that includes a RNA-stabilizing additive in the blood collection tube was used.

TF gene expression patterns are influenced by the genetic background. Polymorphisms have been identified that modulate TF gene expression in endothelial cells and monocytes [22–24]. These polymorphisms are in complete linkage, determined by two haplotypes with almost equal frequency, namely TF-603A and TF-603G. The homozygous TF-603A haplotype has been associated with lower TF transcription rates and a potentially protecting effect regarding venous thromboembolic diseases and myocardial infarction [22–24]. To study the impact of these polymorphisms on the TF expression levels in the present investigations, we routinely genotyped all study patients and healthy controls.

Materials and Methods

Patients and Controls

Blood samples were taken from healthy blood donors and thrombophilic outpatients. All patients had a positive history of at least one idiopathic deep venous thrombosis but showed no acute event in the last 3 months.

Cell Counting

Global white cell counts were done using a Sysmex K-4500 (Sysmex, Norderstedt, Germany). The percent portion of monocytes was determined by flow cytometry using a FACScan and CellQuest software version 3.3 (Becton Dickinson, Basel, Switzerland). EDTA-anticoagulated whole blood (50 µl) was labeled with fluorescent dye-conjugated mouse monoclonal antibodies (10 µl each; Becton Dickinson) against human CD45 (phycoerythrin (PE)-conjugated) and human CD14 (fluorescein-isothiocyanate (FITC)-conjugated). As a negative control, 10 µl each of mouse IgG1 (PE) and IgG2a (FITC) antibodies (Becton Dickinson) were added to 50 µl whole blood. All samples were incubated for 20 min at room temperature in the dark. Afterwards, 500 µl FACS lysis solution (Becton Dickinson) were added, and samples measured directly after complete lysis of red cells. At least 20,000 CD45+ events were recorded, and monocytes were identified by their specific forward and side scatter pattern and present CD14 expression.

Isolation of Total RNA from Whole Blood Samples

PAXgene™ Blood RNA tubes (PreAnalytix, Hombrechtikon, Switzerland) were used for stabilization of the cellular RNA profile *in vitro*. A volume of 2.5 ml of whole blood was directly drawn into the PAXgene Blood RNA tube using a blood collection set or transferred by pipetting from *in vitro* experiments. Isolation of total RNA from the samples was performed using the PAXgene™ Blood RNA Kit (PreAnalytix).

Total RNA from whole blood collected in EDTA tubes was isolated after erythrocyte lysis (Buffer EL, Qiagen, Hilden, Germany) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

TF/Glyceraldehyde-3-Phosphate Dehydrogenase and CD14/Glyceraldehyde-3-Phosphate Dehydrogenase Real-Time RT-PCR

One-step real-time RT-PCR using *in vitro* transcribed RNA calibrators

was performed for quantification of mRNA sequences. For normalization of determined TF and CD14 mRNA values to the total amount of RNA in the samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was quantified in a multiplex configuration. Details and performance characteristics of the applied multiplex real-time RT-PCR assays are given elsewhere [25].

TF-603A/G Genotyping

Genomic DNA was isolated from 200 μ l of whole blood using the QI-Aamp Blood DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The TF-603A/G haplotype polymorphism was identified by PCR-RFLP. The primers used were: 5'-GATGTGAGGCTGCTTCCT-3' (forward) and 5'-GGACCTGCAGCGAAGTAAAC-3' (reverse). PCR mixtures (50 μ l) contained: 1 $\times$ PCR buffer (20 mmol/l Tris-HCl, pH 8.5, 50 mmol/l KCl), 1.5 mmol/l $MgCl_2$, 200 μ mol/l each dNTP, 200 nmol/l each forward and reverse primer, 1.25 U PlatinumTaq DNA polymerase (Invitrogen, Karlsruhe, Germany) and 5 μ l of the sample preparation. Amplification was performed using a PTC 225 thermal cycler (MJ Research, Waltham, MA, USA) and the following temperature profile: 95 $^{\circ}$ C for 5 min followed by 40 cycles of 94 $^{\circ}$ C for 20 s, 58 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 30 s. PCR products (259 bp) were digested overnight at 37 $^{\circ}$ C with ScrF I (CCNGG; New England Biolabs, Frankfurt/M., Germany) and subsequently separated on ethidiumbromide-stained 2% agarose gels. DNA fragment patterns were as follows: TF-603 A/A, 216 and 43 bp; TF-603 G/G, 126, 90 and 43 bp.

Statistical Analysis

All data were analyzed using SPSS 12.0 for Windows software. The Student's t-test was used to determine statistical significance between data sets. Statistical significance was judged at confidence levels greater than 95% ($p < 0.05$).

Results

TF Gene Expression Patterns in LPS-Stimulated Whole Blood

LPS stimulated monocytes were used as an in vitro model for assay evaluation. In order to study the dose-dependent induction of TF gene expression, EDTA-anticoagulated whole blood obtained from 10 healthy volunteers (5 male and 5 female) was stimulated with *Salmonella enteritidis*-derived lipopolysaccharide B (Sigma, St. Louis, MO, USA) in concentrations ranging from 0 to 100 ng/ml. After incubation for 2 h at 37 $^{\circ}$ C, total RNA was isolated from 1 ml of the whole blood samples and analyzed by real-time RT-PCR. A statistically significant increase of TF/GAPDH mRNA ratios was detectable at concentrations from 10 pg/ml LPS (fig. 1). The expression of GAPDH mRNA was not effected by any of the tested LPS concentrations (data not shown).

The kinetics of LPS-induced TF gene expression were determined using the described blood collection system for immediate RNA stabilization. EDTA-anticoagulated whole blood obtained from 10 healthy volunteers (5 male and 5 female) was mixed with LPS to achieve final concentrations of 100 ng/ml and incubated at 37 $^{\circ}$ C. Subsequently, subsamples of 2.5 ml were drawn from the whole blood and transferred to PAXgene Blood RNA tubes. As shown in figure 2, the mean TF transcription rate in the whole blood samples increased by a factor of 250 during the 1st hour of incubation with LPS.

Fig. 1. Concentration-dependent induction of TF gene expression by LPS. Whole blood samples from 10 healthy volunteers were incubated with different concentrations of LPS. There was a statistically significant increase of TF/GAPDH mRNA ratios up from a concentration of 10 pg/ml (* $p < 0.05$).

Fig. 2. Kinetics of LPS-induced TF gene expression. EDTA-anticoagulated whole blood ($n = 10$) was incubated with 100 ng/ml LPS. TF mRNA levels were measured at the indicated time points. Open symbols represent the results obtained with unstimulated samples ($n = 3$). Reprinted with permission from *Clinical Chemistry* 2004;50:245–247.

Unstimulated whole blood samples ($n = 3$) showed no increase but a slightly decrease of the TF/GAPDH ratio to approximately 50% of the basal level during the first 2 hours of incubation.

Fig. 3. Influence of sample handling on RNA stability. Whole blood was collected into PAXgene blood sampling tubes (closed symbols) or EDTA tubes (open symbols) and stored **a** at 4 °C or **b** at room temperature over a total of 7 days. At the indicated time points the yield of RNA transcripts was measured by quantitative RT-PCR. TF (triangles); CD14 (boxes), GAPDH (circles). Reprinted with permission from *Clinical Chemistry* 2004;50:245–247.

Performance of Preanalytical RNA Stabilization

In order to assess the RNA stabilization capacity of the PAXgene additive, whole blood from one healthy donor was drawn in parallel into PAXgene and EDTA tubes. Tubes were stored at room temperature or 4 °C for up to 7 days. Total RNA was isolated at days 0, 1, 3, 5, and 7 after blood collection and stored at –80 °C until quantification of TF, CD14 and GAPDH RNA values was performed. As shown in figure 3a, RNA levels remained stable up to 5 days of storage in PAXgene tubes at 4 °C. This time span was reduced to 1 day if the tubes were stored at room temperature (fig. 3b). In contrast, in EDTA anticoagulated blood the RNA expression profile dramatically changed within the first 24 hours of storage, independent of the storage temperature.

Fig. 4. Levels of monocytic TF gene expression in healthy blood donors (n = 50) and thrombophilic patients (n = 50). Boxes represent the interquartile range, with the median represented by the line inside the box. Whiskers indicate the highest and lowest values, excluding outliers (circles).

Fig. 5. Monocytic TF gene expression according to the TF-603 A/G haplotype polymorphism. Boxes represent the interquartile range, with the median represented by the line inside the box. Whiskers indicate the highest and lowest values, excluding outliers (circles).

Monocytic TF Gene Expression in Thrombophilic Patients and Healthy Volunteers

Whole blood samples (PAXgene) obtained from 50 thrombophilic patients and 50 matched healthy volunteers were analyzed (mean age 33.0 (range 19–65) years) for TF gene expression. Test results were normalized to the number of monocytes in the samples. As shown in figure 4, the mean transcription rate in the patient group (0.0061 ± 0.0028 TF transcripts/monocyte, 95% reference interval: 0.0017–0.0117) was significantly higher if compared to the control group (0.0041 ± 0.0021 , 95% reference interval: 0.0018–0.0095).

TF Gene Expression According to the TF-603A/G Haplotype

The described healthy volunteers and thrombophilic patients were additionally genotyped regarding the TF-603A/G haplotype polymorphism. Altogether, 18 were homozygous and 42 heterozygous for the TF-603G allele. The remaining 40 were homozygous for the TF-603A allele. Determined allele frequencies were similar in both patients and healthy volunteers. Overall monocytic TF gene expression was significantly higher in carriers of the G allele (fig. 5).

Discussion

The level of TF gene expression in monocytes might be a useful marker to indicate a hypercoagulable state in various pathological situations. In order to investigate this hypothesis, a method is needed that allows reliable and sensitive monitoring of monocytic TF mRNA levels. In this report we describe a protocol that fulfils these requirements.

Induction of monocytic TF expression by LPS has been extensively investigated in *in vitro* and *in vivo* studies [9, 26–28]. An *in vitro* LPS model was therefore used to evaluate the methods applied for both *ex vivo* RNA stabilization and subsequent quantification of specific mRNA transcripts. Regarding the dose-dependent induction of TF gene expression by LPS, a concentration of 10 pg/ml was sufficient to induce an increase of monocytic TF transcripts in whole blood as determined by real-time RT-PCR. Thus, the sensitivity of the described TF/GAPDH multiplex assay meets the requirements of *in vivo* studies on monocytic TF gene expression during severe sepsis or septic shock in which median levels of 300 pg/ml of circulating endotoxin have been described [29].

Monocytes are highly reactive cells, and TF gene expression might be induced *ex vivo* [20, 30]. Furthermore, levels of TF transcripts are altered by general degradation of RNA in whole blood samples [18, 21]. To overcome these problems we applied a blood collection system that allowed immediate stabilization and integrated purification of total RNA *ex vivo*. During initial assay evaluation, the described system showed to be a helpful tool for the meticulous determination of monocytic TF gene expression patterns in whole blood after *in vitro* LPS stimulation. We found that increasing amounts of TF transcripts were quickly generated within the 1st hour of exposure to LPS, followed by consistent decrease thereafter. These findings are in good agreement with previously published data on monocytic TF gene expression patterns, confirming the reliability of both total RNA purification and subsequent transcript quantification [31, 32].

Testing the longitudinal performance of whole blood RNA stabilization, we found that *ex vivo* levels of TF, CD14, and GAPDH transcripts were preserved for up to 5 days if samples were stored at 4 °C. Storage at room temperature reduced this time span to 24 h. Generally, the impact of sample storage on RNA test results depends on both the transcript to

be detected and the downstream application used. Thus, storage conditions must be individually determined for each novel application.

To date, *ex vivo* stabilization of the mRNA profile in whole blood cells prior to RNA isolation was described for a number of purposes in transcription analysis [33–36]. In this context also the extended storage of drawn PAXgene Blood RNA tubes at –20 °C was evaluated [21, 37]. However, the main benefit of *ex vivo* RNA stabilization is the possibility of fluently transportation of unfrozen sample material. Furthermore, immediate snap-freezing of anticoagulated whole blood was shown to be a reasonable alternative to more expensive blood collection systems that include RNA stabilizing additives [38].

Increased TF transcription rates in circulating monocytes have been associated with a hypercoagulable state [5–7, 9]. Normalized to the number of monocytes, we found that our thrombophilic patients had higher levels of TF transcripts than a corresponding reference group. However, further studies involving more patients are needed to confirm these data.

It was shown previously that homozygous carriers of the TF-603A haplotype yielded significant lower levels of TF gene expression than carriers of the TF-603G allele [23]. Furthermore, the TF-603G allele was associated with an increased risk for both venous thrombosis and myocardial infarction [22, 24]. Applying the PAXgene Blood RNA system, we confirmed the described allele-depending TF gene expression patterns. However, the total number of investigated subjects was too low to assess the impact of the TF-603G allele on the risk for the development of venous thrombosis in our patients. Generally, normalization of TF gene expression values to the level of co-purified transcripts that are specific for the myelomonocytic cell lineage, such as the LPS receptor CD14 would be more feasible. Studying monocytic CD14 gene expression in whole blood, we demonstrated interindividual variability of CD14 mRNA / monocyte ratios [25]. Furthermore, it was previously shown that monocytic CD14 expression might be altered by various triggers, e.g. LPS or IL-4 [39, 40]. Therefore, the usage of CD14 as marker transcript has to be carefully evaluated for each application. Currently, it is controversially discussed whether or not TF gene expression might also be induced in circulating neutrophils [41, 42]. We therefore normalized our test results to the number of monocytes as determined by FACS analysis.

In conclusion, we demonstrated standardized quantification of TF transcripts from whole blood cells. The described procedure allows the realization of multicenter clinical studies that are needed to further assess the impact of intravascular TF gene expression on various diseases, e.g. venous thrombosis. Moreover, the described performance characteristics and limitations might give helpful information for the future development of comparable assays in the field of clinical diagnosis and research.

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