Thrombosis Research 123 (2009) 851–855

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Expression of the V264M TFPI mutant in endothelial cell cultures may involve mRNA stability

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ARTICLE INFO

Article history:
Received 19 June 2008
Received in revised form 22 August 2008
Accepted 19 September 2008
Available online 13 November 2008

Keywords:
Endothelial cells
Expression
mRNA
Stability
Mutant
TFPI

ABSTRACT

Introduction: Tissue factor (TF) pathway inhibitor (TFPI) is the endogenous inhibitor regulating TF-induced blood coagulation. Several polymorphisms have been identified in the TFPI gene and some of them have been correlated with variations in plasma TFPI levels. The aim of the present study was to characterize the $TFPI_{V264M}$ mutant in comparison with the wild type protein ($TFPI_{WT}$).

Materials and Methods: We have overexpressed the $TFPI_{V264M}$ mutant and $TFPI_{WT}$ in human coronary artery endothelial cells and compared the expression and activity levels of the mutated protein relative to the $TFPI_{WT}$. The protein levels were determined by ELISA, the inhibitory activity of the proteins was assessed with a chromogenic substrate assay. The mRNA level of the two TFPI variants was determined using real time RT-PCR. MFOLD was used to predict mRNA secondary structure.

Results and Conclusions: $TFPI_{V264M}$ displayed increased protein levels and activity compared to $TFPI_{WT}$ accompanied by an increase in mRNA levels of $TFPI_{V264M}$ due to prolonged stability of $TFPI_{V264M}$ mRNA. The specific activity of the $TFPI_{V264M}$ was similar to $TFPI_{WT}$, indicating that the mutation does not affect the enzymatic function of the protein.

Introduction

Tissue factor (TF) pathway inhibitor (TFPI) is a glycoprotein synthesized mainly by the vascular endothelium [1]. Its key role is to regulate TF induced coagulation by modulating the catalytic activity of the factor VIIa (FVIIa)/TF complex and of activated factor X (FXa). Another important feature of TFPI is its anti-angiogenic and anti-metastatic effects (reviewed in [2]). Variations in plasma TFPI levels and/or activity have been correlated to various pathological conditions such as arterial and venous thrombosis, atherosclerosis, stroke, and cancer (reviewed in [3]), and by estrogen therapy [4,5]. The TFPI gene is located on chromosome 2, and recently, a locus on chromosome 2 near the TFPI gene, was found to be associated with variations in TFPI levels [6]. The gene spans 70 kb [7,8] and contains nine exons separated by eight introns. Seven polymorphisms in the promoter and coding region of the TFPI gene have been reported [9–14]. Except for a two nucleotide deletion in the 3′ UTR (E9del) [14], which has only been found in Afro-Americans, the other polymorphisms are caused by single nucleotide transitions. Two of them are located in the 5′ UTR (-287T→C and -399C→T) [11,12], one in intron 7 (-33T→C) [10], and three in the coding region (exon 4, Y56Y; exon 7, P151L; exon 9, V264M) [13,9,10]. The P151L, the V264M, the -287T→C, and the -33T→C polymorphisms have been reported to be associated with altered plasma TFPI levels and/or activity and/or the risk for thrombotic events. The P151L polymorphism was found to be associated with higher risk for venous thrombosis [15]. No differences were, however, detected in TFPI levels/activity in the same study. Conflicting results were later reported in another study where no differences between the distribution of the P151L between patients with venous thrombosis and controls were found, neither was this polymorphism associated with arterial thrombosis [16]. The CC genotype of the -33T→C polymorphism was associated with elevated total plasma TFPI levels in a small group of patients with venous thrombosis [17,18]. The C-allele of the -287T→C polymorphism was found to give reduced expression of a reporter gene in an in-vitro study [19]. A linkage between the -287T→C and the -33T→C has been suggested [20]. The V264M polymorphism has been reported to be associated with reduced plasma TFPI levels but no link has so far been found to thrombotic events [10,13,21]. The P151L polymorphism was recently characterized in vitro [22]. We therefore chose the V264M variant in the present study.

Most studies on the TFPI polymorphisms have been conducted with heterozygous subjects since few homozygous carriers are known. The level of TFPI in plasma is low (70–120 ng/ml). Accordingly,

Abbreviations: TF, tissue factor; TFPI, tissue factor pathway inhibitor; CAT, chloramphenicol acetyltransferase; HCAEC, human coronary artery endothelial cells.

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doi:10.1016/j.thromres.2008.09.003

sufficient amounts of homozygous variants of the protein for further characterizations have not yet been available. In addition to obtain appropriate amounts of TFPI for further biochemical and pathophysiological characterizations, the aim of the present study was to overexpress wild-type TFPI ($TFPI_{WT}$) and the V264M TFPI ($TFPIV_{264M}$) mutant in human coronary arterial endothelial cells focusing on potential differences in expression level and activity. We found that the $TFPI_{V264M}$ mutant has elevated expression level and activity compared to $TFPI_{WT}$ as a result from increased stability of $TFPI_{V264M}$ mRNA. However, the mutated TFPI displayed equal inhibitory activity compared to the wild-type.

Materials and methods

Cells

Human Coronary Artery Endothelial Cells (HCAEC) was obtained from Cambrex Bio Science (Walkersville, MD) at passage s3. The cells were cultured in modified EBM-2 basal medium supplemented with 10% FBS and EGM-2-MV SingleQuots Supplements and Growth Factors (Cambrex Bio Science). The cells were used for experiments at passage 8 with 80% confluency. All experiments were performed with cells of the same origin.

Site-directed mutagenesis

The mutation was introduced into the expression vector pcDNA3.1/V5-His-TOPO containing the full-length cDNA of human TFPI (Iversen et al., unpublished) using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two complementary oligonucleotides containing the mutation were used. The sense primer for the V264M variant was:

5′-GAAAAAGAAAGAAGCAGAGAAAAAATAGCATATGAAG-3′ (nucleotide causing the mutation is *underlined*). The presence of the mutation was confirmed by sequencing. Both the wild-type and the mutant plasmid were amplified and isolated using the Plasmid Giga Kit (Qiagen, Hilden, Germany).

Cell transfections

Cells were transfected using electroporation [23] in Amaxa nucleofector solution specifically formulated for transfection of primary HCAEC (#VPB-1001; Basic Nucleofector Kit, Amaxa GmbH, Cologne, Germany). 20 μg of plasmid was added to 100 μl nucleofector solution containing 1×10^6 cells. After transfection, the cells were immediately plated out in pre-warmed medium into 6 well plates, which were incubated at 37°C in a 5% CO_2 humidified incubator. At indicated time points after transfection, the culture medium was collected. The cells were washed 3 times with cold PBS and lysed in 1 ml protein lysis buffer (supplied in CAT ELISA kit, Roche Molecular Biochemicals, Mannheim, Germany), or 600 μl RNA lysis buffer (Applied Biosystems, Foster City, CA). The samples were stored at -70 °C until analysis. For half-life determinations, actinomycinD (5 μg/ml) was added to the medium 30 min prior to transfection and the incubation with actinomycinD continued 9 h post transfection. Control cells without the presence of actinomycinD were treated equally. The cells were harvested by washing once with PBS followed by lysis of the cells in RNA lysis buffer. The transfection efficiency was evaluated by co-transfection with pRcCMV/CAT. Chloramphenicol acetyltransferase (CAT) activity was measured using CAT-ELISA (Roche) and statistically no significant differences (Mann-Whitney) were observed in lysates from cells transfected with either wild-type or mutant TFPI constructs using two different plasmid preparations of each. The CAT activity (arbitrary units) in cell lysates (median [range]) was 0.112 [0.081-0.159] ($TFPI_{WT}$) and 0.082 [0.077-0.147] ($TFPI_{V264M}$).

TFPI assays

The TFPI free and total antigen were assayed with commercial enzyme-linked immunosorbent assays (Asserachrom® Free TFPI and Asserachrom® Total TFPI; Diagnostica Stago, Asnières, France) as described in detail elsewhere [24]. Inter- and intra-assay variability, measured as CV, was 4.4% and 2.9% for TFPI total antigen, and 4.9% and 3.8% for TFPI free antigen, respectively. Cellular total protein was measured using Lowry assay (Bio-Rad Laboratories, Hercules, CA). The TFPI antigen results were normalized to total protein content within each well. The TFPI chromogenic substrate activity was assayed in duplicate with an in-house two-stage chromogenic substrate assay as described earlier [25]. In this assay, TFPI activity was determined by the quantification of residual TF/FVIIa catalytic activity after the incubation of diluted plasma (containing TFPI) with TF, FVIIa in excess of TF-binding sites, and FXa. Specific activity was calculated by dividing the activity results with the amount of total TFPI antigen present.

Quantitative RT-PCR

Total RNA was isolated from cells after transfection using the ABI Prism™ 6100 Nucleic Acid PrepStation (Applied Biosystems). The purified RNA was finally eluted in a total volume of 100 μl. The quality and quantity of total RNA were estimated using the RNA 6000 NanoChip on the Bioanalyzer 2100 (Agilent Technologies, Walbronn, Germany) and the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE), respectively. Reverse transcription was performed using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time PCR was used to measure the expression levels of mRNA. Primers and dual labelled probes were designed using Primer Express Software, version 2.0 (Applied Biosystems) and purchased from Eurogentec, Belgium. PCR amplification was performed on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The sequences of the TFPI primers and probe were as follows:

forward 5´- ACACACAATTATCACAGATACGGAGTT -3′;reverse 5′-GCCATCATCCGCCTTGAA -3′; and probe 5′-FAM- CCACCACTGAAA CTTATGCATTCATTTTGTGC -TAMRA-3′.

The human phosphomannomutase 1 (PMM1) gene served as endogenous control in the normalization of input amount of RNA and RT efficiency. It was tested and proved constant expression levels despite various types of treatment among the samples. The respective primers and probe for PMM1 were purchased from Applied Biosystems. The PCR-reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations with 300 nM and 200 nM of primers and probes, respectively. The pre-run thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C. Thermal cycling conditions were 40 cycles at 95 °C for 15 s followed by 65 °C for 1 min.

Prediction of mRNA secondary structure

To evaluate the potential effect of the mutation on mRNA structure, the mRNA secondary structure of TFPI was predicted using MFOLD (http://bioweb.pasteur.fr/seqanal/interfacrs/mfold-simple.htlm). Full-length mRNA sequence was entered and the corresponding wild-type mRNA sequence was used as control for comparison.

Statistics

The statistical work was done in GraphPad Prism version 5 using the non-parametric Mann-Whithey test. The results were expressed