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Demonstration of inducible TFPI-2 mRNA synthesis in BeWo and JEG-3 trophoblast cells using a competitive RT-PCR

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Abstract

Tissue factor pathway inhibitor-2 (TFPI-2) displays structural similarities with TFPI-1, the major inhibitor of tissue factor (TF)/ factor VIIa. It is synthesized mostly by syncytiotrophoblast in the placenta, but its physiological functions are not fully understood. We studied the synthesis of TFPI-2 mRNA and that of TFPI-1 and TF in three human trophoblast cell lines, JAR, BeWo, and JEG-3. We first developed specific competitive reverse transcription-polymerase chain reaction (RT-PCR) assays for each gene studied using human umbilical vein endothelial cells (HUVEC). The three trophoblast cell lines strongly synthesized TF mRNA whereas the synthesis of TFPI-1 mRNA was very low. TFPI-2 mRNA was not detected in unstimulated or stimulated JAR cells. In contrast, JEG-3 and, to a lesser extent, BeWo produced significant amounts of TFPI-2 mRNA, which were significantly increased after stimulation with phorbol 12-myristate 13-acetate (PMA). However, tumor necrosis factor-α (TNF-α) had no effect on this synthesis. JEG-3 and BeWo are thus two cell lines that could be used to study TFPI-2 gene regulation and to investigate the role of TF, TFPI-1, and TFPI-2 during trophoblast differentiation.

Keywords: Tissue factor; TFPI-1; TFPI-2; Trophoblast cells; Competitive RT-PCR

1. Introduction

Tissue factor (TF) is a transmembrane cell surface receptor that specifically binds factors VII and VIIa and triggers blood coagulation. TF is abundant in placental extracts [1], but to date it has been mainly found within macrophages, endothelial cells, or fibroblast-like cells of connective tissues and not in trophoblast cells [2,3]. TF has also been detected in human microvilli membranes [4,5] that sheared off differentiated multinuclear syncytiotrophoblast cells, which are themselves derived from the fusion of villous cytotrophoblast cells. Syncytiotrophoblast is in contact with maternal blood in the intervillous spaces and expresses von Willebrand factor and thrombomodulin [6,7], supporting its role in maintaining the haemostatic balance of intervillous spaces, such as endothelial cells lining blood vessels [8,9].

To regulate TF activity in blood vessels, endothelial cells constitutively express tissue factor pathway inhibitor, TFPI-1 [10], also synthesized by monocytes/macrophages [11–13], megakaryocytes [14], and smooth muscle cells [15]. Large amounts of TFPI-1 mRNA have also been detected in placental tissues by Northern blotting [16] but the protein was only localized in macrophages within the villi of term placenta [14]. TFPI-2, a second inhibitor of TF, also named placental protein 5 (PP5), was initially isolated from human placenta extracts [17] and plasma TFPI-2 levels increase during pregnancy [18]. TFPI-2 mRNA has also been detected by Northern blotting in the placenta [19] and more recently localized by in situ hybridization in the

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; PP5, placental protein 5; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcription-polymerase chain reaction; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TNF-α, tumor necrosis factor-α

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syncytiotrophoblast from the first trimester to term placenta while it was not detected in cytotrophoblast [20]. Protein was also detected in syncytiotrophoblast by previous immunohistological studies [21,22], but the role of TFPI-2 in the placenta is still not understood. Despite structural and sequence similarities to TFPI-1 [10,23], TFPI-2 weakly inhibits factor Xa and thrombin activities [23,24] and other functions are likely to be involved in the placenta, which is a particularly invasive and proliferative tissue.

Few techniques are available to isolate trophoblast cells to study TFPI-2 synthesis in normal placenta and they are all difficult and time-consuming. We therefore investigated whether a human continuous cell line could be used as a trophoblast cellular model to study TFPI-2 gene regulation. We first developed competitive and quantitative reverse transcription-polymerase chain reaction (RT-PCR) methods allowing evaluation of TFPI-2 mRNA synthesis in endothelial cells isolated from human umbilical vein (HUVEC) together with that of TF and TFPI-1, and we then applied this procedure to study three different trophoblast cell lines, JAR, BeWo, and JEG-3.

2. Materials and methods

2.1. Cell isolation and culture

Human trophoblast cell lines, JAR [25], BeWo [26], and JEG-3 [27], kindly provided by Dr. G. Chaouat, were grown to confluence in 25-cm^2 flasks (Greiner Labortechnik, Poitiers, France) at 37 °C in an atmosphere of 95% air and 5% CO_2. Cells were cultured in RPMI 1640 medium (Life Technologies, Cergy Pontoise, France) supplemented with 10% endotoxin-free heat-inactivated foetal calf serum (ATGC Biotechnologie, Noisy le Grand, France), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 mM sodium bicarbonate for JAR cell culture or 15 mM sodium bicarbonate and 2 mM glucose for JEG-3 and BeWo cell cultures.

Endothelial cells were isolated from human umbilical cord veins as previously described [5] and grown to confluence at 37 °C in an atmosphere of 95% air and 5% CO_2 in M-199 medium supplemented with 20% endotoxin-free heat-inactivated foetal calf serum, 2 mM L-glutamine, 0.13% sodium bicarbonate, 25 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/ml fungizone. HUVEC were characterized by their typical morphology at confluence and their positive immunofluorescence using a polyclonal antibody specific for von Willebrand factor.

2.2. Cell stimulation

Confluent cells were washed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and detached by 0.05% trypsin–0.02% EDTA. Cell viability was determined by the Trypan blue dye exclusion test and ranged between 90% and 95%. Endothelial cells at the first passage and trophoblast cells were then seeded in 1% gelatin-coated six-well plates (Falcon, Becton Dickinson, Le Pont de Claix, France) at 10^6 cells per well and cultured for 18 h in complete medium. Trophoblast cells were then incubated with 20 ng/ml recombinant human tumor necrosis factor-α (TNF-α, specific activity: 2×10^7 IU/mg, Genzyme, Cambridge, MA, USA) for 90 min at 37 °C for JAR cells or 60 min for JEG-3 and BeWo cells. HUVEC were incubated with 10 ng/ml recombinant human TNF-α for 90 min at 37 °C. Trophoblast and endothelial cells were also stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich Chimie, Saint Quentin Fallavier, France) for 4 h before mRNA extraction.

2.3. Competitive RT-PCR

Simultaneous TF, TFPI-1, and TFPI-2 mRNA expression was investigated using competitive RT-PCR assays. Heterologous DNA competitors were thus constructed for each gene studied using the PCR MIMIC kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Composite primers were designed to contain both the target-specific primer sequence and 20 nucleotides of the v-erbB sequence corresponding to the heterologous DNA fragment to be amplified. Initial PCR was carried out at 50 °C (annealing temperature) for 16 cycles with 2 ng of heterologous DNA and 0.4 μM of each composite primer. Second amplification of 23 cycles at 62 °C was performed with dilution of the first PCR and 0.4 μM of each target-specific primer. The DNA competitors thus generated were then

Table 1
Primer sequences used in RT-PCR

Transcript	Primer sequence 5′–3′	Reference of cDNA sequence	Target PCR product (bp)	Competitor PCR product (bp)
TF	For CTACTGTTTCAGTGTTCAAGCAGTGA Rev CAGTGCAATATAGCATTTGCAGTAGC	[33]	282	428
TFPI-1	For GGAAGAAGATCCTGGAATATGTCGAGG Rev CTTGGTTGATTGCGGAGTCAGGGAG	[31]	230	378
TFPI-2	For CAGATGAAGCTACTTGTATGGGCTTC Rev GGCAAAGCGAAGCTTTGGCATC	[23]	253	398
GAPDH	For ACAGTCCATGCCATCACTGCC Rev GCCTGCTTCACCACCACCTTCTTG	[28]	265	–

For: forward; Rev: reverse.

purified with Chroma Spin columns (Clontech) and quantified at 260 nm on a spectrophotometer or by comparing the electrophoretic band intensities of the target with those of known amounts of size markers (ϕX 174 DNA digested with *Hae*III, Life Technologies) using the Multi Analyst/Macintosh software (Bio Rad, Ivry sur Seine, France). Competitors for TF, TFPI-1, and TFPI-2 yielded PCR products 150 bp larger than the target sequence in order to be easily distinguished after gel electrophoresis (Table 1).

Total mRNA was isolated from 10^6 cells of each trophoblast cell line or from 10^6 HUVEC, stimulated with TNF-α, PMA, or unstimulated, using the Dynabeads mRNA Direct kit (Dynal France, Compiègne, France) according to the manufacturer's instructions. Total mRNA (200 ng for 10^6 cells) was then reverse-transcribed for 1 h at 42 °C in 1 × incubation buffer containing 250 μM of each deoxynucleoside triphosphate, 5 μM oligo$(dT)_{20}$, 25 U of RNase inhibitor, and 20 U of AMV Reverse Transcriptase (Roche Diagnostics, Meylan, France).

Competitive PCR were performed on samples containing a constant volume of first-strand cDNA (0.8–3.2 μl when mRNA expression was very low) and 2 μl of twofold competitor dilutions. PCR was carried out in 1 × Taq buffer (ATGC Biotechnologie) containing 200 μM of each deoxynucleotide triphosphate, 0.4 μM of each reverse- and forward-synthesized oligonucleotide primer (Genset, Paris, France) specific for human TF, TFPI-1, TFPI-2, or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA sequences (Table 1) and 0.5 U of Super Taq DNA polymerase (ATGC Biotechnologie). Primers specific for TF and TFPI-1 mRNA have been previously described [29,30]. The primers used for detection of TFPI-2 mRNA were defined taking into account the similarities between TFPI-1 and TFPI-2 to avoid hybridization to homologous sequences [23,31]. In addition, all primers were designed to have melting temperatures in the same range, allowing simultaneous amplification, and to span one intron to distinguish genomic DNA from cDNA amplifications. PCR was set up in the GeneAmp PCR system 2400 (Applied Biosystems, Courtaboeuf, France) programmed for an initial denaturation step of 3 min at 95 °C, followed by 35 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, and the final extension step was performed at 72 °C for 7 min.

PCR products were then analyzed by electrophoresis through 1.6% agarose gel in TBE buffer (90 mM Tris–HCl, 90 mM borate acid, 2.5 mM EDTA) containing 1 μg/ml ethidium bromide and visualized by UV transillumination (Gel Doc 1000 system, Bio Rad). Band intensities of PCR products were measured by the Multi Analyst/Macintosh software and expressed in arbitrary units corresponding to pixel integration after correction taking into account the size difference between competitor and target cDNA. The logarithm ratio of target to competitor band intensities was plotted as a function of the logarithm of the competitor amount added. The amount of target cDNA was determined when the logarithm of the ratio was equal to zero and was expressed in attomoles for 5×10^4 cells.

In order to ensure that target and competitor cDNA have similar amplification efficiency, equal amounts were coamplified for 29–44 cycles and the intensity values of PCR

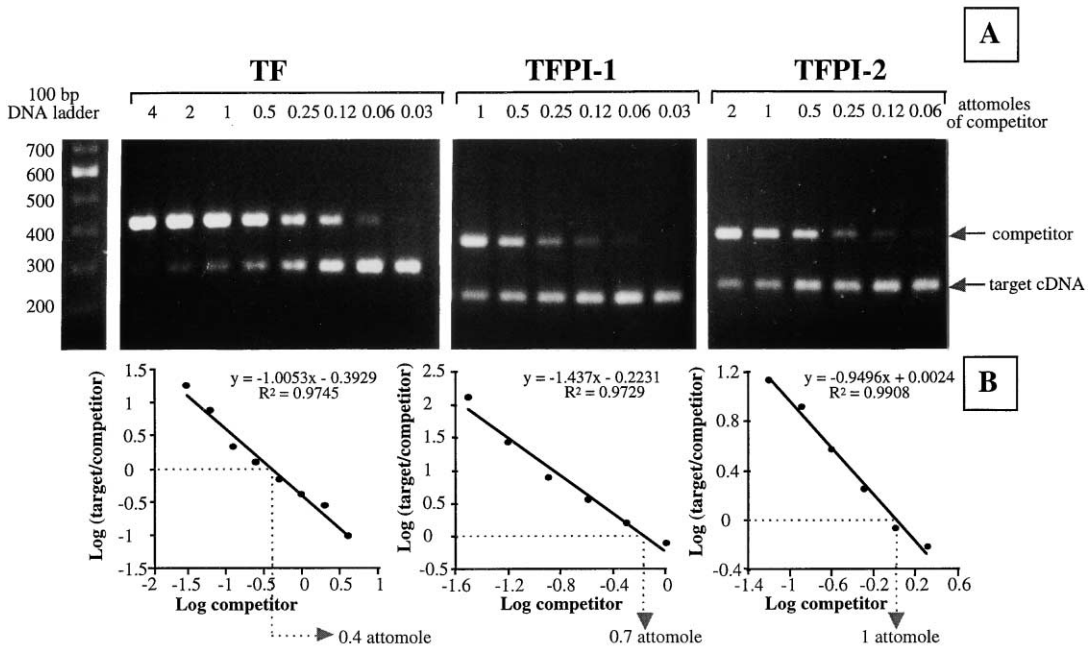

Fig. 1. Competitive RT-PCR assay specific for TF, TFPI-1, and TFPI-2 in HUVEC. (A) Twofold dilutions of competitor were coamplified with constant aliquots of cDNA from 2×10^4 HUVEC stimulated with PMA. (B) The logarithm ratio of target to competitor band intensities was plotted as a function of the logarithm of the amount of competitor added. The amount of target cDNA was calculated with the linear regression curve.

products were then plotted as a function of the cycle numbers. For each gene studied, regression lines with comparable slopes were obtained for competitor and target.

RT-PCR products specific for TF, TFPI-1, and TFPI-2 were automatically sequenced using the dideoxynucleotide chain termination method [32] on a Perkin Elmer Abi Perkin 377 automat (INSERM U316, Tours, France). Sequencing was performed on both strands with the reverse and forward primers specific for TF, TFPI-1, and TFPI-2 used for RT-PCR.

3. Results

3.1. Development of competitive RT-PCR methods

Competitive RT-PCR assays were developed using HUVEC in which we quantified TF, TFPI-1, and TFPI-2 mRNA expression after stimulation by PMA (Fig. 1). Serial dilutions of competitors, starting from amounts of 4, 1, and 2 amol of TF, TFPI-1, and TFPI-2 cDNA competitor, respectively, were coamplified with a constant volume of 0.8 μl first-strand cDNA obtained from PMA-treated HUVEC. For every gene studied, an increase in target PCR products was observed when competitor PCR products decreased (Fig. 1A). Thus, when the intensities of target and competitor PCR products were equal, we could evaluate the amount of target cDNA present in the sample tested. After measuring target and competitor PCR product intensities, the logarithm of their ratio was plotted as a function of the logarithm of the amount of competitor present in the mixture reaction (Fig. 1B). Target cDNA levels were then calculated using the regression curves obtained, which were all linear with correlation coefficients higher than 0.9. Using the above experimental conditions, the amounts of TF, TFPI-1, and TFPI-2 cDNA measured from 2×10^4 HUVEC and stimulated with PMA were 0.4, 0.7, and 1 amol, respectively (Fig. 1B).

3.2. Quantification of TF, TFPI-1, TFPI-2 mRNA expression in trophoblast cell lines

TF, TFPI-1, and TFPI-2 mRNA expression was then studied in JAR, BeWo, and JEG-3 trophoblast cells. The optimal TNF-α and PMA concentrations and incubation times to be used for cell stimulation were first defined, corresponding to 90 min for JAR and 60 min for BeWo and JEG-3 in the presence of 20 ng/ml TNF-α and 4 h with 100 ng/ml of PMA.

Total mRNA was isolated from 10^6 cells and RT-PCR specific for TF, TFPI-1, TFPI-2, and GAPDH was then simultaneously performed using single-strand cDNA from about 5×10^4 cells. RT-PCR products were obtained as single bands of 282, 230, 253, and 265 bp, the expected sizes of TF, TFPI-1, TFPI-2, and GAPDH cDNA, respectively. The sequencing of RT-PCR products was similar to the previously published sequences [23,31,33] and confirmed the specificity of the technique. Cell expression of

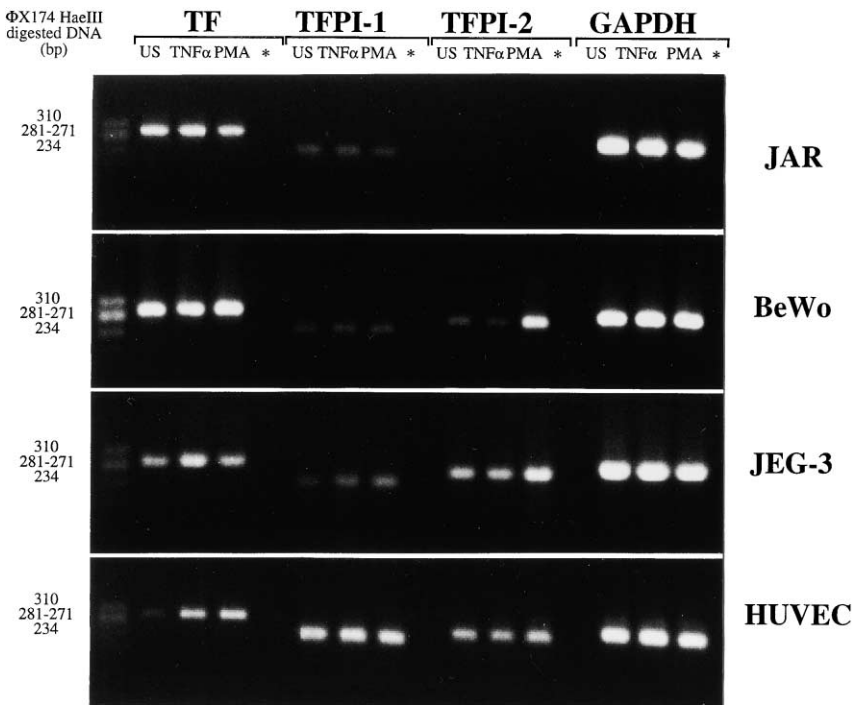

Fig. 2. Detection of TF, TFPI-1, TFPI-2, and GAPDH mRNA in JAR, BeWo, and JEG-3 trophoblast cell lines. Trophoblast cells were unstimulated (US) or incubated with 20 ng/ml TNF-α or 100 ng/ml PMA. Endothelial cells used as control cells were unstimulated or treated with 10 ng/ml TNF-α or 100 ng/ml PMA.

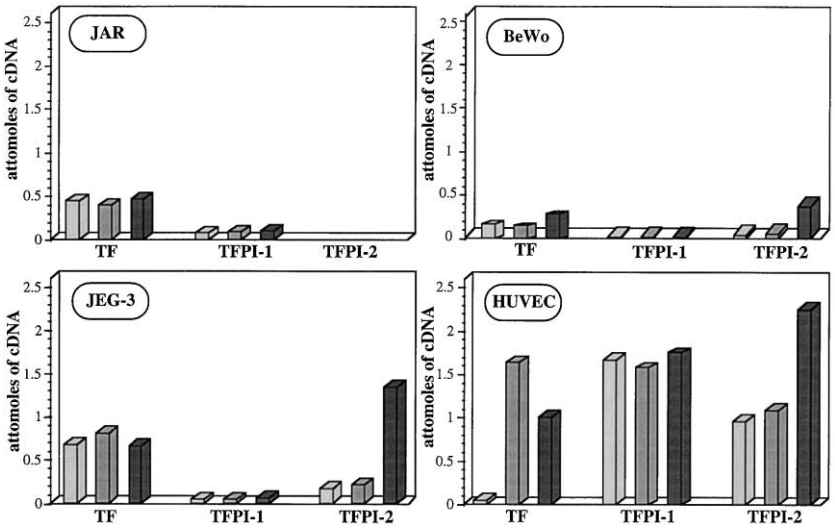

Fig. 3. Quantification of TF, TFPI-1, and TFPI-2 cDNA from JAR, BeWo, JEG-3 trophoblast cell lines and from HUVEC. Results are expressed in attomoles of cDNA obtained from 5×10^4 cells, unstimulated (□), incubated with TNF-α (▤) at 20 ng/ml for trophoblast cells or 10 ng/ml for endothelial cells, or incubated with 100 ng/ml PMA (■). These data are representative of three independent experiments.

GAPDH, used as control, was not affected in any experiment, regardless of cell stimulation (Fig. 2).

Significant amounts of TF mRNA were detected in all trophoblast cells with and without inducer (Fig. 2). In contrast, very low levels of TFPI-1 mRNA were present in all unstimulated trophoblast cells, particularly in JAR and BeWo, and appeared unmodified after incubation with TNF-α or PMA. TFPI-2 mRNA expression was not detected in JAR even after incubation with TNF-α or PMA. In contrast, significant synthesis was detected in unstimulated JEG-3 and BeWo, slightly higher in the former. After stimulation for 4 h with PMA, TFPI-2 mRNA synthesis was clearly increased in these two trophoblast cell lines.

To quantify these results, competitive RT-PCR specific for TF, TFPI-1, and TFPI-2 was then performed, and results obtained from 5×10^4 cells and with twofold dilution of competitor were expressed in attomoles of cDNA (Fig. 3). The three trophoblast cells constitutively expressed strong levels of TF mRNA, with about 0.4, 0.18, and 0.65 amol of TF cDNA from JAR, BeWo, and JEG-3, respectively, and TNF-α or PMA did not significantly modify this synthesis. In contrast, unstimulated HUVEC expressed a very low level of TF cDNA (0.05 amol) and a 32- or 20-fold increase in this amount was obtained when cells were stimulated with TNF-α or PMA, respectively. TFPI-1 cDNA level was lower than 0.1 amol in both unstimulated and stimulated trophoblast cells, in contrast to those measured with unstimulated HUVEC (1.6 amol) that remained unmodified when endothelial cells were treated with TNF-α or PMA. TFPI-2 mRNA was never detected in JAR cells whereas 0.03 and 0.17 amol of TFPI-2 cDNA were quantified in unstimulated BeWo and JEG-3 cells, respectively. No significant effect of TNF-α on TFPI-2 mRNA synthesis was observed for either cell line. In contrast, TFPI-2 cDNA levels rose dramatically when cells were stimulated with PMA, reaching a value of 0.36 amol for BeWo, corresponding to a 10.8-fold increase in synthesis, and 1.3 amol for JEG-3, corresponding to a 7.8-fold increment. By comparison, unstimulated HUVEC expressed 0.9 amol of TFPI-2 cDNA and a 2.6-fold increase in synthesis was measured when cells were incubated with PMA.

4. Discussion

TFPI-2, a 32-kDa Kunitz-type serine proteinase inhibitor, is particularly abundant in the placenta [17,18,20], but its physiological role during pregnancy remains unknown. Apart from endothelial cells [34,35] and monocytes/macrophages [13], trophoblast cells are another potential source of TFPI-2 in the placenta. However, trophoblast cells are difficult to isolate from the normal placenta and this explains why continuous lines were previously employed to study expression of cytokine and hormone related to differentiation and invasion [36–38]. We therefore studied TFPI-2 mRNA synthesis in three different trophoblast cell lines (JAR, BeWo, and JEG-3) using specific competitive RT-PCR techniques adapted from a previously developed method that allowed the simultaneous detection of TF and TFPI-1 mRNA on a limited number of cells [30]. To detect TFPI-2 mRNA, primers were designed taking into account the similarities between TFPI-1 and TFPI-2 cDNA sequences existing particularly in the regions coding the three tandem Kunitz-type protease inhibitor domains, K1, K2, and K3 [23,31]. We therefore chose a TFPI-2 reverse primer hybridizing the region located after the K3 domain and a forward primer corresponding to the end of the K2 domain.

We showed that two of the three trophoblast cell lines studied (i.e., BeWo and JEG-3) produced significant

amounts of TFPI-2 mRNA, particularly when stimulated with PMA. In contrast, no synthesis of TFPI-2 mRNA could be detected in either unstimulated or stimulated JAR cells. In order to quantify TFPI-2 gene expression together with the expression of TF and TFPI-1, we developed competitive RT-PCR assays in HUVEC able to synthesize these three molecules. We prepared nonhomologous DNA competitors for each gene studied that were then coamplified with the target cDNA. Although DNA competitors do not allow checking of the reverse transcription step, they are more stable than RNA competitors and are thus frequently chosen for competitive RT-PCR [39–41]. Using such a competitive RT-PCR approach, we showed that PMA, previously identified to stimulate TFPI-2 expression in HUVEC [34,35,42] and in fibroblast cells [43,44], also increased TFPI-2 mRNA synthesis in trophoblast cells (i.e., 7.8- and 10.8-fold in JEG-3 and BeWo, respectively). In contrast, no significant effect of TNF-α on TFPI-2 mRNA synthesis by HUVEC was shown in our conditions. In a previous work, TFPI-2 mRNA synthesis was not detected by Northern blotting in JAR and BeWo whether cells were stimulated or not [20]. Our results observed with BeWo cells are therefore supporting by the higher sensitivity of RT-PCR to detect TFPI-2 mRNA compared to Northern blotting [29,40].

TF is also produced in large amounts in the placenta, particularly in term syncytiotrophoblast microvilli [4,5]. In agreement with previous findings [5], we showed that TF mRNA was constitutively synthesized in the three cell lines studied, but these results could also be due to the tumoral origin of trophoblast cell lines [45–47]. To regulate procoagulant TF activity, normal endothelial cells expressed TFPI-1 but in the placenta this protein is not significantly produced by trophoblast cells and is only detected within macrophages of the placental villi [14]. Similarly and as shown by RT-PCR, TFPI-1 mRNA was also poorly synthesized in JAR, BeWo, and JEG-3 cells.

In contrast, TFPI-2 is produced in BeWo and JEG-3 at a significant level as well as in normal placenta. The physiological role of this proteinase inhibitor is unknown, but it could be involved in trophoblast invasion. Indeed, TFPI-2 down-regulates the activation of metalloproteinases by inhibiting plasmin and trypsin and also inhibits matrix degradation and invasion by tumoral cells [43,44,48–51]. These properties of TFPI-2 might thus be of importance in the placenta to regulate trophoblast invasion that involves metalloproteinases [52,53]. TFPI-2 might also participate in cell differentiation, leading to the formation of a multinucleated syncytiotrophoblast that is the main source of TFPI-2 in the normal human placenta [20,21].

However, additional studies will be necessary to establish whether or not TFPI-2 is functionally important to regulate invasion and differentiation of trophoblast cells. JEG-3 and BeWo cells should be useful for such studies and also allow to study TFPI-2 gene regulation.

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