Tissue factor and tissue factor pathway inhibitor levels in trophoblast cells: implications for placental hemostasis

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Summary

The placenta is a highly vascularized organ with fetal and maternal blood supply. Syncytiotrophoblasts (STB), which line the placenta villous are possibly involved in local hemostatic mechanisms. The aim of this study was to evaluate the levels of tissue factor (TF) and its inhibitors, tissue factor pathway inhibitor (TFPI, TFPI-2), in STB model within hemostatic and inflammatory environments. Human primary STB cell cultures were characterized by vascular and hormonal markers. TF and TFPI mRNA expression, protein levels and activity were determined and compared to human umbilical vein endothelial cells (HUVEC). High levels of TF were demonstrated in STB cells compared to low levels in HUVEC. In contrast, STB expressed lower TFPI levels than HUVEC. LPS and TNFα increased the high constitutive TF in STB, whereas LPS and IL-1α further reduced TFPI levels. The procoagulant tendency of STB identified by us may reflect the physiological need for immediate inhibition of hemorrhage in the placental inter-villous spaces in basal and inflammatory conditions. This hemostatic balance may be critical for normal placental function and pregnancy outcome.

Keywords

Tissue factor, tissue factor pathway inhibitor (TFPI), placenta, trophoblast, inflammation

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Introduction

The placenta is a highly vascularized organ with a blood supply originating from fetal and maternal circulations. Placental architecture is unique since maternal blood flows in the inter-villous spaces, while fetal blood is confined in the intra villous blood vessels. This presents haemostatic problems, mainly the risk of hemorrhage or on the other hand, placental vascular complications that may be associated with thrombophilia or inflammation (1, 2).

During pregnancy, the placenta is a putative source of tissue factor (TF) (3-5), which is essential for embryogenesis, angiogenesis, implantation and hemostasis (6, 7). However, the localization of TF in the placenta is still a controversial issue (8-10). TF binds to activated factor VII (FVIIa) and forms the complex TF: FVIIa that initiates the coagulation process. Normally, TF is not present in cells that are in contact with blood. Rather, cells such as endothelial cells or monocytes can be induced to express TF (11-13). Tissue factor pathway inhibitor (TFPI), the main TF activation inhibitor (14), is expressed in placental tissues (15). TFPI-2, is identical to placental protein 5 (PP5) (16), has prominent expression in human placenta (17), but its physiological role in the placenta is currently unclear.

Syncytiotrophoblasts (STB) differentiate from cytotrophoblasts (CTB), line the inter-villous spaces and are in contact with maternal blood. Invading cytotrophoblasts acquire vascular endothelial phenotype and express endothelial cells (EC) markers (19). STB cells express TF (9), TFPI (20) TFPI-2 (17) and thrombomodulin (21). Recently, it was found that TF activation at the fetus-maternal interface is involved in fetus abortion of thrombomodulin deficient mice (22). Considering these

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findings, we investigated the presence and balance of TF and the inhibitors TFPI and TFPI-2 in human primary syncytiotrophoblast cultures and compared to the widely characterized macro-vascular endothelial cells (HUVEC) under non-inflammatory and inflammatory conditions.

Materials and methods

Cell cultures

Syncytiotrophoblasts (STB) cell culture

All culture media, buffers and solutions were purchased from Biological Industries, Beit Haemek, Israel. Cells were isolated as described previously (23) with some modifications. Normal human term placentas (36–42 weeks of gestation) were obtained from spontaneous deliveries or cesarean sections and were used within one hour after delivery. Villous tissue from the deciduas side was cut into small pieces. Tissue (50g wet weight) was washed with Dullbecco's PBS (D-PBS) and digested by two steps: incubation with 0.125% trypsin ended by filtration and digestion with 0.4 mg/ml collagenase type II (Sigma, St Louis, MO). Fetal calf serum (FCS) (10%) was added to the supernatant before loading it on Histopaque 1077 (Sigma) and centrifugation (2000 rpm for 20 minutes). Cells in the median layer were separated, washed and plated (~10^5 cells/ml) on 6-well (35-mm) culture plates pre-coated with 10 mg/ml fibronectin. Cells were cultured in medium M199 supplemented with 10% FCS, 8% pooled human serum, 1% antibiotics (10,000 units/ml penicillin 10μg/ml streptomycin, 250 units/ml nyastatin), 3.5 U/ml heparin (Kamada, Israel) and 25 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA). After the first 24 hours, apoptotic STB were washed out and the remaining CTB differentiated and matured in culture into new generation of STB. An enriched culture with three-sub populations (STB, CTB and elongated cells) was usually obtained after 14 days. For most experiments, cells were detached with 0.25% trypsin and seeded in 96 well plate (10^4 cells/ well) or 6 wells (10^6 cells/ well) for 24 hours prior to the experiment.

Human umbilical vein endothelial cells (HUVEC)

HUVEC were isolated from umbilical cords according to the technique of Jaffe et al (24) and grown in a full culture medium. Passages 2-3 were used for experiments.

Antibodies

Antibodies were used for cellular characterization. Vascular markers were: mouse anti-human CD31 (Dako, Glostrup, Denmark) and rabbit anti-human von Willebrand factor (VWF) (Sigma Chemicals, St Louis, MO). Hormonal markers were: rabbit anti-human β choriogonadotropin (βhCG) (Zymed Laboratories, San Francisco, CA) and mouse anti-human placental lacotogen (hPL) (Zymed). Coagulation factors were: mouse anti-human TF (American Diagnostica, Greenwich CT), mouse anti-human TFPI (American Diagnostica) and rabbit anti-human TFPI-2 antibodies, which were developed as described (18). Secondary antibodies were: fluoroscein isothiocyanate conjugated (FITC) rabbit anti-mouse IgG (Sigma), FITC sheep anti-rabbit IgG (Sigma) and phycoerythrin (PE) goat anti-mouse (Sigma). Histostain-Plus Bulk Kit (Broad Spectrum) (Zymed Laboratories).

Immunofluorescence

Differentiated STB, grown on cover slides, were fixed with 2% formaldehyde in PBS and blocked with 3% BSA in D-PBS. Staining was performed by the incubation of slides with primary antibodies followed with secondary antibodies. Hormonal markers staining (intracellular) were performed after cell membranes were perforated with 0.2% triton-X in PBS. Cells were visualized and photographed using inverted fluorescent microscope (Axiovert 135- Zeiss, Oberkochen, Germany). Each staining was performed on three cultures from different placentas.

Flow cytometry analysis (FACS)

STB were washed with D-PBS and then harvested by 0.25% trypsin. The cells were saturated with 10% FCS in D-PBS, labeled by incubation with primary antibodies, followed by washing and incubation with second antibodies. Rabbit IgG or mouse Ig and second antibodies served as controls. Hormonal markers staining was performed after fixation and permeabilization with Fix & Perm Permeabilization Kit (Caltag, Burlingame, CA). After immunofluorescent labeling, cells were washed and suspended in D-PBS containing 0.02% azide and 1% formaldehyde. Cells were scanned by a Becton Dickinson FACS scan flow cytometer (FACScalibur, Becton Dickinson, San-Jose, CA) and the data was analyzed using CellQuest software. At least 5000 cells per sample were analyzed.. The FACS analyses were performed in 6 cultures from different placentas.

Immunohystochemistry

This study was performed on placental formalin-fixed paraffin-embedded sections, utilizing the Histostain-Plus Bulk Kit (Zymed) according to the manufacturers instructions. Briefly, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% H_2O_2, followed by brief rinsing in distilled water and was then washed in PBS. The sections were blocked with serum solution and stained with primary specific antibodies for TF, TFPI and TFPI-2 followed by biotinylated secondary antibody. Enhanced alkaline phosphatase conjugated streptavidin (AP-SA) was then bounded to the secondary antibody. The Fast-Red chromogen substrate solution created an intense color deposit (brown) around the antigen in the sample. The sections were visualized by microscope and photographed using CMS-2 software (computerized microscope systems, Tirat Carmel, Israel). Each staining was performed on three sections from different placentas.

Total RNA isolation and cDNA construction

Total RNA was isolated by Pure Script RNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. RNA concentration and purity were determined by UV absorption at 260 nm and 280 nm. Complementary DNA (cDNA) was prepared from the total RNA. The first mixture contained total RNA (10.5 µl), random primers (1.5 µl) (Invitrogen, Carlsbad, CA), 5mM dNTP (3 µl) (Amersham Biosciences, Piscataway, NJ) and 5 x buffer (6 µl) (Invitrogen). The mixture was incubated for 5 minutes at 90°C using a PTC 200 Thermal Cycler (MJ Research, Waltham MA) and cooled to 25°C for 5 minutes. The second mixture containing DTT (3µl) (Sigma), 40 U/ml Rnasin (0.5µl) (Promega, Madison WI), 200 U reverse transcriptase (RT) (1µl) (Invitrogen) and water (5 µl) was added, incubated for one hour at 42°C and cooled to 4°C.

mRNA expression

Quality RNA expressions of TF, TFPI and TFPI-2 in cells were determined using primer sequences (produced by General Biotechnology, Rehovot, Israel), as previously described (26), Each sample contained cDNA (3 µl), 5mM dNTPs (1 µl) (Amersham Biosciences), 10 x buffer (2.5 µl) and 5 U/µl Super term DNA polymerase (1 µl) (Roche Molecular Biochemicals, Indianapolis IN), 25 pM/µl primers (0.5 µl) and water for PCR reaction (17.5 µl). Amplification was performed by PTC 200 Thermal Cycles for 3 minutes at 95°C, 30 circles of 30 seconds at 95°C, 30 seconds at 62°C and 30 seconds at 72°C and ended with 7 minutes at 72°C. Then, electrophoresis samples were visualized in 2.5% agarose gel containing ethidium bromide.

Quantity mRNA expressions of TF, TFPI, and TFPI-2 gene were evaluated by ABI 7700 ™ quantitative real time PCR system (Applied Biosystems, Foster City, CA) and compared to the human housekeeping glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene. All reagents were obtained from Perkin Elmer (PE)/Applied Biosystems. Primer and probe sequences were either supplied as a ready mix (for human GAPDH), or designed with Primer Express software (PE) using published gene sequences. The probes were labeled at the 5' end with a reporter dyes (VIC™ dye-labeled GAPDH, probe and FAM™ dye-labeled the others probes) and at the 3' end with a quencher dye (TAMRA™). The sequences of the primers and probes were as follows: TF forward primer 5'-CACCGACGAGATTGTGAAGGA-3', TF reverse primers 5'-CCC TGCCGG GTAGGAG-3', TF probe 5'-TGAAGC AGACGTACT TGGCACGGGT-3'. TFPI forward primer 5'-ACAAAACAGTGT GAACGTTTC AAGTAT-3', TFPI reverse primers: 5' GCATTGAGCTGGGTT CCATAA-3', TFPI probe 5'-CCA CCT GGA AAC CAT TCG GAC CAT CTT-3'. TFPI-2 forward primer 5'-TGCCAATGTGACTCGCTA TTATTT-3', TFPI-2 reverse primers 5'-TC ATTCCCTCCACAGCCAGTA -3', TFPI-2 probe 5'-AATCCAAGA TACAGAAC CTGTGATGCTT TCACC-3'.

Each sample contained: cDNA (1µl), Taqman Universal PCR Master Mix (12.5 µl), specific probe (1 µl), GAPDH primer mix (1.25 µl) and 500 pmol protein of interest (300 pmol TFPI-2) reverse and forward primers. Relative gene expression levels were calculated using standard curves generated by serial dilutions of cDNA from cells samples. A strong correlation between PCR efficiency of the internal control (GAPDH) and the targets (TF, TFPI) allowed the use of the delta CT-method to quantify comparable mRNA levels. Expression of TFPI-2 was calculated by using the expression of GAPDH as an external control. Three independent analyses were performed with replicates for each gene. Each sample included pooled RNA samples from three different cell cultures using the same experimental condition.

Protein extraction

Cells (10^4/well) were washed with D-PBS. Lysis buffer (20 mM tris, 150 mM NaCl, 0.5 mM Na_3Vo_4, 1 mM DTT, 1% Triton, 10% Glycerol and complete protease inhibitors EDTA free [Roche Diagnostic, Mannheim, Germany]) was added. The plates were frozen at –20°C, and then thawed for three cycles.

Total protein concentration was measured by the BCA method (Pierce, Rockford, IL). Concentrations of TF and TFPI in cell lysates or in supernatants were determined by an enzyme-linked immunosorbent assay (American Diagnostica). The results of several assays have demonstrated that total TFPI levels in STB cellular lysates were very low; therefore TFPI assays were determined in cell supernatants. The TF, TFPI determinations were performed with 2 replicates, and repeated in 6 cultures obtained from different placentas.

TF activity

TF activity was measured by a one-step clotting assay using 100 µl cell extract. Cells in 96 wells plates were frozen to –20°C and thawed for three cycles. Pooled normal human plasma (100 µl) was added to the cell extract, and incubated for one minute at 37°C. Then 25 mM calcium chloride solution (100 µl) was added and the clotting time was measured. In addition, 100 µl of diluted (1:10 – 1:1000) human recombinant TF (Innovin, Dade Behring Diagnostic, Liedercbach, Germany) was used for clotting time assay. The clotting times were converted to standard curve of 10-1000 arbitrary units of TF (AU/ml) – where 180 seconds of clotting time stand for 1 AU TF. TF activity assays were performed with 3 replicates and repeated in 6 cultures from different placentas. To confirm TF clotting-dependence on the presence of factor VII (FVII), several experiments were performed using reconstituted, lyophilized FVII-deficient plasma (American Diagnostica) as the source of coagulation factors. Clotting times of FVII deficient assays of both cell extracts exceeded 200 seconds as compared to 145 seconds clotting times of the HUVEC control or 40 seconds of STB control.

TFPI activity

TFPI activity was measured in cell lysates and supernatants as described before (25). Briefly, supernatants or lysates (10 µl) were incubated with reagent mixture (100 µl) containing: 0.8 nM activated factor X (FXa) (Chromogenix-IL Milan, Italy), 25 pM FVII (Sigma), 10 mM CaCl2, 1% TF (Innovin) in tris saline citrate buffer (0.05 M tris, 0.1 M NaCL, 0.01 M Na_3 citrate, 0.2% BSA (Sigma), pH 8.0) for 20 minutes at 37^0C, followed by the addition of 0.4U/ml FX (20 ul) (Sigma) and further incubation for 10 minutes. A chromogenic substrate for FXa - 0.72 mM S2765 (Chromogenix), was added and incubated for 1 hour. The reaction was stopped with 50% acetic acid (50 µl). Absorbance was read at 405 nm and compared to values of a standard curve as described. The TFPI activity was expressed as % inhibition of the control. TFPI activity assays were performed with 3 replicates and repeated in 6 cultures from different placentas.

Cell treatment with inflammation mediators

Potential modulation of TF and TFPI by cytokines was repeated in 4 cultures obtained from different placentas. The cells were seeded in 96 well plate for 24 hours in M-199, supplemented with 10% FCS. Following transfer, STB cells did not proliferate, while HUVEC cells were seeded in a confluent way, and their number was not changed during incubation. Cells were exposed to elevated doses of lipopolysaccharides (LPS, Sigma). As STB have a low sensitivity for LPS, high doses of LPS (25 µg/ml) were implied to get a significant effect. TNFα (R&D systems, Minneapolis MN) (100 ng/ml) and IL-1α (R&D Systems) (1 ng/ml) were added for 6 or 20 hours. Following incubation, supernatants were separated and stored, and cell extracts were prepared as described.

Statistical analysis

Statistical analysis was performed by SPSS. The results, presented in the supplementary table, include mean, STD and median values. Intra coefficient of variance (CV) and inter CV were calculated for each test. Differences between groups were analyzed by Mann-Whitney method, a non–parametric independent 2 groups comparison. ANOVA or Kruskal Wallis test, a non-parametric test (distribution free), was used to compare three or more independent groups. P values <0.05 were considered statistically significant.

Results

In order to assess the characteristics of the cell model, growth and differentiation of cultured STB cells were followed for 14

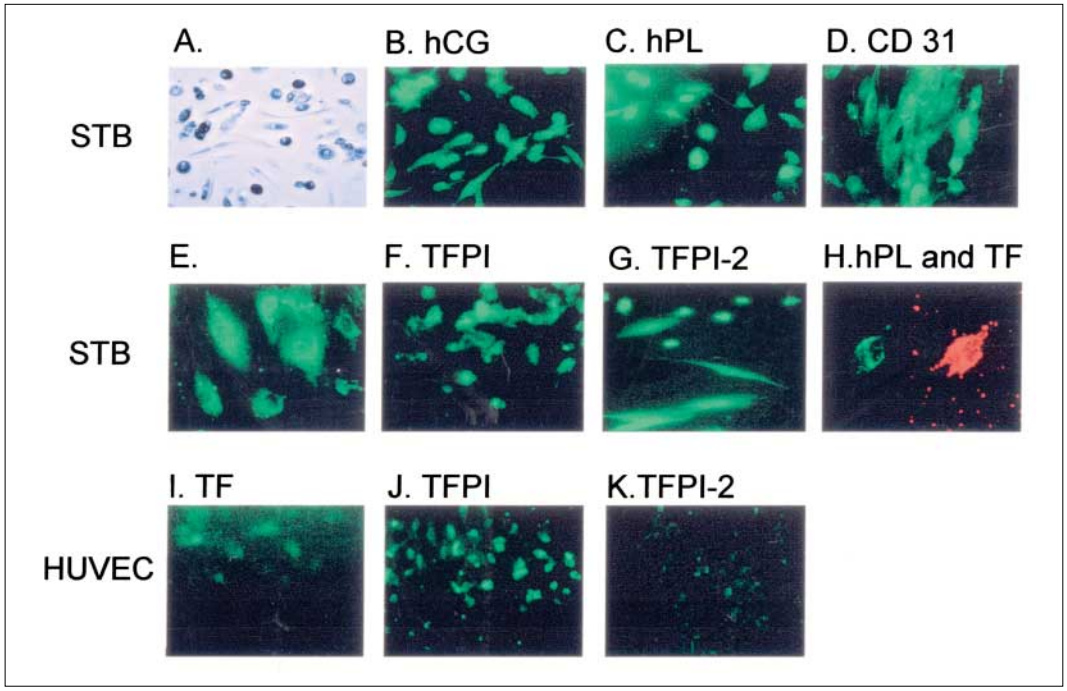

Figure 1: Characterization of STB and HUVEC with endocrine, vascular and coagulation markers. STB cells were incubated with a specific primary antibodies followed by staining with FITC- conjugated secondary antibodies (A). Light microscopy of STB culture. B-G, STB immunofluorescence staining. (B). Staining for the endocrine marker βhCG. (C). Staining for the endocrine marker hPL. (D). Staining for the vascular marker CD31. (E). Staining for TF. (F). Staining for TFPI. (G). Staining for TFPI-2. (H). Double staining for hPL and TF. STB incubated with rabbit anti hPL and mouse anti-human TF followed by staining with FITC-conjugate sheep anti rabbit IgG (green) and PE-conjugate goat anti mouse IgG (red) I-K. HUVEC immunofluorescence staining. (I). Negative staining for TF. (J). Positive staining for TFPI (K). Negative staining for TFPI-2.

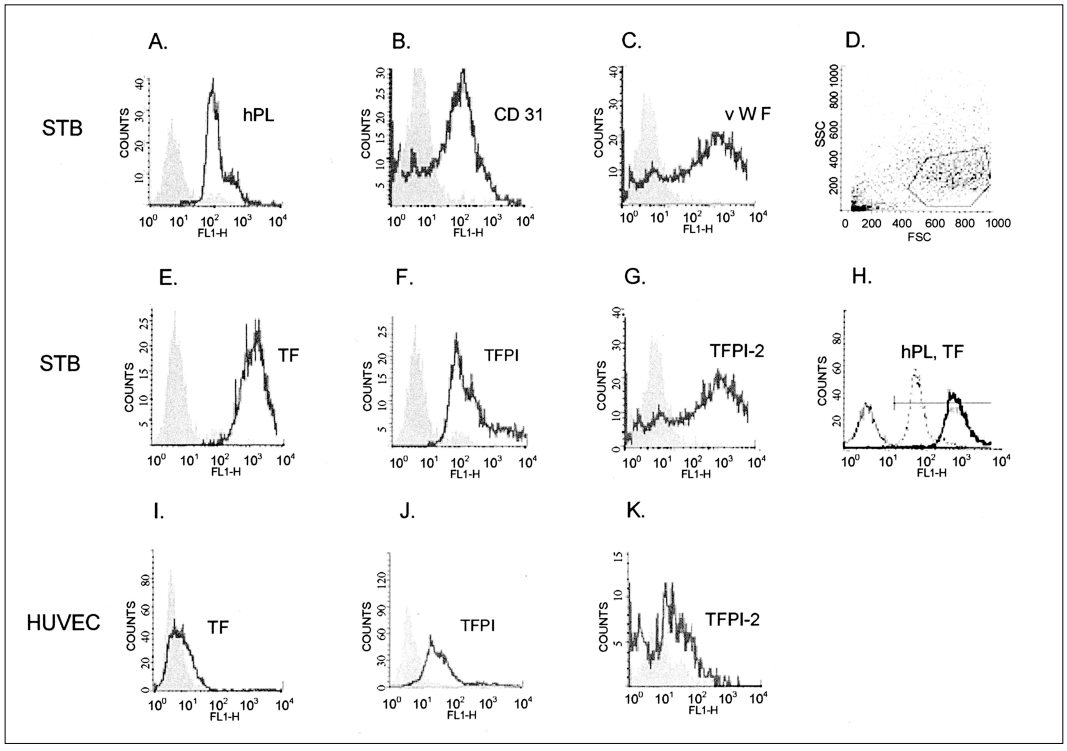

Figure 2: Flow cytometry analysis of TF, TFPI and TFPI-2 expression in STB and HUVEC. STB or HUVE cells were detached, resuspended, stained for specific markers and analyzed by flow cytometry as described in materials and methods. A-H Staining of STB cells. (A). Positive staining for the endocrine marker hPL (95±3.6%). (B). Positive staining for the vascular marker CD31 (92±4.5%). (C). Positive staining for the vascular marker vWF (88±8%). (D) Forward scatter (FSC) of STB cells with chosen gate of large STB population. (E). Positive staining for TF (95±2.9%). (F). Positive staining for TFPI (90%±5). (G). Positive staining for TFPI-2 (90±4%). (H) TF and hPL positive stainings of the gated STB population. (I-K). Staining of HUVEC. (I). Negative staining for TF. (J). Positive staining for TFPI (90%±5). (K). Negative staining for TFPI-2.

days. After 24 hours in culture, apoptotic STB were washed out and the remaining CTB differentiated and matured into STB. Subsequently, spindle shaped attached cells were observed in culture. Ten days later, cells began to proliferate; cytoplasm volume increased, and a fusion process that was verified by time-lapse cinematography (data not shown) occurred, and resulted in multinuclear cells. Enriched unique primary STB cell cultures containing small cells (40-50%), giant multinuclear cells (30-40%) and elongated cells (5-15%) were established (Fig. 1A). The following experiments were performed with these differentiated STB cells.

The syncytiotrophoblast cells were characterized by two immunofluorescence methods. First, cell staining with fluorescent-conjugated antibodies viewed by a fluorescence microscopy (Fig. 1), depicted the three sub populations of the STB culture. Second, flow cytometry (Fig. 2), demonstrated the analysis of large and highly granulated cells in a chosen gate (Fig 2D). The hormonal markers βhCG and hPL were strongly stained in STB cells using the immunofluorescence method (Fig. 1B, 1C), a result which was confirmed by using flow cytometry (Fig. 2A). STB cells also showed vascular markers CD31 (Fig. 1D) and vWF (data not shown) as confirmed by flow cytometry (Fig. 2B, 2C). STB cells demonstrated positive immunofluorescence staining for TF, (Fig. 1E) as confirmed by flow cytometry analysis (Fig. 2E) contrasting the negative TF staining in HUVEC by both methods (Fig. 1I, 2I). Dual immunofluorescence staining showed that the STB cells express both the endocrine marker hPL (green) and the coagulation marker TF (red) (Fig 1H). The same cell population at the FACS gating expressed hPL and TF (2D, 2H).

STB immunofluorescence staining for TFPI (Fig. 1F) revealed a weaker signal than HUVEC (Fig. 1J), while the number of cells expressing TFPI measured by flow cytometry analysis was similar (Fig. 2F, 2J). STB cells had strong immunofluorescence stain for TFPI-2 (Fig. 1G) as confirmed by flow cytometry (Fig. 2G). TFPI-2 could not be detected in HUVEC by immunofluorescence (Fig. 1K) or by flow cytometry analysis (Fig. 2K).

To study the spatial distribution of TF and its inhibitors TFPI, TFPI-2 within the placenta, sections of term human placenta were fixed in paraffin and stained with primary antibodies against TF, TFPI and TFPI-2. Trophoblast cells that line the villous surface were markedly stained for TF (Fig. 3A). The staining for TFPI was stronger in the endothelial cells within the

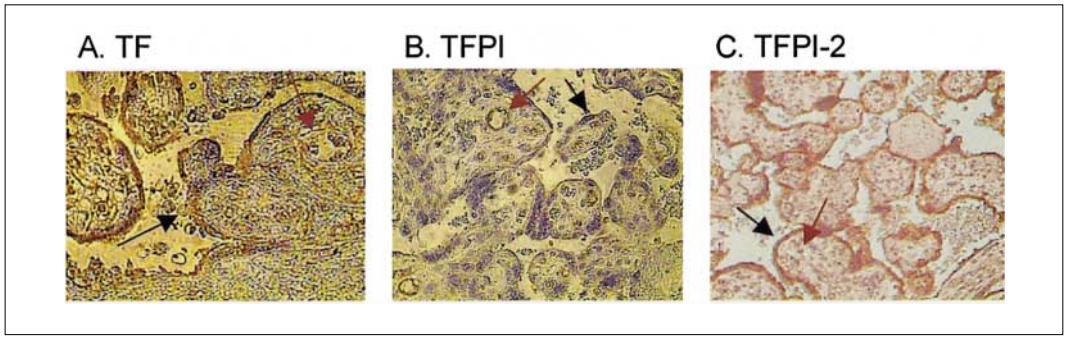

Figure 3: Immunohystochemistry analysis of TF, TFPI and TFPI-2 on placenta sections. Human term placenta sections were fixed in paraffin utilizing the avidin-biotin-peroxidase method (black arrow point on villous surface, red arrow point on intra-villous blood vessel). (A). Staining of TF on trophoblasts cells that line the villous surface. (B). Staining for TFPI in the endothelial cells lining the blood vessels compared to the trophoblasts that line the villous surface. (C). Staining of TFPI-2 on trophoblasts cell on the villous surface but not on the endothelial cells.

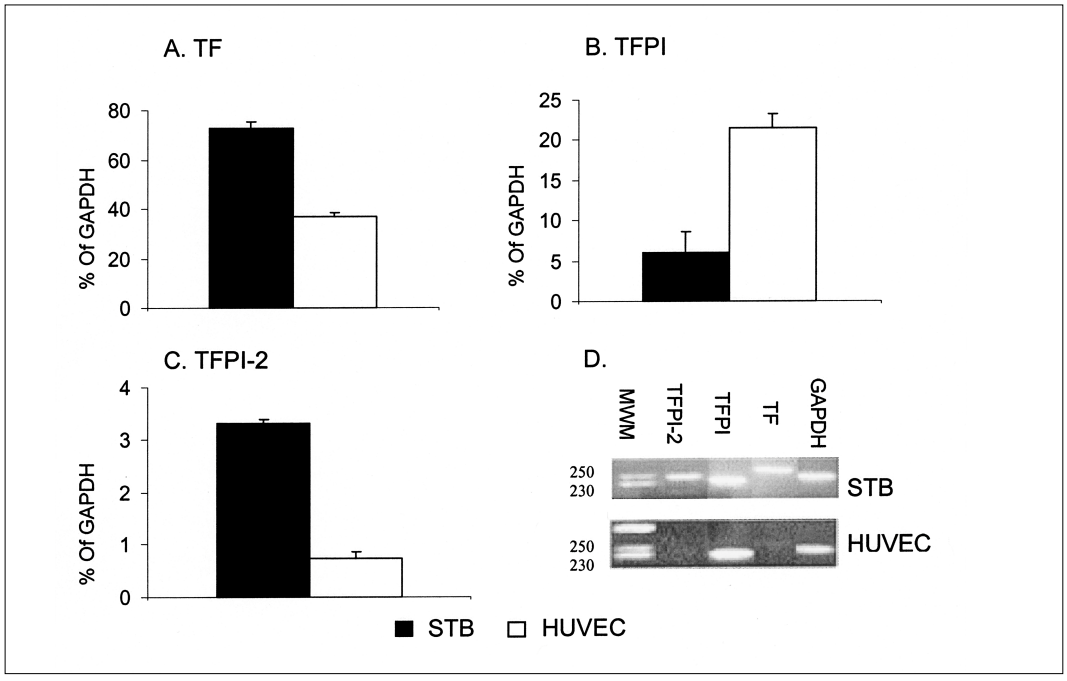

Figure 4: mRNA expression of TF, TFPI and TFPI-2 in STB and HUVE cells. A-C. mRNA levels were determined by ABI 7700™ quantitative PCR system and expressed as % of GAPD. The data presented as mean value with standard deviation (STD) of three tests (A). TF mRNA level measured in STB cells is expressed as % of GAPDH. (B). TFPI mRNA level in STB was relatively low compared to HUVEC. (C). TFPI-2 mRNA expression was higher in STB cells than in HUVEC. (D). TF, TFPI and TFPI-2 RT-PCR products were electrophoresed in agarose gel and viewed by Ehtidium Bromide staining.

intra blood vessels compared to the trophoblasts cells of the villous surface (Fig. 3B). Trophoblasts, but not endothelial cells, were heavily stained for TFPI-2 (Fig. 3C). These results confirmed our earlier findings on isolated STB, indicating high TF and low TFPI expression pattern in trophoblasts lining the placenta villous surface.

In order to assess the haemostatic balance of TF and its inhibitor TFPI in syncytiotrophoblasts culture, mRNA expression, protein levels and activity were evaluated and compared to HUVEC. TFPI-2 expression was evaluated only by mRNA expression. The results, including mean, median, intra and inter CV are summarized in table 1. TF mRNA level in STB was evaluated by quantitative PCR system and was found to be twice as high in STB compared to HUVEC (Fig. 4A). STB cells demonstrated a strong band of mRNA TF on agarose gel containing ethidium bromide, while no TF band was present on HUVEC (Fig. 4D). TF protein measured by ELISA was twice as high in STB extract as compared to HUVEC (Fig. 5A). TF activity level was found as the most prominent difference between STB and HUVEC being ten times higher in STB

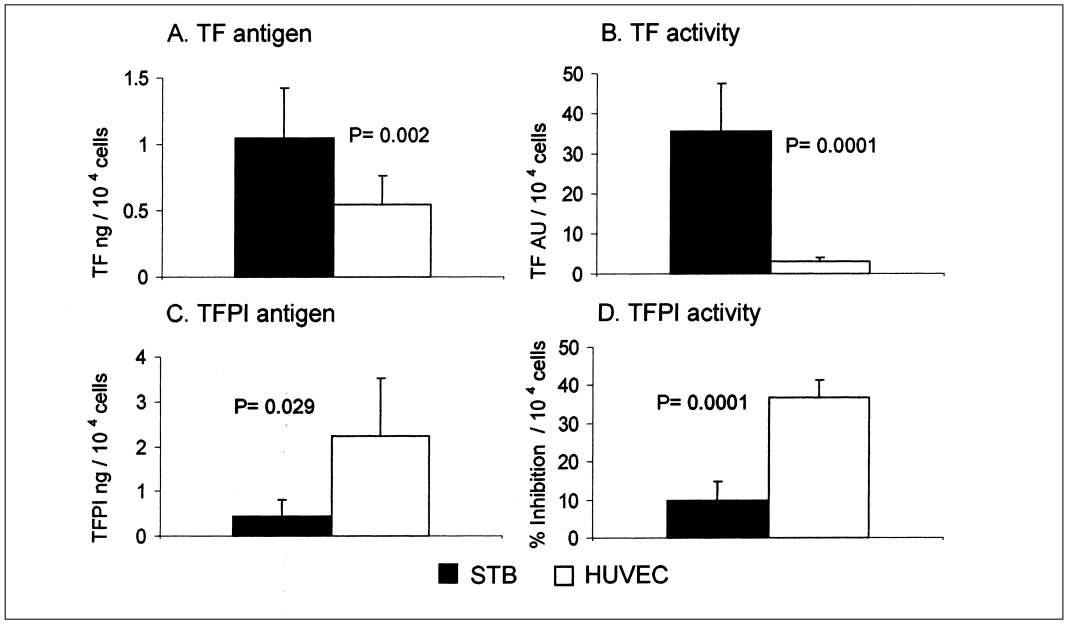

Figure 5: TF and TFPI protein levels and activity in STB and HUVE cells. (A). TF protein was measured by ELISA and expressed as ng antigen/10^4 cells. (B). TF activity was determined by one-step coagulation assay and expressed as AU/10^4 cells. (C). TFPI protein levels were measured by ELISA and expressed as ng antigen/10^4 cells. (D). TFPI activity levels were determined in STB supernatants and expressed as % inhibition/10^4 cells. The data presented as mean value with standard deviation (STD) of six experiments for each test.

Table 1: Statistical analysis of TF, TFPI and TFPI-2 results. The results include mean, standard deviation (STD), median, minimum (min) and maximum (max) values, the inter covariance (CV) between assays and intra assay CV.

STB Tests	Test	Mean	STD	Median	Min	Max	Inter CV	Intra CV
mRNA (% Of GAPDH)	TF	72.7	2.4	73.3	68.8	75.7	3.3%	2.6%
	TFPI	6.48	1.6	6.23	4.47	8.31	24%	11.2%
	TFPI-2	2.32	0.07	2.32	2.27	2.37	3%	0.07%
Protein (ng/10^4 cells)	TF	1.05	0.37	1.01	0.51	1.78	35%	7.6%
	TFPI	0.45	0.35	0.3	0.1	1	90%	10.3%
TF activity (AU/10^4 cells)	Control	35.7	11.7	33	18	58	35%	4.2%
	IL1 1 ng/ml	34.8	10.9	33	18	53	36%	2.8%
	LPS 25 µg/ml	44.6	7.9	40	36	56	20%	4.0%
	TNF 100 ng/ml	58.2	7.1	60	50	75	11%	7.1%
TFPI activity (% Inhibition/10^4 Cells)	Control	9.94	4.8	9.7	4.9	21.6	55%	6.1%
	IL1 1 ng/ml	1.41	1.4	1.2	0.0	4.4	97%	1.9%
	LPS 25 µg/ml	2.44	2.8	0.5	0	6.4	127%	0.4%
	TNF 100 ng/ml	7.48	3.6	5.8	3.6	13.6	49%	5.0%
HUVEC Tests								
mRNA (% Of GAPDH)	TF	36.9	1.6	37.3	35	39.3	4.2%	2.9%
	TFPI	19.5	7.4	22.2	9.6	26.2	38%	11.5%
	TFPI-2	0.74	1.4	0.78	0.57	0.83	180%	9.6%
Protein (ng/10^4 cells)	TF	0.55	0.21	0.51	0.21	0.89	41%	5.5%
	TFPI	2.23	1.3	1.5	1.3	4.5	60%	5.5%
TF activity (AU/10^4 cells)	Control	3.13	0.9	3.2	1.5	4.8	32%	6.9%
	IL1 1 ng/ml	14.8	3.6	14	10	20	25%	11.9%
	LPS 25 µg/ml	12.1	3.1	12	8.8	16	27%	4.6%
	TNF 100 ng/ml	5.1	1.89	4.2	3	8	40%	4.4%
TFPI activity (% Inhibition/10^4 Cells)	Control	36.8	4.4	35.5	32.4	45	12.00%	2.0%
	IL1 1 ng/ml	38	4.9	37	30.6	45.7	18.50%	3.6%
	LPS 25 µg/ml	36	7.5	36	26.4	44.8	22%	4.7%
	TNF 100 ng/ml	42	8.7	42.9	29.6	53.8	23%	3%

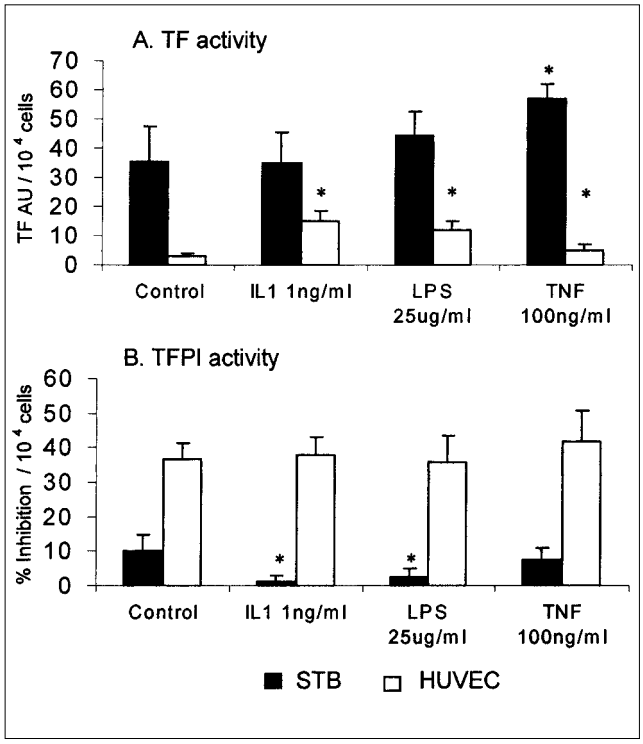

Figure 6: Effects of inflammatory mediators on TF and TFPI levels. (A). STB and HUVEC cells were exposed for 5 hours to 1ng/ml IL1α, 25 μg/ml LPS or 100U/ml TNFα. TF activity in cell extracts was measured by one step coagulation assay and expressed as AU/10^4 cells. TF activity in STB was increased by LPS (non significantly) and by TNFα (* p=0.04). Low TF activity in HUVEC was induced by 1ng/ml IL1α (* p=0.0001), by TNFα (* p=0.04) and by LPS (* p=0.002). (B). STB and HUVEC cells were exposed for 20 hours to 1ng IL1α /ml, 25 μg/ml LPS or 100U/ml TNFα. TFPI in cell supernatants was measured by chromogenic assay. TFPI activity in STB supernatant was low and was further reduced by IL1α (* p=0.01) and by LPS (* p=0.05) and unaffected by TNFα. Prolonged exposure (20 hours) of HUVEC to the mediators did not increase the relatively high TFPI basal activity level. The data presented as mean value with standard deviation (STD) of four experiments of each test.

(Fig. 5B). Additional experiments with FVII-deficient plasma as a source of coagulation factors, presented prolonged clotting times of over 180 sec (data not shown), confirming the TF clotting dependence of STB.

In contrast to the high level of TF, TFPI mRNA level in STB was three times lower than in HUVEC (Fig. 4B). A distinctive band of TFPI mRNA on agarose gel was demonstrated by both cultures (Fig. 4D). TFPI protein level in STB supernatants was five times lower than HUVEC supernatants measured by ELISA (Fig. 5C). TFPI activity level measured by chromogenic activity assay was four times lower in STB supernatants compared to HUVEC supernatants (Fig. 5D). mRNA level of TFPI-2 were relatively low but 2.8 times higher in STB cells than in HUVEC (Fig. 4C, Table 1). STB showed a strong band of TFPI-2 on the agarose gel while no band for TFPI-2 was observed in HUVEC mRNA indicating TFPI-2 specific presence in placental STB (Fig. 4D).

In order to evaluate the influence of inflammatory mediators on the haemostatic balance in STB culture, we exposed differentiated STB cells and HUVEC to LPS, IL-1α and TNFα. We found that the high constitutive levels of TF activity in STB were not affected by IL-1α, but were moderately upregulated by LPS and by TNFα. STB were not sensitive to low doses of LPS (data not shown). In contrast, the inflammatory mediators caused a significant increase in the relatively low TF activity that is normally found in HUVEC: IL-1α induced and LPS induced more than a four-fold increase, while TNFα induced a two-fold increase (Fig. 6A), in accordance with previous results (23).

TFPI activity in STB supernatant was below the detectable limit after a 5 hours incubation period. Prolonged exposure time (20 hours) increased TFPI in STB to detectable levels while a high steady state level in HUVEC was reached. TFPI activity in STB supernatant after 20 hours accumulation was low and was further reduced by IL-1α and by LPS and unaffected by TNFα (Fig. 6B). TFPI activity determined in HUVEC supernatant was non-significantly increased by IL-1α and LPS and unaffected by TNFα after 5 hours exposure. A longer exposure (20 hours) of HUVEC to these inflammatory mediators did not increase the relatively high basal level of TFPI activity (Fig 6B). These results suggest that high expression of TF and low level of TFPI in STB model may be modulated by inflammation.

Discussion

Placental growth depends on CTB proliferation and differentiation. Human STB cells function as a complex barrier between maternal and fetal circulations. The mechanisms that regulate syncytial fusion include early apoptotic events and changes in phospholipid organization in the plasma membrane orientation (28), which may determine the procoagulant nature of the cells. The CTB cells adopt vascular phenotypes as they differentiate and express vascular markers (19). The vascular markers, vWF and CD31, that were found in our cultured STB, indicate that STB cells adopt "endothelial character" while functioning as a blood barrier. However, unlike EC or other cells in contact with blood, we found that STB express high levels of TF.

Several laboratories investigated the presence of coagulation proteins on purely isolated CTB cells immediately after separation. These undifferentiated CTB expressed TFPI and TFPI-2, but not TF or the endocrine markers hCG and hPL (29, 30).

Our study is the first to characterize the hemostatic balance in a primary differentiated STB that express the endocrine markers hCG and hPL. The endocrine markers are the key evidence for the differentiation and maturation of STB cells (31). Maturation process may depict different proportions of cells

bearing both markers representing stages in differentiation as was found by us later (data not shown).

The quantitative measurement of mRNA, antigen level, protein activity and qualitative immunofluorescence analysis provide keen insight on the delicate balance of TF and its inhibitors TFPI, TFPI-2 under distinct growth and inflammatory environments. The present study localizes the TF and TFPI-2 on the villous surface at placenta section, where the syncytiotrophoblast cells are located. TFPI is mainly located in the endothelial intravillous blood vessels. High constitutive levels of TF and low levels of TFPI in the STB culture suggest a procoagulant tendency in these cells, contrasting the anticoagulant profile of HUVEC.

TF as well as other coagulation proteins like thrombomodulin and annexin V were found on syncytial membranes (7, 21, 32). Studies in mice demonstrated that TF is essential for vascular development and angiogenesis (6, 7), $TF^{-/-}$ embryos die between E8.5-10.5 (33). TF activation followed by fibrin deposition and fibrin degradation, caused trophoblast apoptosis and disruption of placental development, while thrombomodulin prevented this process. On the other hand TF, through the protein C system and PAR-1, modulates the growth of trophoblast cells in the placenta (22). In humans, TF is crucial for the prevention of bleeding during trophoblast invasion to the deciduas spiral arteries (4, 34). Nemerson and Giesen suggested that vascular TF is latent (35) and, under normal conditions, cell-surface TF is encrypted (36). The presence of maternal factor VIIa in the intervilli spaces may regulate the activation of coagulation by the high basal level of STB TF found in this study.

The primary sites of TFPI synthesis are the endothelium and extra vascular cells that synthesize TF (13). TFPI was found in placental STB, CTB and vascular endothelium cells from 10 weeks through term by immunohistochemistry staining (20). Total TFPI in placenta sections and myometrium was lower than TF (3). The present study demonstrated that STB TFPI levels remained low through *in-vitro* cell differentiation and TFPI at placenta sections was mainly located on the endothelial cells.

The role of TFPI-2 in the coagulation balance in human placenta as well as in other tissues remains elusive. TFPI-2 is a Kunitz-type proteinase inhibitor that inhibits several proteinases involved in coagulation and fibrinolysis (37). It is a matrix associated serine protease inhibitor (MSPI) (38) that regulates invasiveness of tumor cells (39, 40). The inhibition effect of TFPI-2 on metalloproteinases may play a major role in regulation of trophoblast invasion and differentiation leading to placental implantation and development. Our findings, as well as other studies (18, 29), confirm the presence of TFPI-2 in STB culture and in the placental villous surface, while additional studies are needed to assess this special function. There is no method for distinguishing between TFPI and TFPI-2 inhibition activity of the complex TF-FVIIa, which may be applicable to this study, due to the low TFPI inhibitory activity of trophoblast cells and the difficulty to modulate it. Therefore, the TFPI activity measured in this study may result from both proteins. Several studies suggest that TFPI-2 coagulation inhibitory activity is low (37, 39), we believe that the inhibition activity was mostly related to TFPI. In the present study, TFPI-2 mRNA was higher in STB cells than in HUVEC. These results are consistent with the high levels of TFPI-2 mRNA previously found in CTB cells (29). We have also detected a strong immunofluorescence staining of TFPI-2 in a STB culture and intense immunohystochemistry staining in the trophoblast layer at placental sections, in accordance with other immunohystochemistry studies in placenta sections (18).

STB cells respond to inflammatory stimuli that intensify their procoagulant tendency by increased TF activity and decreased TFPI activity. In contrast, restricted TF and relatively high TFPI maintain the anticoagulant nature of HUVEC while activation by inflammatory cytokines induces an increase in TF followed by an increase of TFPI (27). Cytokines produced by the placenta and its associated membranes are involved in the control of implantation and placental development (41, 42). Lipopolysaccharide (LPS) induces activation of the nuclear factor kappa B (NF-kB) and upregulates cytokines such as TNFα and IL-1α, resulting in an inflammatory response, which induces TF (13, 43). Coagulation and inflammatory signals that accumulate in the placenta affect each other (44-46). In the present study, TF in STB cells was slightly increased by LPS and TNFα, but not by IL-1α, while the three mediators induced TF in HUVEC, as confirmed by gene expression (data not shown). It is therefore apparent that while TF in the STB culture can be differentially modulated by cytokines, it is still not clear whether STB cells respond to these different agonists through common mechanisms or through distinct transcriptions. Further studies on the transcription factors of TF and its inhibitors on STB may lead to a better understanding of these issues.

The TFPI level in STB was low and was further reduced by LPS and IL-1α, while both mediators induced a slightly increased TFPI in HUVEC. TFPI induction depends on cell type or local tissue reactivity towards inflammation (47-49).

In conclusion, the procoagulant tendency of STB cells, which can be enhanced by inflammatory stimuli, may reflect the physiological need for immediate inhibition of bleeding. Its regulation by TFPI and TFPI-2 is not completely understood and other local regulators such as cell-surface protein C system or systemic coagulation proteins like factor VII can modify the TF/TFPI coagulation potential. The unique hemostatic balance demonstrated in STB cells, which can be modulated by cytokines and thrombophilia, may be critical for placenta function and pregnancy outcome.

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