

Blood Coagulation, Fibrinolysis and Cellular Haemostasis

Regulation of chemotaxis by the cytoplasmic domain of tissue factor

Agneta Siegbahn, Matilda Johnell, Brit B. Sorensen, Lars C. Petersen, Carl-Henrik Heldin

Department of Medical Sciences, Clinical Chemistry, Akademiska Hospital and Ludwig Institute for Cancer Research, Biomedical Centre, Uppsala Sweden, Haemostasis Biology, Health Care Discovery, Novo Nordisk A/S, Maaloev, Denmark

Summary

We previously demonstrated that FVIIa bound to tissue factor (TF) induces a hyperchemotactic response towards PDGF-BB. The aim of the present study was to investigate the role of the cytoplasmic domain of TF in cell migration. Porcine aortic endothelial (PAE) cells expressing human PDGF β -receptors (PAE/PDGFR β) were transfected for expression of human wildtype TF (PAE/PDGFR β /TF), a construct lacking the cytoplasmic domain (PAE/PDGFR β /TF Δ cyto), a construct with alanine replacement of serine 258 (PAE/PDGFR β /TFS258A), or a construct with alanine replacement of serine 253, 258 and 263 in the cytoplasmic domain (PAE/PDGFR β /TF3SA). All stably transfected cell lines expressed functional TF. Chemotaxis was analyzed in a modified Boyden chamber assay. PAE/PDGFR β /TF cells stimu-

lated with FVIIa migrated towards a 100-fold lower concentration of PDGF-BB than in the absence of FVIIa, however, hyperchemotaxis was not seen in PAE/PDGFR β /TF Δ cyto cells. PAE/PDGFR β /TFS258A and PAE/PDGFR β /TF3SA cells responded to low levels of PDGF-BB, but migrated a significantly shorter distance than PAE/PDGFR β /TF cells. Thus, hyperchemotaxis towards PDGF-BB is likely to depend in part on phosphorylation of the cytoplasmic domain of TF. We conclude that the cytoplasmic domain of TF plays a pivotal role in modulating cellular migration response. Our results suggest that the FVIIa/TF complex mediates intracellular signaling by alternative signal transduction pathway(s).

Keywords

Tissue factor, platelet-derived growth factor, intracellular signaling, cell migration

Thromb Haemost 2005; 93: 27-34

Introduction

Tissue factor (TF), a 47 kDa transmembrane glycoprotein, is the main initiator of the coagulation protease cascade *in vivo*, via complex formation between TF and factor (F) VII/VIIa. TF is constitutively expressed in cells surrounding blood vessels and organ capsules, thus forming a haemostatic barrier to prevent loss of blood in case of vessel damage. By a number of inflammatory stimuli, TF can also be induced in endothelial cells and monocytes (1). Moreover, many tumor cells have been demonstrated to have a constitutive expression of TF (2–4). In addition to its role in haemostasis, the FVIIa/TF-complex has recently been shown to mediate intracellular signal transduction resulting in gene induction and implicating a possible role in various biological functions, such as embryological development, cell migration, inflammation, apoptosis, angiogenesis and tumor metastasis and invasion (5–8).

The extracellular domain of TF consists of 219 amino acids and share structural homology with type II cytokine receptors, i.e. interferon (IFN) and IL-10 receptors (9). The C-terminal domain of TF consists of a single transmembrane domain and a short cytoplasmic domain of 21 amino acids with three serine residues that are potential phosphorylation sites (9).

Several effects of FVIIa/TF, including activation of coagulation, intracellular signaling inducing Ca²⁺-oscillation, activation of MAP-kinase pathways, stimulation of protein synthesis and induction of a number of genes were all shown to be TF-dependent responses that did not require the presence of the TF cytoplasmic domain (10–16). Accumulating data rather suggest that G protein-coupled protease activated receptor (PAR)-2 mediates these types of FVIIa/TF signaling responses (17, 18). Today, the function of the cytoplasmic domain of TF is incompletely understood. Recently presented data indicate the TF cytoplasmic domain to be involved in cellular signaling associated

Correspondence to:
Agneta Siegbahn
Department of Medical Sciences
Clinical Chemistry
Akademiska Hospital
S-751 85 Uppsala, Sweden
Tel.: +46 18 611 4251, Fax: +46 18 55 25 62
E-mail: agneta.siegbahn@akademiska.se

Received July 2, 2004
Accepted after revision October 21, 2004

Financial support:
Supported by Swedish Research Council, Swedish Cancer Foundation, Swedish Heart and Lung Foundation and King Gustav V and Queen Victoria Foundation

Republished online December 8, 2004 DOI: 10.1160/TH04-07-0405

with inflammatory processes (19). Overexpression of TF by Chinese hamster ovary cells or human melanoma cell lines resulted in enhanced metastatic potential of the cells in murine models and this required proteolytically active FVIIa/TF complex, as well as the cytoplasmic domain of the molecule (20–23). In melanoma cells the cytoplasmic tail of TF appears also to be essential for VEGF production thereby promoting angiogenesis (24). Contradictory results to these findings, however, have been presented (23).

A connection between FVIIa/TF-induced signaling and the cellular response to platelet-derived growth factor (PDGF) was indicated by our study showing that FVIIa/TF profoundly increased the chemotactic response towards PDGF-BB in human vascular smooth muscle cells and fibroblasts (25). This response was dependent on both the proteolytic activity of FVIIa/TF and phospholipase-C (PLC) activity, but was independent of phosphatidylinositol 3'-kinase (PI3'K)(5). The aim of this study was to further investigate the role of the TF cytoplasmic domain in chemotaxis towards PDGF-BB. We report the mandatory function of the cytoplasmic domain for cell migration. In porcine aorta endothelial (PAE) cells expressing PDGF β -receptors and stably transfected with a TF construct, we could show that deletion of the cytoplasmic domain completely abolished the TF-dependent hyperchemotactic response to PDGF-BB.

Material and methods

Reagents

Recombinant human FVIIa and FVIIa blocked in the active site with phenylalanyl-phenylalanyl-arginyl chloromethyl ketone (FFR-VIIa) were obtained from NovoNordisk A/S (Maaloev, Denmark). Zymogen FX, FXa and thrombin were from Enzyme Research laboratory (South Bend, IN). Hirudin was purchased from Sigma (St. Louis, MO). The MEK-inhibitor PD98059 was from Cell Signaling Technology Inc. (CST.; Beverly, MA). PAR-2 antibodies (SAM 11) were from Zymed Laboratories Inc. (San Francisco, CA) and PAR-2 agonist human peptide SLIGKV-NH₂ from Sigma. Polyclonal neutralizing antibody of PAR-2 were kindly provided by Dr. Wolfram Ruf, Scripps Research Institute, La Jolla, CA. The antibody inhibited specific responses mediated by PAR-2 agonist peptides, but not by PAR-1. PAR-1 agonist human peptide SFLLRN was from Bachem AG (Bubendorf, Switzerland). PDGF-BB was provided by Amgen Inc. (Thousand Oaks, CA). Chromozyme X from Roche Diagnostics (Hvidovre, Denmark).

Cell lines and cell cultures

PAE cells stable transfected with PDGF β -receptors (26) with and without wild type TF or TF with the cytoplasmic domain deleted or with intracellular serine residues mutated, were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin, 100 μ g/ml streptomycin (Gibco, Invitrogen, Carlsbad, CA). The cells were cultured at 37°C and 5% CO₂ in a humidified incubator. The cells were cultured to near confluency before use, detached by trypsinization (2.5 mg/ml trypsin for 5 minutes at 37°C) or Cell dissociation solution from Sigma, washed in Hanks balanced salt solution and resuspended in complete medium.

Transfection of PAE cells with TF, TF Δ cyto, TFS258A, and TF3SA

Full-length human TF cDNA was inserted into the mammalian expression vector pcDNA3.1Hygro+ (Invitrogen). A cDNA encoding the TF mutant lacking amino acid residues (248–263) (TF Δ cyto) was prepared by a modified version of reverse polymerase chain reaction with the polymerase Pwo as described earlier (12). An expression plasmid for this mutant was prepared by inserting the cDNA into the expression plasmid pcDNA3.1Hygro+. A TF mutant lacking the three possible phosphorylation sites in the cytoplasmic part of TF (S253A, S258A, S263A) was prepared as follows, the 3'-end of human TF was amplified by PCR using the forward primer 5'-GAATTCAGAGAAATATTCTACATC-3' and the mutagenic reverse primer 5'-GAATTCCTTATGCAA-CATTCAGTGGGGCGTTCTCCTTCCAGGCCTGCCCCAC TCCTGCCTTTCTAC-3'. The 129 bp PCR-product was inserted *EcoRI-EcoRI* into the plasmid, hTF/pcDNA3 replacing the wildtype 3' end. The fragment encoding mutant tissue factor was subcloned, *BamHI-NotI*, into pcDNA3.1Hygro+ to form the plasmid hTF(S253A, S258A, S263A)/ pcDNA3.1Hygro+. The DNA sequence was verified by sequence analysis. A TF mutant lacking the possible phosphorylation site S258 in the cytoplasmic part of TF was prepared by site-directed mutagenesis using QuickChange® kit (Stratagene, La Jolla, CA, USA). The following primer was used to introduce the S258A mutation (only sense primers given) with base substitutions in italics and the altered codon underlined: 5'-GCTGGAAGGAGAACGCCCCACTGAATGTTTCA-3'. Plasmid was prepared using the plasmid DNA midi kit and QIAfilter (Qiagen, Valencia, CA, USA). The entire cDNA was verified by sequencing to confirm that the primary structure only contained the desired mutation.

PAE cells stably transfected with the PDGF β -receptor were subsequently transfected with either wild type TF (PAE/PDGFR β ,TF), TF Δ cyto (PAE/PDGFR β ,TF Δ cyto), TF(Ser258Ala) (PAE/PDGFR β ,TFS258A), or TF(Ser 253,258,263Ala) (PAE/PDGFR β ,TF3SA). Transfections were performed using FuGENE™ 6 Transfection Reagent according to the manufacturers' procedures. Cells with stably integrated TF-constructs were selected with 200 μ g/ml Hygromycin (PAE cells transfected with the PDGF β -receptor were in advance selected with 0.7 mg/ml Geneticin), and clones were selected by limited dilution.

Measurements of cell surface FVIIa/TF-mediated FX activation and FVIIa binding capacity

Surface-exposed TF was determined by FVIIa-mediated FXa generation and measurements of ¹²⁵I-FVIIa binding as described previously (27). Briefly, for FX activation assay confluent monolayers in 48-well tissue culture plates were washed once with buffer A (10 mM HEPES, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) supplemented with 5 mM EDTA and then once with buffer A and once with buffer B (buffer A supplemented with 1 mg/ml bovine serum albumin (BSA) and 5 mM Ca²⁺). Cells were incubated with various concentrations (range 0.5 pM to 50 nM) of FVIIa in buffer B (final volume 150 μ l) at 37°C for 15 minutes to allow saturation of ligand binding to the cell surface TF. The activation of FX was initiated by adding 135 nM FX (final concentration). After 15 min, 100 μ l aliquots were re-

moved from each well and added to 50 μ l of ice-cold stopping buffer (Buffer A supplemented with 10 mM EDTA and 1 mg/ml BSA). The amount of FXa generated was determined in a chromogenic assay by transferring 50 μ l of the above mixture to a microtiter plate well and adding 25 μ l Chromozyme X (1.25 mg/ml) to the well. The absorbance at 405 nm was measured continuously in a microplate reader (Molecular Devices) and the initial rates of color development were converted to FXa concentrations using a FXa standard curve (85 pM-11 nM).

For 125 I-FVIIa radioligand binding assay, confluent monolayers of cell cultures seeded in 24-well tissue culture plates, were washed once with buffer A and once with buffer B. The monolayers were incubated for 2 h at 4°C with varying concentrations of 125 I-FVIIa in buffer B in a final volume of 300 μ l. At the end of the incubation, the unbound material was removed; the cells were washed 4 times with ice-cold buffer B and lysed with lysis buffer (200 mM NaOH, 1 % SDS and 10 mM EDTA). Radioactivity was measured in a gamma counter (Packard Instruments, Cobra). For all experiments, non-specific binding was determined in parallel duplicate wells in which the monolayers were preincubated for 15 min with 1 μ M FVIIa before the addition of radioligand. TF-specific binding was determined by subtracting non-specific binding from total binding.

Curve-fitting to a single-binding site model using Grafit 4 (Erithacus Software Limited) program was applied for determination of functional K_d in the FXa formation assay, as well as the affinity of 125 I-FVIIa and maximum binding sites, B_{max} .

Flow cytometry

The surface expression of PAR-2 receptors was analysed by immunofluorescence with a flow cytometer (Coulter Epics XL-MCL; Beckman Coulter, Fullerton, CA). The instrument was calibrated daily with Flow Check calibration beads (Beckman Coulter). For indirect immunofluorescence experiments PAE-cells stably transfected with PDGF β -receptors and TF, TF Δ cyto or TFS258A were incubated for 30 minutes in the dark, on ice with the primary antibody, a human PAR-2 antibody (SAM 11). Thereafter the cells were washed twice with phosphate-buffered saline (PBS) containing 0.1% BSA, incubated for 30 minutes in the dark, on ice with fluorescein-isothiocyanate (FITC)-labelled rabbit anti-mouse (Dakopatts A/S, Glostrup, Denmark), and finally washed twice. The anti-Aspergillus niger glucose oxidase monoclonal IgG1 (DAK-G01; Dakopatts) was used as a negative control. Mean channel intensity and percentage of positive cells were determined for each sample.

SDS-PAGE gel and Western blotting

After 48 h culture of PAE cells in DMEM/F12 supplemented with 10% FBS, 1% PG, fresh medium with 0.5% FBS was added to reduce basal levels of phosphorylation and the cells were further cultured for two hours. Stimulation with 10% FBS, 50 nM FVIIa and 0.1 ng/mL PDGF-BB for 15 min was preceded by 50 μ M PD98059 (CST) for one hour in those wells where inhibition of MEK1/2 was wanted. Cell lysates were run on a Bis-Tris gel 4–12% (NuPAGE Novex, Invitrogen) and electrotransferred to a nitrocellulose membrane for immunoblotting. The membrane was blocked with 1% BSA (Sigma) in Tris buffered saline with 0.1% Tween-20 (TBS-T) (Invitrogen) for one

hour. For detection of the phosphorylated protein the membrane was incubated overnight with phospho-p44/42 MAPK (Thr202/Tyr204) antibody (CST) diluted 1:1000 in TBS-T with 5% BSA followed by incubation with a HRP-conjugated secondary antibody (CST) 1:2000 in blocking buffer for one hour. Finally, the membrane was incubated with LumiGLO (CST) and chemiluminescence was detected by Fluor-S MAX (Bio-Rad Laboratories, Hercules, CA).

Chemotaxis assay

The chemotactic response of PAE cells expressing PDGF β -receptors and wild type TF or TF receptor mutants was assayed by means of the leading front technique in a modified Boyden chamber as described previously (25, 28). The two compartments in the Boyden chamber were separated by a 150 μ m thick micropore filter (Millipore Corporation, Bedford, MA), which was precoated with type-1 collagen (Vitrogen 100; Collagen Corporation). The cells were preincubated for 10 minutes with or without FVIIa, FFR-FVIIa, FXa, FVIIa plus FX, thrombin, SFLLRN or SLIGKV before assay. One hundred microliters of the cell suspension (2×10^5 cells/ml) was added above the filter in the Boyden chamber. PDGF-BB was diluted in the assay media (DMEM/F12 with 10% FBS) and added below the filter in the chamber. The cells migrated in the filter for 6 hours at 37°C in a humidified chamber containing 95% air/5% CO₂. All agonists were present during the entire experiment. The filters were then removed, fixed in ethanol, stained with Mayers hemalum, and mounted. Migration was measured as the distance of the two farthest migrating cell nuclei of one high-power field (12.5x 24) in focus. The migration distance in each filter was calculated as the mean of the readings of at least three different parts of the filter. Experiments were performed with two to four separate filters for each concentration of chemoattractant and repeated at least in three separate experiments. For each set of experiments, the motility response of each cell clone towards the assay medium without ligand served as control and was referred to as 100% (stimulated random migration or chemokinesis).

When the MEK inhibitor PD98059, PAR-2 neutralizing antibody, or thrombin inhibitor, Hirudin, were used, PAE cells expressing PDGF β -receptors and wild type TF were preincubated for one hour or 15 minutes (Hirudin) with these agents, then with or without FVIIa, before the chemotaxis assay was performed. The MEK inhibitor, PAR-2 antibody and Hirudin were also present during the entire migration experiment.

Statistical analysis

Statistica for Windows (StatSoft, Tulsa, OK) was used for all statistical analysis. Results are presented as mean and standard error of the mean (SEM). Student's t-test was used for comparison between samples within the series or groups.

Results

TF surface expression/function in transfected PAE cells

The PDGF β -receptor induces chemotaxis, both in the homodimeric form or in the heterodimeric form with the α -receptor. Porcine aortic endothelial cells do not normally express PDGF α - or β -receptors or TF, but these cells stably transfected with PDGF

Table 1: Surface exposed TF on transfected PAE cells determined by FVIIa/TF-mediated FXa generation and measurement of ¹²⁵I-FVIIa binding. FXa generation was measured in the presence of 135 nM FX and various fixed concentrations (0.1 pM – 50 nM) of FVIIa. The data was fitted to a non-linear one-site model. The values for the amount of total TF sites per cell (B_{max}) and K_d for FVIIa were determined by saturation radioligand binding assay and data was fitted to a single-site ligand binding equation using the software Grafit 4. Data presented are mean \pm STD.

Cell line	FXa generation assay			Saturation binding of ¹²⁵ I-FVIIa and Scatchard transformation		
	n	K_d (pM)	Max FXa (nM FXa/min)	n	K_d (nM)	B_{max} (sites/cell)
PAE/PDGFR β	2	-	BD		NA	NA
PAE/PDGFR β ,TF	5	10.6 \pm 3.3	1.0 \pm 0.3	5	6.5 \pm 3.3	360 000 \pm 130 000
PAE/PDGFR β ,TF Δ cyto	6	24 \pm 12.2	1.1 \pm 0.2	5	4.2 \pm 2.7	360 000 \pm 190 000
PAE/PDGFR β ,TFS258A	3	20 \pm 10.9	1.2 \pm 0.5		NA	NA
PAE/PDGFR β ,TF3SA	4	8.3 \pm 3.3	1.4 \pm 0.6	4	3.5 \pm 2.1	420 000 \pm 210 000

NA: not analyzed; BD: below detection limit

β -receptors have been used in a number of studies to investigate various biological functions, among these chemotaxis, mediated by PDGF receptors (26, 29). Thus, double transfected PAE cells provide a suitable model system to further the investigations of the role of TF-mediated regulation of PDGF β -receptor-induced chemotaxis. Untransfected PAE cells as well as cells transfected with the PDGF β -receptor, (PAE/PDGFR β) cells, did not express detectable levels of functional TF (Table 1). To study the effect of FVIIa/TF-induced signaling in PDGF-BB-mediated migration, PAE/PDGFR β cells were stably transfected with cDNA encoding wildtype human TF (PAE/PDGFR β ,TF). To further elucidate the functional implications of the TF cytoplasmic domain PAE/PDGFR β cells were also stably transfected with a human TF lacking the cytoplasmic domain (PAE/PDGFR β ,TF Δ cyto), a construct with serine 258 changed to alanine (PAE/PDGFR β ,TFS258A) and a construct in which the phosphorylatable serines 253, 258, and 263 were all changed to alanine residues (PAE/PDGFR β ,TF3SA). The functional characteristics and binding properties of TF in the transfected cell lines were examined by a FXa generation assay and a ¹²⁵I-FVIIa binding assay. Results are shown in Table 1. The cell lines expressed comparable amounts of TF or TF mutants, both with respect to functional TF and number of TF molecules per cell.

Effects of FVIIa on directed migration to PDGF-BB in double transfected PAE cell lines

Random migration, i.e. the migration distance to assay medium only, in the absence of FVIIa, was similar in all transfected PAE cell lines. Also, FVIIa did not enhance the random migration of any of the cell lines. PAE cells double transfected with wtTF and PDGF β -receptors stimulated with 100 nM FVIIa migrated towards a concentration of PDGF-BB which was 100-fold lower than that required to induce migration in the absence of FVIIa stimulation (Fig. 1A). A significant migration response ($p < 0.001$) was achieved at concentrations of 0.01–0.1 ng/ml of PDGF-BB. The catalytic activity of FVIIa was mandatory for the hyperchemotactic response, since FFR-FVIIa did not induce enhanced chemotaxis (Fig. 1A). To substantiate the physiological relevance of the observed FVIIa/TF-induced chemotactic response, we next stimulated the transfected PAE/PDGFR β ,TF

cells with 10 nM FVIIa, a concentration equivalent to plasma concentrations of FVII. A significant stimulation ($p < 0.001$) of the chemotactic response towards 0.01–0.1 ng/ml PDGF-BB was obtained also under these conditions (Fig. 2). Thus, in TF-expressing cells, FVIIa induce migration at physiological concentrations.

The hyperchemotactic response is dependent on the cytoplasmic domain of TF

PAE cells expressing PDGF β -receptors and human TF lacking the cytoplasmic domain (TF Δ cyto) were incubated with 10 or 100 nM FVIIa and assayed for migration towards different concentrations of PDGF-BB (0.01–100 ng/ml). The migration of these cells in the presence of FVIIa was identical to the migration in the absence of FVIIa (Fig. 1B and 2), and also of PAE cells only expressing PDGF β -receptors [data not shown (26)].

Three serines in the cytoplasmic domain of TF are putative phosphorylation sites. Since the enhanced chemotactic response towards PDGF-BB was dependent on the TF cytoplasmic domain, we examined the effect of alanine-substitution of these serine residues. PAE/PDGFR β ,TF3SA cells were stimulated with 100 nM FVIIa and FFR-FVIIa. At 0.01–0.1 ng/ml of PDGF-BB the chemotactic response to FVIIa was significantly ($p < 0.01$) enhanced compared to the chemotactic response in the absence of FVIIa. However, a closer examination of the migration revealed that in comparison with cells expressing wtTF, the PAE/PDGFR β ,TF3SA cells migrated a significantly shorter distance in the filters, (wtTF versus TF3SA; $p = 0.0041$ at 0.01 ng/ml; $p = 0.0033$ at 0.1–1 ng/ml PDGF-BB) (Fig. 3). We therefore specifically analyzed the importance of serine 258 in the cytoplasmic domain. The migration pattern of PAE/PDGFR β ,TFS258A cells towards PDGF-BB was similar to PAE/PDGFR β ,TF3SA cells (wtTF versus TFS258A; $p < 0.001$ at 0.01–0.1 ng/ml; $p = 0.004$ at 1 ng/ml PDGF-BB, and TF Δ cyto versus TFS258A; $p = 0.01$) (Fig. 3).

The hyperchemotactic response is mediated by PAR-2 but not PAR-1 activation

Controversy exists whether FVIIa/TF-transduced signaling involves the activation of PAR-1, PAR-2 or an unknown PAR-re-

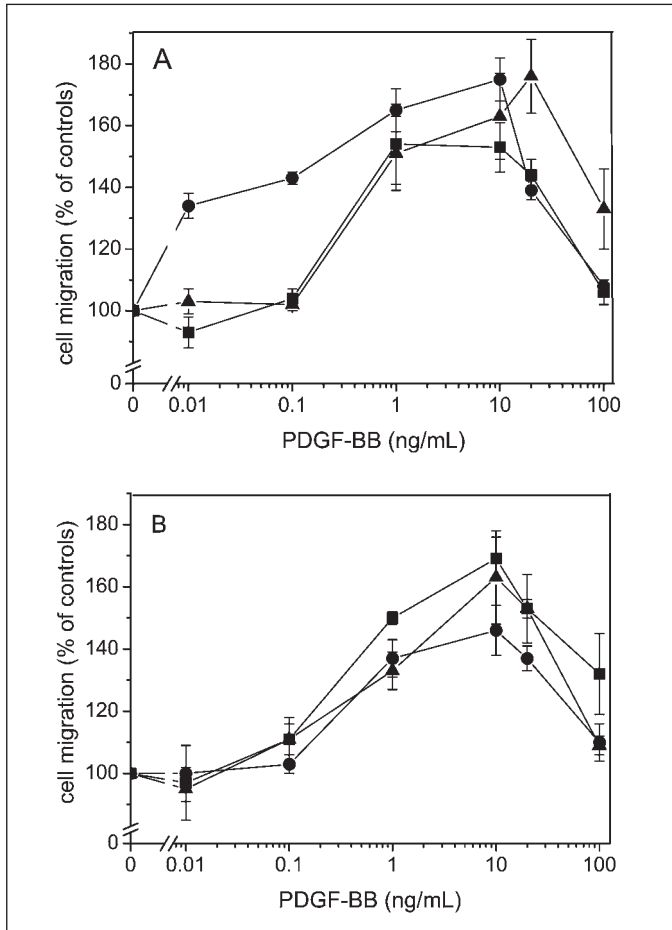


Figure 1: Effects of FVIIa and FFR-FVIIa on PDGF-BB-induced chemotaxis in PAE cells. The cells are transfected with human wtPDGFR β and human wtTF (PAE/PDGFR β ,TF) (A) or human TF with deleted cytoplasmic tail (PAE/PDGFR β ,TF Δ cyto) (B). The chemotactic response at different concentrations of PDGF-BB was determined in the absence (square) or presence of 100 nM FVIIa (circle) or 100 nM FFR-FVIIa (triangle). Results are mean \pm SEM of five separate experiments.

ceptor (17, 18, 30, 31). To investigate whether activation of the PAR-1 receptor contributed to FVIIa/TF-induced hyperchemotaxis, we incubated PAE/PDGFR β ,TF cells with thrombin (9 nM). Neither thrombin ($p=0.14$ versus control) nor 5 U/ml of the specific thrombin inhibitor hirudin affected the migration in response to PDGF-BB and FVIIa (Fig. 4). Incubation of the cells with 50 μ M of the PAR-1 agonist SFLLRN had a slight, but non-significant, inhibitory effect on the migration response towards 0.1 ng/ml PDGF-BB (Fig. 4).

Formation of the ternary TF-FVIIa-FXa complex is reported under some conditions to further enhance FVIIa/TF-mediated cellular responses by a presumed PAR-1-dependent mechanism (31, 32). We therefore studied the chemotactic response of PAE/PDGFR β ,TF cells at a low concentration (0.1 ng/ml) of PDGF-BB and compared the stimulation with a) 100 nM FVIIa, b) 20 nM FXa and c) a combination of 5 nM FVIIa and 100 nM FXa to create the ternary complex. Neither FXa alone, nor the ternary TF-FVIIa-FXa complex affected the chemotactic response significantly (Fig. 4). To investigate the possible involvement of

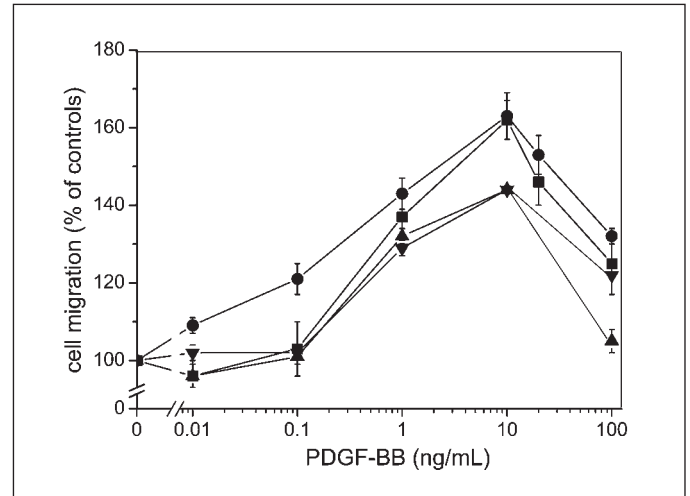


Figure 2: Effects of physiological concentration of FVIIa on PDGF-BB-induced chemotaxis in PAE cells transfected with human wild-type TF or human TF with deleted cytoplasmic tail. The chemotactic response at different concentrations of PDGF-BB was determined in the absence (PAE/PDGFR β ,TF square or PAE/PDGFR β ,TF Δ cyto up triangle) or presence of 10 nM FVIIa (PAE/PDGFR β ,TF circle or PAE/PDGFR β ,TF Δ cyto down triangle). Results are mean \pm SEM of three separate experiments.

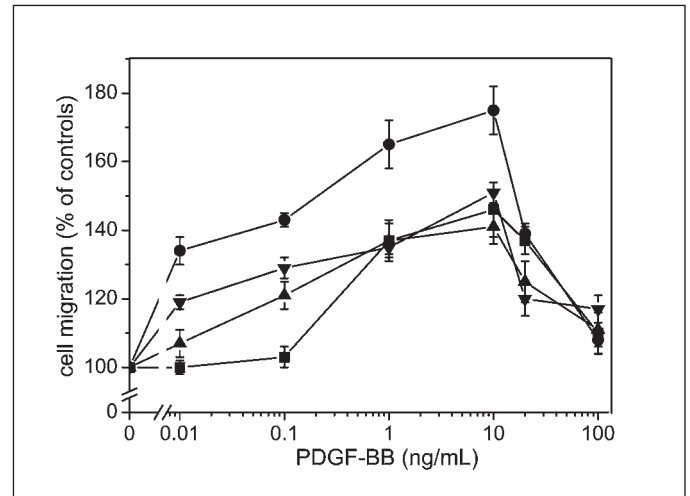


Figure 3: Importance of serine residues in the cytoplasmic tail of TF on the chemotactic response to PDGF-BB influenced by FVIIa in transfected PAE cells. All cells were incubated with 100 nM FVIIa. Chemotaxis was induced by different concentrations of PDGF-BB. The PAE cells were transfected with human wtPDGFR β and wtTF (circle), TF with intracellular serine residues mutated (PAE/PDGFR β ,TF3SA, down triangle) TF with serine 258 changed to alanine (PAE/PDGFR β ,TFS258A, up triangle) or TF with deleted cytoplasmic tail (PAE/PDGFR β ,TF Δ cyto, square). Results are mean \pm SEM of three separate experiments.

PAR-2, the receptor surface expression on the PAE/PDGFR β ,TF,TF Δ cyto and TFS258A cells was analyzed by flow cytometry using the PAR-2 antibody SAM 11. Data from these experiments show comparable PAR-2 expression on the PAE cell-clones, but at a low level (Fig. 5A). The PAR-2 agonist SLIGKV did enhance

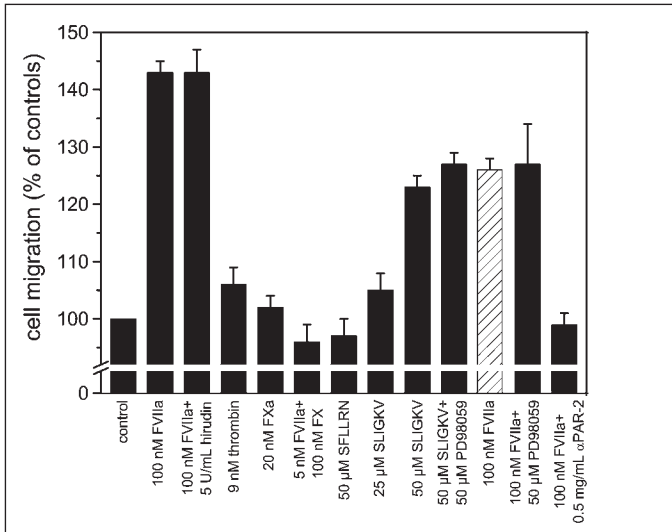


Figure 4: Effects of stimulation of PAR-1/2 on PDGF-BB-induced chemotaxis in double transfected PAE cells. PAE/PDGFR β cells were transfected with wild-type TF. Cells were stimulated by (i) 100 nM FVIIa, (n=10), (ii) 100 nM FVIIa in the presence of 5 U/mL Hirudin, (iii) 9 nM thrombin, (iv) 20 nM FXa, (v) 5 nM FVIIa+100 nM FX, (n=8), (vi) 50 μ M SFLLRN, (vii) 25 μ M or 50 μ M SLIGKV, (viii) 50 μ M SLIGKV and 50 μ M PD98059 (ix) 100 nM FVIIa in the absence (striated staple) or presence of 50 μ M PD98059, (x) 0.5 mg/ml PAR-2 neutralizing ab plus 100 nM FVIIa. In all experiments cells migrated towards 0.1 ng/ml of PDGF-BB. For each set of experiments, the motility response of the cells to 0.1 ng/ml PDGF-BB in the absence of ligand-stimulation, served as a control. Each data point represents three separate experiments if not stated otherwise. Number of experiments = n. The results are mean \pm SEM.

chemotaxis towards 0.01–1 ng/ml PDGF-BB in a dose-dependent way, significant at 50 μ M, ($p < 0.001$ versus control) (Fig. 4). We moreover incubated the PAE/PDGFR β ,TF cells with a PAR-2 neutralizing antibody and stimulated with 100 nM FVIIa in an experiment where the cells migrated towards 0.1 ng/ml of PDGF-BB. The hyperchemotactic response was totally abolished by the antibody (Fig. 4). The results with the PAR-2 agonist and with the PAR-2 neutralizing antibody were confirmed in similar experiments replacing PAE cells with human TF-expressing fibroblasts, 1064Sk (33)(data not shown).

The hyperchemotactic response is independent on Erk/MAPK activation

Previous results have shown that FVIIa stimulation resulted in phosphorylation of the Erk/MAP kinase and that this was totally blocked by 50 μ M of MEK-inhibitor PD98059 (11). Furthermore, the Erk/MAP kinase activation was demonstrated to occur independent of the presence of TF cytoplasmic domain (12). To investigate whether the PAR-2-dependent FVIIa/TF-induced chemotactic response involved the activation of Erk/MAP kinase pathway, we preincubated PAE/PDGFR β ,TF cells with 50 μ M of the MEK inhibitor PD98059 for one hour, and subsequently stimulated the cells with 100 nM FVIIa or 50 μ M SLIGKV. We then analyzed the chemotactic response after 6 hours migration towards a concentration gradient of 0.01–100 ng/ml of PDGF-BB in the Boyden chamber. The enhanced mi-

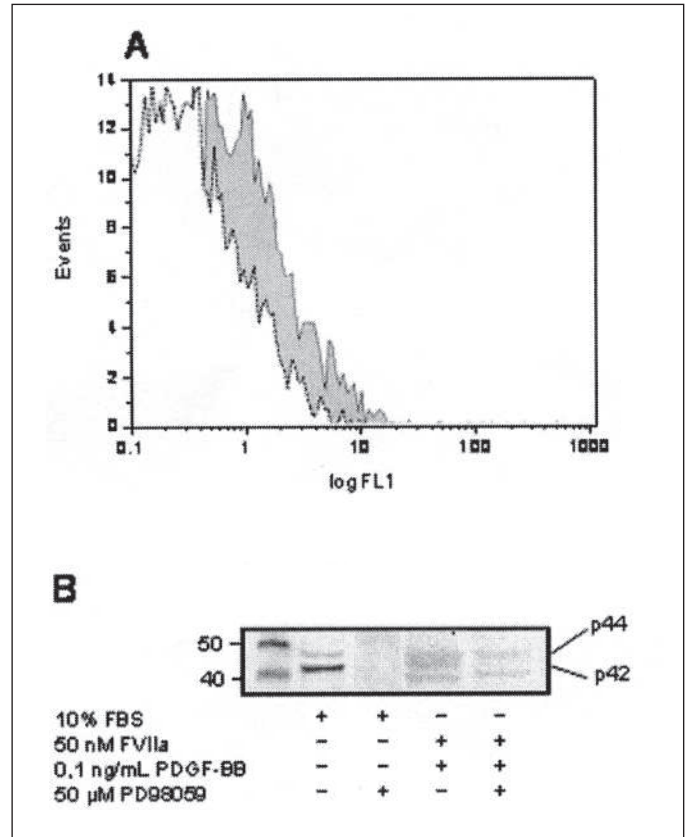


Figure 5: Flow cytometric analysis of PAR-2 expression and Erk/MAPK phosphorylation in in double transfected PAE cells. (A) PAE/PDGFR β ,TF, PAE/PDGFR β ,TF Δ cyto or PAE/PDGFR β ,TFS258A cells were stained with either a monoclonal anti-IgG1 antibody that was used as a negative control (unfilled area) or a human PAR-2 antibody (filled area). All clones expressed similar levels of PAR-2. (B) PAE/PDGFR β ,TF cells were incubated with FBS, FVIIa and PDGF-BB with or without preincubation by PD98059. Erk/MAPK phosphorylation was determined by Western blotting with a phosphorylation-specific antibody. The results are one out of three representative experiments.

gration response of cells induced by FVIIa/TF or SLIGKV was unaffected by PD98059 (Fig. 4). PAE/PDGFR β ,TF cells incubated with 50 nM FVIIa plus 0.1 ng/ml PDGF-BB induced Erk/MAPK phosphorylation (Fig. 5B), which was totally blocked by the MEK inhibitor PD98059.

Discussion

In this study we used PAE/PDGFR β cells transfected with human wild-type TF to demonstrate that FVIIa induces signal transduction resulting in stimulation of a chemotactic response to PDGF-BB. At the physiological concentration of 10 nM, FVIIa induced chemotaxis towards PDGF-BB at a 100-fold lower concentration than in the absence of FVIIa. This observation confirms our previous results with human fibroblasts and human vascular smooth muscle cells (25). Taken together, these data suggest that stimulation of chemotaxis towards PDGF-BB by the proteolytically active FVIIa/TF-complex may constitute a more general mechanism of importance for the biology of TF-expressing cells involved in tissue repair, cancer invasion, angio-

genesis and atherosclerosis. Cell migration plays an active role in these processes and many cells involved express both TF and PDGF β -receptors and also secrete PDGF-BB (1, 29). We now report for the first time that the cytoplasmic domain of TF is required to potentiate the chemotactic response to low concentrations of PDGF-BB. This was evident by the absence of a FVIIa-dependent enhancement effect on the migration of PAE/PDGFR β cells transfected with a construct lacking the intracellular part of TF. The two serine residues 253 and 258 in the cytoplasmic domain of TF have been shown to be rapidly phosphorylated upon stimulation with PMA in a protein kinase C (PKC)-dependent way (34, 35). The functional significance of TF phosphorylation and the biological effects mediated by the cytoplasmic domain are, however, largely unknown. In our experimental model, with PAE/PDGFR β cells transfected with a TF with alanine substitutions for serine 258, the migration response was significantly reduced. This is in line with previous studies which indicated a distinct function of the cytoplasmic domain of TF in both angiogenesis and metastasis (20–24). For full hematogenous metastatic effect of TF-transfected melanoma cells, the cytoplasmic domain of TF was required and at least one of the phosphorylatable serine residues in the cytoplasmic domain (23). Concerning tumor-associated angiogenesis, transfection of melanoma cells with full length TF resulted in VEGF-production, whereas, in contrast, transfection with a cytoplasmic TF mutant missing the serine residues at positions 253, 258 and 263 resulted in little or no VEGF-production (24). Thus, it seems that FVIIa/TF-induced VEGF production in these cells is mediated by the cytoplasmic part of TF in a PKC-dependent manner. Mice with the cytoplasmic domain of TF deleted (TF $^{\delta CT/\delta CT}$) develop normally and have normal coagulation function (36). In contrast the results obtained upon injection of endotoxin in these mice, clearly indicate that the cytoplasmic domain of TF contributes to leukocyte recruitment and activation (37). Finally, in a very recent report Yang et al. (19) showed that arthritis severity, including synovitis, was significantly reduced in TF $^{\delta CT/\delta CT}$ mice. The results generated so far concerning the significance of TF cytoplasmic domain-mediated biological responses, suggest a role in processes where cell migration is affected, such as tissue repair, inflammation, angiogenesis and cancer cell invasion.

The Erk/MAP kinase signaling pathway has been identified as a possible route for FVIIa/TF-mediated gene transcription (15, 18, 38). Signal transduction by the Erk/MAP kinase pathway is induced by the proteolytic activity of FVIIa. Signaling is prevented by an inhibitor of the upstream MAP kinase kinase, MEK, but is independent of the TF cytoplasmic domain (12, 36). In the present study, inhibition of MEK, although it prevented Erk/MAP kinase phosphorylation, was without an effect on migration, suggesting that activation of this pathway was not required for the FVIIa/TF-dependent stimulation. In contrast, inhibition of PAR-2 cleavage by the agonist abolished hyperchemotaxis. We have previously shown that the hyperchemotactic response induced by FVIIa/TF complex is dependent on PLC activity (25). The combination of these results are consistent with a new report, where PAR-2 at a fairly low expression level, was shown to induce sustained PKC α membrane recruitment leading to TF cytoplasmic domain phosphorylation (32). Also, phosphorylation of the TF cytoplasmic domain seems to promote

PAR-2-dependent angiogenesis (39). Thus, a possible cross-talk between PAR-2 signaling and the TF cytoplasmic part may influence the downstream signaling that leads to processes involving cell migration. Stimulation of PAR-1 by thrombin, FXa and FXa generated on the cell surface by FVIIa/TF, had no effect on the chemotactic response. In this context, a slight but constant reduction of the chemotactic response to all concentrations of PDGF-BB was obtained with the PAR-1 agonist. Notably, activation of PAR-1 was recently identified to inhibit cellular migration (40, 41). Whether PAR-1 activation affects the mechanisms that regulate cellular migration remains to be elucidated.

The requirement of the TF cytoplasmic domain in FVIIa/TF-induced hyperchemotaxis could be linked to the interaction with the cytoskeleton. TF is endowed with high affinity binding sites for the actin-binding protein (ABP)-280, which has been shown to be recruited to the tail upon extracellular ligation leading to reorganization of the actin cytoskeleton (42–44). Molecular interaction between TF and ABP-280 subsequently induces phosphorylation of focal adhesion kinase (FAK), a protein kinase crucial for cell movement (45, 46). Further downstream candidate signaling molecules remain to be identified. Based on this, we hypothesize that an alternative 'TF receptor' signal transduction pathway promotes the enhanced motility response to PDGF-BB.

Cell migration and invasion are essential in a variety of physiological and pathophysiological processes in the body, such as embryogenesis, angiogenesis, wound healing, inflammation, cancer and cardiovascular diseases. Much the same molecular mechanisms that move the cells are utilized in these motility processes (47). In our experimental set-up for chemotaxis, directed cell migration, we use a 150 μ m thick nitrocellulose filter to separate the two compartments of the Boyden chamber. Cells are stimulated by FVIIa and put above the filter, with the chemoattractant, PDGF-BB, in the lower compartment below the filter. With this type of filters the concentration gradient of the chemoattractant remains for more than 6 hours as measured by radiolabeled PDGF-BB (manuscript in preparation). The cells migrate towards the gradient and stay into the filters during the experiment. Calculation of cell migration is a translocation over a clearly defined distance (48). Other groups have demonstrated migration response to FVIIa alone or FVIIa in complex with TF as chemoattractants (41, 49, 50). In the assays used in these reports the cells migrated for 6–24 hours through a 8 μ m thin filter of polycarbonate with straight pores. The adherent cells on the lower side of the filters were counted. One of these reports found that the combination of FVIIa and FXa promoted migration in a breast cancer cell line, but not physiological concentrations of FVIIa alone (41). With another breast cancer cell line, the migration response was entirely dependent on FVIIa/TF-induced IL-8 production (50). Thus, it is important to point out that our results do not contradict those of other authors. Rather, considering all these observations, it appears that extracellular ligation of TF can elicit at least three different signal transduction pathways, dependent and independent of the generation of activated coagulation factors, resulting in the process of migration/invasion.

In conclusion, the present results provide evidence that the TF cytoplasmic domain is critical for migration induced at physiological concentrations of FVIIa bonded to TF. Our data are also in accordance with the idea that the proteolytic activity of

the FVIIa/TF complex links to phosphorylation of the TF cytoplasmic domain and cytoskeletal reorganization. Future studies will elucidate the cellular functions and processes induced by this signaling pathway. Therapy concepts with molecular targeting of FVIIa/TF pathways leading to cell migration may be of potential benefit in a number of diseases.

References

- Camerer E, Kolsto AB, Prydz H. Cell biology of tissue factor, the principal initiator of blood coagulation. *Thromb Res* 1996; 81: 1–41.
- Rao LV. Tissue factor as a tumor procoagulant. *Cancer Metastasis Rev* 1992; 11: 249–66.
- Hamada K, Kuratsu J, Saitoh Y, et al. Expression of tissue factor correlates with grade of malignancy in human glioma. *Cancer* 1996; 77: 1877–83.
- Shigemori C, Wada H, Matsumoto K, et al. Tissue factor expression and metastatic potential of colorectal cancer. *Thromb Haemost* 1998; 80: 894–8.
- Siegbahn A. Cellular consequences upon factor VIIa binding to tissue factor. *Haemostasis* 2000; 30 S2: 41–7.
- Morrissey JH. Tissue factor: an enzyme cofactor and a true receptor. *Thromb Haemost* 2001; 86: 66–74.
- Chen J, et al. Tissue factor – A receptor involved in the control of cellular properties, including angiogenesis. *Thromb Haemost* 2001; 86: 334–45.
- Sorensen BB, et al. Antiapoptotic effect of coagulation factor VIIa. *Blood* 2003; 102: 1708–15.
- Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci U S A* 1990; 87: 6934–8.
- Camerer E, Rottingen JA, Iversen JG, et al. Coagulation factors VII and X induce Ca²⁺ oscillations in Madin-Darby canine kidney cells only when proteolytically active. *J Biol Chem* 1996; 271: 29034–42.
- Poulsen LK, Jacobsen N, Sorensen BB, et al. Signal transduction via the mitogen-activated protein kinase pathway induced by binding of coagulation factor VIIa to tissue factor. *J Biol Chem* 1998; 273: 6228–32.
- Sorensen BB, Freskgard PO, Nielsen LS, et al. Factor VIIa-induced p44/42 mitogen-activated protein kinase activation requires the proteolytic activity of factor VIIa and is independent of the tissue factor cytoplasmic domain. *J Biol Chem* 1999; 274: 21349–54.
- Camerer E, et al. Coagulation factors VIIa and Xa induce cell signaling leading to up-regulation of the *egr-1* gene. *J Biol Chem* 1999; 274: 32225–33.
- Pendurthi UR, et al. Binding of factor VIIa to tissue factor induces alterations in gene expression in human fibroblast cells: up-regulation of poly(A) polymerase. *Proc Natl Acad Sci U S A* 1997; 94: 12598–603.
- Pendurthi UR, Allen KE, Ezban M, et al. Factor VIIa and thrombin induce the expression of Cyr61 and connective tissue growth factor, extracellular matrix signaling proteins that could act as possible downstream mediators in factor VIIa x tissue factor-induced signal transduction. *J Biol Chem* 2000; 275: 14632–41.
- Versteeg HH, Sorensen BB, Slofstra SH, et al. VIIa/tissue factor interaction results in a tissue factor cytoplasmic domain-independent activation of protein synthesis, p70, and p90 S6 kinase phosphorylation. *J Biol Chem* 2002; 277: 27065–72.
- Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci U S A* 2000; 97: 5255–60.
- Ruf W, et al. Specificity of coagulation factor signaling. *J Thromb Haemost* 2003; 1: 1495–503.
- Yang YH, Hall P, Milenkovski G, et al. Reduction in arthritis severity and modulation of immune function in tissue factor cytoplasmic domain mutant mice. *Am J Pathol* 2004; 164: 109–17.
- Mueller BM, Reisfeld RA, Edgington TS, et al. Expression of Tissue Factor by Melanoma-Cells Promotes Efficient Hematogenous Metastasis. *Proc Natl Acad Sci U S A* 1992; 89: 11832–6.
- Mueller BM, Ruf W. Requirement for binding of catalytically active factor VIIa in tissue factor-dependent experimental metastasis. *J Clin Invest* 1998; 101: 1372–8.
- Bromberg ME, Konigsberg WH, Madison JF, et al. Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. *Proc Natl Acad Sci U S A* 1995; 92: 8205–9.
- Bromberg ME, Sundaram R, Homer RJ, et al. Role of tissue factor in metastasis: functions of the cytoplasmic and extracellular domains of the molecule. *Thromb Haemost* 1999; 82: 88–92.
- Abe K, Shoji M, Chen J, et al. Regulation of vascular endothelial growth factor production and angiogenesis by the cytoplasmic tail of tissue factor. *Proc Natl Acad Sci U S A* 1999; 96: 8663–8.
- Siegbahn A, et al. Binding of factor VIIa to tissue factor on human fibroblasts leads to activation of phospholipase C and enhanced PDGF-BB-stimulated chemotaxis. *Blood* 2000; 96: 3452–8.
- Ronnstrand L, et al. Overactivation of phospholipase C-gamma 1 renders platelet-derived growth factor beta-receptor-expressing cells independent of the phosphatidylinositol 3-kinase pathway for chemotaxis. *J Biol Chem* 1999; 274: 22089–94.
- Sorensen BB, et al. Incorporation of an active site inhibitor in factor VIIa alters the affinity for tissue factor. *J Biol Chem* 1997; 272: 11863–8.
- Siegbahn A, et al. Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. *J Clin Invest* 1990; 85: 916–20.
- Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Phys Rev* 1999; 79: 1283–316.
- Petersen LC, et al. Exclusion of known protease-activated receptors in factor VIIa-induced signal transduction. *Thromb Haemost* 2000; 83: 571–6.
- Riewald M, Ruf W. Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. *Proc Natl Acad Sci U S A* 2001; 98: 7742–7.
- Ahamed J, Ruf W. Protease-activated receptor 2-dependent phosphorylation of the tissue factor cytoplasmic domain. *J Biol Chem* 2004; 279: 23038–44.
- Malarstig A, Tenno T, Jossan S, et al. A quantitative real-time PCR method for tissue factor mRNA. *Thromb Res* 2003; 112: 175–83.
- Zioncheck TF, Roy S, Vehar GA. The cytoplasmic domain of tissue factor is phosphorylated by a protein kinase C-dependent mechanism. *J Biol Chem* 1992; 267: 3561–4.
- Dorflautner A, Ruf W. Regulation of tissue factor cytoplasmic domain phosphorylation by palmitoylation. *Blood* 2003; 102: 3998–4005.
- Melis E, Moons L, De Mol M, et al. Targeted deletion of the cytosolic domain of tissue factor in mice does not affect development. *Biochem Biophys Res Commun* 2001; 286: 580–6.
- Sharma L, et al. The cytoplasmic domain of tissue factor contributes to leukocyte recruitment and death in endotoxemia. *Am J Pathol* 2004; 165: 331–40.
- Camerer E, Gjernes E, Wiiger M, et al. Binding of factor VIIa to tissue factor on keratinocytes induces gene expression. *J Biol Chem* 2000; 275: 6580–5.
- Belting M, Dorrell MI, Sandgren S, et al. Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nat Med* 2004; 10: 502–9.
- Kamath L, et al. Signaling from protease-activated receptor-1 inhibits migration and invasion of breast cancer cells. *Cancer Res* 2001; 61: 5933–40.
- Jiang X, Bailly MA, Panetti TS, et al. Formation of tissue factor-factor VIIa-factor Xa complex promotes cellular signaling and migration of human breast cancer cells. *J Thromb Haemost* 2004; 2: 93–101.
- Ott I, et al. A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280. *J Cell Biol* 1998; 140: 1241–53.
- Cunningham CC, Gorlin JB, Kwiatkowski DJ, et al. Actin-binding protein requirement for cortical stability and efficient locomotion. *Science* 1992; 255: 325–7.
- Stossel TP, Condeelis J, Cooley L, et al. Filamins as integrators of cell mechanics and signalling. *Nat Rev Molecular Cell Biol* 2001; 2: 138–45.
- Ilic D, Furuta Y, Kanazawa S, et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 1995; 377: 539–44.
- Goldmann WH. p56(lck) controls phosphorylation of filamin (ABP-280) and regulates focal adhesion kinase (pp125(FAK)). *Cell Biol Int* 2002; 26: 567–71.
- Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. *Cell* 1996; 84: 359–69.
- Manske M, Bade EG. Growth factor-induced cell-migration – biology and methods of analysis. *Int Rev Cytol* 1994; 155: 49–96.
- Marutsuka K, Hatakeyama K, Sato Y, et al. Protease-activated receptor 2 (PAR2) mediates vascular smooth muscle cell migration induced by tissue factor/factor VIIa complex. *Thromb Res* 2002; 107: 271–6.
- Hjortoe GM, et al. Tissue factor-factor VIIa specific up-regulation of IL-8 expression in MDA-MB-231 cells is mediated via PAR-2 and results in increased cell migration. *Blood* 2004; 103: 3029–37.