Suppression of Breast Cancer Progression by Tissue Factor

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Tissue Factor (TF) is the cell surface receptor that activates coagulation by binding the serine protease coagulation factor VIIa (VIIa). The activation of the coagulation cascade leads to thrombin generation, fibrin formation and platelet activation which together may aide tumor growth and metastasis. TF is released from tumor cells by shedding or TF comes in contact with coagulation factors when tumor cells enter the blood stream, leading to a hypercoagulable state and its clinical manifestation of spontaneous thrombosis (Trousseau’s Syndrome) that occurs in several types of cancer. This provides clear evidence that TF is a frequent marker of advanced cancer. This project proposes to elucidate mechanisms that underlie the seemingly paradoxical observation that TF which supports metastasis under certain conditions may actually delay tumor development and local spread in breast cancer. The basic hypothesis pursued here is that the complex signaling functions of the TF cytoplasmic domain contribute to this paradox. We hope to discover new avenues to specifically interrupt breast cancer progression by unraveling the role of TF cytoplasmic domain signaling in cancer cells.
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Introduction

Tissue Factor (TF) is the cell surface receptor that activates coagulation by binding the serine protease coagulation factor VIIa (VIIa). The activation of the coagulation cascade leads to thrombin generation, fibrin formation and platelet activation which together may aide tumor growth and metastasis. TF is released from tumor cells by shedding or TF comes in contact with coagulation factors when tumor cells enter the blood stream, leading to a hypercoagulable state and its clinical manifestation of spontaneous thrombosis (Trousseau’s Syndrome) that occurs in several types of cancer. This provides clear evidence that TF is a frequent marker of advanced cancer. This project proposes to elucidate mechanisms that underlie the seemingly paradoxical observation that TF which supports metastasis under certain conditions may actually delay tumor development and local spread in breast cancer. The basic hypothesis pursued here is that the complex signaling functions of the TF cytoplasmic domain contribute to this paradox. We hope to discover new avenues to specifically interrupt breast cancer progression by unraveling the role of TF cytoplasmic domain signaling in cancer cells.

Body

This application has proposed two specific aims. Aim 1 is to test in A7 melanoma cells whether TF suppresses tumor growth by TF cytoplasmic domain signaling and whether WW-domain proteins are involved in this suppression by binding to the phosphorylated TF cytoplasmic domain. The review committee was concerned by the use of a melanoma cell line to study mechanisms of breast cancer development. We have responded to this conceptual weakness in continuing our survey to identify a TF-negative breast cancer cells line. We were fortunate to identify two such clones that were established in the laboratory of our colleague Dr. B. Felding-Habermann here at Scripps. These breast cancer lines are known to produce tumors in immuno-deficient mice and metastasize aggressively. Theses biological properties suggest that these cell lines may be a suitable model to study mechanisms by which TF suppresses the development of breast cancer. We made the decision to continue our studies with these cell lines, instead of melanoma cells. Fig. 1 shows FACS analysis with anti-TF antibody of these cell lines, named BMS and BCM1. Both cell lines were transfected, as proposed originally, with an expression plasmid for human TF that can be regulated by tetracycline. Stable lines were successfully generated and addition of tetracycline suppressed TF expression in BMS/TF cells by 95% and in BCM1/TF cells by 84% (Fig. 1). Tumor growth experiments are currently ongoing in mice that do or do not receive
tetracycline in their drinking water. In this experiment, tumor growth curves for both cell lines are established and tumors are isolated upon termination of the observation period to confirm TF expression levels in the tumors. This is important to exclude a survival advantage of TF negative escape mutants. The suppression of TF expression by tetracycline administration is further confirmed.

We have recently applied our phosphorylation-specific antibody against the TF cytoplasmic domain (1) to the analysis of human pathological specimens. In ocular, diabetic neovascularization we have made the discovery that TF phosphorylation is a hallmark of TF expressed under pathological conditions, but not in normal tissues (2). We also demonstrated in vitro that the TF cytoplasmic domain becomes phosphorylated when breast cancer cells are stimulated through PAR2 (3), established in vivo as the signaling receptor for TF (2). Thus, tools are available to directly test whether TF becomes phosphorylated in breast cancer cells. Therefore, specimens of the currently growing tumors are also embedded for frozen sections and the phosphorylation status of TF in our breast cancer model is determined. We are continuing the proposed studies with these newly established breast cancer models with the hope to have a model of highest relevance for breast cancer biology.

Aim 2 is to generate tumor prone animals by crossing hormone sensitive C3-TAg mice with TF cytoplasmic domain deleted (TF$^{ACD/ACD}$) mice. These crosses are progressing according to schedule. We have recently obtained male founders that lack the TF cytoplasmic domain and carry the C3-TAg transgene (Fig. 2). We have initiated crosses of these mice with ~20 female TF$^{ACD/ACD}$. Breeding of the control group was also initiated. To this end, C3-TAg wild-type mice are crossed with wild-type mice from our colony. We expect to establish a cohort of sufficient size for observation of tumor incidence and development in the upcoming year, as proposed.

**Key Research Accomplishments**

- Identified TF negative breast cancer cells
- Established breast cancer cell lines with tetracycline regulated TF expression
- Generated TF$^{ACD/ACD}$/C3-TAg founders and initiated breeding program
Reportable Outcomes

Our recent paper has established that the TF cytoplasmic domain is phosphorylated downstream of PAR2 signaling in breast cancer cells (Ahamed & Ruf. J.Biol.Chem. 279:23038, 2004)

Conclusions

We have made progress in identifying a novel cell model of potential utility to study the role of TF in breast cancer. We continue further studies in this model to conform with the recommendations of the review committee. Generation of breast cancer prone mouse strains progresses on schedule.

References


Protease-activated Receptor 2-dependent Phosphorylation of the Tissue Factor Cytoplasmic Domain*

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Tissue factor (TF) is the physiological activator of the coagulation cascade that plays pathophysiological roles in metastasis, angiogenesis, and inflammation. Downstream in coagulation, thrombin is the central protease that signals through G protein-coupled, protease-activated receptors (PARs). However, the TF-VIIa-Xa complex upstream in coagulation also activates PAR1 and 2. Here, we address the question of whether signaling of the TF initiation complex is a relevant pathway that leads to TF cytoplasmic domain phosphorylation. In heterologous expression systems and primary endothelial cells, we demonstrate that the ternary TF-VIIa-Xa complex induces TF phosphorylation specifically by activating PAR2 but not PAR1 signaling. In addition, TF cytoplasmic domain phosphorylation is induced only by TF-dependent signaling but not by other coagulation factors in endothelial cells. Phosphorylation of the Pro-directed kinase target site Ser258 is dependent on prior phosphorylation of Ser253 by protein kinase C (PKC) α. TF phosphorylation is somewhat delayed and coincides with sustained PKCa activation downstream of PAR2 but not PAR1 signaling. Phosphatidylinositol-choline-dependent phospholipase C is the major pathway that leads to prolonged PKCa recruitment downstream of PAR2. Thus, PAR2 signaling specifically phosphorylates TF in a receptor cross-talk that distinguishes upstream from downstream coagulation protease signaling.

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1 The abbreviations used are: PAR, protease-activated receptor; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; NAP, nematode anticoagulant protein; TF, tissue factor; ERK, extracellular signal-regulated kinase; TFCD, TF cytoplasmic domain; HUVEC, human umbilical vein endothelial cells; PI, phosphatidylinositol; PC, phosphatidylcholine; PLC, phospholipase C.
phase of coagulation and thus distinguishes PAR signaling in the upstream and downstream coagulation reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Vili, inactive Ser<sup>165</sup> to Ala Vili (Vili), factors Xa, X and Xa, thrombin, and PAR agonist peptides TFLLRNPDNK (PAR1) and SLIGRL (PAR2) were as described (8, 9, 23). A polyclonal goat antibody to the human TF extracellular domain and rabbit antibodies to the phosphorylated (α-TFCD) of nonphosphorylated (α-TFCD) TF cytoplasmic domain were characterized in detail (22). Sequencing grade Hispidin were purchased from Calbiochem (San Diego, CA). Anti-PAR1 antibodies ATAP2 and WEDE15 were kindly provided by Dr. L. F. Brass (24). Recombinant mouse anticoagulant proteins 5 (NAP5) and ε2 (NAPε2) (25, 26) were kindly provided by Dr. G. Vlaskov (Corvas International, San Diego, CA). Wild-type and kinase inactive PKCa (K368N) constructs were gifts from Dr. Alex Toker (Boston, MA).

**Cell Culture and Transient Transfection**—Chinese hamster ovary (CHO) cells expressing full-length TF (CHO/TF cells) (27) were transiently transfected by electroporation. Cells (2 × 10<sup>5</sup>) in 200 μl of Dulbecco's modified Eagle's medium, 2% fetal bovine serum, 1 mM EDTA aprotinin (5 μg/ml), leupeptin (10 μg/ml), 1 mM Tris-HCl, pH 7.6, 1 mM EDTA, and the Xa concentrations were determined by hemoglobin assay using equal protein loading for Western blotting with anti-PKCa antibodies. The experiments were performed 48 h after transfection.

**Adenovirus Vectors and Transduction of Endothelial Cells**—Ad5 adenovirus particles were used to transduce endothelial cells (HUVEC) obtained from Clonetics (San Diego, CA) or Cascade Biologics (Portland, OR) and cultured in EGM medium containing 2–5% fetal bovine serum, 1 mg/ml hydrocortisone, 10 μg/ml human epidermal growth factor, and 3 mg/ml bovine brain extract. HUVECs were transiently transfected with either human PARI or human PAR2. We have shown previously that PARI or PAR2 expression in CHO cells is sufficient to induce TF cytoplasmic domain phosphorylation only in cells that expressed cleavage-sensitive PAR2. Thus, cleavage of PARI by the ternary TF-VIIa-Xa complex induces TF cytoplasmic domain phosphorylation, and simply forming the ternary complex is not sufficient to induce TF cytoplasmic domain phosphorylation in PAR2-expressing cells.

**RESULTS**

**TF Cytoplasmic Domain Phosphorylation Is Dependent on PAR2 Signaling**—Because endogenous PAR1 was co-expressed in PAR2-transfected CHO cells, it remained unclear whether PAR1 is required, albeit not sufficient to trigger TF phosphorylation. To address this question, fibroblasts from PAR1-deficient mice that also lack other PARs (9, 19) were used. PMA-stimulated TF cytoplasmic domain phosphorylation is negatively regulated by palmitoylation of the cytoplasmic Cys<sup>382</sup> residue in endothelial cells (22), and mutation of Cys<sup>382</sup> greatly facilitates the detection of TF cytoplasmic domain phosphorylation. PAR-1-deficient fibroblasts were co-transduced with adenovirus to express human Cys<sup>382</sup> to Ser mutated TF together with PAR2 or PAR1, achieving expression levels shown in Fig. 2A. The cells were stimulated with PAR1 or PAR2 agonist peptides, and with thrombin, trypsin, or ternary complex as relevant proteases that activate PARs. No TF phosphorylation was observed in PAR1-expressing cells, but TF cytoplasmic domain phosphorylation was observed in PAR2-transduced cells stimulated with appropriate PAR2 agonists (Fig. 2B). These data demonstrate that endogenous PAR1 is not required in the signaling pathway that phosphorylates TF. Note the more quickly migrating form of TF in the loading.
PAR2-mediated TF Phosphorylation

Fig. 1. TF cytoplasmic domain phosphorylation is mediated by PAR2 signaling in heterologous expression systems. A, serum-starved CHoT cells were stimulated with 10 µM PAR1 agonist, 100 µM PAR2 agonist, or PMA (20 ng/ml) for 1 h. Immunoprecipitated TF was detected by Western blotting with anti-phosphorylated TF cytoplasmic domain antibody (α-TFCD) or pan-specific anti-TF cytoplasmic domain antibody (αTFCD). B, CHO/TF cells stably expressing a noncleavable mutant of PAR2 (CHO/TF/PAR2/P1) or wild-type PAR2 (CHO/TF/PAR2) were stimulated with PAR2 agonist peptide or NAPc2-stabilized ternary TF-VIIa-Xa complex (VIIa/Xa/NAPc2 10/20/100 nM) for 1 h, followed by Western blotting for TF phosphorylation. Loading was controlled by reprobing with pan-specific anti-TF cytoplasmic domain antibody. C, transiently transfected CHO/TF cells were analyzed for human PAR expression by flow cytometry using WEDE15 or SAMI1 monoclonal antibodies to PARI or PAR2, respectively. Controls were stained with isotype matched primary antibody. D, CHO/TF cells transiently expressing human PAR1 or PAR2 were stimulated with ternary complex or PAR agonists for 10 min for ERK1/2 phosphorylation or for 1 h for TF cytoplasmic domain phosphorylation. The loading control in the top panel was reprobed and the anti-ERK1/2 blot were performed on separate transfers. E, left panel, quantitation of receptor internalization after 30 min (37 °C) stimulation by ternary complex or PAR agonist of CHO/TF cells transiently transfected with human PARI or PAR2 (means and standard deviations, n = 3). Right panel, the increase in TF cytoplasmic domain phosphorylation, as shown in D, was quantified by laser densitometry (means and standard deviations, n = 3). FITC, fluorescein isothiocyanate.

control that was performed by reprobing with pan-specific anti-TF cytoplasmic domain antibody. We had previously found in PMA-stimulated cells that certain N-linked carbohydrate isoforms of TF cannot be phosphorylated (22). The lack of phosphorylation of the more quickly migrating forms of TF in this and other experiments shown below indicates that Golgi-dependent carbohydrate modification, and thus presumably a certain subcellular protein trafficking route, is also important for phosphorylation of the TF cytoplasmic domain in response to PAR2 activation.

TF Cytoplasmic Domain Phosphorylation of Endogenously Expressed TF—To provide evidence that endogenously expressed TF can be phosphorylated downstream of PAR2 signaling, we studied MDA-MB-231 cells. Under our culture conditions, this breast cancer cell line expressed fairly low levels of cell surface-expressed PAR2 relative to PARI. Thus, in cells that express endogenous TF, PAR1, and PAR2, TF cytoplasmic domain phosphorylation occurs specifically downstream of PAR2 signaling.

TF Cytoplasmic Domain Phosphorylation Is Specific for Upstream Coagulation Signaling in Primary Cells—The relative importance of PAR1 and PAR2 signaling in TF cytoplasmic domain phosphorylation was analyzed in primary HUVECs transduced with adenovirus to express TF and variable levels of PAR1 and PAR2. HUVECs express endogenous PAR1 (Fig. 3A, UT) and very little PAR2 (data not shown). HUVECs were adenovirus transduced to achieve comparable expression levels of PAR1 or PAR2. PAR activation was confirmed by measuring internalization by flow cytometry and stimulation with the NAPc2 stabilized ternary complex similarly induced internalization of both receptors (Fig. 3B). With both PAR1 and PAR2, internalization by TF-dependent signaling was somewhat less efficient when compared with the cleavage by thrombin and trypsin, respectively.

Consistent with data in the CHO cell model, activation of...
endogenous PAR1 (not shown) or overexpressed PAR1 did not induce TF phosphorylation (Fig. 3C). In contrast, introducing PAR2 with TF resulted in TF cytoplasmic domain phosphorylation after 10 min of stimulation with ternary complex, PAR2 agonist, or trypsin. Co-expression of PAR2 with Cys²⁴⁵ to Ser mutated TF at similar levels as wild-type TF resulted in significantly enhanced TF cytoplasmic domain phosphorylation after 10 min of stimulation (Fig. 3C) and PAR1 stimulation was also not sufficient to induce phosphorylation of this mutant (data not shown). The differences in TF phosphorylation between wild-type and mutant TF were quantified by densitometry, using normalization for slight differences in expression levels. TF was phosphorylated ~7-fold more efficiently when thioester modification of Cys²⁴⁵ was prevented by mutagenesis (n = 3).

The specificity of TF cytoplasmic domain phosphorylation in response to other coagulation proteases was analyzed in HUVECs expressing PAR2 and Cys²⁴⁵ to Ser mutated TF. The cells were stimulated for 1 h to increase the sensitivity for detecting inefficient responses, but high concentrations of factor IXa and activated protein C as well as thrombin did not induce TF phosphorylation (Fig. 3D). These are important data, because thrombin-cleaved PAR1 has been shown to cross-activate PAR2 (24). Whereas the NAPc2 stabilized TF-VIIa-Xa ternary complex triggered TF cytoplasmic domain phosphorylation with efficiency similar to that of a direct PAR2 agonist, signaling by VIIa (50 nM) alone produced less efficient TF phosphorylation in HUVEC monolayers. In contrast, stimulation with VIIa and X, which mimics activation of coagulation, efficiently induced TF cytoplasmic domain phosphorylation. In these experiments, Xa activity was blocked by adding a Xa inhibitor (NAP5) before free Xa reached levels that could activate PAR2.

The efficiency of ternary complex signaling is further illustrated in the middle panel of Fig. 3D with cells stimulated for only 10 min. The reaction with VIIa and X generated <10 nM Xa in this time period but lead to more efficient TF phosphorylation in comparison with free Xa at concentrations as high as 50 nM. In the right panel, the cells were stimulated with VIIa and X in the presence of the potent Xa inhibitor NAP5. Blocking Xa activity prevented TF cytoplasmic domain phosphorylation, confirming our previous conclusions (9) that in the ternary complex signaling pathway following the initiation of coagulation, TF phosphorylation is induced by Xa and not VIIia. Taken together, these experiments provide evidence that phosphorylation of the TF cytoplasmic domain occurs as a result of the activation of a highly specific signaling pathway following the initiation of coagulation.

PAR2 Signaling Induces Sustained PKCa Activation—The phosphorylation-specific antibody to TF recognizes the conformational change induced by phosphorylation of the cytoplasmic Ser²⁵⁵ residue that probably is the target for a Pro-directed kinase (20-22). However, PKCo-dependent phosphorylation of Ser²⁵⁵ appears to be required for PMA-induced Ser²⁵⁵ phosphorylation in HUVEC (22). Mutation of either the PKC phosphorylation site Ser²⁵⁵ or the PKC consensus recognition residues Lys²⁵⁶ abolished TF phosphorylation induced by PAR2-dependent ternary complex signaling (Fig. 4A). TF cytoplasmic domain phosphorylation was also blocked by inhibitors for conventional PKCs (α, β, and γ), Go6976, and Go6983, as well as the PKC-selective inhibitor Safingol but not the PKCβ-inhibitor Hispidin (Fig. 4B). To further demonstrate PKC specificity, we overexpressed wild-type PKCo or kinase-inactive, Lys²⁵⁶ to Asn mutated PKCo constructs. Ternary complex-induced TF cytoplasmic domain phosphorylation was significantly inhibited in cells expressing dominant negative, inactive PKCs, as compared with vector control or wild-type PKCs transfected cells (Fig. 4C). Thus, PKCo is important for TF cytoplasmic domain phosphorylation downstream of PAR2, presumably by targeting the PKC phosphorylation site at Ser²⁵⁵.

Thrombin-mediated PAR1 signaling activates PKCo in endothelial cells to increase permeability (29, 30), but PAR2-mediated PKCo activation is poorly characterized. TF cytoplasmic domain phosphorylation in response to PAR2 agonists in fibroblasts and breast cancer cells. A, PAR-deficient M611 fibroblasts from PAR1 knockout mice were co-transduced with Cys²⁴⁵ to Ser mutated TF and PAR1 or PAR2. Cell surface expression of TF, PAR1, and PAR2 was determined by flow cytometry, as in Fig. 1C. M611 fibroblasts were co-transduced with Cys²⁴⁵ to Ser mutated TF with PAR1 or PAR2 were stimulated with the indicated agonists and analyzed for TF cytoplasmic domain phosphorylation. Reprobing of the same blot with anti-TFCD was used to control for loading. C and D, phosphorylation of endogenously expressed TF in MDA-MB-231 human breast cancer cells downstream of PAR2 signaling. C, expression of TF, PAR1, and PAR2 in EDTA-lifted MAD-MB-231 cells was analyzed by flow cytometry. D, MDA-MB-231 cells were passaged by EDTA detachment, and grown for 24 h. Cells were stimulated by addition to the medium of PAR1 or PAR2 agonist peptides for the indicated times. TF phosphorylation was determined by Western blotting with phosphorylation specific antibody and loading control by reprobing with pan-specific anti-TF cytoplasmic domain antibody. FITC, fluorescein isothiocyanate.
obtained at the chosen virus dose.
PARI and PAR2 internalization in viral transduction, and PAP2 and PAR2 was detected by flow cytometry 48 h after adenoviral transduction. PARI (UT) depicts PARI expression levels without adenoviral transduction, and PAR1 and PAR2 are typical expression levels obtained at the chosen virus dose. A, co-expression of TF and PARI or PAR2 in HUVECs. Surface expression of TF, PARI, PAR2 were changed to serum-free medium and equilibrated for 2 h, to quench Xa activity immediately. TF cytoplasmic domain phosphorylation and PKCa activation, we determined by flow cytometry (means and standard deviation of residual mean fluorescence, n = 3). C, HUVECs co-transduced as in A to express either wild-type TF or Cys246 to Ser mutated TF and PAR1 or PAR2 were stimulated for 10 min with the indicated agonists and analyzed for TF phosphorylation by Western blotting. D, left panel, TF cytoplasmic domain phosphorylation is specific for upstream coagulation protease signaling. HUVECs transduced with Cys246 to Ser mutated TF and PAR2 were changed to serum-free medium and equilibrated for 2 h, followed by the addition of the indicated agonists for 1 h. 1 μM NAPs was added after 10 min to prevent free Xa signaling in cells stimulated with VIIa/Xa. Middle and right panels, cells expressing Cys246 to Ser mutant TF and PAR2 were stimulated with factor X (100 nM) and VIIa (10 nM) or free Xa for 10 min. NAPs was added at 1 μM just prior to the addition of VIIa and Xa to quench Xa activity immediately. TF cytoplasmic domain phosphorylation was determined by Western blotting. Controls for TF expression were performed on separate Western blots. For all panels, PAR1 agonist (10 μM), PAR2 agonist (100 μM), ternary complex IVla/ Xa/NAPsc2 (10/20/100 nM), thrombin (20 nM), activated protein C (10 nM), trypsin, VIIa alone, and IXa (50 nM) were used. For adenosinergic co-transduction, wild-type and Cys246 to Ser mutated TF (200 virus particles/cell) and PAR1 and PAR2 (600 virus particles/cell) were used.

**DISCUSSION**

In this study, we show that PAR2 signaling specifically targets the TF cytoplasmic domain by inducing TF phosphorylation. Our data demonstrate that the ternary complex VIIa-Xa complex similarly activates PAR1 or PAR2, based on efficiency of PAR internalization and ERK phosphorylation, but only PAR2 signaling leads to TF phosphorylation. TF cytoplasmic domain phosphorylation is dependent on PKCa activation. The presented mutational data support the concept that phosphorylation of the PKC consensus Ser253 is necessary for subsequent Ser256 phosphorylation, which is recognized by the phosphorylation-specific antibody to TF. Rapid PKCa membrane recruitment downstream of PAR1 or PAR2 demonstrates efficient receptor activation by the ternary complex. However, only PAR2 signaling induced sustained PKCa membrane localization, which coincides with the somewhat delayed kinetics of TF phosphorylation. Pharmacological blockade shows that PAR1 and PAR2 activations recruit PKCa through distinct PLC pathways. Sustained activation of PKCa downstream of PAR2 is dependent on PLC, which is known to induce prolonged...
Fig. 4. Role of PKCa in PAR2-dependent TF cytoplasmic domain phosphorylation. A, HUVECs were co-transduced with C245S, K255A, or S253A mutants of TF and PARK with the same virus dose as described for Fig. 2. After 48 h, the cells were transferred to serum-free medium for 2 h and stimulated with ternary complex for 1 h. TF cytoplasmic domain phosphorylation was determined by Western blotting and reprobing with α-TFCD. B, cells expressing Cys245Ser TF with PARK were serum-starved (2 h) and pretreated for 30 min with conventional PKC inhibitors, Go6983 (1 μM), Go6983 (1 μM), or isoform specific inhibitors for PKCa, Safingol, (50 μM), and PKCβ, Hipsin, (10 μM), followed by stimulation with ternary complex for 1 h at 37 °C. C, cells expressing C245S TF and PARK were co-transfected with 2 μg of pcdNA3.1 (vector control), FLAG-tagged PKCa or a catalytically inactive PKCa (K368N) constructs using FuGENE 6. After 48 h, the cells were serum-starved and stimulated with ternary complex for 1 h. Top panel, TF cytoplasmic domain phosphorylation by α-TFCD blot. Second panel, TF expression by reprobing with α-TFCD. Bottom two panels, PKCa expression by anti-PKCa and anti-FLAG blot.

Fig. 5. PAR2-dependent TF cytoplasmic domain phosphorylation requires PC-PLC-mediated sustained PKCa activation. A, time course of ternary complex-induced TF cytoplasmic domain phosphorylation. HUVECs co-expressing C245S TF and PAR2 were transferred to serum-free medium for 2 h and stimulated with ternary complex for the indicated time. TF cytoplasmic domain phosphorylation was determined by Western blotting and reprobing with α-TFCD. B, C245S TF and PAR2- or PAR1-expressing cells were serum-starved (4 h) and stimulated with ternary complex for 1 or 10 min followed by preparation of membrane and cytosolic fractions. Similar loading of membrane fractions is confirmed by reprobing with α-TFCD. C, quantitation of PKCa membrane recruitment by ternary complex signaling in PAR2- or PAR1-expressing cells. Fold inductions of translocation were determined by densitometry (means and standard deviations, n = 3). D, effect of PC- and PI-PLC-specific inhibitors on TF cytoplasmic domain phosphorylation. HUVECs expressing C245S TF and PARK2 were transferred to serum-free medium for 2 h and preincubated with the indicated concentration of the PC-PLC-specific inhibitor D609 or the PI-PLC inhibitor U73122 (10 μM) for 30 min following by stimulation with agonist for 1 h. In the right panel, D609 was used at 20 μM TF cytoplasmic domain phosphorylation was determined by Western blotting, and equal TF expression was confirmed on separate blots. E, HUVECs pretreated with 20 μM D609 or 10 μM U73122 were stimulated, followed by membrane isolation. PKCa membrane recruitment was determined by Western blotting with α-PKCa and reprobing for confirmation of equal protein loading with α-TF. For all panels ternary complex (VIIa/Xa/NaF)2 (10/20/100 nM) and PMA (20 ng/ml) were used.

The role of the TF cytoplasmic domain has previously been analyzed in heterologous transfection systems. Deletion of the entire cytoplasmic domain has little influence on the procoagulant function of TF (27, 38, 39) and on TF-VIIa proteolytic signaling, as measured by immediate early gene induction or phosphorylation events downstream of G protein-coupled receptor signaling (40–42). Although the relevant PARs that transmit TF-dependent signaling in these cell models are incompletely defined, these studies are consistent with an interpretation that the TF cytoplasmic domain is not required for PAR activation. Our data show that TF ternary complex signaling in the case of both PAR1 and PAR2 signaling is rapid, leading to PKCa membrane recruitment and PKCa phosphorylation within 1 min. However, TF cytoplasmic domain phosphorylation is a delayed response, and it is therefore not surprising that the TF cytoplasmic domain played apparently no role in early responses of TF-VIIa signaling. In addition, TF cytoplasmic domain phosphorylation appears to be more efficient than PAR2- or PAR1-Xa in comparison with TF-VIIa stimulation, at least under our experimental conditions, which may indicate that the TF cytoplasmic domain can play a regulatory role in signaling associated

membrane translocation of PKCa (32). The fact that PKCα stimulation, independent of PAR2, induces both TF phosphorylation and sustained PKCa membrane recruitment provides further support for an essential role of prolonged PKCa signaling in TF cytoplasmic domain phosphorylation.

This study adds to emerging evidence (10, 12, 13) that PAR1 and PAR2 signaling elicit specific and only partially overlapping responses in endothelial cells. More importantly, only TF-dependent signaling, but not the signaling by other coagulation factors, induced cytoplasmic domain phosphorylation. The phosphorylation of the TF cytoplasmic domain thus represents a marker for upstream coagulation signaling through PAR2. Both PAR2 and TF are induced by inflammatory mediators in endothelial cells in tissue culture (33, 34), and TF has been detected in vivo in splenic endothelial cells in severe systemic inflammation (35) or in breast cancer-associated endothelium (36). Moreover, PAR2 is found to be up-regulated upon differentiation of monocytes into macrophages (37), which also express TF. Thus, PAR2-dependent phosphorylation of TF may play regulatory roles in the biology and pathology of vascular cell types that co-express TF and PAR2.
with activation of the coagulation pathways.

TF is important for metastasis (27, 43), cell adhesion, and migration (44–47). Whereas PAR1 signaling potently induces Rho activation (12, 13), PAR2 only transiently activates Rho while activating Rac and recruiting a β-arrestin scaffolding complex for ERK localization (14, 15). These pathways promote cell motility, a cellular function that is possibly supported by Rho activation (12, 13), PAR2 only transiently activates Rho

Migration (44-47). Whereas PARI signaling potently induces cell motility, a cellular function that is possibly supported by Rho activation (12, 13), PAR2 only transiently activates Rho