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GENE TARGETING IN HEMOSTASIS. TISSUE FACTOR

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1. ABSTRACT

Tissue factor (TF) is the primary cellular initiator of blood coagulation. At sites of vascular injury, formation of a TF:FVIIa complex leads to the generation of FXa, thrombin and the deposition of fibrin to limit hemorrhage. In contrast to its beneficial role in hemostasis, TF initiates life-threatening intravascular thrombosis in sepsis, atherosclerosis and cancer. More recently, TF has been proposed to play a role in other biological processes, including tumor-associated angiogenesis, metastasis and inflammation. Indeed, gene targeting of TF resulted in embryonic lethality, which appeared to be due to a defect in the yolk sac vasculature.

2. INTRODUCTION

Tissue factor (TF) is the primary cellular initiator of the coagulation protease cascades. It is a transmembrane protein that is expressed at extravascular sites and limits bleeding in the event of vascular injury (for reviews see (3;23;37)). TF is the high-affinity receptor for plasma FVII/VIIa. The TF:FVIIa complex activates both FX and FIX, leading to thrombin generation and fibrin deposition. In sepsis, TF expression by vascular cells, such as monocytes and endothelial cells, initiates life-threatening disseminated intravascular coagulation. In atherosclerosis, TF expression by foam cells within the atherosclerotic lesion initiates blood coagulation and thrombosis after plaque rupture.

3. SECTIONS

3.1. Structure of the human TF protein

The human TF cDNA was cloned by four independent groups in 1987 (25;42;61;66). The cDNA encodes a protein of 263 amino acids preceded by a 32 amino signal peptide. The TF protein consists of a 219 amino acid extracellular domain, a 23 amino acid transmembrane domain and a 21 amino acid intracellular domain, as shown in figure 1. The TF extracellular domain contains two disulphide linkages and 3 N-linked glycosylation sites. TF has been crystallized and the structure solved by two groups (28;44;45). These studies demonstrated that the TF extracellular domain is composed of two C2-type immunoglobulin-like modules that each contain two beta sheets. Mutagenesis of TF has mapped the amino acids that contribute to the binding of FVII/VIIa (36;59). The single cysteine in the TF intracellular domain serves as an acceptor for the covalent linkage of palmitate or stereate (2), which probably enhances membrane anchoring of the TF molecule. Several studies have investigated the role of the TF intracellular domain in different processes. Cellular stimulation with protein kinase C activators and incubation with cell lysates with these activators leads to phosphorylation of serine residues in the TF intracellular domain (40;77), suggesting a role for these amino acids in signaling, as shown in figure 1. Indeed, one study showed that the TF intracellular domain was required for FVIIa-induced intracellular calcium fluxes (16). However, a second study indicated that FVIIa induction of the ERK MAP kinase pathway was independent of the TF

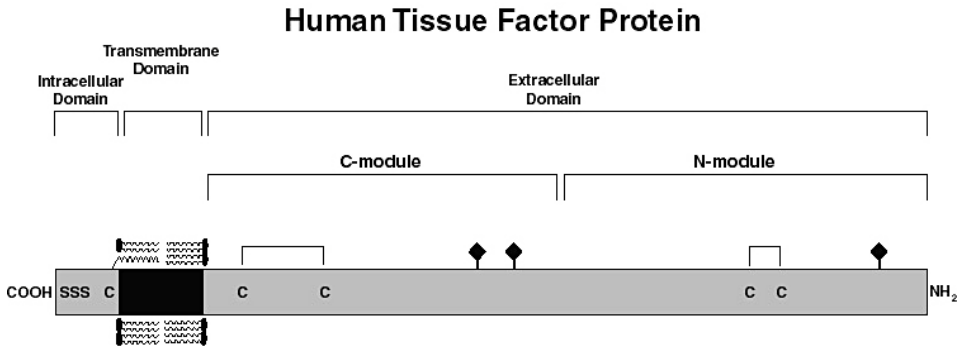

Figure 1. Human TF protein. Three N-linked glycosylation sites (diamonds) and two disulphide linkages are present in the extracellular domain. The cysteine (C) residue in the intracellular domain is palmitylated. The three serine (S) residues in the intracellular domain are sites of phosphorylation.

intracellular domain (65). In addition, the TF intracellular domain is not required for de-encryption of TF (12;73).

3.2. Regulation of the TF gene

TF is expressed constitutively by epithelial cells and advential fibroblasts surrounding blood vessels to limit bleeding after vascular injury (20;26). TF is also expressed in cardiomyocytes in the heart and astrocytes in the brain and may serve functions beyond blood coagulation. In sepsis, TF expression is induced in vascular cells, including endothelial cells and monocytes (21;41). In addition, arterial smooth muscle cells increase TF expression in response to balloon injury and growth factors (35). In 1989, we isolated the complete human TF gene to initiate studies on TF gene regulation (34). Many studies have identified binding sites for various transcription factors that regulate basal and inducible TF gene expression in different cell types, as shown in figure 2. Basal TF expression is regulated by Sp1, whereas inducible expression is regulated by c-Fos/c-Jun, c-Rel/p65 and Egr-1 ((reviewed in (32)). LPS induction of TF gene expression in monocytes required the cooperative interaction of c-Fos/c-Jun and c-Rel/p65 (33;46;47). More recent studies showed that vascular endothelial growth factor (VEGF) induction of TF expression in endothelial cells was mediated by both Egr-1 and NFAT (30;39). Regulation of TF expression by inflammatory mediators and angiogenic factors suggests that TF may contribute to both inflammation and angiogenesis.

3.3. Cell biology of TF and signaling

FVIIa binding to TF on various cell types has been shown to induce intracellular Ca^{2+} oscillations (16;58), transient tyrosine phosphorylation in monocytes (38), activation of the ERK MAP kinase pathway (56), VEGF expression in fibroblasts (49), up-regulation of the urokinase receptor in tumor cells (70) and *Egr-1* expression in HaCaT cells (9). TF also appears to enhance proinflammatory functions of macrophages (16). More recent studies have used cDNA arrays to study the differential gene expression in response to FVIIa binding to TF on a human keratinocyte cell line and on human fibroblasts (7;53). Many genes were up-regulated in the keratinocyte cell line, whereas only five genes were up-regulated in human fibroblasts. Interestingly, connective tissue growth factor (CTGF) was up-regulated in both cell types. These data suggest that the TF:VIIa complex may affect various biological processes by inducing expression of various downstream effector proteins.

Generation of the product, FXa, by the TF:FVIIa complex permits the formation of a stable quarternary complex with cell-bound tissue factor pathway inhibitors (TFPI-1 and TFPI-2). On endothelial cells, this quarternary complex traffics from anionic parts of the plasma membrane to caveolae (62), which may provide assembly-dependent signals to the cell. Caveolae are enriched in G-protein-coupled signaling receptors and non-receptor tyrosine kinases.

Cellular activation requires the FVIIa to be proteolytically active (10), suggesting that signaling is mediated by a protease activated receptor (PAR). An early study implicated PAR-2 as the signaling receptor for FVIIa (9). Another study excluded known PARs in FVIIa signaling (54). Nevertheless, TF:FVIIa signaling is complex because this system also generates downstream proteases, such as FXa and thrombin. For instance, it was shown that TF:FVIIa signaling is mediated by the generation of FXa and the activation of PAR-2 (8). Signaling through PAR-2 may be important in inflammation because PAR-2 expression is induced by TNFalpha and IL-1 in endothelial cells. Another study indicated that TF:FVIIa induction of VEGF in fibroblast is mediated by FXa and thrombin (49). Thrombin is

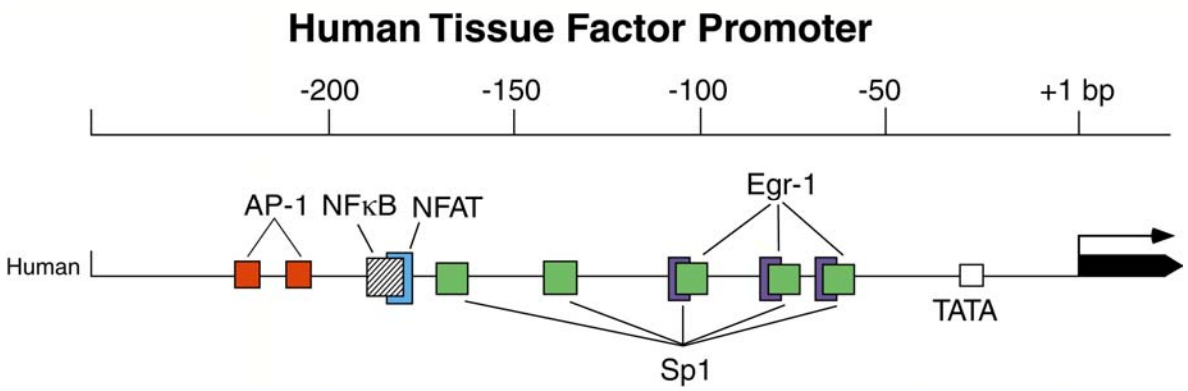

Figure 2. Human TF promoter. The human TF promoter contains binding sites for Sp1, Egr-1, c-Rel/p65, c-Fos/c-Jun and NFAT. The TATA box and start site of transcription (bent arrow) are shown.

known to stimulate cell activation and inflammation by cleavage of PAR-1, 3 and 4 (14). The availability of mice deficient in the various PARs will be useful in understanding their roles in TF:FVIIa signaling.

3.4. TF and angiogenesis

An earlier study showed that tumor cells transfected to overexpress TF generated larger and more vascularized tumors than control transfectants (76). TF-positive tumor cells expressed more VEGF than controls (76), which suggested that TF regulates the angiogenic properties of tumor cells. A more recent study demonstrated a significant correlation between TF and VEGF production in human malignant melanoma cell lines (1). Several studies have shown that TF and VEGF colocalize in human tumors (31;64;69). *In vitro* studies showed that TF-dependent VEGF production in human fibroblasts required FVIIa and was mediated by FXa and thrombin (48;49). Another study suggested that the TF intracellular domain was required for increased VEGF production (1).

3.5. TF and metastasis

Tumor cells expressing procoagulant activity exhibit enhanced metastasis ((for review see (60)). It is proposed that procoagulant tumor cells are encapsulated in clots that facilitates arrest in capillaries. Several studies have shown that TF activity enhances metastasis of human melanomas (4;5;29;43). Despite some controversy, it appears that the TF extracellular domain and protease generation is required for TF-dependent metastasis (43). Deletion of the TF intracellular domain abolished the enhanced metastasis (4;43). A yeast two-hybrid screening identified actin-binding protein 280 (ABP280) as a ligand for the TF intracellular domain (50). Studies are ongoing to determine the mechanism by which the TF intracellular domain contributes to tumor metastasis.

3.6. Inactivation of the murine TF gene

In 1996, the murine TF gene was inactivated by three independent groups (figure 3) (6;11;72). Mice heterozygous for the inactivated TF allele were phenotypically normal. However, homozygous TF-/- pups were very rare in crosses between heterozygous mice, which suggested that TF-/- embryos were dying in utero. Timed-breedings indicated that the majority of TF-/-embryos (~90%) died at embryonic day 10.5 (6;11;72). A large number of gene deletions cause embryonic lethality at this time (19;22;27;75). Like the TF-/- embryos, the embryos are often characterized by growth retardation, pallor, distended pericardia, yolk sac abnormalities and hemorrhage. Therefore, it is difficult to distinguish primary events causing mortality from secondary changes due to morbidity. Two mechanisms have been proposed to explain the embryonic lethality of *TF*$^{-/-}$embryos. First, the lethal phenotype may result from hemorrhaging of embryonic blood from both extra-embryonic and embryonic vessels (6). Second, loss of TF may lead to disruption of the yolk-sac vasculature, possibly due to defective formation of contacts between endodermal and mesodermal cell layers (11). However, the specific defect in the yolk sac vasculature and/or the *TF*$^{-/-}$embryos themselves is still to be defined. TF-deficient humans have never been identified, which is consistent with the high rate of embryonic lethality of murine *TF*$^{-/-}$embryos.

Interestingly, in a 129/SvJ or a mixed 129/SvJ:NIH Black swiss genetic background, *TF*$^{-/-}$ embryos do not survive beyond embryonic day 10.5 (6;71). In contrast, between 1 and 4% of *TF*$^{-/-}$embryos survive beyond embryonic day 10.5 in a 129/SvJ:C57BL/6 genetic background (11;71;72). At present, it is unclear if this compensatory effect is derived transplacentally or from the embryos. The birth of *TF*$^{-/-}$pups has been observed by three independent

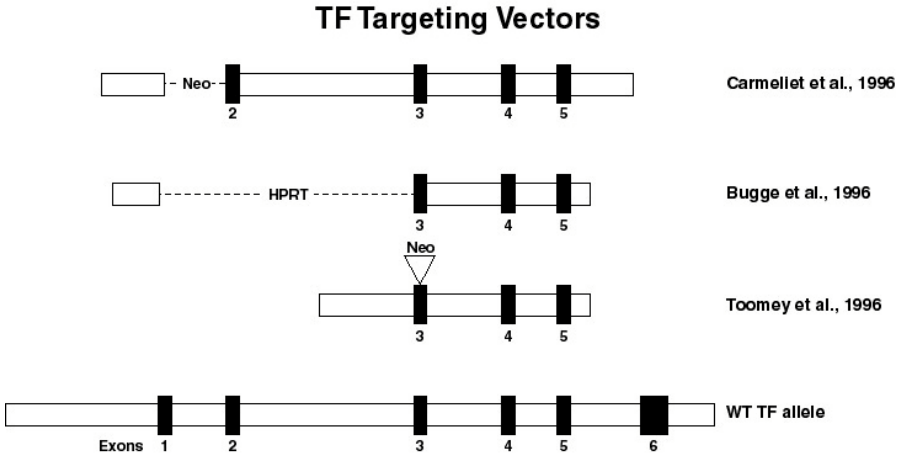

Figure 3. TF targeting vectors. The three targeting vectors used to inactivate the *TF* gene are shown (6;11;72). Exons are numbered and shown as black rectangles.

groups indicating that TF is not essential for development of the embryo itself. Indeed, one *TF*$^{-/-}$ mouse survived 30 days before succumbing to a fatal hemorrhage (Mackman, unpublished). In summary, the majority of *TF*$^{-/-}$embryos (~90-95%) die at embryonic day 10.5, which appears to be due to a defective yolk sac vasculature. The remainder (5-10%) die shortly after birth due to abdominal hemorrhage induced by birth.

The survival of all *FVII*$^{-/-}$ embryos to birth (57)contrasts to the death of *TF*$^{-/-}$ embryos. FX deficiency resulted in partial embryonic lethality with about 33% of the *FX*$^{-/-}$ embryos dying at embryonic day 11.5-12.5 (18). The survival of *FVII*$^{-/-}$ and *FX*$^{-/-}$ embryos may be due to the transfer of small amounts of maternal FVII and FX to null embryos. In addition, the survival of *fibrinogen*$^{-/-}$ embryos and embryos deficient in the transcription factor NF-E2, suggest that fibrin deposition, platelets, and hemostasis are not essential for embryonic development (55;63;67). It is provocative that embryos deficient in FV, prothrombin, and PAR-1 also die at embryonic day 10.5 with defects in the yolk-sac vasculature (13;15;17;68;74). These results suggest a link between TF-dependent activation of the coagulation protease cascade and PAR-1 signaling.

3.7. Rescue of murine TF$^{-/-}$embryos with a human TF minigene

We have successfully rescued murine *TF*$^{-/-}$ embryos with a *TF* minigene expressing human TF from the human TF promoter (51). Quantitation of the levels of TF functional activity in rescued mice indicated that human TF was expressed at 1% of the levels of murine TF. We called this mouse line "low TF" to reflect this low level of human TF expression. Despite this low expression, low TF mice developed normally, exhibited no excessive hemorrhage from tail transection and were fertile (51). However, we observed 14-18% fatal hemorrhage in low TF female mice postpartum (24), suggesting that TF plays an important role in uterine hemostasis postpartum. In addition, either a complete deficiency of murine TF in the embryos or a low level of human TF in the *TF*$^{-/-}$ embryos was associated with disruption of the placenta barrier and maternal hemorrhage into the placenta (24). At present, the molecular basis for this defect has not been defined.

3.8. Role of the TF intracellular and extracellular domains in embryogenesis

To investigate the role of TF in embryogenesis, we made mutant human TF minigenes whose products either bound FVII/VIIa at a reduced level or lacked the intracellular domain (52). Two independent transgenic lines expressing the human TF intracellular domain mutant (TF mutID) rescued the embryonic lethality of murine *TF*$^{-/-}$ embryos, indicating that the intracellular domain of TF is not required for embryogenesis. Carmeliet and colleagues have used cre-lox technology to generate mice expressing murine TF without the intracellular domain. These mice are viable and support our conclusion that the TF intracellular domain is not required for embryogenesis. In contrast to these results, we found that two independent transgenic lines expressing the human TF extracellular domain mutant (TF mutED) failed to rescue the embryonic lethality of murine *TF*$^{-/-}$ embryos, suggesting that FVII/VIIa binding to TF and/or proteolytic activity of the TF:FVIIa complex is required for embryogenesis (52). Our current hypothesis is that TF may function in embryogenesis to generate thrombin, which then activates PAR-1-dependent intracellular signaling in the visceral yolk sac. We

propose that the TF-thrombin-PAR-1 pathway is required for the maintenance of the integrity of yolk sac blood vessels. At present, it is unclear why there is a greater level of lethality observed with *TF*$^{-/-}$embryos (90%) compared with the level of lethality of *PAR-1*$^{-/-}$ embryos (50%). This may be due to signaling by clotting proteins upstream of thrombin, such as FVIIa and FXa, or compensation for the loss of PAR-1 by other PARs.

4. PERSPECTIVE

Gene targeting of *TF* revealed an unexpected mid-gestational embryonic lethality. This lethality appears to be due to a defect in the yolk sac vasculature. Similar yolk sac defects were observed in embryos deficient in FV, prothrombin, and PAR-1. A human *TF* minigene expressing TF with a deleted intracellular domain successfully rescued *TF*$^{-/-}$embryos, whereas a TF extracellular mutant with reduced FVII/FVIIa binding failed to rescue *TF*$^{-/-}$ embryos. Taken together, these data suggest that TF expression is required for thrombin generation and PAR-1 signaling in the yolk sac during mid-gestation. Future studies should better elucidate the role of TF in tumor angiogenesis, metastasis, placentation and inflammation. These studies will be facilitated by the generation of mice containing a floxed TF gene, which will permit selective deletion of TF in various cell types in adult mice.

5. ACKNOWLEDGMENT

I would like to acknowledge that gene targeting of TF was performed in collaboration with P. Carmeliet and D. Collen at the Center for Transgene Technology and Gene Therapy, Leuven. In my laboratory, G. Parry rescued *TF*$^{-/-}$ embryos with a human *TF* minigene and analyzed the role of the TF extracellular and intracellular domains in embryogenesis, and J. Erlich characterized the uterine and placental defects in the low TF mice. I would also like to acknowledge T. Luther at the Technical University of Dresden for advice and expertise in analysis of mice. Funds for these studies were provided by the National Institutes of Health.

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