The role of blood coagulation in cancer, inflammation and embryonic development
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The capacity to produce TF in all cells is not required for development although preference for TF bearing cells is evident in specific tissues

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(submitted)
Abstract

Tissue Factor (TF) is essential for normal embryonic development and survival to term. Low TF levels are, however, sufficient to rescue the otherwise lethal phenotype of TF deficiency. It is unknown which cells are required to express TF for normal embryogenesis to take place, and whether there is selective preference for TF bearing cells to form specific tissues. To address these issues, chimaeric mice were created using TF wildtype (wt) blastocysts and TF knock-out (-/-) embryonic stem cells. Using semi-quantitative PCR we could show that each organ of the chimaeric mice contained approximately equal amounts of genomic TF(-/-) and TF(wt) DNA. We did not observe a net overall preference for TF(wt) DNA in the major arteries and veins (aorta, vena cava inferior and superior). Immunohistochemical analysis revealed that TF was mainly expressed (in a constitutive manner) in capillary endothelial cells of the brain, the collecting tubules of the kidney, the outer plexiform layer of the retina, mature spermatogonia and epithelial cells of the intestine. We did not observe any difference in TF protein expression between chimaeric and wildtype animals indicating that TF bearing cells are preferred when these cell types emerge. In conclusion, our data show that TF chimaeric mice are viable and develop without vascular malformations. Moreover, the capacity to produce TF on every single cell is not required for normal embryogenesis, but TF seems essential for the development of those cell types that constitutively express TF.
Introduction

Tissue factor is a 263–amino acid transmembrane glycoprotein that serves as the cellular receptor and cofactor for plasma FVII/VIIa [1-2]. In addition to its essential role in blood coagulation [3], it is evident that TF serves an important role in angiogenesis, tumor metastasis, induction of a pro-inflammatory response in several physiological processes and during embryonic development [4].

Important with respect to the role of TF in embryonic development are data showing that knocking out the murine TF gene leads to embryonic lethality between embryonic day (ED) 9.5 and 11.5 of gestation [5-7]. TF-deficient blood vessels lack a proper muscular wall, they are too fragile and have a tendency to leak when blood pressure rises [5-7]. The early embryonic death in TF-deficient mice probably results from haemorrhage and leakage of blood from both extra-embryonic and embryonic vessels [5]. Although TF knock-out mice can survive to birth if delivered by Caesarean section, they die after just 2–3 weeks as a result of major bleeding [7]. In addition, some TF(-/-) embryos have survived beyond ED 10.5 in a 129/SvJ:C57Bl/6 genetic background in contrast to the 129/SvJ or 129/SvJ:NIH Black Swiss background in which all embryos died at and around ED10.5 [7]. A small number of embryos survived into late gestation, and died then due to hemorrhage. It is thus clear that the genetic background has an influence on this phenotype, but the genetic compensatory mechanism remains unknown. Consistent with the severe bleeding phenotype and consequent embryonic lethality of TF deficiency in mice, no TF deficient human individuals are known, implying that TF is essential for human life. Interestingly, very low TF levels (less than 1% of wt levels) seem sufficient for embryonic development and survival in mice [8], although these animals typically die at around 8 months of age from cardiac fibrosis and left ventricular dysfunction, caused by haemorrhage from cardiac vessels [9]. In these studies a human TF minigene (containing the human TF promoter and cDNA) was used. In alternative studies the presence of the cytoplasmic or extracellular domain of TF was evaluated [10-11]. Mutants lacking either domain were created of which the lines expressing the human TF extracellular mutant failed to rescue embryonic lethality of murine TF deficient embryos. Lines expressing the human TF cytoplasmic domain mutant (containing a normal extracellular domain) on the other hand rescued embryonic lethality. This indicates that embryogenesis requires the TF extracellular domain, probably to support TF/FVIIa protease activity. Studies using human melanoma cell lines suggested, however, that the cytoplasmic domain of TF is essential for vascular endothelial growth factor (VEGF) production [12].

In the developing embryo, blood cell formation and vasculogenesis begins at ED 7.5 in the blood islands of the yolk sac [13]. The endothelial and haematopoietic cells of these islands originate from a common precursor, termed the haemangioblast. The primary vascular plexus formed in the yolk sac will form the large vitelline blood vessel. In TF deficient yolk sacs, the mesenchyme derived smooth muscle cells (i.e. pericytes) fail to accumulate and differentiate around endothelial cell-lined capillaries [6]. In both human and mouse organogenesis, there
is marked expression of TF in cardiovascular and central nervous systems [14]. This pattern of early expression supports the notion that TF serves as an important morphogenic factor during embryogenesis.

In this study we focused on the role of TF in embryogenesis. Pluripotent embryonic stem (ES) cells are able to differentiate \textit{in vivo} into all cell types of the fetal and adult organism and \textit{in vitro} they can differentiate into a variety of cell types. In order to examine and compare the developmental potential of TF(-/-) ES cells, we exposed them to an environment that is permissive for the development of all cell types of the embryo, namely the mouse pre-implantation blastocyst. To this end, we created TF chimaeric mice to investigate whether the capacity to produce TF is obligatory for every single cell in order to develop normally and whether there is selective pressure on TF bearing cells to form specific tissues.

**Materials and Methods**

**Embryonic stem cell culture**

TF deficient embryonic stem (ES) cells (129SV/C57Bl/6), derived from TF knock-out mice, [6] were kindly provided by Dr. Dewerchin (Belgium, Center for Transgene Technology and Gen Therapy, KULeuven, Belgium). The ES cells were cultured in complete medium (DMEM, high glucose, w/o Na-pyruvate-Gibco, 15% fetal calf serum (FCS), 2mM glutamine, 1 mM Na-pyruvate, non-essential amino acids, 0.1 mM β-mercapto-ethanol and 1x10^3 U/ml Leukemia Inhibitory Factor (LIF)) on irradiated (25 Gy), and thus mitotically inactive, mouse embryonic fibroblasts. Prior to blastocyst injection, ES cells were trypsinized and resuspended in complete medium without β-mercapto-ethanol and LIF.

**Generation of chimaeric mice**

Mouse stocks used for embryo donors were six week old C57Bl/6 (Harlan, The Netherlands), maintained under controlled lightening conditions (14 hours light, 10 hours dark). The females were superovulated by the injection of Follicle Stimulating Hormone (5 I.U. PMSG; Folligon, Intervet Co., The Netherlands). Forty-eight hours after PMSG injection of Luteinizing Hormone (5 I.U hCG; Chorulon: Intervet Co., The Netherlands) was administered and mating was concomitantly initiated. The presence of a vaginal plug was examined 16 hours after hCG injection and blastocysts were collected after 72 hours. Chimaeric mice were generated by microinjection of the TF(-/-) ES cells into C57Bl/6 blastocysts as described [15]. After injection with ES cells, blastocysts were incubated for 30 minutes at 37°C to allow proper remodeling. Subsequently, the blastocysts were reimplanted into pseudo-pregnant FVB foster mothers using standard techniques [16].

All mice were maintained at the animal care facility at the Academic Medical Center according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of
Laboratory Animals. All mice were housed in the same temperature-controlled room with alternating 12h light/dark cycles.

**Tissue collection and processing**

Chimaeric mice were anesthetized with Hyponorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, The Netherlands), organs were collected and blood was sampled from the inferior vena cava (VCI). One part of each tissue was used to collect genomic DNA. To this end, tissues were lysed in lysis buffer (100mM Tris, pH 8.3, 5 mM EDTA, 200 mM NaCl, 0.2% SDS and 100 μg/ml proteinase K) at 55°C and DNA was extracted using the standard phenol/chloroform extraction procedure. The other part of the organ was fixed 24 hours in 4% formaldehyde and embedded in paraffin after dehydration using graded alcohols and butanol, using routine methods.

**Quantitative Polymerase Chain Reaction**

The PCR reaction was carried out in a total volume of 21 μl of PCR mix (0.5 U AmpliTaq polymerase (PerkinElmer, Norwalk, CT), 1.25 mM dNTPs, 2.1 μl 10x Pol buffer (0.67 M Tris-HCl pH 8.8, 67 mM MgCl₂, 0.1 M β-mercapto-ethanol, 67 μM EDTA, and 0.166 M (NH₄)₂SO₄), 0.5 mg/ml BSA, and 4 μg/ml of each primer) on a thermocycler (PerkinElmer) with the following sequence: 92°C for 4 min (one cycle), followed immediately by 91°C for 1 min, 55°C for 1 min, and 57°C for 2 min (32 cycles), and a final extension phase of 72°C for 7 min. The primer sequences were as follows: Neo-cassette forward: Neopf3: 5’TGC TCC AGA CTG CCT TGG GAA AAG 3’, TF forward: 5’ GCA TTC CAG AGA AAG CGT TTA ATT 3’, TF reverse: 5’AAC CGA CTT CAA AGA GTC CCT ATT 3’. The PCR products were separated on a 2% agarose gel and visualized by UV illumination.

**Tissue Factor Immunostaining**

Paraffin slides were deparaffinized using xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 1.5% H₂O₂ in phosphate buffered saline (PBS) for 30 minutes and then washed in PBS. Non-specific binding sites were blocked with TENG-T (10mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (v/v) Tween-20, pH 8.0) for 30 minutes. After washing with PBS, slides were incubated overnight at 4°C with a primary rabbit anti-mouse TF polyclonal antibody [17] diluted in 1% Bovine Serum Albumin (BSA) in PBS or with a rabbit IgG negative control antibody. After washing with PBS, slides were incubated with biotinylated goat anti-rabbit Ab (DAKO, Glostrup, Denmark) diluted in PBS/1% BSA for 1.5 hours at room temperature. Slides were washed using PBS, incubated with streptavidin-biotin-horseradish peroxidase (DAKO, Glostrup, Denmark) for 30 minutes and washed again with PBS. The slides were subsequently preincubated in acetate buffer, pH 5.0 for 2 minutes and stained with filtered (0.45 μm) 3-amino-9-ethylcarbazole (AEC, 0.2 gram/l) and 1.5% H₂O₂ in acetate buffer for 5-15 minutes. The reaction was stopped in dH₂O. Finally, slides were counterstained with hematoxylin and eosin. Examination of immunohistochemical stained slides was performed on coded samples in a blinded fashion.
Results

Chimaeric mice

After microinjection of wildtype blastocysts with TF(-/-) ES cells, five mice were found to be chimaeric for TF expression. They were healthy and vital and developed without vascular or other malformations. Moreover, they were able to reproduce (every single chimaera produced offspring with expected nest-size) and to live a normal lifespan (the chimaeras were sacrificed at around an age of 1 year). So no overt phenotype was observed in the chimaeric mice.

Quantification of TF chimaerism

To determine the ratio between TF(-/-) and TF(wt) cells in the chimaeras, DNA was isolated from different organs and tissues (aorta, bone, brain, colon, ear, eye, gonads, heart, kidney, liver, lung, muscle (hind leg), skin, small intestine, spleen, tail, thymus, toes, vena cava superior and inferior) and the percentage of TF(-/-) cells was determined. As shown in table 1, most organs have approximately equal amounts of TF(-/-) DNA compared to TF(wt) DNA (with a range between 38 and 58% TF(-/-) DNA). Please note that in the large arteries and veins (aorta and vena cava inferior (VCI) and superior (VCS)) no net overrepresentation was observed for TF(wt) cells as again approximately 50% of DNA was derived from TF(-/-) ES cells. Occasionally, lower percentages of TF(-/-) were seen, like in the gonads, but such lower values were not consistently observed in all chimaeras.

Immunohistochemistry for TF in chimaeric mice

In order to determine whether the chimaeric mice also show chimaerism in constitutive TF expression, we performed TF immunohistochemistry on paraffin sections from different tissues. This analysis showed that TF was expressed in most of the organs that were selected for this analysis but not in all. For example, TF was not expressed in the heart muscle, in the muscles of the thigh or in the liver (data not shown). TF was, however, expressed in all capillary endothelial cells of the brain (figure 1), the collecting tubules of the kidney (figure 2), and outer plexiform layer of the retina (figure 3). In addition, TF expression was observed in mature spermatogonia and epithelial cells of the intestine (data not shown). In fact we did not observe any difference in the distribution of TF expression between chimaeric and wildtype animals (figures 1-3), indicating that cells that constitutively express TF are derived from wildtype cells only.

Discussion

The embryonic lethality of TF deficient mice in combination with the notion that TF deficiency is incompatible human life suggests that TF is essential for normal embryonic development and survival [5-7]. As TF deficient mice suffer from massive bleeding complications, it is hypothesized that vascular development is impaired in TF deficient mice.
The capacity to produce TF in all cells is not required for development

Expression levels as low as 1% are sufficient to rescue embryonic lethality [8]. This latter study however does not address the question which cells critically express TF in order to support normal embryogenesis. Our data show that TF needed to be present on only a minority of cell types, such as the capillary endothelial cells of the brain, the epithelium of collecting tubules of the kidney, and the outer plexiform layer of the retina.

In the current study, we generated TF chimaeric mice consisting of about 50% wildtype and 50% TF(-/-) cells. As an exception, the gonads of 2 out of 5 chimaeric mice were almost completely TF(wt) derived (90%, see Table 1). We are not certain of the reason for this, but the fact that the other 3 chimaeric mice did not show bias towards wildtype cells suggests that this may have been a chance finding.

Table 1: Percentage of TF knock-out DNA in different organs of the chimaeric mice, as a measurement of the percentage of TF(-/-) cells. Analyzed by quantitative PCR. - : No DNA available. Please note that the testis only had 10% knock-out cells. VCI/VCS: vena cava inferior and vena cava superior.

<table>
<thead>
<tr>
<th></th>
<th>Mouse 1</th>
<th>Mouse 2</th>
<th>Mouse 3</th>
<th>Mouse 4</th>
<th>Mouse 5</th>
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<tr>
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<td>54.5</td>
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<td>Bone</td>
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<td>60.0</td>
<td>58.3</td>
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<td>54.5</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>52.4</td>
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<td>-</td>
<td>61.5</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Tail</td>
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<td>54.5</td>
<td>40.0</td>
<td>50.0</td>
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<tr>
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<td>56.5</td>
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<td>-</td>
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<tr>
<td>Toe</td>
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<td>58.3</td>
<td>54.5</td>
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<td>-</td>
</tr>
<tr>
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<td>50.0</td>
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</tr>
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<td><strong>54.2</strong></td>
<td><strong>37.8</strong></td>
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</table>
Figure 1: Immunohistochemical TF staining of brain sections from chi- maeric mice (panels A and B) and wildtype controls (panels C and D). Shown is a TF positive blood vessel in the cortex. All cells of the vessel are TF positive, whereas all other cells present in the cortex do not express TF constitutively. Panels A and C: 40x magnification; panels B and D: 100x magnification. (For color figure see page 196)

Figure 2: Immunohistochemical TF staining of kidney sections from chimaeric mice (A and B) and wildtype controls (C and D). The collecting tubules in the kidney all constitutively express TF, whereas other cells of the kidney marrow are TF negative. Panels A and C: 40x magnification; panels B and D: 100x magnification. (For color figure see page 196)

Figure 3: Immunohistochemical TF staining of eye sections from chimaeric mice (A and B) and wildtype controls (C and D). Shown is a cross section of the eye of which only the outer plexiform layer of the retina stains positive for TF. All other layers like for instance the ganglion cell layer, the inner plexiform layer and the inner nuclear layer do not constitutively express TF. Panels A and C: 40x magnification; panels B and D: 100x magnification. (For color figure see page 196)
The capacity to produce TF in all cells is not required for development. This finding is perhaps not that surprising as not all cells express TF, but urged us to determine whether TF expression also shows a chimaeric pattern in the chimaeric mice. As shown in figures 1-3, only a limited number of cell types express TF constitutively. As shown previously, the capillary endothelial cells of the brain [17,18], the collecting tubules of the kidney [17] and outer plexiform layer of the retina express high amount of TF. In addition, TF is also expressed by a subset of spermatogonia, especially the mature cells [14,19], and by differentiated intestinal epithelium [14,19]. Interestingly, as opposed to scattered TF expression in the spermatogonia and the intestine, all cells of the capillary endothelial cells of the brain, collecting tubules and outer plexiform layer of the retina were TF positive. Especially in these latter structures TF expression was distinct in all cells, and a chimaeric TF expression pattern would have been easy to detect by immunohistochemistry. The detection of chimaerism in spermatogonia and epithelial cells of the intestine was more difficult to document as these structures already display a chimaeric-like pattern in wildtype mice. As shown in figures 1-3, however, TF expression is indistinguishable between wildtype and chimaeric animals suggesting that TF expression is also essential for the development (and probably function) of these structures. The rationale for the selective pressure on TF in the capillary endothelial cells of the brain, the collecting tubules and the outer plexiform layer of the retina is not known. One could speculate that TF in the capillary endothelial cells of the brain serves to protect against fatal embryonic bleeding [20-22], although it is not evident why we did not observe selective pressure for TF expression in other vessels.

There are several potential limitations to our study. First, the immunohistochemical analysis was performed with a single TF antibody. Although TF expression determined using this antibody correlated particularly well with patterns obtained with other antibodies [17], we can not exclude subtle discrepancies with antibodies used elsewhere. Notably, we did not observe TF expression in heart, in agreement with observations by Fleck [23], but some other studies do not only show TF expression in heart tissue [18] (mRNA [14]), but also claim a prominent role of cardiomyocyte TF [24]. Second, our study only addresses constitutive and not induced TF expression. It would be interesting to assess whether induced TF expression (i.e. induced by inflammatory mediators like LPS [25,18,26] or by cancer cells [27,28]) is also indistinguishable between wildtype and chimaeric mice. The generation of chimaeric mice is unfortunately rather laborious, which severely compromises the generation of large numbers of chimaeric animals to perform such studies. Third, immunohistochemistry does neither discriminate between active (coagulant) TF and encrypted [1] or signal transduction [29] TF, nor does it allow quantitative analysis of TF expression. It is thus difficult to establish with certainty whether TF coagulant or TF signaling properties (or both) are essential for the development of collecting tubules, the capillary endothelial cells of the brain and the outer plexiform layer of the retina. Finally we performed our studies with mice on a mixed 129/Sv/C57Bl/6 background. As genetic background is a rather important determinant of the TF(-/-)
phenotype (see introduction), we cannot exclude that on other genetic backgrounds there would have been selection for TF expression in additional vessels (or cell types).

In conclusion, the capacity to express TF on every single cell is not obligatory for normal development although selective pressure on TF bearing cells is evident in specific tissues.
The capacity to produce TF in all cells is not required for development

References
