Chapter 12

Differentiation of tissue factor knock-out embryoid bodies

Lois W. Brüggemann[1], Pieter H. Reitsma[1,2] and C. Arnold Spek[1]

[1]Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam The Netherlands, [2]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands

Abstract

The embryonic lethality of tissue factor (TF) deficient mice suggests that TF is essential for normal embryonic development and survival. Since TF deficient embryos suffer from massive bleeding, it has been suggested that vascular development is impaired in TF deficient mice. Our previous studies using TF chimaeric mice did, however, not provide solid evidence for a role of TF in vascular development. To further study the role of TF in the early formation of blood vessels, we generated wildtype and TF deficient embryoid bodies and determined the capacity of these embryoid bodies to differentiate into endothelial cells and smooth muscle cells. We could show that wildtype TF embryoid bodies indeed differentiated into smooth muscle cells and endothelial cells and that these cells came in close contact, eventually forming vessel-like structures. Heterozygous and homozygous TF deficient embryoid bodies were also able to differentiate into endothelial cells and smooth muscle cells and again these cells formed vessel-like structures that were indistinguishable from those obtained from wildtype embryoid bodies. In conclusion, by studying *in vitro* embryoid body development, we did not obtain evidence that TF is involved in early vascular development.

Introduction

Tissue factor (TF), the cellular receptor and cofactor for plasma FVII/VIIa [1-2], serves an important role in embryonic development [3]. This notion is based on the fact that knocking out the murine TF gene leads to embryonic lethality between embryonic day (ED) 9.5 and 11.5 of gestation [4-5]. TF-deficient blood vessels lack a proper muscular wall, they are fragile, and they have a tendency to leak when blood pressure rises [4-5]. The early embryonic death in TF-deficient mice probably results from haemorrhage and leakage of blood from both extra-embryonic and embryonic vessels [4].

Very low TF levels (less than 1% of wt levels) seem sufficient for embryonic development and survival in mice [6], although animals with low TF typically die at around 8 months of age from cardiac fibrosis and left ventricular dysfunction caused by haemorrhage from cardiac vessels [7]. We recently showed that TF chimaeric mice, consisting of 50% wildtype and 50% TF deficient cells on average, develop normally and no net overall preference for TF(wt) DNA in the major arteries and veins was observed [8]. Importantly, no overt malformations in the vascular integrity of the chimaeras were evident.

In the developing embryo, blood cell formation and vasculogenesis begins at ED 7.5 in the blood islands of the yolk sac [9]. The endothelial cells (ECs) and haematopoietic cells of these islands originate from a common precursor termed the hemangioblast. 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 (SMCs)/pericytes are thought to fail to accumulate and differentiate around EC-lined capillaries [10].

In this study we focus on TF and its role in vasculogenesis and especially on the capacity of pluripotent stem cells with different TF genotypes to differentiate into ECs and SMCs. Pluripotent embryonic stem cells (ESCs) are able to differentiate *in vivo* into all cell types of the fetal and adult organism. *In vitro* ESCs are able to differentiate into a variety of cell types and after these assemble into embryoid bodies, vascular development occurs. This suggest that ESCs produce all the factors needed for the induction of vasculogenesis [11-12] and can serve as a simple model for early vascular development, particularly well suited for loss of function analysis of genes required for embryogenesis [13]. In order to examine and compare the developmental potential of TF deficient ESCs and to investigate the necessity of the presence of TF in order to form blood vessels we used embryoid bodies of three different genotypes (TF(+/+), TF(+/-) and TF(-/-)) and followed differentiation into blood vessel components.

Materials and Methods

Embryonic stem cell culture

TF deficient embryonic stem (ES) cells (129SV/C57Bl/6), derived from TF knock-out mice, [10] were kindly provided by Dr. Dewerchin (Belgium, Center for Transgene Technology and Gen Therapy, KU Leuven, Belgium). The ES cells were cultured in complete medium (DMEM,

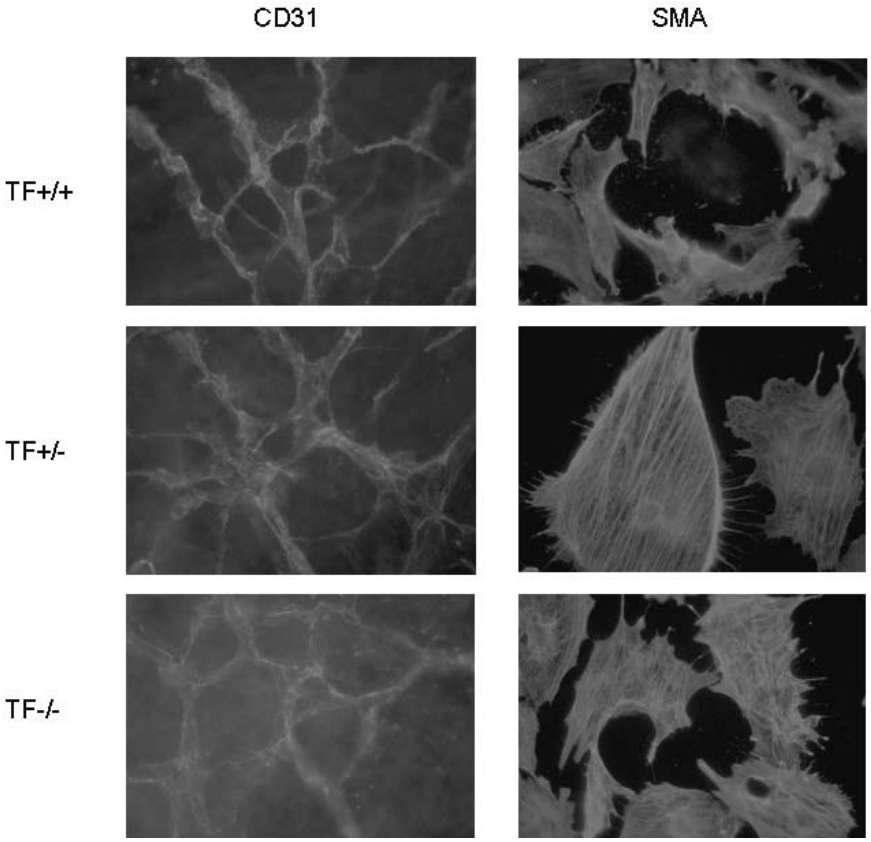

Figure 1: Immunofluorescence of wildtype and TF deficient embroid bodies. The left panel is stained for the endothelial marker CD31. The right panel is stained for smooth muscle actin, a smooth muscle marker. Genotypes as indicated on the left. Magnification: 63x. Please note that the overall appearance of the cells and the distribution of CD31 and smooth muscle actin are the same for all genotypes. (*For color figure see page 197*)

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 $1 \cdot 10^3$ 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 embryoid bodies

To generate embryoid bodies the "hanging drop" method was followed [14]. In short, 800 ES cells in 20µl drops (DMEM as described above without LIF) were placed on the lid of bacteriological grade Petri dishes, containing phosphate buffered saline (PBS) for 4 days. Differentiation was evoked by bringing the embryoid bodies into culture, one embryoid body

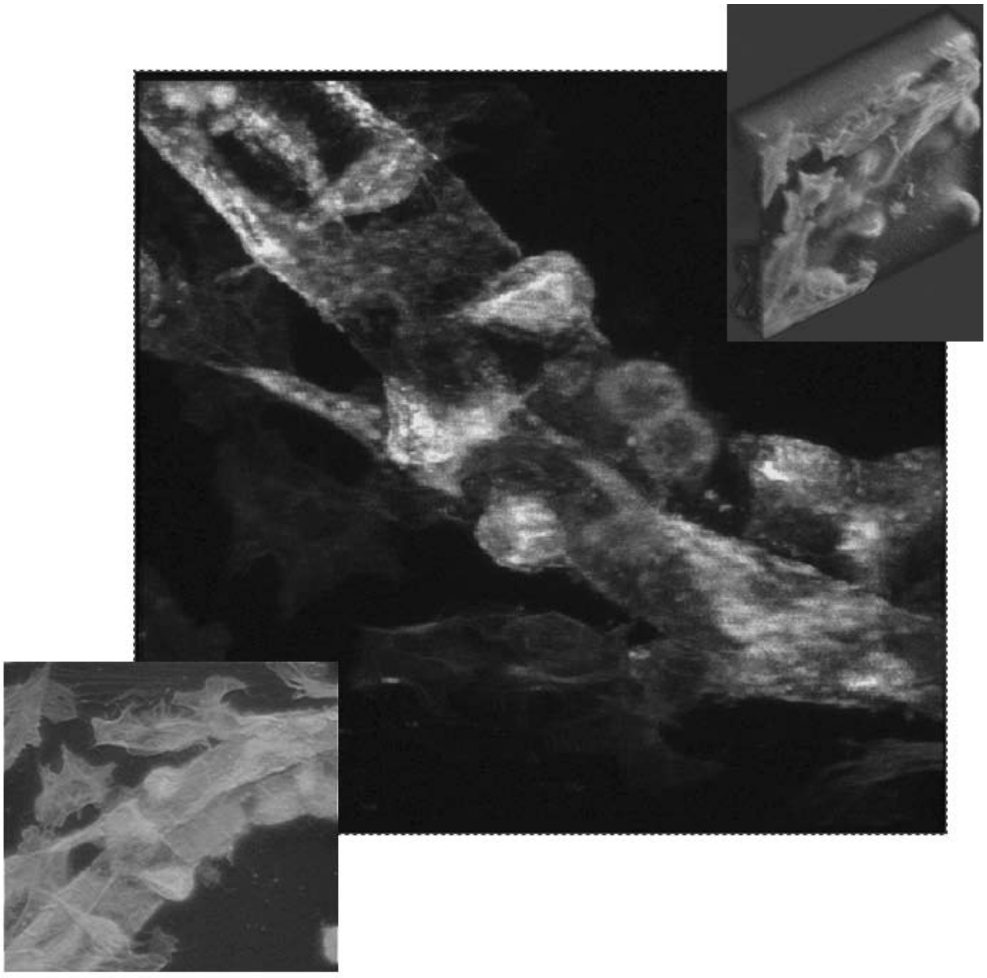

Figure 2: Immunofluorescence staining of the sprouting of a TF -/- embroid body using a smooth muscle actin marker together with a lectin marker. The lower insert shows a lectin marked endothelial vessel-like structure (green) surrounded by smooth muscle cells (red). The upper insert shows a 3D representation of the blood vessel-like structure. The main figure is a red and green reconstruction of the vessel-like structure to be viewed with red/green spectacles for a 3D impression. (*For color figure see page 198*)

per culture well (24-well plate, COSTAR, diameter ca. 1.5 cm) on coverslips coated with a layer of 0.1% gelatine or 0.1% collagen in medium with 10 ng/ml VEGF.

Immunofluorescence

The embryoid bodies grown on coverslips were washed using PBS and fixated in acetone/methanol (1:1) for 5 minutes. After rinsing with PBS the first antibody (M390-CD31/PECAM Rat α Mouse, smooth muscle cell actin Mouse α Mouse or BS-1 Lectin-green fluorescent) was added and incubated for 45 minutes at room temperature. After rinsing again the slides were

incubated 30 minutes with the second antibody (Goat α Rat-Cy3 or Goat α Mouse-Cy3). When using Goat α Rat-Cy3 a third antibody was used to magnify the signal (Donkey α Goat-Cy3). Subsequently the slides were rinsed in PBS (without Ca^{2+} and Mg^{2+}) and water, imbedded in vectashield and dried. Nuclei were stained using Hoechst when needed. Slides were studied using fluorescence and confocal microscopy.

Results and Discussion

Since there is controversy whether TF is compulsory for blood vessel formation and for the interaction between endothelial en smooth muscle cells, we set up a model using differentiating embryoid bodies to study the influence of TF deficiency on the capacity to form blood vessels. As shown in figure 1, wildtype embryoid bodies did differentiate into endothelial cells (as shown by CD31 and lectin staining) and smooth muscle cells (as shown by smooth muscle actin staining). It appeared as if the smooth muscle cells lay mostly on top of the endothelial cells, suggesting that these two cell types indeed interacted (not shown). As also shown in figure 1, embryoid bodies which are either heterozygous deficient for TF or homozygous deficient differentiated normally into endothelial cells and smooth muscle cells. Similarly to wildtype embryoid bodies, deficient embryoid bodies also formed vessel-like structures. Figure 2 shows a 3D composition for a TF (-/-) vessel-like structure composed of endothelium surrounded by smooth muscle cells. This reconstruction was exactly the same as for a normal embryoid body (not shown)

Previously, we showed that TF chimaeric mice, consisting of 50% wildtype and 50% TF deficient cells on average, develop normally and no net overall preference for TF(wt) DNA in the major arteries and veins was observed [8]. Such chimeras represent a rather complicated model system and it is hard to draw firm conclusions. Therefore, we aimed in this particular study at individual cells and their capacity to differentiate towards SMCs and ECs. The embryoid body model used is a standardized model [12] which is very suited to answer questions related to early vascular development [13]. Using this model, we did not obtain any evidence that TF is involved in vasculogenesis. However, it is known that TF knock-out mice do die in utero due to bleeding complications. Most likely, these bleeding problems do not result from inappropriate vessel development, but from small bleedings during stress in utero. Such bleedings normally do not cause problems, but might be hazardous to the TF deficient embryos.

Overall, our data show that embryoid bodies without functional TF differentiate into vessel like structures as do wildtype embryoid bodies. These findings do not support a major role for TF expression in early vessel formation.

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