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A combined deficiency of tissue factor and PAR-4 is associated with fatal pulmonary hemorrhage in mice

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ABSTRACT

Introduction: Mice with a complete absence of tissue factor (TF) die during embryonic development whereas mice with low levels of TF (Low-TF mice) survive to adulthood. Low-TF mice exhibit spontaneous hemorrhage in various organs, including the lung. In contrast, mice can survive without protease-activated receptor (PAR)-4, which is the major thrombin receptor on mouse platelets. We determined the effect of combining a deficiency PAR-4 (primary hemostasis) with a deficiency in TF (secondary hemostasis) on embryonic development and survival of adult mice.
Materials and methods: Low-TF mice ($mTF^{-/-}$, $hTF^{+/+}$) were crossed with PAR-$4^{-/-}$ mice to generate heterozygous mice ($mTF^{+/-}$, $hTF^{+/-}$, PAR-$4^{+/-}$). These mice were intercrossed to generate Low-TF mice lacking PAR-4. Mice surviving to wean were genotyped and survival was monitored for 6 months.
Results: We observed the expected number of Low-TF,PAR-$4^{-/-}$ mice at wean indicating survival in utero and after birth. However, an absence of PAR-4 was associated with premature death of all Low-TF,PAR-$4^{-/-}$ mice in the 6 month observational period. This compares with 40% death of the Low-TF,PAR-$4^{+/+}$ mice ($p = 0.003$). Low-TF,PAR-$4^{+/-}$ mice had an intermediate phenotype with 55% of the mice dying within 6 months. The primary cause of mortality of Low-TF,PAR-$4^{-/-}$ mice was pulmonary hemorrhage.
Conclusions: Low-TF,PAR-$4^{-/-}$ mice survive into adulthood, but combining a deficiency of primary hemostasis (PAR-4 deficiency) with secondary hemostasis (low levels of TF) leads to premature death primarily due to pulmonary hemorrhage.

1. Introduction

Tissue factor (TF) is the major physiological activator of coagulation. After vessel injury, thrombin generated by the coagulation cascade cleaves fibrinogen to fibrin that stabilizes the platelet clot. Previous studies have shown that embryos with a complete deficiency of TF die at embryonic day ~E9.5 due to a defect in yolk sac vascular development [1–3]. Similarly, a complete absence of other components of the clotting cascade, namely FVII, FV, FX, and prothrombin, leads to death due to either mid-gestational yolk sac vascular defects in embryos or hemorrhage in neonates after birth [4–8]. A deficiency of fibrinogen is not associated with any mid-gestational death, although there is a variable degree of death (10–50%) due to abdominal bleeds after birth depending on genetic background [9,10].

In mice, thrombin also activates platelets via protease-activated receptor (PAR)-4 [11]. PAR-4 deficient mice survive to adulthood but have increased bleeding after tail transection [11]. However, a combined deficiency of both fibrinogen and PAR-4 results in fatal neonatal bleeding similar to the absence of prothrombin [12]. Similar results were observed when a deficiency of fibrinogen was combined with thrombocytopenia caused by an absence of the transcription factor NF-E2 [13]. These results indicate that thrombin has two major functions: cleavage of fibrinogen to fibrin and activation of platelets [12–14].

Various mice have been generated with reduced levels of procoagulant proteins. For instance, Low-TF mice express low levels (~1% of wild-type levels) of human TF from a minigene on a mouse TF null background [15]. The majority of Low-TF mice (~80%) survive to wean but exhibit spontaneous bleeding in the lung, heart, testis and brain later in life, and also have hemostatic defects in the placenta and uterus [15–19]. Mice have been generated with 5–10% of wild-type prothrombin and live to adulthood without excess bleeding except after tail transection or in traumatic situations, such as fighting [20]. A transgene expressing FV from an albumin promoter at <0.1% of wild-type FV activity was able to support the survival of adult mice but was unable to

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rescue FV null embryos [21]. In addition to the generation of mice with defects in hemostasis, other mice have been generated that are prothrombotic, such as mice lacking the anticoagulant tissue factor pathway inhibitor (TFPI), as well as mice with mutations in FV (FV Leiden) and thrombomodulin (TM^{pro}) [22–24].

Mice with pro-hemorrhagic or pro-coagulant phenotypes have been intercrossed to either rebalance thrombin generation or exacerbate a mild phenotype [8,25]. For example, combining FV Leiden mice with $TFPI^{+/-}$ mice is lethal [26]. A deficiency of TFPI leads to intrauterine death due to a consumptive coagulopathy [22]; however, the embryonic lethality of TFPI deficient mice can be rescued by reducing levels of TF using Low-TF mice [27]. The chronic lung bleeding and fatal pulmonary hemorrhages that are observed in adult Low-TF mice were also markedly reduced when TFPI was absent. Similarly, other mutations that lead to intrauterine death, such as endothelial cell protein C receptor, thrombomodulin, and antithrombin deficiency can be rebalanced by decreasing levels of TF [28,29]. Recently, it was shown that a deficiency in PAR-4 rescued ~40% of TFPI null embryos [30]. These mice lived to adulthood but were more prothrombotic with fibrin deposition in the liver and higher susceptibility to TF-induced pulmonary embolism.

In this study we analyzed the effect of combining a defect in primary hemostasis (PAR-4 deficiency) with a defect in secondary hemostasis (low levels of TF) on development during embryogenesis and survival in adulthood.

2. Material and methods

2.1. Mice

All studies were performed in accordance with the guidelines of the animal care and use committees of UNC-Chapel Hill and comply with National Institutes of Health guidelines. Low-TF mice ($mTF^{-/-}$,$hTF^{+/+}$) were generated as previously described [15] and were backcrossed 6 generation to a C57BL/6 J genetic background. We observed ~80% of the expected number of Low-TF mice at wean on this genetic background [27]. Genotyping for the wild-type mTF allele and the hTF transgene was performed by polymerase chain reaction (PCR) as described previously [15]. The mutant TF allele was identified using a primer in the promoter (mTF-1097) and a primer in PGK-NEO. We use the notation $^{+/-}$ to describe the status of the mTF alleles in Low-TF mice. All Low-TF mice have at least one copy of the hTF transgene. $PAR4^{-/-}$ breeding pairs on a C57BL/6 J background were a gift from Dr. Shaun Coughlin, University of California, San Francisco [11]. Low-TF mice ($mTF^{+/-}$,$hTF^{+/+}$) mice were crossed with $PAR4^{+/-}$ mice and mice with the genotype $mTF^{+/-}$,$hTF^{+/-}$,$PAR\text{-}4^{+/-}$ were intercrossed.

2.2. Postmortem analysis of mice

All dead mice that were not decomposed were analyzed for bleeding. Fatal bleeding was mostly observed in the lungs or brain. Lungs were fixed and sectioned for histological analysis.

2.3. Data analysis

Statistical analysis of the distribution of genotypes was analyzed by Chi-square test assuming the null hypothesis. Log-rank analysis was used to determine statistical significance of the survival of the different mice. Differences were determined to be statistically significant at a P value of <0.05.

3. Results

3.1. Generation of Low-TF,$PAR\text{-}4^{-/-}$ mice

Previously, we have observed ~80% survival of Low-TF mice at wean on this C57BL/6 J genetic background (backcrossed 7 generations) [27]. We examined the frequency of Low-TF,$PAR\text{-}4^{-/-}$ mice in two different breeding strategies. First, we intercrossed $mTF^{+/-}$,$hTF^{+/-}$,$PAR\text{-}4^{+/-}$ mice. We generated 69 mice and found that at wean the number of Low-TF,$PAR\text{-}4^{-/-}$ mice did not differ significantly from the expected number ($P = 0.90$) (Table 1). As expected from our earlier study [27], all three Low-TF groups had slightly lower numbers than expected due to ~20% embryonic lethality. In a second breeding we crossed $mTF^{-/-}$,$hTF^{+/-}$, $PAR\text{-}4^{+/-}$ mice with $mTF^{+/-}$,$hTF^{+/-}$,$PAR\text{-}4^{+/-}$ mice. We generated 39 mice in this breeding. As expected from the first breeding, the number of Low-TF,$PAR\text{-}4^{-/-}$ mice observed at wean did not differ from the expected number ($P = 0.62$) (Table 1). These results indicate that an absence of PAR-4 does not affect the survival of Low-TF mice during embryonic development and early adulthood.

3.2. Survival of Low-TF mice with different levels of PAR-4

In a previous study [27] we observed a relatively high rate of mortality of Low-TF mice on the same C57BL/6J background as used in this study (backcrossed 7 generations) within 6 months. We monitored mice surviving to wean over a 6-month period to determine if reducing levels of PAR-4 affect the survival of Low-TF mice. We used $mTF^{+/+}$,$hTF^{+/-}$,$PAR\text{-}4^{-/-}$ mice as controls because these mice exhibit normal survival [11]. We observed that 100% of the Low-TF,$PAR\text{-}4^{-/-}$ mice died during the observation period of 6 months (Fig. 1). The median survival time of these mice was 83 days. In contrast, only 40% of the Low-TF,$PAR\text{-}4^{+/+}$ mice died during the observational period

Table 1
Expected and observed numbers of mice surviving to wean. Low-TF status is associated with a ~20% lethality on this C57BL/6 J background [27], which is also observed in our study when comparing the observed/expected survival ratio of all $mTF^{-/-}$ mice to all $mTF^{+/+}$ mice. Chi-square test did not show any additional effect of PAR-4 status on survival in any of the groups. hTF^{+} indicates that at least one allele of human TF is present ($hTF^{+/-}$ or $hTF^{+/+}$).

Breeding pair	Genotype mTF	hTF	PAR-4	Expected %	Observed %	Observed #	Obs/E %
$mTF^{+/-}$,$hTF^{+/-}$,$PAR\text{-}4^{+/-}$ × $mTF^{+/-}$,$hTF^{+/-}$, $PAR\text{-}4^{+/-}$ (breeding 1)	−/−	+	−/−	6	4	3	70
	−/−	+	±	13	12	8	93
	−/−	+	+/+	6	3	2	46
	±	+	−/−	13	12	8	93
	±	+	±	25	38	26	151
	±	+	+/+	13	9	6	70
	+/+	+	−/−	6	4	3	70
	+/+	+	±	13	13	9	104
	+/+	+	+/+	6	6	4	93
$mTF^{-/-}$,$hTF^{+/-}$,$PAR\text{-}4^{+/-}$ × $mTF^{+/-}$,$hTF^{+/-}$,$PAR\text{-}4^{+/-}$ (breeding 2)	−/−	+	−/−	13	10	4	82
	−/−	+	±	25	28	11	113
	−/−	+	+/+	13	3	1	21
	±	+	−/−	13	21	8	164
	±	+	±	25	26	10	103
	±	+	+/+	13	13	5	103