Rescue of the early vascular defects in Tek/Tie2 null mice reveals an essential survival function

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Disruption of the signaling pathways mediated by the receptor tyrosine kinase Tek/Tie2 has shown that this receptor plays a pivotal role in vascularization of the developing embryo. In this report, we have utilized the tetracycline-responsive binary transgenic system to overcome the early lethal cardiovascular defects associated with the tekΔsp null allele in order to investigate the role of Tek in later stages of vessel growth. We show for the first time in vivo that synchronized loss of tek expression correlates with rapid endothelial cell apoptosis in hemorrhagic regions of the embryo, demonstrating an ongoing requirement for Tek-mediated signal transduction in vascular maintenance.

INTRODUCTION

Assembly of a functional vascular system requires coordinated signaling events between a variety of endothelial cell (EC) growth factors and receptors. In addition to the vascular endothelial growth factor (VEGF) family, a novel family of growth factors known as the angiopoietins has begun to emerge and these unique ligands appear to bind exclusively to the Tek/Tie2 receptor (Davis et al., 1996; Maisonpierre et al., 1997). Gene targeting studies in mice have revealed that the Tek receptor and its ligands appear to be required for the angiogenic remodeling and vessel stabilization processes that occur subsequent to the initial vasculogenic actions of the VEGF receptors. Embryos lacking Tek signaling pathways die between embryonic day 9.5 (E9.5) and E12.5 as a consequence of impaired cardiac function and vascular hemorrhage (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996; Maisonpierre et al., 1997). Myocardial trabecular projections are absent in these mutant embryos and they display a general simplification of the vessel branching pattern as well as a progressive reduction in EC number (Dumont et al., 1994). Ultrastructural analysis of the vessels in embryos lacking tek or ang1 has also revealed a paucity of peri-endothelial support cells in regions of deficient vessel branching (Patan, 1998). Collectively these experiments have provided evidence for a physiological role of Tek signaling in vessel stability, however, the early embryonic lethality seen in these mice has precluded the analysis of the precise function of the receptor during later stages of blood vessel development.

RESULTS AND DISCUSSION

To investigate the role of Tek in the mature vasculature, we have utilized a binary transgenic approach based on the bacterial tetracycline repressor (Gossen et al., 1995; Kistner et al., 1996) to specifically regulate the temporal expression of Tek during mouse embryonic development. This system allows expression of a tek transgene in ECs which can be rapidly extinguished with oral administration of a tetracycline analog known as dox. In this strategy, two independent transgenic mouse lines were developed (Figure 1A). The first line, commonly referred to as the ‘driver line’, was generated by injection of a transgene which has the EC-specific tek promoter (Dumont et al., 1994) driving the expression of a tetracycline-responsive transactivator, ITA (Sarao and Dumont, 1999). The EC-specific expression pattern of this driver line as well as its response to dox has been described previously (Sarao and...
Tek/Tie2 in endothelial cell survival

**Fig. 1.** The dox-repressible binary transgenic system. (A) The driver transgene contains the tek promoter driving the expression of the dox responsive transactivator, tTA. The responder transgene contains the tTA binding site (tetos) upstream of the tek cDNA. The regions corresponding to the RNase protection probe and the RT–PCR oligos are depicted. (B) RNase protection assay from total RNA prepared from E8.5 embryos harvested from matings between hemizygous driver and responder transgenic lines which are wildtype for the tek locus. The 300 bp transgene-specific message and the 200 bp endogenous tek transcript are indicated (tek). (C) RT–PCR analysis on untreated E8.5 embryos or E9.5 embryos that have been treated with dox for 1 day (8.5–9.5) using oligos specific for the transgene or for β-actin demonstrates the presence of a 140 bp transgene-specific fragment in untreated embryos alone. (D) Schematic of the genotypes of the mice used in these studies. Mice heterozygous for the tekΔsp allele and homozygous for either of the transgenes were crossed. The genotype of the desired double transgenic tekΔsp-rescued offspring is indicated. (E) Southern blot analysis of DNA extracted from E12.5 embryos obtained from the mating outlined above. The presence of the mutant and wildtype alleles is indicated. An asterisk above the lane indicates the presence of a homozygous-tekΔsp embryo. All embryos were hemizygous for each transgene as determined by PCR (data not shown).
The second transgenic line in this study, which is referred to as the ‘responder line’, was generated by injection of a transgene which contains the tetracycline DNA binding operator sequences (tet<sup>C</sup>) upstream of the tek cDNA (Figure 1A). The driver and responder transgenic lines were mated to determine whether tTA could mediate expression of tek. RNase protection assays using total RNA isolated from E8.5 double transgenic embryos revealed that expression levels of tek from the responder transgene are comparable to endogenous tek levels and that expression of the responder transgene is solely dependent on the presence of the driver transgene (Figure 1B). To ensure that the driver transgene could respond to dox to regulate tek-responder transgene expression in double transgenic embryos, mothers were treated with dox for 1 day beginning at E8.5 (8.5–9.5). RT–PCR analysis on total RNA isolated from untreated embryos at E8.5 or from treated embryos at E9.5 demonstrated that expression of the tek-responder transgene could be abolished by a single overnight treatment with dox (Figure 1C).

Suitable driver and responder transgenic lines were individually mated with heterozygous tek<sup>Δsp</sup> mice to obtain mice that were homozygous for each transgene and heterozygous for the tek<sup>Δsp</sup> allele (Figure 1D). The appropriate mice were subsequently bred to produce offspring that were double transgenic and homozygous for the tek<sup>Δsp</sup> allele (Figure 1D). These double transgenic tek<sup>Δsp</sup>-null embryos are herein denoted ‘rescued’ embryos. To date, we have not obtained any live rescued offspring suggesting that our rescue approach was incomplete. Dissection of embryos at various time points during gestation revealed that we were able to rescue null mice beyond E9.5, which is the point where tek<sup>Δsp</sup>/tek<sup>Δsp</sup> embryos normally die (Dumont et al., 1994). This rescue was completely dependent upon expression from the tek-responder transgene since double transgenic tek<sup>Δsp</sup>-null embryos obtained from mothers maintained on dox throughout gestation did not survive beyond E9.5 (data not shown). Southern blot analysis of DNA extracted from the yolk sacs of one litter harvested on E12.5 demonstrated that five embryos were homozygous for the tek<sup>Δsp</sup> allele and were positive for the presence of both transgenes (Figure 1E and data not shown). Interestingly, these embryos were all phenotypically indistinguishable from wildtype littersmates.

Remarkably, embryos obtained 1 day later on E13.5 exhibited signs of massive vascular hemorrhaging into the lower limbs and tail although they were still alive as demonstrated by beating hearts (Figure 2A). This extensive bleeding was accompanied by an underdeveloped liver and severe anemia. Embryos harvested on E14.5 and E15.5 were anemic and exhibited signs of necrosis upon histological examination (Figure 2B and data not shown). The comparable size of rescued and wildtype littermates on E12.5 through to E15.5 suggested that five embryos were homozygous for the tek<sup>Δsp</sup> allele and were positive for the presence of both transgenes (Figure 1E and data not shown). Interestingly, these embryos were all phenotypically indistinguishable from wildtype littersmates. The lethality in these embryos may be due to a progressive loss of tek promoter activity after E12.5 due to the absence of an enhancer element (Slaeger et al., 1997; M. Puri and D.J. Dumont, unpublished results) and as consequence, this would result in reduced tTA levels and loss of expression of the tek responder transgene. This notwithstanding, the dramatic differences observed between E12.5 and E13.5 rescued embryos implied that the onset of vascular failure was a relatively rapid process. To examine the possibility that loss of Tek might be responsible for this vascular hemorrhage, we attempted to accelerate the onset of this phenotype through repression of the

Fig. 2. E13.5 and E14.5 rescued embryos present with signs of vascular hemorrhage and anemia. (A) Homozygous tek<sup>Δsp</sup> double transgenic (rescued) embryos (−/−) obtained on E13.5 are anemic and the livers are reduced in size when compared to double transgenic wildtype (+/+ ) littersmates. Extensive hemorrhaging in the tail and lower limbs of rescued embryos can also be observed. (B) Similar defects are found in rescued embryos obtained on E14.5. (C) RT–PCR analysis on untreated (12.5) or dox–treated (11.5–12.5) E12.5 wildtype or rescued embryos with oligos specific for the transgene or for β-actin demonstrates repression of tek-responder transgene expression with overnight dox treatment. Scale bars: (A), 450 μm; (B), 625 μm.
tek-responder transgene. Mothers were treated with dox for 1 day beginning on E11.5 prior to being killed on E12.5. Rescued embryos from these mothers presented with many of the vascular defects of E13.5 rescued embryos, such as bleeding into the lower extremities, reduced liver size and anemia (data not shown). Importantly, these defects were not observed in dox-treated double transgenic wildtype littermates. Repression of tek-responder transgene expression in these treated embryos was confirmed by RT–PCR to demonstrate suppression of mRNA production (Figure 2C) and by immunohistochemistry to verify the absence of Tek protein (see Figure 6K). Collectively these results suggest that the repression of Tek-mediated signal transduction in dox-treated E12.5 double transgenic tekΔsp-null embryos led to a massive loss of vascular integrity.

The embryonic lethality seen in mice that lack Tek signaling pathways is likely to be a result of impaired cardiac function due to incomplete development of ventricular trabeculae. This aspect of the null phenotype is completely penetrant in these embryos and is presumed to arise as a result of poor interactions between the endocardium and the underlying myocardium (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996; Maisonpierre et al., 1997; Patan, 1998). An absolute requirement for Tek in early stages of heart morphogenesis has also been elucidated from mosaic analysis of tekΔsp-null embryos mixed with wildtype embryos where tek-deficient cells were excluded from the endocardium of E10.5 chimeras (Puri et al., 1999). Despite our inability to rescue tekΔsp-null embryos, rescued embryos, which have been harvested on E13.5 or on E12.5 following a day of dox treatment, present with hearts with a normal looking architecture comparable to that seen in wildtype littermate controls (Figure 3 and data not shown). These results suggest that Tek signaling may only be required during a defined window of early cardiac development and is dispensable once the myocardium and endocardium have been induced to adhere tightly together. The possibility of such a restricted requirement for Tek signaling in the developing heart awaits endocardial- and myocardial-specific inducible expression of the receptor and its ligands, respectively.

The rapid onset of vascular hemorrhage between E12.5 and E13.5 in rescued embryos prompted us to examine the structural integrity of blood vessels in these embryos. Parasagittal thin sections prepared from E13.5 rescued and wildtype embryos and yolk sacs were stained with antibodies recognizing the EC markers von Willebrand factor (vWF) and platelet and endothelial cell adhesion molecule 1 (PECAM-1) (data not shown) and the myogenic lineage marker smooth muscle actin (SMA). Immunohistological analysis of rescued embryos revealed that several vessels had lost the inner EC lining while retaining the surrounding smooth muscle cells (SMC). Examination of sections taken from the yolk sacs of E13.5 rescued embryos revealed that there were few detectable vWF- or PECAM-1-positive cells lining the lumens of the vasculature in any of the yolk sacs examined despite the presence of positive SMA-staining mesenchymal support cells (Figure 4A and B). In contrast, sections prepared from the yolk sac of a double transgenic wildtype littermate stained positive for all markers (Figure 4C and D). Similarly, in the embryo proper, vessel-like structures containing blood surrounded by rings of SMA-positive cells could be observed but many of these structures had few vWF-positive cells lining the lumen of the vessel (Figure 4G–J) and in some cases, the remaining vWF-positive cells appeared rounded and detached from the luminal lining (Figure 4K–L). Interestingly however, a subset of blood vessels in rescued embryos contained both ECs and SMCs (Figure 4E and F), indicating that

**Fig. 3.** Cardiac development in double transgenic rescued and wildtype embryos. H & E stained sections of the heart region of E13.5 embryos indicate extensive ventricular trabeculation in rescued embryos (A) comparable to that seen in wildtype littermates (B). Heart sections prepared from an independent litter stained with hematoxylin alone demonstrate that the general architecture of the heart in rescued embryos (C) is indistinguishable from normal littermates (D). Arrowheads indicate loose endothelium in both rescued and wildtype embryos. Scale bars: (A–D), 600 μm.
vasculogenesis and early angiogenesis had proceeded normally in these embryos.

The organization of SMA-positive cells in E13.5 rescued embryos indicated the prior existence of established blood vessels although there appeared to be some insufficiency in the maintenance of ECs in a number of these vessels. In earlier reports, we have proposed that the progressive loss of endothelium in tekΔsp-null embryos may be attributed to an active cell death process, although we were unable to detect cells undergoing apoptosis in these compromised embryos, possibly due to the overall deterioration of the embryo (Dumont et al., 1994). In an effort to capture the rapid event responsible for vascular failure, we synchronized the onset of vessel hemorrhage via repression of tek and examined these dox-treated E12.5 embryos.
for the presence of apoptotic cells. Loss of vessel integrity in these embryos was especially evident when the vasculature surrounding the central nervous system was examined. When compared with wildtype littermates, the leptomeninges lining the brain and spinal cord in the region of the lower neural tube of rescued embryos do not seem well adhered and there are focal regions where extravasated blood can be observed (Figure 5A and B). TUNEL assays performed on adjacent sections revealed a large number of apoptotic cells in dox-treated rescued embryos that localized predominately to the surface of the neural tube in the same region where the leptomeninges were detached from the tube (Figure 5C). In contrast, very few apoptotic cells were observed in sections taken from dox-treated wildtype littermates (Figure 5D). Comparison of the number of TUNEL-positive cells in rescued embryos versus normal littermates revealed that there was an ~20-fold statistically significant increase in apoptosis in rescued embryos (Table I). It is likely that the combination of apoptosis and loss of vessel integrity results in extensive bleeding from this structure although the restriction in pooling of blood to the lower limbs is unclear.

To further characterize the identity of these TUNEL-positive cells, sections were double-labeled using antibodies specific for the EC markers vWF and Tek and the TUNEL reagent. Confocal microscopy images captured from sections prepared from untreated E10.5 rescued and wildtype littermates revealed intact vessels which were positive for both EC markers and were not positive for TUNEL (Figure 6A–D). Similarly, in E12.5 untreated rescued embryos, TUNEL staining was virtually undetectable in ECs although Tek protein expression could be detected on the cell surface (Figure 6E–H). In both cases, TUNEL staining was observed in other areas of the embryo (data not shown). Upon repression of responder transgene expression beginning at

**Table I. Analysis of apoptotic cells in dox-treated rescued and wildtype double transgenic littermate embryos**

<table>
<thead>
<tr>
<th>Gestational agea (days)</th>
<th>tekb genotype</th>
<th>Average No. of TUNEL-positive cells</th>
<th>No. of embryo sections examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>E12.5 –/–</td>
<td></td>
<td>63</td>
<td>6</td>
</tr>
<tr>
<td>E12.5 +/+</td>
<td></td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>E13.5 –/–</td>
<td></td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>E13.5 +/+</td>
<td></td>
<td>1.5</td>
<td>8</td>
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*Mothers were fed doxycycline 24 h prior to being killed.
E11.5, dox-treated E12.5 rescued embryos (E11.5–E12.5) exhibited extensive TUNEL staining that colocalized with vWF expression (Figure 6I), suggesting that the apoptotic cells detected by TUNEL staining were in fact ECs. In contrast, very few TUNEL-positive ECs were detected in dox-treated E12.5 wildtype littermates (Figure 6J). As anticipated, adjacent sections confirmed the absence of Tek on the surface of apoptotic ECs in rescued embryos with repressed tek-responder transgene expression although Tek-positive cells were readily observed lining the vessels in double transgenic wildtype embryos (Figure 6K and L).

Conditional expression of tek in embryonic ECs demonstrates that Tek is required throughout vascular development and in its absence, embryos quickly manifest disruptions within their blood vessels which result in massive hemorrhaging and death. Vascular hemorrhage appears to be secondary to the impaired survival of the endothelium since the onset of hemorrhage is concurrent with the temporal loss of tek expression in ECs undergoing apoptosis. It has been hypothesized that the vascular defects observed in embryos lacking tek or ang1 may result from a failure to properly recruit SMC precursors to developing vessels (Suri et al., 1996; Patan, 1998) as Tek may stimulate the release or activation of chemoattractant growth signals (Folkman and D’Amore, 1996). This hypothesis has been challenged by mosaic experiments of Puri et al. (1999) where it was demonstrated that the presence of large numbers of tek-deficient cells in developing chimeric vessels did not lead to abnormal perivascular cell recruitment. Instead, a selection against tek-deficient cells in late stage chimeric embryos and adults was observed, suggesting that Tek plays a role in EC maintenance or survival (Puri et al., 1999). Our findings support this premise since inhibition of tek transgene expression in rescued embryos through administration of dox results in a rapid onset of EC apoptosis. Persistent expression and phosphorylation of Tek in quiescent adult endothelium is consistent with a role for Tek in transducing an ongoing survival stimulus to ECs (Wong et al., 1997). The finding that Ang1 can initiate an anti-apoptotic signal transduction cascade involving phosphatidylinositol 3-kinase and Akt supports this conclusion (Kontos et al., 1998; Jones et al., 1999; Kim et al., 2000). VEGF also functions as a strong survival factor for ECs (Gerber et al., 1998) suggesting that ECs have evolved to respond to two distinct survival cues. Although early vascular patterning appears to be independent of Ang1 owing to the actions of VEGF, Ang1 may become the predominant survival factor in particular vascular beds where VEGF expression is downregulated. A stringent requirement for Ang1-dependent survival is especially evident in the region surrounding the leptomeninges where VEGF expression is virtually undetectable at E12.5 (Dumont et al., 1992) and repression of tek leads to the rapid manifestation of massive apoptosis. The leptomeninges have previously been shown to express high levels of tek (Dumont et al., 1995), thus it is plausible that Tek signaling is required to prevent these cells from undergoing programmed cell death. Temporal and spatial overlap between VEGF and Ang1-mediated EC survival may therefore need to be considered in the therapeutic control of pathological angiogenesis.

METHODS

Generation and genotyping of transgenic mice. Construction of the driver transgene, ptektTA, and the plasmid ptek*, have been described previously (Sarao and Dumont, 1999). To generate the responder transgene, ptek*tek, a 4183 bp EcoRI fragment corresponding to mouse Tek cDNA (amino acids 1–1122) was inserted into the EcoRI sites of ptek*. Transgenic lines were
maintained and genotyped as previously described (Sarao and Dumont, 1999). The ptekTA driver transgene was amplified using the primer set described in Sarao and Dumont (1999). The ptekTet responder transgene was amplified using the forward primer 5′-ACCATGTCATAGCCTCTT-3′ and the reverse primer 5′-GTGCTGGTCACTATTAGCTT-3′. Wildtype tek and tekΔsp alleles were amplified using the primer sets described in Puri et al. (1999).

Transgene expression analysis. RNase protection analysis was performed essentially as described in Dumont et al. (1995). The ptekTet cRNA probe was derived from sequences within the tek cDNA and the β-globin exon. RNA was isolated using the RNeasy system and RT–PCR was carried out using the OneStep RT–PCR system (both Qiagen). PtekTet was detected using the forward primer 5′-CGATCCTGAGAACTTCAGG-3′ and the reverse primer 5′-AGAGAACTAAGCCGGCTAAA-3′. The β-actin primers were from Clontech.

Morphological and histological analysis. Whole-mount embryos were examined and photographed on a dissecting microscope (Leica). For histology, embryos were isolated and fixed for 4 h at room temperature in Histochoice (Amresco). Paraffin and cryoembedding, sectioning and staining were performed using standard techniques.

Immunohistochemical staining. Immunohistochemistry was performed essentially as described in the Vectastain Elite ABC Kit (Vector Laboratories) using the following primary antibodies: anti-PECAM-1 (rat; MEC 13.3, Pharmingen), anti-Ś MA (mouse) and anti-vWF (rabbit) (both Dako) and anti-Tek (rat; TEK4, a kind gift of Toshiro Suda, see Yano et al., 1997). For confocal analysis, vWF and Tek4 primary antibodies were detected with Cy3-conjugated secondary antibodies (Jackson Laboratories). Slides were analyzed on a Leica DMR compound microscope or a Zeiss Axiostep 100M confocal microscope. Image processing was performed using the Adobe Photoshop v5.02 program (Adobe Systems Inc.).

TUNEL assays. TUNEL staining was performed according to the manufacturer’s instructions as outlined in the in situ Cell Death Detection Kit, POD (Boehringer Mannheim) using 1% non-fat milk powder as a blocking agent. For quantitation of apoptotic cells, 10 fields per section were counted and data was analyzed using a Student’s t-test with a p value of 0.0005.

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