Tie1 deletion inhibits tumor growth and improves angiopoietin antagonist therapy


The endothelial Tie1 receptor is ligand-less, but interacts with the Tie2 receptor for angiopoietins (Angpt). Angpt2 is expressed in tumor blood vessels, and its blockade inhibits tumor angiogenesis. Here we found that Tie1 deletion from the endothelium of adult mice inhibits tumor angiogenesis and growth by decreasing endothelial cell survival in tumor vessels, without affecting normal vasculature. Treatment with VEGF or VEGFR-2 blocking antibodies similarly reduced tumor angiogenesis and growth; however, no additive inhibition was obtained by targeting both Tie1 and VEGF/VEGFR-2. In contrast, treatment of Tie1-deficient mice with a soluble form of the extracellular domain of Tie2, which blocks Angpt activity, resulted in additive inhibition of tumor growth. Notably, Tie1 deletion decreased sprouting angiogenesis and increased Notch pathway activity in the postnatal retinal vasculature, while pharmacological Notch suppression in the absence of Tie1 promoted retinal hypervascularization. Moreover, substantial additive inhibition of the retinal vascular front migration was observed when Angpt2 blocking antibodies were administered to Tie1-deficient pups. Thus, Tie1 regulates tumor angiogenesis, postnatal sprouting angiogenesis, and endothelial cell survival, which are controlled by VEGF, Angpt, and Notch signals. Our results suggest that targeting Tie1 in combination with Angpt/Tie2 has the potential to improve antiangiogenic therapy.

Introduction

Angiogenesis, the sprouting of new blood vessels from preexisting ones, is required for a variety of physiological processes, such as embryonic development, reproduction, wound healing, and organ regeneration in adults. Furthermore, angiogenesis is involved in several pathological processes, including age-related macular degeneration and cancer (1, 2), and compounds that inhibit the VEGF/VEGFR-2 pathway are in clinical use for the treatment of these diseases. However, targeting the VEGF/VEGFR-2 pathway is often insufficient to control tumor growth. Thus, novel approaches to further develop antiangiogenic therapies for cancer are needed (3).

The angiopoietin (Angpt) growth factors Angpt1 and Angpt2 and the Tie receptors Tie1 and Tie2 form another endothelial receptor tyrosine kinase signaling system with a pivotal role in embryonic vessel morphogenesis and vascular homeostasis (4). Constitutive deletion of Tie1, Tie2, or Angpt1 results in embryonic lethality (5–8). In adults, Angpt1-mediated signals are important for stabilization of the vascular endothelium after angiogenic processes (8). Angpt2 is required for lymphatic development, and postnatally, both Angpt1 and Angpt2 contribute to the development of the retinal vasculature (9, 10).

The Angpt-Tie pathway also regulates tumor angiogenesis (4, 11). Angpt2 levels are increased in many human cancers, and its blockage inhibits tumor growth and angiogenesis (12–14). Blocking Angpt2 also inhibits tumor metastasis via hematogenous and lymphatic routes (13, 15).

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DNA, mRNA, and protein levels in all PGK-Cre/Tie1flox/flox (referred to herein as Tie1Δ/Δ) embryos analyzed (Supplemental Figure 1, C–F, and Supplemental Figure 2). Tie1 mRNA and protein levels in samples of PGK-Cre/Tie1flox/+ (Tie1Δ+/+) embryos were about half of those seen in WT and Tie1flox/flox control samples (Supplemental Figure 1, E and F). At E14.5, Tie1Δ/Δ embryos exhibited a pronounced edematous phenotype and hemorrhages (Supplemental Figure 1A), resembling transgenic embryos in which ubiquitous Tie1 deletion is achieved by insertion of the LacZ gene into the Tie1 locus (5, 21). The Tie1Δ/Δ mice died by E17.5 (data not shown).

Examination of histological sections of the thoracic region, stained for the blood vascular endothelial marker endomucin and the lymphatic endothelial markers Prox1 and Lyve1, showed the presence of mispatterned first lymphatic structures that have been previously described as lymph sacs, in Tie1Δ/Δ but not control embryos at E14.5 (Supplemental Figure 1A), resembling transgenic embryos in which ubiquitous Tie1 deletion is achieved by insertion of the LacZ gene into the Tie1 locus (5, 21). The Tie1Δ/Δ mice died by E17.5 (data not shown).

Endothelial Tie1 deficiency inhibits tumor growth. (A) Tie1 expression in the LLC (Tie1, green; endomucin, red; DNA, blue) and B16F1 (X-gal, blue) tumor vasculatures in Tie1+/+ and Tie1lacZ/+ mice, respectively. (B) Tie1 mRNA expression analyzed by quantitative RT-PCR (P < 10−5) and Tie1 and Hsc70 Western blots from control and Tie1ECΔ/Δ lung extracts. Numbers denote relative Tie1 levels. n = 7–10 lungs/genotype. (C) Growth curves of LLC, EL4, and B16F10 (B16) tumors. (D) Tumor weight at termination. Scale bars: 20 μm (A); 10 mm (C). Error bars denote SEM. Significant differences are shown by asterisks, with P values indicated.

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Endothelial Tie1 deletion inhibits tumor angiogenesis and growth. We next assessed the function of Tie1 during tumor angiogenesis. Previous in situ hybridization studies have shown elevated Tie1 mRNA expression in the vascular endothelium of malignant human tumors compared with normal vessel endothelium (19,
We confirmed Tie1 expression in angiogenic tumor vessels by immunohistochemical staining and by using heterozygous Tie1ΔEC/Δ mice, in which the endogenous Tie1 locus drives LacZ gene expression and thus unambiguously marks transgene-expressing cells (Figure 1A). Interestingly, immunohistochemical staining showed variable levels of Tie1 expression in the tumor vasculature. We crossed Tie1fl/fl mice with Cdh5(PAC)-cre/ERT2 and Pdgfb-icre/ERT2 mice (Supplemental Methods). This enabled inducible Tie1 deletion by endothelial-specific Cre recombinase activation after tamoxifen administration (Figure 1B and Supplemental Table 1).

We implanted Lewis lung carcinoma (LLC), B16F10 melanoma (B16), or EL4 leukemia/lymphoma (EL4) cells to the Tie1iEC mice and found significantly reduced tumor growth compared with controls (Figure 1, C and D).

Immunohistochemical staining indicated that Tie1 expression and blood vessel density were reduced in tumors grown in Tie1iEC/Δ mice versus control mice (Supplemental Figure 4 and Figure 2, A and B). Notably, whereas LLC tumor vessels in control mice tended to grow in a branching honeycomb-like pattern, those in Tie1iEC/Δ mice showed significantly decreased density of angiogenic sprouts and filopodial extensions (Figure 2, A and B). These results indicated that Tie1 is crucial for tumor growth and angiogenesis.

As Tie1 has been reported to promote endothelial cell survival in the late phase of angiogenic capillary growth during mouse embryonic development (21), we assessed endothelial cell apoptosis in the tumor vasculature. Apoptosis was significantly increased in Tie1iEC/Δ tumor vessels (Figure 3, A–F). Electron microscopy indicated that the degenerating endothelial cells were occasionally associated with small intravascular fibrin deposits in Tie1iEC/Δ tumors (Figure 3, G–J, and Supplemental Figure 5). However, we found no significant differences in endothelial cell proliferation between Tie1iEC/Δ and control tumors (Supplemental Figure 6).

We also analyzed parameters of vessel maturation, integrity, and functionality in the tumors. The percentage of blood vessels coiled with NG2 proteoglycan–positive pericytes or α-SMA–positive smooth muscle cells was similar in Tie1iEC/Δ and control tumors (Supplemental Figure 7). Intravascular perfusion with Lycopersicon esculentum (tomato) lectin showed a decreased proportion of perfused tumor vessels, and pimonidazole hydrochloride staining indicated a trend toward increased tumor hypoxia, in Tie1iEC/Δ versus control tumors (Figure 4, A–F). Furthermore, tumor cell apoptosis was increased in Tie1iEC/Δ mice (Figure 4, G–L). Thus, inhibition of tumor angiogenesis by Tie1 deletion was associated with reduced endothelial cell viability in the tumor vessels as well as increased tumor hypoxia and tumor cell death.

Endothelial Tie1 has been suggested to play a role in inflammation, and inflammatory cells regulate tumor angiogenesis and growth (20, 26). Therefore, we investigated whether Tie1 deletion affects endothelial expression of leukocyte adhesion receptors or leukocyte recruitment to the tumor stroma. P-selectin and intercellular adhesion molecule 1 (ICAM1) expression were reduced in Tie1iEC/Δ tumor endothelium, but there were no significant differences in leukocyte (CD45), monocyte/macrophage (F4/80), or granulocyte (Ly6b/Gr-1) recruitment to the tumors (Supplemental Figures 8 and 9). These results suggest that inflammatory cells do not contribute substantially to the decreased tumor growth in Tie1iEC/Δ mice.

Tie1 deletion does not harm the healthy vasculature or renal function. It was previously shown that stringent blocking of VEGF signaling in healthy adult mice for 2 weeks results in significant capillary regression in certain organs (27, 28). As adult Tie1iEC/Δ mice exhibited increased endothelial cell apoptosis in tumor isografts, we next analyzed the effect of Tie1 deficiency on normal healthy vessels. We used the Pdgfb-icre/ERT2 deleter strain to induce endothelial Tie1 deletion in 8- to 10-week-old mice. After a deletion period of 3 weeks (corresponding to that in the tumor models), we analyzed the blood vasculature in the lungs, kidneys, heart, thyroid, salivary glands, small intestine, trachea, and skin. Quantitation of Pecam1 and endomucin staining indicated that blood vessel density was not significantly different in Tie1iEC/Δ versus control tissues (Supplemental Figure 10, A and B). Analysis of interendothelial adhesion junctions of blood capillaries immunostained for cadherin 5 (Cdh5; also known as VE-cadherin) and quantitation of the diam-
The ultrastructural features of renal glomerular capillaries, such as endothelial cell fenestrations, basement membrane thickness, or podocyte foot processes were not altered by either endothelial-specific or ubiquitous (Rosa26/creERT2 deleter; ref. 29) Tie1 deletion (3 weeks and 5 months, respectively; Supplemental Figure 11A, Supplemental Table 1, and Supplemental Methods). In line with this, Tie1 deletion did not alter blood urea nitrogen or creatinine concentrations in serum. Moreover, no significant changes were observed in polypeptides in urine when comparing control versus Tie1-deficient mice (Supplemental Figure 11, B and C), which suggests that Tie1 is not necessary for the maintenance of kidney function under basal conditions. Thus, despite the effects of Tie1 deficiency on tumor angiogenesis, normal vessels were not significantly affected in various adult Tie1iECΔ/Δ mouse tissues after a 3-week deletion period.

**Endothelial Tie1 deficiency does not improve antiangiogenic therapy directed against VEGF or VEGFR-2.** Recent results have suggested that blocking Angpt2 increases the efficacy of antiangiogenic therapy directed against the VEGF/VEGFR-2 pathway (30–32). We tested whether endothelial Tie1 deletion in combination with blocking antibodies against VEGF or VEGFR-2 provides additive inhibition of tumor angiogenesis and growth. The VEGFR-2 blocking antibody DC101 (33) and the VEGF blocking antibody mcr84 (34) were both capable of significantly inhibiting angiogenesis and growth of LLC, EL4, and B16F1 tumors in control mice. Although Tie1 deletion was equally effective in reducing tumor growth, no additive antiangiogenic effects were seen when either antibody was used in combination with endothelial Tie1 deletion (Supplemental Figure 12). Interestingly, when VEGF was injected and allowed to circulate for 10 minutes, decreased VEGFR-2 protein was detected in Tie1iECΔ/Δ versus control lung lysates. The decreased VEGFR-2 levels may help to explain the reduced endothelial cell survival in the tumors and the ineffectiveness of VEGF signaling inhibition in Tie1iECΔ/Δ mice (Supplemental Figure 13).

Previous studies assessing the safety of very-high-affinity anti-VEGF antibodies showed some renal and hepatic damage, including glomerulosclerosis and loss of glomerular structural integrity (35). We thus analyzed kidney glomeruli in the tumor-bearing mice treated with placebo or mcr84 antibody, which has a low toxicity profile in mice (34). Morphological and immunohistochemical analysis showed no statistically significant differences in glomerular tufts, Bowman’s space, or vascular density in the kidney glomeruli (Supplemental Figure 14). Furthermore, the liver histology in H&E staining was unaltered, and total body weights were similar in
Tie2 (referred to herein as mTie2-ECD; Figure 5A), which binds observations), we used an adeno-associated virus (AAV) vector to that is sensitive to anti-Angpt2 antibodies (our unpublished and growth. Because we could not identify a tumor isograft model (39, 40). We thus tested whether combinatorial targeting of Tie1 Angpt2, which is produced by the angiogenic tumor endothelium. The dominant Tie2 ligand in tumors is (38), was Tie2 at Y1106, which is known to bind the Dok-R protein and to mental Figure 15A). Interestingly however, phosphorylation of lungs of Tie1-deleted mice, even in the presence of VEGF, which total Tie2 phosphotyrosine was not significantly altered in the vitro studies have suggested that silencing of endothelial Tie1 increases Angpt2-induced Tie2 phosphorylation and that VEGF enhances Tie2 responsiveness to Angpt1 by modulating the Tie1/ Angpt1 and Angpt2 and blocks their activity (13, 41). In agreement with previous reports (32, 41), mTie2-ECD treatment inhibited the growth of LLC tumor isografts in mice (Figure 5, B and C). However, the antitumor effect was significantly greater when mTie2-ECD was used in combination with endothelial Tie1 deletion (Figure 5, B and C). Compared with mTie2-ECD alone, the combinatorial targeting decreased the length and increased the width of the vessels in the tumor sections and tended to increase pericyte recruitment (Figure 5D and data not shown).

**Effect of Tie1 deletion on Tie2 signaling pathways in vivo.** Previous in vitro studies have suggested that silencing of endothelial Tie1 increases Angpt2-induced Tie2 phosphorylation and that VEGF enhances Tie2 responsiveness to Angpt1 by modulating the Tie1/ Tie2 ratio on the cell surface (36, 37). However, in our experiments, total Tie2 phosphotyrosine was not significantly altered in the lungs of Tie1-deleted mice, even in the presence of VEGF, which was injected i.v. and allowed to circulate for 10 minutes (Supplemental Figure 15A). Interestingly however, phosphorylation of Tie2 at Y1106, which is known to bind the Dok-R protein and to promote Angpt1-mediated endothelial cell migration (38), was decreased when Tie1 was deleted (Supplemental Figure 15B).

**Combinatorial targeting of Tie1 and angiopoietins results in additive tumor growth inhibition.** The dominant Tie2 ligand in tumors is Angpt2, which is produced by the angiogenic tumor endothelium (39, 40). We thus tested whether combinatorial targeting of Tie1 and Angpts provides additive inhibition of tumor angiogenesis and growth. Because we could not identify a tumor isograft model that is sensitive to anti-Angpt2 antibodies (our unpublished observations), we used an adeno-associated virus (AAV) vector to deliver a soluble form of the extracellular domain (ECD) of mouse Tie2 (referred to herein as mTie2-ECD; Figure 5A), which binds all mice throughout the experiment (data not shown). Thus, in our tumor model, the combination of endothelial Tie1 deletion and mcr84 antibody treatment did not induce kidney or liver damage (Supplemental Figure 14 and data not shown).

**Figure 4**

Vessel perfusion, hypoxia, and apoptosis in tumors of control and Tie1iEC/Δ mice. (A and B) LLC tumor sections from mice injected i.v. with biotinylated *L. esculentum* lectin 10 minutes before tumor excision at day 18 after tumor cell implantation. Lectin (green) staining marks functional tumor blood vessels. (C) Vessel perfusion, determined as percent of lectin-positive area relative to percent of endomucin-positive area per field. *n* = 3–6 tumors/genotype (Pdgfb-icre/ERT2 deleter). (D and E) LLC tumor sections from mice injected i.p. with pimonidazole (hypoxia marker) 1.5 hours before tumor excision. Pimonidazole (green) staining marks the hypoxic area. (F) Percent hypoxic area relative to total field area. *n* = 9 tumors/genotype (both Cdh5(PAC)-cre/ERT2 and Pdgfb-icre/ERT2 deletors). Note the trend toward increased hypoxia in Tie1iEC/Δ tumors. (G and H) Staining for TUNEL (red), endomucin (green), and DAPI (blue). (J and K) TUNEL staining alone from G and H, shown for better visualization. (I and L) Tumor cell apoptosis indices, calculated as ratio of (I) cleaved Caspase-3 and (L) TUNEL fluorescent cells to total DAPI-positive tumor cells per field. *n* = 3–6 tumors/genotype (both Cdh5(PAC)-cre/ ERT2 and Pdgfb-icre/ERT2 deletors). Scale bars: 100 μm. Error bars denote SEM. Significant differences are shown by asterisks, with *P* values indicated.
tistically significant decrease in vessel area, vessel branchpoints, and the distance of the migratory angiogenic vessel front from the optic nerve (Supplemental Figure 17). Notably, no significant retinal phenotype was observed in pups in which the Tie1 deletion was only 80%–85% complete (data not shown).

To maximize the extent of Tie1 deletion, we combined Tie1 conditional and constitutive targeted alleles (Figure 6, C–I). Tamoxifen-induced Tie1 deletion in Cdh5(PAC)-cre/ERT2;Tie1flox/– and Pdgfb-icre/ERT2;Tie1flox/– pups (referred to herein as Tie1 iECΔ/–) resulted in near-complete Tie1 ablation, and the resulting retinal phenotype was severely aggravated compared with that of Tie1iECΔ/Δ pups (Figure 6, C–I, and Supplemental Figure 17). Interestingly, in some Tie1iECΔ/– pups, we also observed the presence of endothelial cell tufts (Figure 6E), a feature not seen in Tie1iECΔ/Δ pups. Furthermore, the filopodial extensions of the tip cells were significantly reduced, and in some Tie1 iECΔ/– pups, the migratory front of the retinal vasculature was almost devoid of endothelial sprouts (Figure 6H and Supplemental Figure 18). Further analysis of the vessels revealed a few pericyte-positive (i.e., NG2 staining), but endothelium-negative (i.e., lacking isolectin B4 [IB4] staining) cellular structures, particularly in capillary branches extending from the arterioles of the Tie1iECΔ/– retinas (Supplemental Figure 18), suggestive of occasional vessel branch regression. Nevertheless, the retinal vasculature seemed normal when Tie1 was deleted between P7 and P9 and analyzed at P17 (data not shown). These results indicate that Tie1 deficiency delays normal development of the postnatal retinal vasculature during angiogenesis, but does not affect the vascular remodeling phase. Furthermore, the reduced vascular area and decreased number of angiogenic sprouts and filopodial extensions observed in the Tie1-deficient postnatal retinas resembled changes observed in the tumor vasculature in the Tie1-deficient adult mice.

The Notch pathway is activated in Tie1-deficient retinal endothelial cells. The expression of Tie1 in retinal endothelial tip cells and the retinal phenotype of Tie1-deleted mice prompted us to analyze whether the Tie1 deletion is associated with changes in Notch ligand Delta-like 4 (Dll4) signaling. Dll4 is mainly expressed in the angiogenic tip cells, and it activates Notch signaling in the stalk cells to suppress their migration and sprouting (46, 47). Quantitative RT-PCR using retinal extracts showed that the Tie1iECΔ/Δ and Tie1iECΔ/– retinas expressed significantly increased amounts of mRNA encoding Dll4 and the downstream Notch signaling molecules Hairy enhancer-of-split related with YRPW motif 1 (Hey1) and Notch-regulated ankyrin repeat protein (Nrarp), as well as the hypoxia-regulated angiogenic factors Vegf and Angpt2 (Figure 7A). Dll4 mRNA and protein levels, and the intracellular domain of Notch (NCID), which is cleaved upon its activation, were also significantly upregulated in Tie1iECΔ/– retinas versus control retinas at P6 (Figure 7, B and H, and Supplemental Figure 19). Furthermore, whole-mount staining indicated significantly increased endothelial Dll4 abundance in Tie1iECΔ/– versus control retinas at P6 (Figure 7, C–E). These results indicate that the Notch pathway is activated in the Tie1-deficient retinas, consistent with the suppression of the migratory and sprouting phenotype of the retinal vasculature.

Figure 5 Combinatorial targeting of Tie1 and Angpts results in greater inhibition of tumor growth than targeting either alone. (A) Experimental settings for induction of Tie1 deletion by tamoxifen (TAM) pellets, soluble extracellular domain of murine Tie2 (mTie2-ECD) or soluble murine Fc gamma receptor (mFc) expression, and LLC tumor implantation. (B) LLC tumor growth curves. (C) Tumor volume and weight at 18 days. (D) Vascular area, vessel length, and vessel width (expressed as a percentage of control plus mFc). Error bars denote SEM. *P < 0.05 versus control plus IgG; †P < 0.05 versus control plus mTie2-ECD.
To further analyze the involvement of the Notch pathway in the Tie1-deficient phenotype, we used the $\gamma$-secretase inhibitor DAPT, which inhibits Notch cleavage and signaling (46). Interestingly, Tie1iEC$^{Δ/–}$ and control pups had an almost identical retinal hypervascularization phenotype at P6, after a 48-hour treatment with DAPT (Figure 7, F and G). DAPT treatment also prevented the increase of NCID in Tie1iEC$^{Δ/–}$ pups (Figure 7H). These results suggest that increased Notch activation contributes to the Tie1-deficient retinal phenotype.

**Figure 6**
Endothelial Tie1 deficiency inhibits postnatal retinal angiogenesis. (A) $β$-galactosidase staining of a Tie1lacZ/+ retina at P6. (B) A Tie1-expressing tip cell. (C–E) IB4–stained P6 retinas. a, arteriole; v, venule. Arrowheads denote vascular tufts. (F) Quantitative RT-PCR and Western blot of lungs from control and Tie1iEC$^{Δ/–}$ (Cdh5(PAC)-cre/ERT2 and Pdgfb-icre/ERT2 deletors) P6 pups. Numbers denote mean densitometric readouts of relative Tie1 levels. n = 3–4 lungs/genotype. (G and H) High-magnification images of the angiogenic front. Dots denote filopodia. (I) Quantification of vascular area, branchpoints, radial distance from optic nerve, sprouts, and filopodia. Scale bars: 100 μm (A, C–E, G, and H); 50 μm (B). Error bars denote SEM. Significant differences are shown by asterisks, with P values indicated.

Combination targeting of Tie1 and Angpt2 results in additive inhibition of retinal angiogenesis. Anti-Angpt2 blocking antibodies inhibit retinal angiogenesis, and Angpt2 expression is highly selective for retinal endothelial tip cells, where we found Tie1 expression, but little or no Tie2 expression (13, 48, 49). We tested whether Tie1 deletion in combination with anti-Angpt2 blocking antibodies could provide additive inhibition of retinal angiogenesis. We deleted Tie1 in pups as described above and simultaneously administered anti-Angpt2 or IgG isotype control antibodies. At P5, as expected, both IgG-treated Tie1iEC$^{Δ/–}$ pups and anti-Angpt2–treated control pups showed a statistically significant decrease in the distance of the vascular front from the optic nerve compared with IgG-treated controls (Figure 8, A–E). Surprisingly however, significant additive inhibition of vascular front migration was observed when anti-Angpt2 antibodies were used in Tie1iEC$^{Δ/–}$ pups (Figure 8, B, D, and E). These results showed that the combinational treatment with anti-Angpt2 blocking antibodies increases the antiangiogenic effects of Tie1 deletion on retinal sprouting angiogenesis, in line with our results on tumor angiogenesis and growth, in which combinational targeting of Tie1 and Angpt2 via soluble murine Tie2-ECD were additive.

**Discussion**
Here we report that deleting Tie1 specifically from the vascular endothelium inhibited tumor angiogenesis and growth, to the same extent as blocking antibodies targeted against VEGF or its major signal transducing receptor, VEGFR-2. To our knowledge, this is the first study showing that Tie1, a receptor for which no growth factor ligands have yet been identified, functions as a positive regulator of pathological angiogenesis and that Tie1 targeting has beneficial antitumor effects. Tumors grown in Tie1-deficient mice had decreased vessel density, vascular perfusion, and endothelial cell survival, and this was associated with decreased
inhibit physiological retinal angiogenesis (9, 13, 49). Although the success of the anti-VEGF approach has been remarkable, some concerns exist about the long-term effects of VEGF antagonism in the eye (52). Therefore, new targets and their combinations for safer and more effective therapy are still needed. To our knowledge, our report is the first to show that targeting Tie1 significantly inhibits retinal angiogenesis and that, importantly, combinatorial treatment with anti-Angpt2 blocking antibodies increases this effect.

Notch and VEGF pathway interactions have been thoroughly studied in retinal sprouting angiogenesis, but only 1 study thus far has analyzed the Dll4/Notch crosstalk with angiopoietin/Tie2 signaling (53). We showed here that Dll4/Notch signals were not involved in tumor cell survival. The tumor vessels in Tie1<sup>Δ/Δ</sup> mice showed reduced numbers of angiogenic sprouts and filopodial extensions, resembling the delayed development of the postnatal retinal vasculature in Tie1<sup>Δ/Δ</sup> mice.

VEGF- and Angpt2-mediated angiogenesis is involved in retinal and choroidal diseases, the most common causes of loss of vision in the developed countries (50). Angpt2 is expressed in the mouse retina between P0 and P7, when the superficial retinal vessels develop; after P7, its expression shifts to the regions of the developing intermediate and deep capillary vascular beds (9, 50, 51). Retinal vascular development is defective in Angpt2-knockout mice, and we and others have recently shown that anti-Angpt2 blocking antibodies inhibit physiological retinal angiogenesis (9, 13, 49). Although the success of the anti-VEGF approach has been remarkable, some concerns exist about the long-term effects of VEGF antagonism in the eye (52). Therefore, new targets and their combinations for safer and more effective therapy are still needed. To our knowledge, our report is the first to show that targeting Tie1 significantly inhibits retinal angiogenesis and that, importantly, combinatorial treatment with anti-Angpt2 blocking antibodies increases this effect.

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The body has already entered clinical phase III trials for the treatment of ovarian cancer, and Angpt2 blocking antibodies are tested in phase I/II trials in many different cancers (www.clinicaltrials.gov). The development of Tie1 inhibitors would be a worthwhile goal, as they would probably synergize with the Angpt blocking agents in tumor therapy.

Tie1 has been shown to interact with Tie2 in endothelial cell-cell and cell-substratum contacts, where Angpt1 mediates activation of the complex (16–18). Although some reports have suggested that Tie1 may inhibit Tie2 activation, we did not find significantly increased Tie2 phosphorylation in lung lysates from the Tie1-deleted mice. Instead, phosphorylation of Tie2 at Y1106 appeared to be reduced in Tie1-deleted versus control mice. The Tie2 Y1106 phosphopeptide is known to bind Dok-R, which mediates Angpt1-induced endothelial cell migration (38), suggesting that reduced Tie2 phosphorylation at Y1106 may contribute to the phenotype of the retinal and tumor blood vessels in Tie1-deleted mice. In line with this, Angpt1 was recently found to be required for postnatal retinal vascularization (10). However, as exogenous Angpt2 did not affect Tie2 phosphorylation at Y1106 in our experiments, blocking Angpt2 may inhibit another signaling pathway, for example, one mediated by the integrins (49). It should be noted that increased in the absence of Tie1, inhibiting retinal angiogenesis even in the presence of increased amounts of the main hypoxia-induced angiogenic drivers, such as VEGF and Angpt2. Furthermore, blocking the Notch pathway abolished the Tie1 deletion phenotype in the developing retina, which suggests that increased Notch signaling is, at least in part, responsible for the inhibition of retinal angiogenesis in the Tie1-deleted mice. This is consistent with previous studies showing that vascular endothelial cells transfected with Dll4 grow poorly even in the presence of VEGF and that upregulation of the Notch pathway in the tumor vessels results in inhibition of tumor neovascularization and growth (54).

The combination of Tie1 inhibition with Angpt2 antagonists could potentially aid the development of drugs not only for the treatment of ocular diseases, but also for tumor therapy. In line with this, Tie1EC Δ/Δ mice bearing LLC tumors treated with a soluble form of the Tie2 receptor, which blocks Angpt activity, resulted in a stronger antitumor effect than targeting Tie1 or Angpt alone. Considering that the Angpt1/Angpt2 balance in the context of the tumor environment is strongly shifted toward Angpt2 expression (55, 56), one could argue that the additive antitumor effects are likely due to the combinatorial targeting of Tie1 and Angpt2. Notably, the Angpt1/Angpt2 blocking peptide has already entered clinical phase III trials for the treatment of ovarian cancer, and Angpt2 blocking antibodies are tested in phase I/II trials in many different cancers (www.clinicaltrials.gov). The development of Tie1 inhibitors would be a worthwhile goal, as they would probably synergize with the Angpt blocking agents in tumor therapy.

Tie1 has been shown to interact with Tie2 in endothelial cell-cell and cell-substratum contacts, where Angpt1 mediates activation of the complex (16–18). Although some reports have suggested that Tie1 may inhibit Tie2 activation, we did not find significantly increased Tie2 phosphorylation in lung lysates from the Tie1-deleted mice. Instead, phosphorylation of Tie2 at Y1106 appeared to be reduced in Tie1-deleted versus control mice. The Tie2 Y1106 phosphopeptide is known to bind Dok-R, which mediates Angpt1-induced endothelial cell migration (38), suggesting that reduced Tie2 phosphorylation at Y1106 may contribute to the phenotype of the retinal and tumor blood vessels in Tie1-deleted mice. In line with this, Angpt1 was recently found to be required for postnatal retinal vascularization (10). However, as exogenous Angpt2 did not affect Tie2 phosphorylation at Y1106 in our experiments, blocking Angpt2 may inhibit another signaling pathway, for example, one mediated by the integrins (49). It should be noted that...
endogenous Angpt2 is produced by endothelial cells in hypoxic tissues, and autocrine Angpt2 stimulation of Tie2 may mediate a proangiogenic function in conditions of vascular stress (57, 58).

The current antiangiogenic inhibitors based on targeting VEGF signaling are effective in treating age-related macular degeneration, but thus far, their utility in cancer has been limited to prolonging disease-free survival in patients with a few tumor types (3). Our present results indicate that Tie1 targeting inhibits tumor angiogenesis by decreasing endothelial cell survival and subsequently decreasing vascular density and vessel perfusion, while not affecting the normal vessels in various organs. The overall healthy condition of the Tie1-deleted mice is consistent with the conclusion that their possible vascular changes would have to be relatively subtle. Furthermore, recent experiments have shown that atherosclerosis is inhibited by partial Tie1 deletion (20), which argues that decreased Tie1 function might be beneficial not only in cancer, but also in atherosclerosis patients. Thus, our present findings elucidate a previously unknown regulatory system for angiogenesis and propose Tie1 as a potential new target for anti-angiogenic therapy that potentially holds clinical relevance now, more than 20 years after its isolation (59), when we first suggested — based on its enhanced expression pattern in the tumor vasculature (19) — the possible involvement for this interesting receptor in tumorigenesis and other angiogenesis-dependent diseases, such as diabetic retinopathy, atherosclerosis, and arthritis.

Methods

Further information can be found in Supplemental Methods.

Statistics. At least 3 mice were analyzed per time point and treatment. 2-tailed paired or equal-variance Student’s t test or 1-way ANOVA was used for statistical analysis. A P value less than 0.05 was considered statistically significant.

Study approval. Mice were maintained in the Meilahti Experimental Animal Center and in the Laboratory Animal Centre of University of Oulu according to IACUC and IRB guidelines. The National Animal Experiment Board in Finland approved animal experiments used in this study.

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