Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2

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Summary

Angiopoietin-2 (Ang2) exhibits broad expression in the remodeling vasculature of human tumors but very limited expression in normal tissues, making it an attractive candidate target for antiangiogenic cancer therapy. To investigate the functional consequences of blocking Ang2 activity, we generated antibodies and peptide-Fc fusion proteins that potently and selectively neutralize the interaction between Ang2 and its receptor, Tie2. Systemic treatment of tumor-bearing mice with these Ang2-blocking agents resulted in tumor stasis, followed by elimination of all measurable tumor in a subset of animals. These effects were accompanied by reduced endothelial cell proliferation, consistent with an antiangiogenic therapeutic mechanism. Anti-Ang2 therapy also prevented VEGF-stimulated neovascularization in a rat corneal model of angiogenesis. These results imply that specific Ang2 inhibition may represent an effective antiangiogenic strategy for treating patients with solid tumors.

Introduction

Conventional cancer therapies often fail to control disease and are toxic by design. To avoid these drawbacks, pharmaceutical efforts are increasingly focusing on therapies that target molecular abnormalities specific to tumorigenesis. While some success has been achieved with such targeted drugs (e.g., STI571, Rituximab, Trastuzumab, Gefitinib, and Erbitux), none of the commercially approved targeted therapies is capable of treating broad populations of cancer patients (Rothenberg et al., 2003). Moreover, while the targeted therapies are generally less toxic than conventional cancer therapies, clinical safety issues have been noted for these drugs. It has been proposed that antiangiogenic cancer therapy might be more broadly effective and safer than either targeted or traditional therapies aimed at cancer cells (Folkman, 1998). Moreover, because antiangiogenic therapy is designed to target blood vessels perfusing tumors, rather than tumor cells themselves, traditional impediments to successful cancer therapy, such as drug resistance and inadequate drug delivery, might be averted. Promising results have been observed in clinical trials employing such agents (Hurwitz et al., 2003; Kabbinavar et al., 2003; Raymond et al., 2002; Yang et al., 2002), particularly with drugs targeting the VEGF-VEGFR pathway, such as Bevacizumab (a humanized VEGF-A binding antibody that was recently approved for the treatment of colon cancer) and SU11248 (a small molecule that inhibits the activity of several protein kinases, including KDR, a VEGF receptor). Nonetheless, clinical efficacy has been mixed for drugs targeting the VEGFs or their receptors (Anonymous, 2002; Miller et al., 2002; Zangari et al., 2004), and significant toxicities have been observed, including fatigue, headaches, hypertension, clotting abnormalities, and life-threatening hemorrhages (DeVore et al., 2000; Kabbinavar et al., 2003; Miller et al., 2002).

While the VEGFs and their receptors have been among the most extensively targeted molecules in the angiogenesis field, preclinical efforts targeting the more recently discovered angio-

SIGNIFICANCE

It has been proposed that therapies targeting the tumor vasculature may be more broadly efficacious and less toxic than conventional cancer treatments. Rational design of such therapies involves the identification and characterization of candidate drug targets whose functions are largely restricted to the process of angiogenesis. Ang2, a secreted factor whose expression is upregulated at sites of angiogenesis, holds promise as one such candidate. To evaluate the in vivo effects of Ang2 neutralization, we have developed Ang2-selective inhibitors. Systemic anti-Ang2 therapy inhibited tumor growth and angiogenesis in preclinical pharmacology models, and it was well tolerated. Whether Ang2-selective inhibitors will mediate similar effects in humans awaits the evaluation of these therapies in clinical trials.

poietin-Tie2 pathway are now gaining strength (Qian et al., 2002). Both protein families involve ligand-receptor interactions, and both include members whose functions are largely restricted postnatally to endothelial cells (and some hematopoietic stem cell lineages [Iwama et al., 1993]) by virtue of the selective expression of their respective receptors on those cell types (Gale and Yancopoulos, 1999; Mustonen and Alitalo, 1995). Tie2 is a receptor tyrosine kinase with four known ligands, angiopoietin-1 (Ang1) through angiopoietin-4 (Ang4), the best studied being Ang1 and Ang2 (Gale and Yancopoulos, 1999). Whereas Ang1 clearly stimulates phosphorylation of Tie2, Ang2 interaction with Tie2 has been shown to both antagonize and agonize Tie2 receptor phosphorylation, under varying conditions (Davis et al., 1996; Kim et al., 2000; Maisonpierre et al., 1997; Mochizuki et al., 2002; Teichert-Kuliszewska et al., 2001). The phenotypes of mouse Tie2 and Ang1 knockouts are similar and suggest that Ang1-stimulated Tie2 phosphorylation mediates both remodeling and stabilization of developing vessels in utero through maintenance of endothelial cell:support cell adhesion (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). The role of Ang1 in vessel stabilization is thought to be conserved in the adult, where the gene is expressed widely and constitutively (Hanahan, 1997; Zagzag et al., 1999). In contrast, normal postnatal Ang2 expression is highest at sites of vascular remodeling (ovary, placenta, and uterus), where it has been proposed to block Ang1 function, thereby inducing a state of vascular plasticity conducive to angiogenesis (Hanahan, 1997; Holash et al., 1999; Maisonpierre et al., 1997). Vessel-selective Ang2 induction has also been demonstrated in disease states associated with angiogenesis. These pathological conditions include cancer, macular degeneration, rheumatoid arthritis, osteoarthritis, and psoriasis (Bunone et al., 1999; Etoh et al., 2001; Fearon et al., 2003; Hangai et al., 2001a; Holash et al., 1999; Kuroda et al., 2001; Otani et al., 1999; Shahrara et al., 2002; Stratmann et al., 1998; Tanaka et al., 1999; Yoshida et al., 1999; Yuan et al., 2000; Zagzag et al., 1999).

Elevated Ang2 expression at sites of normal and pathological postnatal angiogenesis circumstantially implies a proangiogenic role for Ang2 (Maisonpierre et al., 1997). However, experiments designed to functionally confirm this conclusion have yielded ambiguous and contradictory results. For example, ectopic expression of Ang2 in cancer cells has been shown to promote tumor growth and vascularization in xenograft and chorioallantoic membrane angiogenesis models (Ahmad et al., 2001; Etoh et al., 2001; Tanaka et al., 1999). In contrast, Ang2 overexpression has also been reported to inhibit both local tumor growth and metastasis and thereby prolong the survival of mice injected with these tumors (Yu and Stamenkovic, 2001). Experiments using transgenic mice ectopically expressing VEGF, Ang1, and Ang2 in cardiac muscle have demonstrated that Ang2 stimulates VEGF-mediated angiogenesis, whereas Ang1 suppresses it (Visconti et al., 2002). Further supporting the hypothesis that Ang1 and Ang2 play mutually antagonistic roles in developmental vascular remodeling, Ang2 transgenic mice (in which Ang2 expression is driven by the Tie2 promoter) and Ang1 knockout mice share a similar phenotype, consisting of defective vascular patterning, vessel discontinuity, and detachment of the endocardium from the myocardium (Maisonpierre et al., 1997). But since this phenotype implies defects in both vascular remodeling and stabilization, two seemingly contradictory processes, it remains unclear whether either Ang1 or Ang2 are playing pro- and/or antiangiogenic roles in this context. Bolstering the hypothesis that Ang2 is proangiogenic, Ang2 knockout mice display early postnatal vascular remodeling defects in the eye (Gale et al., 2002; Hackett et al., 2002), as well as developmental lymphatic defects; curiously, the latter deficiency, but not the former, can be rescued by replacing the Ang2 gene with the Ang1 gene. Blocking angiopoietin function pharmacologically in a postnatal setting by sequestration with a soluble Tie2 receptor has been shown to have an inhibitory effect on tumorigenesis and neovascularization (Das et al., 2003; Hangai et al., 2001b; Lin et al., 1997, 1998; Siemeister et al., 1999). However, given that all four angiopoietins bind to Tie2, it is not clear whether this effect was mediated by inhibition of Ang1, Ang2, Ang3, Ang4, or a combination thereof. To enable a more definitive evaluation of postnatal Ang2 function, we have generated agents capable of selectively neutralizing the activity of this factor. Here, we show that systemic administration of these Ang2-selective inhibitors is sufficient to suppress both tumor angiogenesis and corneal angiogenesis, thus supporting the notion that Ang2 plays a proangiogenic role postnatally.

Results

Identification and characterization of Ang2-selective inhibitors

Two types of Ang2-selective inhibitors, peptide-Fc fusion proteins and antibodies, were generated as reagents to interrogate angiopoietin function (Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/6/4/507/DC1/). Phage display peptide and Fab libraries were panned against human Ang2, and the resulting Ang2 binding clones were converted into peptide-Fc fusion proteins (by expressing the active peptides in *E. coli* as fusions to the Fc portion of human IgG1) or fully human antibodies (by expressing the active Fabs in CHO cells on a complete IgG1 framework). Peptide-Fc fusion proteins and antibodies were then screened by ELISA for their ability to neutralize the interaction between Tie2 and angiopoietins. A subset of the peptide-Fc fusion proteins were affinity-matured to enhance their ability to neutralize Ang2. Two peptide-Fc fusion proteins (2xCon4 (C) and L1-7 (N)) and one antibody (Ab536) were chosen for further study, based on their ability to potently neutralize the human Ang2:human Tie2 interaction (IC_{50} values of 23, 54, and 140 pM, respectively [Table 1A]). All three reagents exhibited similar IC_{50} values against Ang2 from other species (Table 1A), enabling their evaluation in preclinical pharmacology models. Furthermore, the three inhibitors exhibited between 30-fold and $>$4500-fold selectivity for Ang2 over other angiopoietin family members (Table 1A). Human IgG1 Fc alone exhibited no angiopoietin-neutralizing activity in this ELISA. None of these four agents had activity in a VEGF-VEGFR neutralization assay (Table 1A), whereas Bevacizumab inhibited this interaction with an IC_{50} of 2 nM (data not shown).

To determine practical dosing frequencies and routes of administration for Ang2-blocking agents, pharmacokinetic profiles were generated in rodents for 2xCon4 (C), L1-7 (N), and Ab536 (Table 1B and Supplemental Experimental Procedures). The resulting pharmacokinetic parameters supported the feasibility of daily to weekly subcutaneous dosing.

Ang2 antagonism inhibits tumor growth

To evaluate the effect of Ang2 inactivation on tumor growth, nude mice bearing subcutaneous A431 human epidermoid tu-

Table 1. Peptide-Fc fusion proteins and antibodies as competitive inhibitors of the angiopoietin:Tie2 interaction

A:	Agent	hAng2 IC_{50} (nM)	mAng2 IC_{50} (nM)	rAng2 IC_{50} (nM)	hAng1 IC_{50} (nM)	mAng1 IC_{50} (nM)	hAng4 IC_{50} (nM)	mAng3 IC_{50} (nM)	hVEGF IC_{50} (nM)
	2xCon4 (C)	0.023	0.021	0.049	0.9	0.64	>100	>100	>100
	L1-7 (N)	0.054	0.071	0.160	>100	>100	>100	>100	>100
	Ab536	0.140	0.061	0.270	>100	>100	>100	>100	>100
	Fc	>100	>100	>100	>100	>100	>100	>100	>100

B:		Mouse		Rat	
	Agent	$t_{1/2}$ (hrs)	Dose-normalized AUC_{inf} (nM×hr/mg/kg)	$t_{1/2}$ (hrs)	Dose-normalized AUC_{inf} (nM×hr/mg/kg)
	2xCon4 (C)[a]	97	15097	85	8733
	L1-7 (N)[a]	56	6967	47	4637
	Ab536[b]	83	3367	102	12702

A: Peptide-Fc fusion proteins, antibody, and human IgG1 Fc were evaluated by ELISA for their ability to neutralize angiopoietin:Tie2 interactions. Candidate inhibitory agents were titrated from 100 nM to 0.4 pM in the presence of 1 nM Tie2 and subsequently added to a panel of angiopoietins. The degree of angiopoietin:Tie2 neutralization (IC_{50}) was determined by comparison against a Tie2 standard curve (the binding activity of serially diluted Tie2 in the absence of competitor). As an additional control for specificity, Ang2-inhibitory agents were also tested in an analogous fashion for their ability to neutralize the hVEGF:KDR interaction (last column of table). Bevacizumab (a neutralizing anti-hVEGF antibody) displayed a 2.1 nM IC_{50} in this assay (data not shown).
B: Pharmacokinetic parameters of Ang2 inhibitors in mice and rats. Terminal half-lives ($t_{1/2}$) and areas under the serum concentration-time curves from time 0 to infinity (AUC_{inf}) are shown. AUC_{inf} values were normalized to a 1 mg/kg dose.
[a] $t_{1/2}$ and AUC_{inf} were calculated from the composite mean serum concentration-time data, n = 2–3/time point.
[b] $t_{1/2}$ and AUC_{inf} were calculated from the mean serum concentration-time data of 6 mice or 3 rats.

mor xenografts were treated with a range of Ab536 dose levels (thrice weekly) beginning 3 days after tumor challenge (Figure 1A). A431 xenografts, like other tumor models, display vessel-selective Ang2 expression (Figure 1C and Supplemental Experimental Procedures). Ab536 significantly inhibited tumor growth relative to control IgG when dosed at 2 and 6 mg/kg, but not at lower dose levels (Figure 1A). A longer-term study employing Ab536 at 2 mg/kg (the optimal biological dose) showed more dramatic inhibition of tumor growth (Figure 1B). Ab536, as well as the other Ang2 inhibitors, failed to suppress the growth of cultured A431 cells (Figure 1D), consistent with the hypothesis that the effects of Ab536 on tumor growth in vivo were conferred indirectly through an antiangiogenic mechanism, rather than through a direct effect on tumor cells.

We next sought to determine whether the tumor growth inhibition observed in the A431 model could be reproduced in other tumor models. Administration of 2xCon4 (C), L1-7 (N), or Ab536 to mice bearing subcutaneous Colo205 human colorectal tumor xenografts resulted in tumor growth inhibition, indicating that the observed antitumor effects were not cancer type specific or reagent specific (Figure 2A). Vascular Ang2 expression was confirmed by in situ hybridization (Figure 2B and Supple-

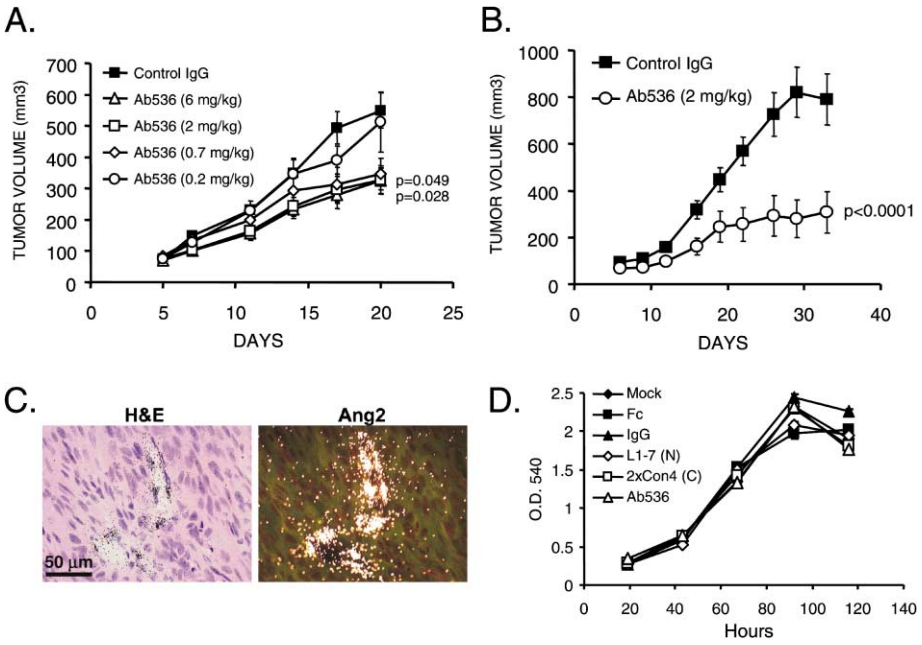

Figure 1. Effect of anti-Ang2 agents on the growth of A431 tumor xenografts and cultured A431 tumor cells

A and B: Inhibition of A431 tumor xenograft growth with a systemically administered Ang2-neutralizing agent. Treatment was initiated 3 days postinjection of tumor cells. Ab536 was dosed thrice weekly.
C: Paired brightfield and darkfield images of Ang2 in situ hybridization, demonstrating Ang2 mRNA expression in endothelial cells lining a blood vessel within the mass of an A431 tumor xenograft from an untreated animal. The horizontal bar indicates scale.
D: In vitro growth of A431 cells in the presence of Ang2 inhibitors. Cells were seeded in 96-well plates and treated with the indicated concentrations of human Fc, human IgG, L1-7 (N), 2xcon4 (C), and Ab536. Cells were fixed and assayed at 24, 48, 72, 96, and 120 hr of treatment.

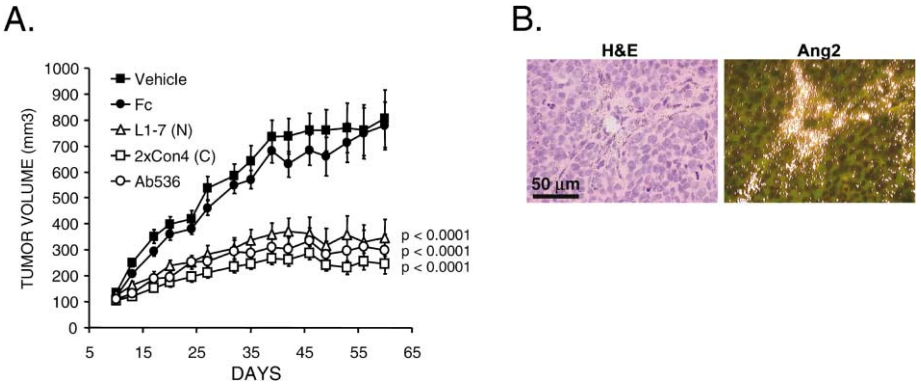

Figure 2. Effect of anti-Ang2 agents on the growth of Colo205 tumor xenografts

A: Inhibition of Colo205 tumor xenograft growth with systemically administered Ang2-neutralizing agents. Treatment was initiated 3 days postinjection of tumor cells.

B: Paired brightfield and darkfield images of Ang2 in situ hybridization, demonstrating Ang2 mRNA expression in endothelial cells lining a blood vessel within the mass of a Colo205 tumor xenograft from an untreated animal. The horizontal bar indicates scale.

mental Experimental Procedures). Ang2 inhibition was shown to mediate similar tumor growth inhibition in the HT29 xenograft model (Supplemental Figure S1).

Two additional Colo205 tumor xenograft experiments were performed to evaluate peptide-Fc fusion protein dose-response relationships. Twice weekly administration of 0.6 or 15 mg/kg 2xCon4 (C) yielded equivalent levels of tumor growth inhibition (Figure 3A), whereas dosing of 0.12 or 0.024 mg/kg 2xCon4 (C) on the same schedule resulted in a progressive reduction in effect relative to the 0.6 mg/kg dose (Figure 3B). The same optimal biological dose of 0.6 mg/kg twice weekly was observed with L1-7 (N) (data not shown).

In the tumor xenograft studies discussed thus far, dosing began 3 days after tumor cells were injected. To better replicate conditions of established disease, Colo205 tumors were allowed to grow for 28 days before 2xCon4 (C) dosing was initiated (Figure 4A). A separation in growth rates between the vehicle and treated groups could be observed within 3 days of treatment initiation, and the gross tumor volume of the treatment group remained static over the 21 days of therapy, indicating that anti-Ang2 therapy could inhibit the growth of established tumors. To examine drug effects at the histological level, cohorts of animals in this study were sacrificed weekly for tumor evaluation. A progressive reduction in viable tumor fraction was observed in the treatment group relative to the vehicle group (Figures 4B–4D and Supplemental Experimental Procedures), indicating that volume-based tumor measurements, which fail to distinguish viable tumor from necrotic tissue, underestimated peptide-Fc fusion protein-mediated antitumor effects. Thus, anti-Ang2 treatment mediated regression of viable tumor.

Even more dramatic regression, apparent even by gross external measurements, was observed when the initiation of therapy was further delayed, and this regression appeared to be reversible following serum clearance of the Ang2-neutralizing agent (Figure 5). In this experiment, 2xCon4 (C) was administered on either a twice weekly schedule (0.6 mg/kg) starting on Day 21 ("narrow-interval dosing") or in individual doses (25 mg/kg) on Days 45 and 116 ("wide-interval dosing"). Dosing on a wide interval allowed 2xCon4 (C) to be cleared from the serum between doses. Rapid tumor regression was observed in the wide-interval group following the Day 45 dose. This regression was sustained until Day 69, at which point tumor regrowth began to occur, suggesting that 2xCon4 (C) exposure had fallen below effective levels. Resumption of dosing on Day 116 resulted again in tumor regression, indicating that tumors had not acquired resistance to anti-Ang2 therapy. While this study showed reversibility of the treatment-mediated antitumor effect, growth inhibition was not observed to be reversible in experiments in which dosing was initiated by experimental Day 28 (data not shown).

To further evaluate the long-term effects of Ang2 inhibition on tumor growth, several groups of animals harboring A431 and Colo205 tumor xenografts were treated continuously with 2xCon4 (C) or Ab536 for at least 10 weeks (Supplemental Table S1 and Supplemental Experimental Procedures). Although various dosing levels and schedules were employed, all were predicted to result in serum trough levels exceeding the exposure required for optimal effect. In 13 of 15 groups, anti-Ang2 therapy eliminated all measurable tumor in a subset of animals. In three of these groups, drug was withdrawn after the complete responses were achieved, and no tumor regrowth was observed

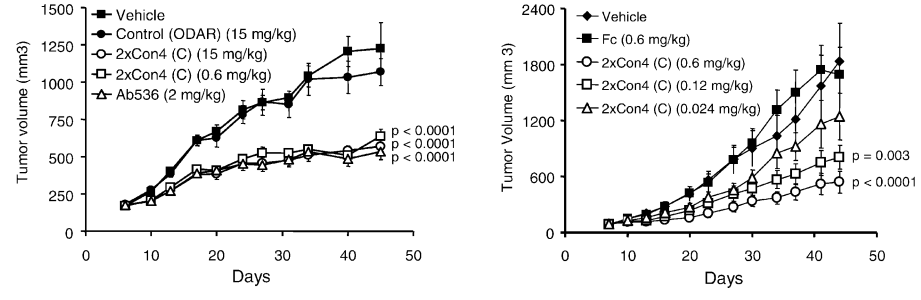

Figure 3. Dose-response effect of systemically administered 2xCon4 (C) in the Colo205 xenograft model

A: 2xCon4 (C) dose levels of 15 and 0.6 mg/kg (twice weekly) had similar antitumor effects to one another and to Ab536 dosed at 2 mg/kg (thrice weekly).

B: Reducing the 2xCon4 (C) doses levels to 0.6, 0.12, and 0.024 mg/kg (twice weekly) resulted in a clear dose-response effect. While maximal effect was observed with 0.6 mg/kg, a partial inhibitory effect was observed with the intermediate dose of 0.12 mg/kg. The low dose of 0.024 mg/kg was ineffective.

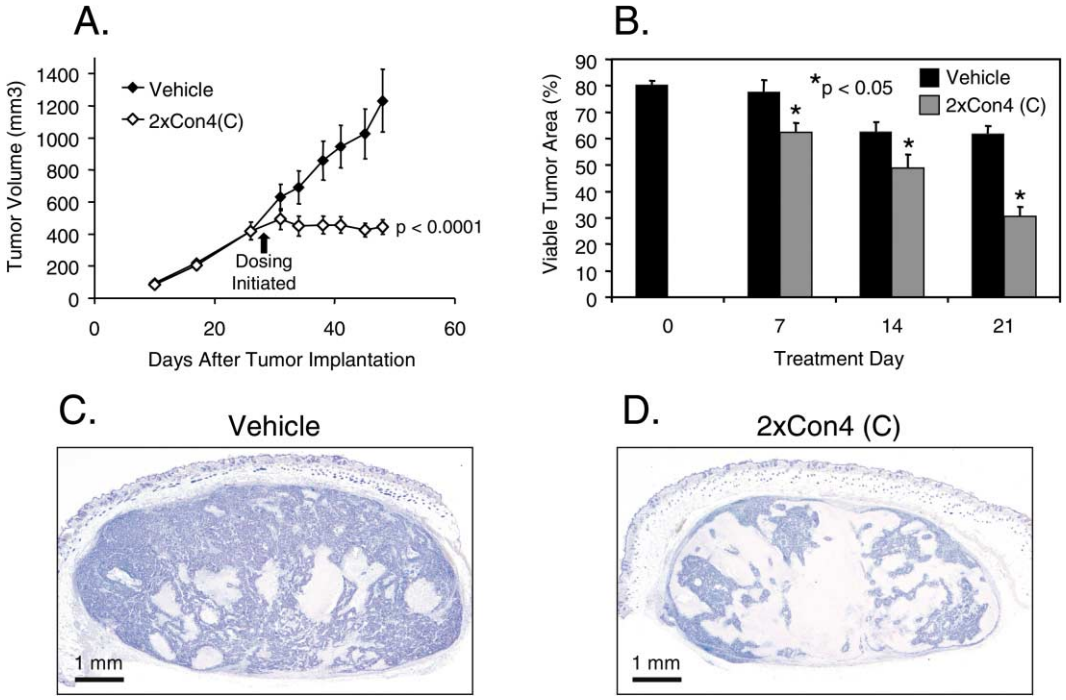

Figure 4. Effect of 2xCon4 (C) treatment on viable tumor fraction in the Colo205 xenograft model

A: Mice were treated twice weekly with 15 mg/kg 2xCon4 (C) starting on day 28 postinjection of Colo205 tumor cells. Tumors were harvested at different time points following treatment initiation for the histological analysis described below. Representative tumor volumes from mice treated with 2xCon4 (C) or vehicle for 21 days are shown.

B: Analysis of tumor viable area fraction comparing 2xCon4 (C)-treated mice and vehicle-treated mice. Eight tumors per group were measured on posttreatment days 0, 7, 14, and 21. A significant reduction in tumor viability was demonstrated following 7 days of 2xCon4 (C) treatment.

C and D: Representative hematoxylin-stained histological sections of bisected tumors following 21 days of treatment with vehicle or 2xCon4 (C), respectively. The horizontal bars indicate scale.

in these animals (average follow-up 15.3 weeks; range 6–27 weeks).

Upon completion of tumor xenograft studies, mice were sacrificed to search for possible adverse effects in major organ systems attributable to Ang2 neutralization (Supplemental Experimental Procedures). The 2xCon4 (C) protein had no significant adverse effects on xenograft-bearing mice when dosed either at 14 mg/kg twice per week for 11 weeks (monitoring hematology values, serum chemistry values, or histology) or 8 mg/kg daily for 11 weeks (monitoring organ weights and histology). Similarly, L1-7 (N) had no untoward effects on any of the endpoints evaluated (hematology values, serum chemistry, or histology) in xenograft-bearing mice when dosed at 14 mg/kg twice per week for 7 weeks.

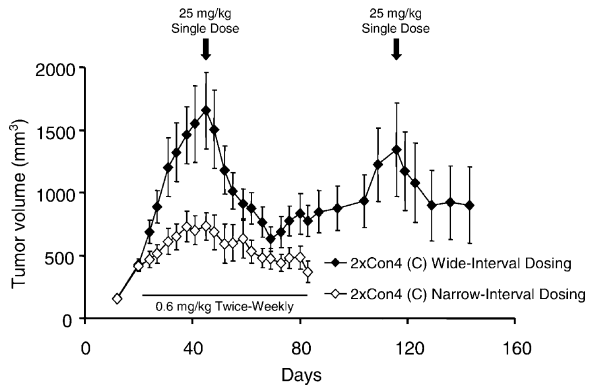

Figure 5. Systemically administered 2xCon4-C induces regression of large established tumors

One group of mice was treated twice weekly with 15 mg/kg 2xCon4 (C) beginning on Day 21 postinjection of cells ("Narrow-Interval Dosing" group). The other group was treated with vehicle until Day 44 and then with 25 mg/kg doses of 2xCon4 (C) on Days 45 and 116 ("Wide-Interval Dosing" group).

Ang2 inhibition suppresses the proliferation of tumor endothelial cells

To begin investigating the mechanism through which Ang2 neutralization mediates tumor growth inhibition, Colo205 xenografts from mice treated with 2xCon4 (C) or vehicle were evaluated at the cellular level. FACS analysis revealed that 2xCon4 (C) treatment resulted in a progressive reduction in endothelial cell proliferation, followed by a reduction in tumor cell proliferation (Figure 6A and Supplemental Experimental Procedures), suggesting that the 2xCon4 (C)-mediated antitumor effects occurred secondary to a drop in tumor perfusion. That is, while 24 hr of 2xCon4 (C) treatment was sufficient to inhibit endothelial cell proliferation, 72 hr of 2xCon4 (C) treatment was required to achieve a reduction in tumor cell proliferation that deviated below the aggregate mean tumor cell proliferation of all vehicle-

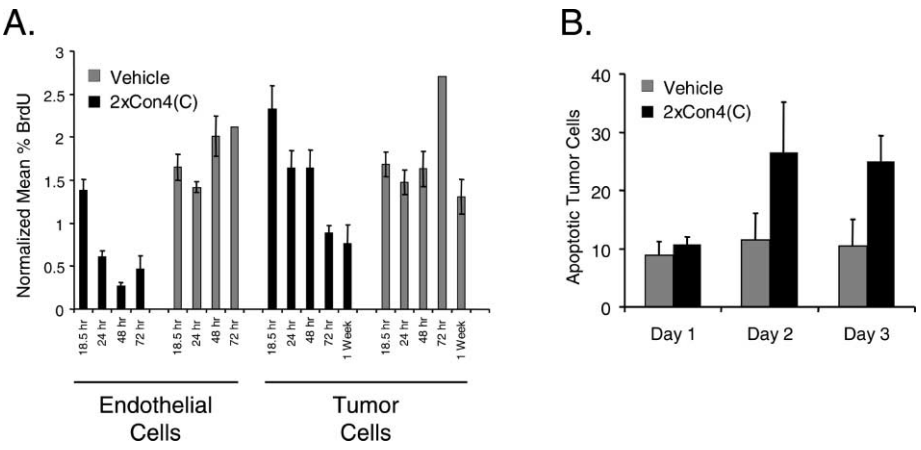

Figure 6. Cell-based mechanism-of-action studies

A: Effect of 2xCon4 (C) on BrdU uptake in tumor cells and endothelial cells derived from Colo205 xenografts. Tumor-bearing mice were treated with a single dose of 15 mg/kg 2xCon4 (C) or vehicle at study day 28. Tumors were collected at different times posttreatment and digested enzymatically, and cells were extracted for FACS analysis. 18 hr prior to each tumor harvest, mice were implanted subcutaneously with osmotic minipumps (Zymed Labs 2001D, 8 μl/hr, 1 day) containing 3 mg/ml BrdU. Dissociated cells were stained with anti-mouse CD45-FITC and CD31-PE antibodies, followed by fixation and staining with anti-BrdU-alexa647 antibodies. Endothelial cell ($CD45^-/CD31^+$ mouse diploid population) and tumor cell ($CD45^-/CD31^-$/FSC tetraploid population) BrdU uptake was normalized to total diploid mouse cell BrdU uptake for each tumor sample. The bar graph represents normalized mean BrdU ratios (n = 4, except for 72 hr vehicle group [n = 1] and 72 hr 2xCon4 group [n = 2]).

B: Apoptotic tumor cell counts per 20× objective field (135,600 μm^2), comparing 2xCon4 (C)-treated and vehicle-treated mice. Eight tumors per group were evaluated following 1, 2, or 3 days of treatment. This analysis demonstrated a significant increase in tumor cell apoptosis following 2 days of 2xCon4 (C) treatment.

treated groups (tumor cell proliferation in the vehicle-treated groups remained relatively constant over time). Additional analyses by histological methods demonstrated that 2xCon4 (C) induced tumor cell apoptosis in Colo205 xenografts (Figure 6B), an effect that is consistent with the reduced viable tumor fraction observed in 2xCon4 (C)-treated mice (Figures 4B–4D). The temporal and causative relationships between 2xCon4 (C)'s effects on tumor cell proliferation and apoptosis are not yet known.

Direct antiangiogenic effect mediated by Ang2 neutralization

We next sought further support for the hypothesis that anti-Ang2 therapy mediates a direct antineovascular effect in vivo. The rat corneal angiogenesis model was chosen for this purpose, as it allows neovascularization to be studied outside the context of tumorigenesis. In this model, a VEGF-soaked nylon disk is placed in the normally avascular cornea at a fixed distance from the surrounding limbal vessels. Neovessels subsequently sprout from the limbal vessels toward the developing VEGF gradient. In situ hybridization (ISH) was used to demonstrate that Ang2 was expressed in the VEGF-induced corneal neovasculature (Supplemental Figure S2 and Supplemental Experimental Procedures). Ang2 mRNA levels were shown to be upregulated during VEGF-induced corneal angiogenesis in a punctate pattern similar to that observed in tumor vessels. Ang2 ISH signal was identified only in neovessels sprouting from the limbus, and no signal could be observed in the absence of exogenous VEGF stimulation. The consequences of inactivating Ang2 in this model were then evaluated. Systemic administration of 2xCon4 (C) over a range of 0.125 to 4 mg/kg twice weekly resulted in a dose-dependent inhibition of VEGF-mediated corneal angiogenesis (Figure 7A), with reduction of angiogenesis to negative control levels observed at the 1 mg/kg dose level. L1-7 (N) and Ab536 were also shown to inhibit corneal angiogenesis to background levels (Figure 7B), further demonstrating that the effects of anti-Ang2 therapy were not reagent specific.

Treatment of rats with L1-7 (N) and 2xCon4 (C) at subcutaneous doses as high as 200 mg/kg twice weekly for 2 weeks was well tolerated and revealed no significant adverse effects (Supplemental Experimental Procedures). A pharmacological effect on epiphyseal growth plate thickness was observed in male 2xCon4 (C)-treated rats dosed at 75 mg/kg (2 of 3 rats) and 200 mg/kg (3 of 3 rats). No growth plate abnormalities were observed in identically treated rats that were subsequently withdrawn from drug for 1 month, suggesting that these effects were reversible. Epiphyseal plate thickening is considered to be a pharmacological consequence of antiangiogenic therapy and has been seen in growing rodents treated with VEGF-neutralizing antibodies (Gerber et al., 1999). This phenomenon would not be expected to occur in adult animals treated with antiangiogenic agents, as endochondral bone formation is completed before adulthood. Also of note, a multifocal infiltration of mononuclear cells was seen on the exterior surface of the splenic capsule in some treated rodents, but not primates (data not shown). The finding had no known functional consequences, and its etiology is under investigation. Together, the accumulated preclinical pharmacology data support the conclusion that Ang2 inactivation suppresses angiogenesis in both neoplastic and nonneoplastic settings, without untoward effects on animal health.

Discussion

Previous work in this field has highlighted the complexity of Ang2 function. In vitro and in vivo experiments have suggested that Ang2 can either agonize or antagonize (Davis et al., 1996; Kim et al., 2000; Maisonpierre et al., 1997; Mochizuki et al., 2002; Teichert-Kuliszewska et al., 2001) the Tie2 receptor, depending on context. Ang2 has been shown to play opposing roles at the cellular and organismal levels as well, promoting either vascular sprouting or regression, depending on whether VEGF is present (Maisonpierre et al., 1997). The phenotype of the recently published Ang2 knockout mouse is informative in that it convincingly implicates Ang2 as a required factor for early postnatal retinal neovascularization (Gale et al., 2002; Hackett et al., 2002). However, these studies also highlight the context-dependent nature

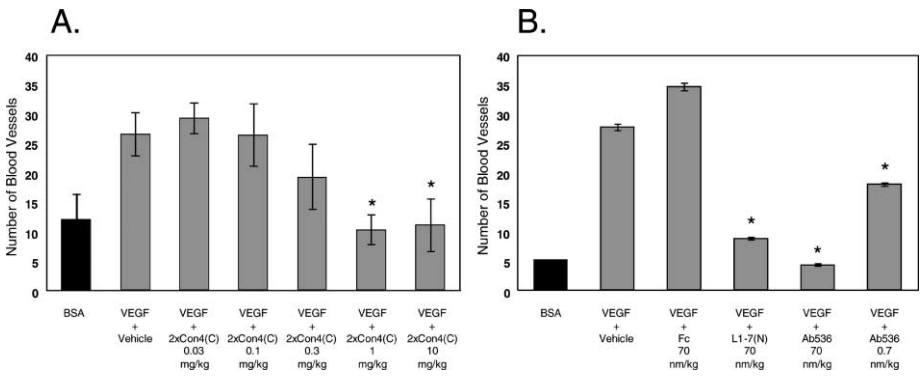

Figure 7. Ang2 antagonists inhibit VEGF-induced corneal angiogenesis

Angiogenesis was induced by implanting VEGF- or BSA-soaked nylon discs into the corneal stroma of female CD rats. Treatment with 2xCon4(C) (**A**), Fc control or L1-7(N) (**B**) was initiated 1 week prior to corneal implantation and continued twice per week. Animals receiving Ab536 (**B**) were injected on the day of surgery and three days later. For all groups, angiogenesis was evaluated 1 week after disc implantation. Values are the group mean ± standard error of the mean (n = 8). *$p < 0.02$.

of Ang2 dependency in angiogenesis; Ang2 was shown in these same experiments to be dispensable for developmental neovascularization and for pathological angiogenesis in the abnormally retained hyaloid vasculature of rare knockout mice surviving to day 25 (Gale et al., 2002). In tumor models, ectopic Ang2 expression has been shown to both stimulate and inhibit tumor growth and tumor angiogenesis, thus adding to the confusion regarding Ang2 function. The only published attempt to selectively neutralize Ang2 in vivo using a pharmacological agent (an RNA aptamer) resulted in incomplete angiogenic inhibition, even in the context of local administration, thus leaving unanswered the question of whether Ang2 inhibition alone is sufficient to fully inhibit angiogenesis (White et al., 2003). The cellular mechanism through which selective Ang2 inhibition mediated angiogenic suppression also remains to be addressed. Panangiopoietin inhibition (with soluble Tie2) has also been shown to mediate suppression of tumor growth and angiogenesis, but none of these effects can be attributed specifically to Ang2 inactivation (Das et al., 2003; Hangai et al., 2001b; Lin et al., 1997, 1998; Siemeister et al., 1999). It therefore remains unclear exactly what molecular, cellular, and phenotypic roles Ang2 plays in postnatal life in normal or pathological vascular processes, and particularly in the setting of cancer.

To begin investigating this question, we generated Ang2-selective peptide-Fc fusion proteins and antibodies, enabling controlled pharmacological inactivation of endogenous Ang2. This approach to Ang2 inhibition circumvents the neonatal lethality observed in Ang2 knockout studies (Gale et al., 2002) and avoids the ambiguous interpretations inherent in studies using panangiopoietin inhibitors (Das et al., 2003; Hangai et al., 2001b; Lin et al., 1997, 1998; Siemeister et al., 1999). Our studies demonstrate that specific inactivation of endogenous Ang2 inhibits tumor angiogenesis in mice and corneal angiogenesis in rats, thus establishing that Ang2 plays a proangiogenic role in these processes. A combination of volume-based tumor measurements and histological analysis indicate that selective Ang2 inhibition mediates regression of viable tumor. Moreover, a subset (~20%) of tumor-bearing mice completely responded to long-term anti-Ang2 therapy, in that no residual tumor could be measured. This result suggested that even established tumor vessels have the capacity to respond to Ang2 inhibition.

No significant adverse effects were observed in rodents following long-term Ang2 inhibition, despite clear Ang2 expression by ISH in several organs of 10-week-old mice (data not shown). This result is reminiscent of the Ang2 knockout mouse phenotype (Gale et al., 2002), in which no prenatal vascular defects were observed, despite strong embryonic expression of Ang2 in and around large vessels in wild-type mice. Thus, expression of Ang2 does not necessarily confer functional dependence on this factor. Nonetheless, additional studies of longer duration and in the context of other angiogenesis-associated processes (such as wound healing and estrous cycling) would be necessary to fully assess the effects of Ang2-neutralizing agents.

While this study sheds light on Ang2 biology and the therapeutic implications of Ang2 inhibition, it also stimulates additional questions. First, the cellular and molecular consequences of Ang2 neutralization require further investigation. It remains to be determined, for example, how Ang2 neutralization blocks endothelial cell proliferation, if it alters pericyte/cell matrix coverage of capillaries, or how it might alter tumor perfusion. Of note, ectopically administered Ang2 has been shown to promote endothelial cell proliferation and sprouting of new blood vessels in the papillary membrane of the eye, and these effects were associated with increased capillary diameter, basal lamina remodeling, and enhanced endothelial cell migration (Lobov et al., 2002). It is attractive to consider that these same associated characteristics might be oppositely affected by Ang2 inhibition. At the molecular level, it remains unclear how Ang2 neutralization affects Tie2 phosphorylation in individual vessels. Attempts to address this question by immunohistochemistry using anti-phospho-Tie2 antibodies have not been successful, as the reagent antibodies generated for this purpose have not been sufficiently sensitive. Evaluating this question in a physiologically relevant setting is critical to elucidating the true biochemical role of Ang2.

A second question that remains to be addressed is whether selective inhibition of Ang1 would be advantageous or deleterious in terms of efficacy and safety. If, for example, Ang1 and Ang2 were both capable of stimulating tumor angiogenesis, then greater tumor growth inhibition might be achievable by neutralizing both angiopoietins, rather than inhibiting Ang2 alone. Alternatively, if Ang1 and Ang2 function in a mutually antagonistic manner, then selective Ang1 neutralization might actually stimulate tumor growth. From the standpoint of safety, one might predict, a priori, that Ang1 inhibition could globally destabilize normal quiescent blood vessels, as Ang1 appears to play a role in normal vessel maintenance. Systemically administered soluble Tie2 has been reported to be efficacious and safe in tumor models (Lin et al., 1998), but interpretation of

these studies is confounded by the fact that Ang1 and Ang2 inhibition could not be evaluated in isolation. While 2xCon4(C) selectively inhibits Ang2, it too displays some inhibitory activity against Ang1. Despite this Ang1-neutralizing activity, 2xCon4(C) shares a similar response profile with the more Ang2-selective reagents (L1-7(N) and Ab536) evaluated herein, suggesting that (1) Ang2 may be the dominant angiopoietin in promoting postnatal angiogenesis and (2) inhibiting Ang1 may confer no deleterious effects. Nonetheless, definitive evaluation of the consequences of inhibiting Ang1 awaits the generation of high-potency Ang1-selective inhibitors and has implications for pharmaceutical strategies involving targeted inactivation of the angiopoietins or their receptor.

A final question involves determining the clinical value of selectively inhibiting Ang2. Ang2 upregulation has been observed not only in cancer, but also in macular degeneration, rheumatoid arthritis, osteoarthritis, and psoriasis (Bunone et al., 1999; Etoh et al., 2001; Fearon et al., 2003; Hangai et al., 2001a; Holash et al., 1999; Kuroda et al., 2001; Otani et al., 1999; Shahrara et al., 2002; Stratmann et al., 1998; Tanaka et al., 1999; Yoshida et al., 1999; Yuan et al., 2000; Zagzag et al., 1999), suggesting that an effective Ang2 inhibitor might have wide applicability in treating human disease. Whether Ang2-selective inhibitors will confer similar activity in humans to that observed in rodent models awaits the introduction of these agents into clinical trials.

Experimental procedures

Angiopoietin:Tie2 neutralization ELISA

96-well microtiter plates were coated with recombinant angiopoietins in 293T cell conditioned media (DMEM/50 μg/ml BSA) at 37°C for 1 hr. The conditioned media were used at angiopoietin concentrations that conferred 75% of maximally achievable binding to 1 nM hTie2-Fc (R&D Systems, catalog #313-TI). Plates were washed with PBS/0.1% Tween-20 and then blocked for 2 hr at room temperature with PBS/5% BSA. Peptide-Fc fusion proteins and antibodies titrated from 100 nM to 0.4 pM in a solution of PBS/1% BSA/1 nM Tie2 were added to the angiopoietin-coated plates, which were incubated overnight at room temperature and then washed with PBS/0.1 percent Tween-20. Mouse-derived anti-Tie2 antibody (Pharmingen Inc., catalog #557039) was added to each plate at a final concentration of 1 μg/ml (1 hr incubation at room temperature), after which plates were washed in PBS/0.1% Tween-20. Goat anti-mouse-IgG-HRP (Pierce, catalog #31432) was added at a dilution of 1:10,000 in PBS/1% BSA (1 hr incubated at room temperature), after which plates were washed several times with PBS/0.1% Tween-20. TMB substrate (Sigma, catalog #T8665) was added, OD 370 nm absorbance was measured, and degree of angiopoietin:Tie2 neutralization was determined by comparison against a Tie2 standard curve.

VEGF/KDR neutralization ELISA

96-well microtiter plates were coated with recombinant human VEGF (0.1 ml per well of 5 μg/ml hVEGF in PBS/1% BSA) at 37°C for 1 hr. The recombinant VEGF was used at concentrations that conferred 80% of maximally achievable binding to 1 nM human KDR-Fc (R&D Systems, catalog #357-KD/CF). Plates were washed with PBS/0.1% Tween-20 and then blocked for 2 hr at room temperature with PBS/5% BSA. Peptide-Fc fusion proteins and antibodies titrated from 1000 nM to 0.5 pM in a solution of PBS/1% BSA/1 nM KDR-Fc were added to the VEGF-coated plates, which were incubated overnight at room temperature and then washed with PBS/0.1% Tween-20. Mouse-derived anti-KDR antibody (R&D Systems, catalog #MAB3573) was added to each plate at a final concentration of 1 μg/ml (1 hr incubation at room temperature), after which plates were washed in PBS/0.1% Tween-20. Goat anti-mouse-IgG-HRP (Pierce, catalog #31432) was added at a dilution of 1:5000 in PBS/1% BSA (1 hr incubated at room temperature), after which plates were washed several times with PBS/0.1% Tween-20. TMB substrate (Sigma, catalog #T8665) was added, OD 370 nm absorbance was measured, and degree of VEGF:KDR neutralization was determined by comparison against a KDR-Fc standard curve.

Pharmacology studies

8- to 10-week-old female CD1 nude mice (Charles Rivers Laboratories) were used in all studies. Mice were injected subcutaneously with 0.2 ml of tumor cell suspension in RPMI, containing 1×10^7 A431 cells (ATCC), 2×10^6 Colo205 cells (ATCC), or 5×10^6 HT29 cells mixed with 2:1 volumes matrigel:cells (BD Bioscience). Subconfluent cells were harvested prior to injection. Treatment with peptide-Fc fusion proteins, antibodies, or vehicle (PBS) was initiated as early as 3 days and as late as 45 days postinjection of cells. Peptide-Fc fusion proteins were administered subcutaneously, and antibodies were administered intraperitoneally at the indicated doses and schedules. Tumor volumes and body weights were recorded at regular intervals. Tumor volume was calculated as length × width × height in mm^3. Results are expressed in mean ± standard error. The data were statistically analyzed with factorial ANOVA followed by Scheffe's post hoc analysis for repeated measurements (StatView v5.0.1, SAS Institute). Mice were euthanized with CO_2 asphyxiation, and in some experiments tumors were collected and fixed for histological analysis. All experiments were conducted in accordance with institutional guidelines, which include mandates for sacrificing mice when tumors exceed size thresholds.

In vitro cell growth assay

A431 cells were seeded in 96-well tissue culture plates at 2000 cells per well in 200 μl of DMEM supplemented with 10% fetal bovine serum (FBS). 16 hr postseeding, the medium was replaced with 100 μl per well of fresh DMEM/10% FBS containing 6.7 μM Ang2-neutralizing agents or controls (all in triplicate). Cells were fixed at 24, 48, 72, 96, and 120 hr post treatment by adding 100 μl 10% trichloroacetic acid (TCA). All plates were stored in 10% TCA at 4C and assayed together. The 10% TCA was shaken out, and the wells were rinsed 5 times with water. The cells were then stained with 100 μl of 0.4% sulforhodamine B (Sigma S-9012) in 1% acetic acid (Sigma A-6283) for 10 min at room temperature and then washed 5 times with 1% acetic acid. The plates were then air-dried. The dye was solubilized with 300 μl of 20 mM unbuffered Tris (pH > 10) for 2 hr on a rotary shaker. Optical density (OD) was then read at 540 nm on a microtiter plate reader.

Tumor cell apoptosis assay

Tumor cell apoptosis was examined at days 1, 2, and 3 for both 2xCon4 (C)-treated and vehicle-treated controls, from the same study as the Viable Tumor Fraction analysis. Sections were stained for cleaved Caspase-3 (Cat#-Asp175, Cell Signaling Technologies) using peroxidase localization with DAB substrate, and a light hematoxylin counterstain. Five widely spaced fields were selected in a blinded fashion from the circumferential region of eight tumor sections per group, avoiding the overlying capsule and areas of frank necrosis. The number of apoptotic tumor cells was counted in each 20× objective field of roughly 135,600 μm^2.

Corneal angiogenesis model

Corneal angiogenesis was induced in adult female CD rats weighing 250–300 g (n = 8 per group) as described (Coxon et al., 2002). Briefly, 0.6 mm diameter circular discs of Nyalflo filter paper (Gelman, Ann Arbor, MI) were incubated in PBS containing either 0.1% bovine serum albumin (BSA) or 0.1% BSA with 10 μM vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN) for 1 hr at 4°C. Rats were anesthetized with isoflurane and a single disc was inserted into a pocket in the corneal stroma. The margin of each disc was located ~1.8 mm from blood vessels of the lateral limbus.

Treatment with 2xCon4 (C) (0, 0.03, 0.1, 0.3, 1.0, or 10 mg/kg), Fc control (70 nM/kg), L1-7(N) (70 nM/kg), or vehicle (PBS) was initiated 1 week prior to corneal implantation and continued twice per week. Rats treated with Ab536 (Ab536 0.7 or 70 nM/kg) received one injection on the day of surgery and another 3 days later. All treatments were injected subcutaneously.

7 days after surgical implant, the corneas were photographed at 25× using a Nikon SV-3 Ophthalmic Slit Lamp (Nikon Ophthalmic) equipped with a Nikon D-1 digital camera back. A reference stage micrometer was photographed for calibration. Numerical data were generated from the digital images using Metamorph image analysis software (v4.5; Universal Imaging,

Downingtown, PA). For each corneal image, the number of vessels intersecting the midpoint between the disc and the limbus was measured. Values represent the group mean ± standard error of the mean. Statistical significance was assessed by analysis of variance followed by Fisher's exact test.

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References

Ahmad, S.A., Liu, W.B., Jung, Y.D., Fan, F., Wilson, M., Reinmuth, N., Shaheen, R.M., Bucana, C.D., and Ellis, L.M. (2001). The effects of angiopoietin-1 and-2 on tumor growth and angiogenesis in human colon cancer. Cancer Res. *61*, 1255–1259.

Anonymous. (2002). Bevacizumab. Anti-VEGF monoclonal antibody, avastin, rhumab-VEGF. Drugs R D. *3*, 28–30.

Bunone, G., Vigneri, P., Mariani, L., Buto, S., Collini, P., Pilotti, S., Pierotti, M.A., and Bongarzone, I. (1999). Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. Am. J. Pathol. *155*, 1967–1976.

Coxon, A., Bolon, B., Estrada, J., Kaufman, S., Scully, S., Rattan, A., Duryea, D., Hu, Y.L., Rex, K., Pacheco, E., et al. (2002). Inhibition of interleukin-1 but not tumor necrosis factor suppresses neovascularization in rat models of corneal angiogenesis and adjuvant arthritis. Arthritis Rheum. *46*, 2604–2612.

Das, A., Fanslow, W., Cerretti, D., Warren, E., Talarico, N., and McGuire, P. (2003). Angiopoietin/Tek interactions regulate MMP-9 expression and retinal neovascularization. Lab. Invest. *83*, 1637–1645.

Davis, S., Aldrich, T.H., Jones, P.F., Acheson, A., Compton, D.L., Jain, V., Ryan, T.E., Bruno, J., Radziejewski, C., Maisonpierre, P.C., and Yancopoulos, G.D. (1996). Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell *87*, 1161–1169.

DeVore, R., Fehrenbacher, L., Herbst, R., Langer, C., Kelly, K., Gaudreault, J., Holmgren, E., Novotny, W., and Kabbinavar, F. (2000). A randomized phase II trial comparing Rhumab VEGF (recombinant humanized monoclonal antibody to vascular endothelial cell growth factor) plus carboplatin/paclitaxel (CP) to CP alone in patients with stage IIIB/IV NSCLC. ASCO *Abstract 1896*.

Dumont, D.J., Gradwohl, G., Fong, G.H., Puri, M.C., Gertsenstein, M., Auerbach, A., and Breitman, M.L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev. *8*, 1897–1909.

Etoh, T., Inoue, H., Tanaka, S., Barnard, G.F., Kitano, S., and Mori, M. (2001). Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: possible in vivo regulation via induction of proteases. Cancer Res. *61*, 2145–2153.

Fearon, U., Griosios, K., Fraser, A., Reece, R., Emery, P., Jones, P.F., and Veale, D.J. (2003). Angiopoietins, growth factors, and vascular morphology in early arthritis. J. Rheumatol. *30*, 260–268.

Folkman, J. (1998). Antiangiogenic gene therapy. Proc. Natl. Acad. Sci. USA *95*, 9064–9066.

Gale, N.W., and Yancopoulos, G.D. (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev. *13*, 1055–1066.

Gale, N.W., Thurston, G., Hackett, S.F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M.H., Jackson, D., et al. (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. Dev. Cell *3*, 411–423.

Gerber, H.P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z., and Ferrara, N. (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. *5*, 623–628.

Hackett, S.F., Wiegand, S., Yancopoulos, G., and Campochiaro, P.A. (2002). Angiopoietin-2 plays an important role in retinal angiogenesis. J. Cell. Physiol. *192*, 182–187.

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. Science *277*, 48–50.

Hangai, M., Murata, T., Miyawaki, N., Spee, C., Lim, J.I., He, S.K., Hinton, D.R., and Ryan, S.J. (2001a). Angiopoietin-1 upregulation by vascular endothelial growth factor in human retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. *42*, 1617–1625.

Hangai, M., Yuen Sung, M., Kitaya, N., Chan, C.K., Wu, D.Y., Peters, K.G., Ryan, S.J., and Hinton, D.R. (2001b). Systemically expressed soluble Tie2 inhibits intraocular neovascularization. Hum. Gene Ther. *12*, 1311–1321.

Holash, J., Maisonpierre, P.C., Compton, D., Boland, P., Alexander, C.R., Zagzag, D., Yancopoulos, G.D., and Wiegand, S.J. (1999). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science *284*, 1994–1998.

Hurwitz, H., Fehrenbacher, L., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Griffing, S., Novotny, W., Holmgren, E., and Kabbinavar, F. (2003). Bevacizumab (a monoclonal antibody to vascular endothelial growth factor) prolongs survival in first-line colorectal cancer (CRC): results of a phase III trial of bevacizumab in combination with bolus IFL (irinotecan, 5-fluorouracil, leucovorin) as first-line therapy in subjects with metastatic CRC. ASCO *Abstract 3646*.

Iwama, A., Hamaguchi, I., Hashiyama, M., Murayama, Y., Yasunaga, K., and Suda, T. (1993). Molecular cloning and characterization of mouse TIE and TEK receptor tyrosine kinase genes and their expression in hematopoietic stem cells. Biochem. Biophys. Res. Commun. *195*, 301–309.

Kabbinavar, F., Hurwitz, H.I., Fehrenbacher, L., Meropol, N.J., Novotny, W.F., Lieberman, G., Griffing, S., and Bergsland, E. (2003). Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J. Clin. Oncol. *21*, 60–65.

Kim, I., Kim, J.H., Moon, S.O., Kwak, H.J., Kim, N.G., and Koh, G.Y. (2000). Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Oncogene *19*, 4549–4552.

Kuroda, K., Sapadin, A., Shoji, T., Fleischmajer, R., and Lebwohl, M. (2001). Altered expression of angiopoietins and Tie2 endothelium receptor in psoriasis. J. Invest. Dermatol. *116*, 713–720.

Lin, P., Polverini, P., Dewhirst, M., Shan, S., Rao, P.S., and Peters, K. (1997). Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. J. Clin. Invest. *100*, 2072–2078.

Lin, P., Buxton, J.A., Acheson, A., Radziejewski, C., Maisonpierre, P.C., Yancopoulos, G.D., Channon, K.M., Hale, L.P., Dewhirst, M.W., George, S.E., and Peters, K.G. (1998). Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. Proc. Natl. Acad. Sci. USA *95*, 8829–8834.

Lobov, I.B., Brooks, P.C., and Lang, R.A. (2002). Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proc. Natl. Acad. Sci. USA *99*, 11205–11210.

Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N., et al. (1997). Angiopoietin-2, a natural antagonist for tie2 that disrupts in vivo angiogenesis. Science *277*, 55–60.

Miller, K.D., Rugo, H.S., Cobleigh, M.A., Marcom, P.K., Chap, L.I., Holmes, F.A., Fehrenbacher, L., Overmoyer, B.A., Reimann, J.D., Vassel, A.V., and Langmuir, V.K. (2002). Phase III trial of capecitabine (Xeloda(R)) plus bevaci-

zumab (AvastinTM) versus capecitabine alone in women with metastatic breast cancer (MBC) previously treated with an anthracycline and a taxane. Paper presented at: 25th Annual San Antonio Breast Cancer Symposium, San Antonio, TX, December 11–14, 2002.

Mochizuki, Y., Nakamura, T., Kanetake, H., and Kanda, S. (2002). Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. J. Cell Sci. *115*, 175–183.

Mustonen, T., and Alitalo, K. (1995). Endothelial receptor tyrosine kinases involved in angiogenesis. J. Cell Biol. *129*, 895–898.

Otani, A., Takagi, H., Oh, H., Koyama, S., Matsumura, M., and Honda, Y. (1999). Expressions of angiopoietins and Tie2 in human choroidal neovascular membranes. Invest. Ophthalmol. Vis. Sci. *40*, 1912–1920.

Qian, X.-D., George, J.S., Moskey, M.D., Nelson, C.M., Smith, P.A., Bousquet, P.F., Seshadri, T., Mazdiyasni, H., Arnold, L.D., and Barlozzari, T. (2002). Inhibition of angiogenesis by BSF 466895 (A-422885.66), a potent and selective low molecular weight Tie-2 kinase inhibitor. Paper presented at: 93rd Annual Meeting of the American Association for Cancer Res., San Francisco, CA, April 6–10, 2002.

Raymond, E., Faivre, S., Vera, K., Delbaldo, C., Robert, C., Brega, N., Achour, A., Massimini, G., Schigalla, P., and Armand, J.P. (2002). First results of a phase I and pharmacokinetic study of SU011248, a novel oral anti-angiogenic agent, in patients with advanced solid tumours. Eur. J. Cancer *38*, 17.

Rothenberg, M.L., Carbone, D.P., and Johnson, D.H. (2003). Opinion: improving the evaluation of new cancer treatments: challenges and opportunities. Nat. Rev. Cancer *3*, 303–309.

Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature *376*, 70–74.

Shahrara, S., Volin, M.V., Connors, M.A., Haines, G.K., and Koch, A.E. (2002). Differential expression of the angiogenic Tie receptor family in arthritic and normal synovial tissue. Arthritis Res. *4*, 201–208.

Siemeister, G., Schirner, M., Weindel, K., Reusch, P., Menrad, A., Marme, D., and Martiny-Baron, G. (1999). Two independent mechanisms essential for tumor angiogenesis: Inhibition of human melanoma xenograft growth by interfering with either the vascular endothelial growth factor receptor pathway or the Tie-2 pathway. Cancer Res. *59*, 3185–3191.

Stratmann, A., Risau, W., and Plate, K.H. (1998). Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. Am. J. Pathol. *153*, 1459–1466.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., and Yancopoulos, G.D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell *87*, 1171–1180.

Tanaka, S., Mori, M., Sakamoto, Y., Makuuchi, M., Sugimachi, K., and Wands, J.R. (1999). Biologic significance of angiopoietin-2 expression in human hepatocellular carcinoma. J. Clin. Invest. *103*, 341–345.

Teichert-Kuliszewska, K., Maisonpierre, P.C., Jones, N., Campbell, A.I., Master, Z., Bendeck, M.P., Alitalo, K., Dumont, D.J., Yancopoulos, G.D., and Stewart, D.J. (2001). Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. Cardiovasc. Res. *49*, 659–670.

Visconti, R.P., Richardson, C.D., and Sato, T.N. (2002). Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). Proc. Natl. Acad. Sci. USA *99*, 8219–8224.

White, R.R., Shan, S., Rusconi, C.P., Shetty, G., Dewhirst, M.W., Kontos, C.D., and Sullenger, B.A. (2003). Inhibition of rat corneal angiogenesis by a nuclease-resistant RNA aptamer specific for angiopoietin-2. Proc. Natl. Acad. Sci. USA *100*, 5028–5033.

Yang, J.C., Haworth, L., Steinberg, S.M., Rosenberg, S.A., and Novotny, W. (2002). A randomized double-blind placebo-controlled trial of bevacizumab anti-VEGF antibody demonstrating a prolongation time to progression in patients with metastatic renal cancer. Proc. Am. Soc. Clin. Oncol. *21*, 15.

Yoshida, Y., Oshika, Y., Fukushima, Y., Tokunaga, T., Hatanaka, H., Kijima, H., Yamazaki, H., Ueyama, Y., Tamaoki, N., Miura, S., and Nakamura, M. (1999). Expression of angiostatic factors in colorectal cancer. Int. J. Oncol. *15*, 1221–1225.

Yu, Q., and Stamenkovic, I. (2001). Angiopoietin-2 is implicated in the regulation of tumor angiogenesis. Am. J. Pathol. *158*, 563–570.

Yuan, K., Jin, Y.T., and Lin, M.T. (2000). Expression of Tie-2, angiopoietin-1, angiopoietin-2, ephrinB2 and EphB4 in pyogenic granuloma of human gingiva implicates their roles in inflammatory angiogenesis. J. Periodontal Res. *35*, 165–171.

Zagzag, D., Hooper, A., Friedlander, D.R., Chan, W., Holash, J., Wiegand, S.J., Yancopoulos, G.D., and Grumet, M. (1999). In situ expression of angiopoietins in astrocytomas identifies angiopoietin-2 as an early marker of tumor angiogenesis. Exp. Neurol. *159*, 391–400.

Zangari, M., Elias, A., Stopeck, A., Morimoto, A., Tan, N., Lancet, J., Cooper, M., Hannah, A., Garcia-Manero, G., Faderl, S., et al. (2004). Phase II study of SU5416, a small molecule vascular endothelial growth factor tyrosine kinase receptor inhibitor, in patients with refractory multiple myeloma. Clin. Cancer Res. *10*, 88–95.