# Pharmacologic Blockade of Angiopoietin-2 Is Efficacious against Model Hemangiomas in Mice

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Hemangioma of infancy is the most common neoplasm of childhood. While hemangiomas are classic examples of angiogenesis, the angiogenic factors responsible for hemangiomas are not fully understood. Previously, we demonstrated that malignant endothelial tumors arise in the setting of autocrine loops involving vascular endothelial growth factor (VEGF) and its major mitogenic receptor vascular endothelial growth factor receptor 2. Hemangiomas of infancy differ from malignant endothelial tumors in that they usually regress, or can be induced to regress by pharmacologic means, suggesting that angiogenesis in hemangiomas differs fundamentally from that of malignant endothelial tumors. Here, we demonstrate constitutive activation of the endothelial tie-2 receptor in human hemangioma of infancy and, using a murine model of hemangioma, bEnd.3 cells; we show that bEnd.3 hemangiomas produce both angiopoietin-2 (ang-2) and its receptor, tie-2, *in vivo*. We also demonstrate that inhibition of tie-2 signaling with a soluble tie-2 receptor decreases bEnd.3 hemangioma growth *in vivo*. The efficacy of tie-2 blockade suggests that either tie-2 activation or ang-2 may be required for *in vivo* growth. To address this issue, we used tie-2-deficient bEnd.3 hemangioma cells, which, surprisingly, were fully proficient in *in vivo* growth. Previous studies from our laboratory and others have implicated reactive oxygen-generating nox enzymes in the angiogenic switch, so we examined the effect of nox inhibitors on ang-2 production *in vitro* and on bEnd.3 tumor growth *in vivo*. We then inhibited ang-2 production pharmacologically using novel inhibitors of nox enzymes and found that this treatment nearly abolished bEnd.3 hemangioma growth *in vivo*. Signal-transduction blockade targeting ang-2 production may be useful in the treatment of human hemangiomas *in vivo*.

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## INTRODUCTION

Hemangiomas are the most common tumor of infancy and childhood and account for a disproportionate number of visits to pediatricians and dermatologists (Chiller *et al.*, 2003). Histologically, hemangiomas consist of clusters of endothelial cells surrounding vascular lumens of varying diameter. The natural history of hemangiomas begins with a *proliferative* phase, characterized by rapid growth of the tumor and endothelial division, followed by an *involuting* stage, which is marked by endothelial apoptosis and decreasing tumor size, and finally ends with an *involuted* stage, during which the original tumor is replaced by a connective tissue scar (Takahashi *et al.*, 1994). While these tumors usually resolve spontaneously, large tumors can compromise the function of vital organs by compression, and may even lead to high-output cardiac failure (Drolet *et al.*, 1999).

Studies have established the clonal nature of hemangiomas and suggested that growth factors may play a role in the pathogenesis of hemangiomas (Boye *et al.*, 2001; Yu *et al.*, 2001). Our laboratory has previously shown that autocrine production of vascular endothelial growth factor (VEGF) by endothelial cells results in malignant transformation to angiosarcoma (Arbiser *et al.*, 2000; McLaughlin *et al.*, 2000). Therefore, we postulated that another factor, which acts as an endothelial chemoattractant and survival factor, is responsible for autocrine growth in hemangiomas. Here, we demonstrate that the tie-2 receptor is constitutively phosphorylated in human hemangioma, implicating either constitutive activation of tie-2 or deregulated production of angiopoietin-2 (ang-2) as a causative factor in hemangiomagenesis. To elucidate the functional role of these agents in the pathogenesis of hemangioma, we used a murine model of

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*Abbreviations: Ang, angiopoietin; DPI, diphenyliodonium; VEGF, vascular endothelial growth factor*

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hemangioma, bEnd.3 cells (Bautch *et al*., 1987; Williams *et al*., 1989), and found that these cells express both tie-2 and its ligand, ang-2. We also demonstrate that functional blockade of tie-2 using a soluble receptor inhibits the growth of bEnd.3 hemangiomas *in vivo*. Surprisingly, tie-2-deficient endothelial cells were also capable of initiating hemangiomas *in vivo*, implicating aberrant ang-2 production as a potential cause of hemangiomas. Nicotinamide adenine dinucleotide phosphate (reduced form) oxidase (*Nox*) genes have previously been linked to the angiogenic switch, and have been known to regulate ang-2 (Arbiser *et al*., 2002; Krikun *et al*., 2002). As we were unable to inhibit ang-2 stably using small interfering RNA, we discovered novel inhibitors of ang-2 production through blockade of *Nox* genes. These inhibitors nearly abolish bEnd.3 hemangioma growth *in vivo*. Thus, our data suggest that neutralization of ang-2 through Nox inhibition may be an effective therapy for hemangiomas of infancy.

## RESULTS

### Tie-2 and ang-2 are highly expressed in bEnd.3 hemangiomas *in vivo*

In order to determine whether bEnd.3 hemangiomas exhibit potential autocrine loops involving tie-2 and angs, we performed *in situ* hybridization of lesions in mice. We found that bEnd.3 hemangiomas exhibit expression of both ang-2 and tie-2. Vascular endothelial growth factor receptor 1 and 2 were expressed at high levels, consistent with active endothelial remodeling, and small quantities of VEGF mRNA were observed (Figures 1 and 2). Use of control sense probes for VEGF did not reveal hybridization. No significant hybridization for ang-1 was observed (data not shown).

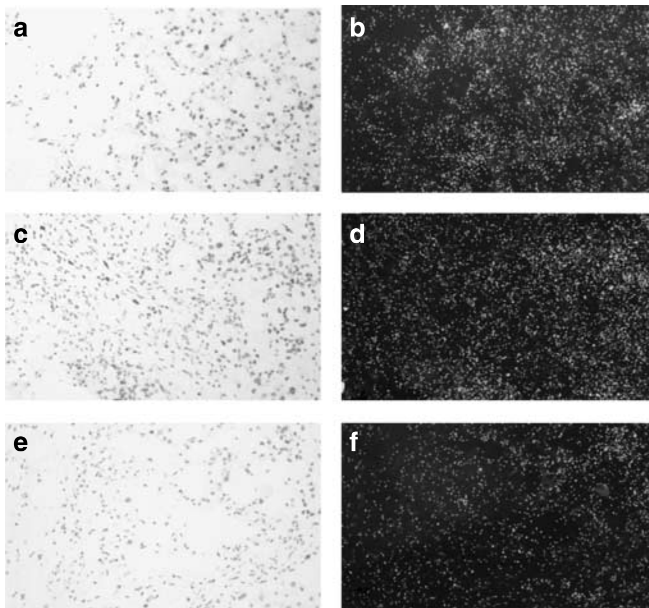


**Figure 1. Ang-2 and tie-2 are expressed in bEnd.3 hemangioma tissue *in vivo*.** *In situ* hybridization studies of subcutaneous bEnd.3 tumors. Tumor cells express mRNAs for ang-2 (**a**, bright field; **b**, corresponding dark field), (**c**, **d**) tie-1, and (**e**, **f**) tie-2. Original magnification ×200.

### Inhibition of ang-2 using a soluble receptor inhibits bEnd.3 growth *in vivo*

To determine whether inhibition of ang-2 expression by blockade of its receptor, tie-2, was needed for hemangioma formation *in vivo*, we infected bEnd.3 cells with adenoviruses encoding either soluble tie-2/Fc or adenoviruses encoding Fc fragment alone, 24 hours before injection. This treatment resulted in an approximately 66% decrease in tumor volume, compared to control adenovirus treatment (Figure 3). No toxicity was observed as a consequence of infection in any of the three animals that were used in each group. Attempts to generate stably infected bEnd.3 cells with lentiviral small interfering RNA for ang-2 were unsuccessful, possibly owing to a requirement for ang-2/tie-2 signaling for longer term growth.

### Tie-2 is not required for *in vivo* growth of hemangiomas

Given that functional blockade of the tie-2 receptor significantly reduced hemangioma formation *in vivo*, we wanted to determine whether this was the result of impaired ang-2 signaling or tie-2 inhibition. Polyoma-expressing endothelial cells derived from tie-2-deficient mice were

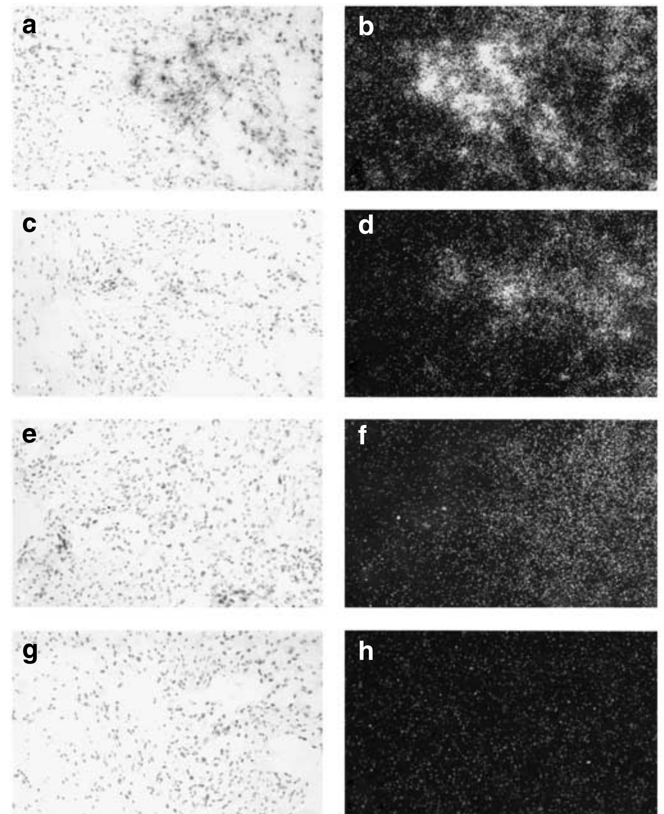


**Figure 2. Expression of VEGF receptors and low-level VEGF expression in bEnd.3 hemangioma tissue *in vivo*.** Tumors are located in the center and right side of the field, with normal subcutaneous tissue to the left-hand side of the field. Tumor cells strongly express Flt-1 (**a**, bright field; **b**, corresponding dark field) and (**c**, **d**) Flk-1 mRNAs. (**e**, **f**) A lower level of expression of VEGF mRNA was seen. (**g**, **h**) A low background level of grains is seen with control VEGF sense probe. Original magnification ×200.

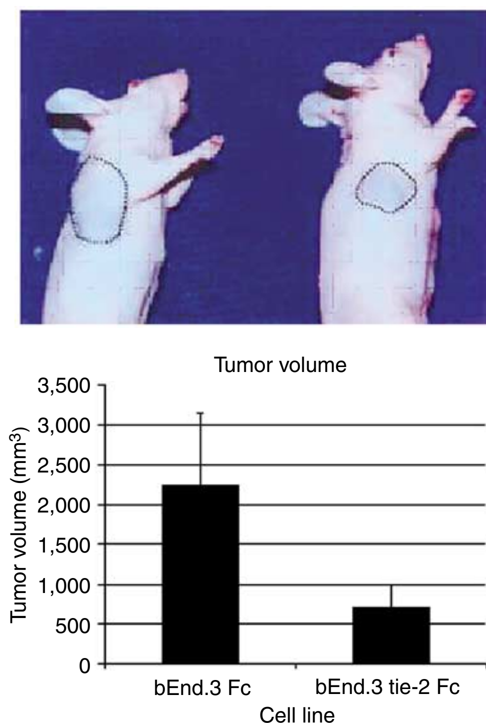


**Figure 3. Inhibition of ang-2 signaling by a soluble tie-2 receptor inhibits bEnd.3 growth *in vivo*.** The top panel shows mice with control Fc-treated tumor (left) and tie-2/Fc-treated hemangioma (right). The bottom panel shows that hemangioma volume in mice treated with tie-2/Fc differs significantly from control mice ($P<0.05$). Three mice were used in each group, and the error bars represent the standard error of the mean.

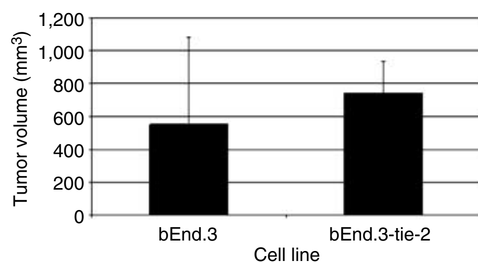


**Figure 4. Tie-2 activation is not required for *in vivo* growth of bEnd.3 cells.** Wild-type and tie-2-deficient polyoma-transformed cells were tested in mice for their ability to form hemangiomas in mice. No significant difference in tumor volume was observed.

injected into nude mice, and compared to controls, no significant difference in tumor volume or histology was noted (Figure 4). Efforts to generate bEnd.3 clones expressing small interfering RNA to ang-2 were unsuccessful, perhaps owing to severe growth disadvantages.

### Triphenylmethane dyes inhibit nox activity

We examined triphenylmethane dyes for activity against nox enzymes because they have chemical similarity to diphenyliodonium (DPI), a specific Nox inhibitor (Figure 5). Additionally, brilliant green and gentian violet have a long history of animal and human exposure, and gentian violet is Food and Drug Administration approved for human use. Brilliant green and gentian violet inhibited Nox2 and Nox4, the species of nox enzymes that are known to be expressed in endothelial cells, in a dose-dependent manner (Figure 6).

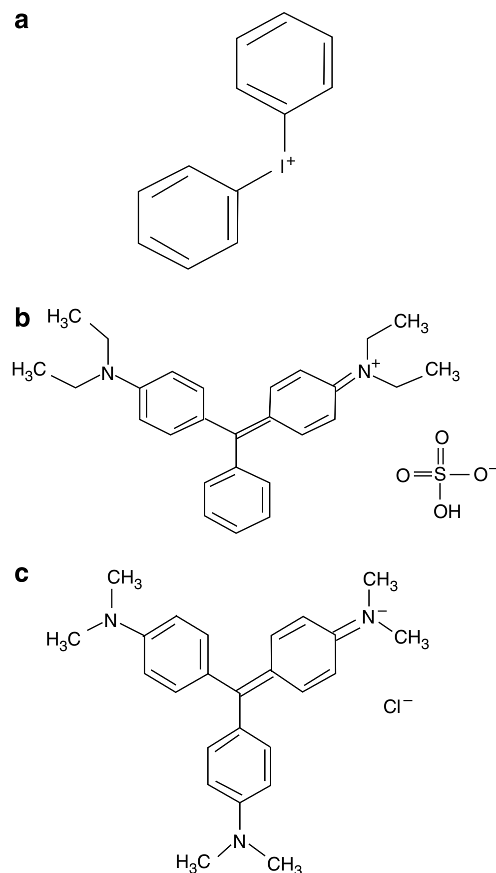


**Figure 5. Chemical structures of Nox enzyme inhibitors.** Structural similarity between known inhibitor of *Nox* genes, (**a**) DPI, and novel inhibitors, (**b**) brilliant green, and (**c**) gentian violet. The common structural feature is a cation surrounded by two or more aromatic rings.

### DPI and triphenylmethane dyes such as brilliant green and gentian violet inhibit ang-2 *in vitro*

Because DPI, brilliant green, and gentian violet all inhibit *Nox* genes, we wanted to see if they had similar effects on ang-2 mRNA expression. bEnd.3 cells were treated for 6.5 hours with either DPI or varying concentrations of brilliant green or gentian violet, and quantitative reverse transcription-PCR revealed a statistically significant decrease in ang-2 expression in all three treatment groups. Treatment with 10 $\mu$M DPI resulted in an 80% decrease in ang-2 expression, compared to control (data not shown), whereas treatment with brilliant green had a marked dose-dependent effect on ang-2 production, such that 0.75 $\mu$M concentrations were sufficient to render ang-2 mRNA undetectable (Figure 7a). Gentian violet increased ang-2 expression at both the 1 and 5 $\mu$M concentrations, compared to control, but higher concentrations effectively inhibited ang-2 mRNA by 70–90% (Figure 7b).

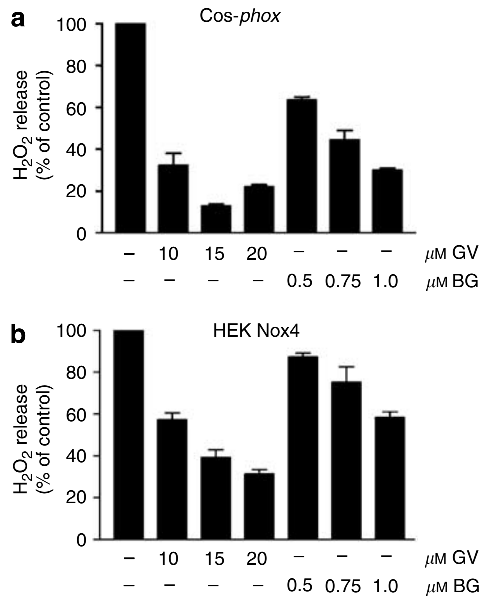


**Figure 6. Brilliant green (BG) and gentian violet (GV) inhibit (a) Nox2 (Cos-*phox*) and (b) Nox4 activity, along with hydrogen peroxide ($H_2O_2$) production in a dose-dependent manner in Cos-*phox* and HEK293 Nox4-11 cells.** Cells were treated with different concentrations of vehicle control, BG, or GV; Cos-*phox* cells were additionally either left unstimulated or stimulated with phorbol 12-myristate 13-acetate. After 1 hour incubation at 37°C, the reaction was stopped and $H_2O_2$ production was measured using the homovanillic acid assay. The abililty of these drugs to inhibit production of $H_2O_2$ is shown as a percentage relative to the untreated control (100%). Cox-*phox* cells did not produce $H_2O_2$ without phorbol 12-myristate 13-acetate stimulation with or without the addition of BG or GV (not shown). Optimal Nox2 activity requires phorbol 12-myristate 13-acetate stimulation whereas Nox4 activity is constitutive.

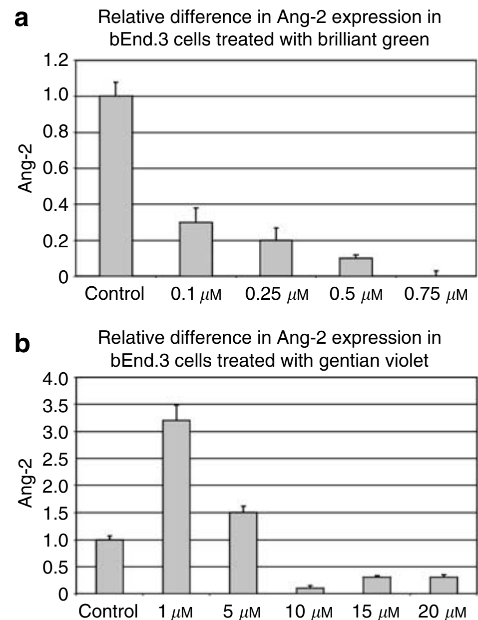


**Figure 7. Treatment of bEnd.3 cells with (a) brilliant green and (b) gentian violet decreases levels of ang-2 mRNA (corrected for 18S RNA).** Bars shown represent the average of triplicate experiments, and error bars indicate the standard error of the mean.

### Brilliant green and gentian violet inhibit hemangioma formation *in vivo*

In order to determine if compounds that inhibit ang-2 formation *in vitro* would ameliorate hemangioma formation *in vivo*, we injected one million bEnd.3 cells subcutaneously into nine nude mice. Intralesional treatment of hemangiomas with either vehicle control, brilliant green, or gentian violet resulted in a 95.7 and 92.6% decrease in tumor size and arrest of tumor progression in both the brilliant green and gentian violet treatment groups, respectively, compared to control (Figure 8a and b). Neither local nor systemic toxicity was observed in any of the nude mice as a result of treatment.

## DISCUSSION

Hemangiomas are the most common cutaneous vascular lesions of childhood and are present in 5% of infants at 1 year of age. They may grow to large sizes and may result in compression of vital structures or high-output cardiac failure. Treatment of large hemangiomas may require lengthy courses of steroids or alpha IFN, which induces endothelial apoptosis, or surgery. These treatments are associated with a high level of morbidity, including growth retardation, infection, and irreversible neuropathy (Barlow *et al.*, 1998). A significant number of these hemangiomas do not respond to treatment, resulting in death (Paller *et al.*, 1983; Mulliken *et al.*, 1982; Blei *et al.*, 1998; Enjolras, 1998; Williams III *et al.*, 2000). Thus, novel therapies are needed for hemangiomas in humans.

The growth factors required for hemangioma formation have not been fully elucidated. We have previously shown that angiosarcomas express VEGF, and overexpression of VEGF leads to the development of angiosarcoma (Arbiser *et al.*, 2000). Hemangiomas express VEGF protein but little VEGF RNA, and it is highly likely that the VEGF protein may arise in surrounding cells, such as overlying skin (Cerimele *et al.*, 2003). Similarly, we have recently demonstrated that verruga peruana, a hemangioma-like condition caused by endothelial infection with the bacterium *Bartonella bacilliformis*, exhibits high-level expression of ang-2 *in vivo*, and that infection results in the induction of ang-2 *in vitro,* whereas VEGF expression is limited to the overlying epidermis (Cerimele *et al.*, 2003). Tie-2 expression has also been demonstrated in oral hemangiomas, but its functional role is unknown (Sato, 2002).

We thus postulated that other receptor tyrosine kinases may be important in the pathogenesis of hemangioma of infancy. Hemangiomas differ biologically from angiosarcomas in that they regress rather than cause progressive growth and metastasis.

Murine models of hemangioma, including the bEnd.3 model we used, exist through infection of neonatal endothelial cells with polyoma virus or polyoma middle T antigen. These models differ from angiosarcoma in that they grow through recruitment of host endothelium rather than active mitosis and they do not metastasize as murine SVR cells do (Williams *et al.*, 1989; Arbiser *et al.*, 1997). In this study, we

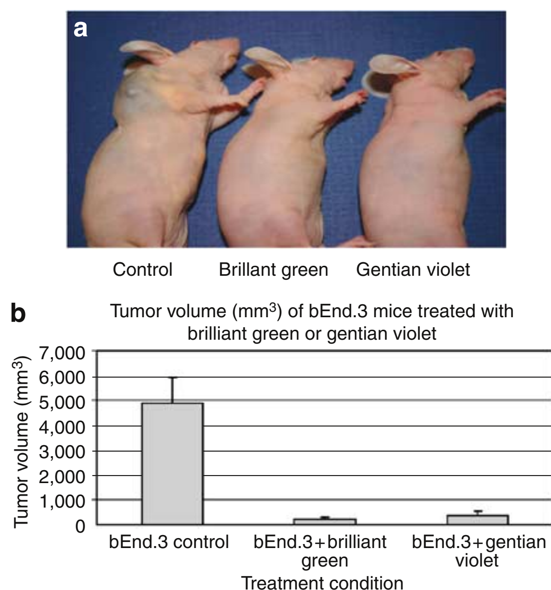


**Figure 8. Effect of brilliant green and gentian violet on bEnd.3 hemangiomas *in vivo*.** For each treatment condition, three mice were injected with 1,000,000 bEnd.3 cells and received intralesional injection with either vehicle control, brilliant green or gentian violet (from left to right in photo) on days 9 and 14. Animals were euthanized on day 20, secondary to tumor burden in the control animals. (**a**) Photos above represent average tumor burden in each of the three groups and tumor volume (mm$^3$) is graphically depicted. (**b**) Error bars represent the standard error of the mean.

show that tie-2 is activated *in vivo* in human hemangioma tissue, suggesting a physiologic role. We also demonstrate for the first time that bEnd.3-derived hemangioma cells, like human hemangiomas, produce ang-2 and tie-2, with a small contribution of VEGF (Figures 2 and 3).

Both ang-1 and -2 are required for viability in mice, as knockouts cause lethal vascular abnormalities *in utero* (Suri *et al.*, 1996; Maisonpierre *et al.*, 1997). Each binds and activates the tie-2 receptor, leading to downstream events such as activation of phosphoinositol-3 kinase (Suri *et al.*, 1996), and both peptides have been shown to promote angiogenesis in the presence of VEGF. However, they have opposing effects *in vivo*. Transgenic overexpression of ang-1 leads to non-permeable vessels, but ang-2 expression leads to leaky vessels (Suri *et al.*, 1998; Thurston *et al.*, 1999). Ang-1 is preferentially expressed by stromal cells, whereas ang-2 is highly expressed by tumor cells (Tanaka *et al.*, 1999) and, as we demonstrate in this study, hemangioma model cells. Overexpression of ang-1 in tumor cells leads to increased vessel maturation and decreased *in vivo* growth (Hawighorst *et al.*, 2002; Stoeltzing *et al.*, 2002). Thus, it is unlikely that ang-1 plays a predominant role in the pathogenesis of proliferative vascular lesions such as hemangioma.

Our results suggest novel therapies for hemangioma of infancy and demonstrate similarities between the polyoma-induced hemangiomas, which have been known to induce hemangiomas through the recruitment of host endothelial cells or endothelial precursor cells, and human hemangiomas (Whitman *et al.*, 1985; Williams *et al.*, 1989; Dahl *et al.*, 1998). Soluble receptors antagonizing both ang-1 and -2 may have the benefit of inhibiting not only the remodeling effect of ang-2 but also the antiapoptotic effect of ang-1. Our study with tie-2-deficient endothelium suggests that aberrant expression of ang-2, rather than constitutive activation of tie-2, is required for hemangiomagenesis. Constitutively active mutations in tie-2 have been found in vascular malformations, but not in hemangiomas, and these activating mutations in tie-2 are associated with phosphoinositol-3 kinase (Vikkula *et al.*, 1996). Naturally occurring mutations of tie-2 have also been shown to be transforming when introduced into immortalized endothelial cells, likely through prevention of apoptosis. This differs from endothelial cells present in human hemangiomas, which undergo apoptosis with age. These findings support our hypothesis that aberrant production of ang-2, rather than constitutive tie-2 activation, is required for hemangioma growth *in vivo*. Interestingly, introduction of a vascular malformation-associated tie-2 allele into immortalized endothelial cells leads to malignant transformation (Wang *et al.*, 2004). Our results differ in that *in vivo* growth of hemangiomas is dependent on ang-2, rather than tie-2, and may reflect basic biologic differences between hemangiomas and vascular malformations. Attempts to generate bEnd.3 clones expressing small interfering RNA to ang-2 were unsuccessful, perhaps reflecting a requirement of ang-2 for hemangioma growth.

We have previously shown that reactive oxygen induces angiogenesis and that blockade of *Nox* genes results in decreased angiogenesis (Arbiser *et al.*, 2002). Based upon this observation, we examined the ability of DPI, a known Nox inhibitor, to donwregulate ang-2 expression. DPI is structurally similar to the triphenylmethane dye family, as it forms a cation directly attached to multiple aromatic rings. Triphenylmethane dyes such as gentian violet and brilliant green have a long history of human and veterinary use. We demonstrate that like DPI, gentian violet and brilliant green decrease expression of ang-2 *in vitro* and, consistent with this activity, decrease growth of bEnd.3 hemangiomas *in vivo*. Inhibitors of *Nox* genes may have therapeutic utility in the treatment of hemangiomas.

## MATERIALS AND METHODS

All experiments contained herein were approved by the Emory University Institutional Review Board.

### Cells

bEnd.3 cells (ATCC CRL 2299) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (4,500 mg glucose/l; Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum, L-glutamine (14 ml/l), recombinant mouse VEGF (10 ng/ml; R&D Systems, Minneapolis, MN), and antibiotic/antimycotic (14 ml/l; Sigma-Aldrich). HEK293-Nox4–11 cells and COS-*phox* cells have been described previously (Price, 2002; Martyn, 2006).

### *In situ* hybridization

*In situ* hybridization was performed on 4 mm sections of formalin-fixed, paraffin-embedded tissue. Details of *in situ* hybridization have

been reported previously (Arbiser *et al.*, 2000; McLaughlin *et al.*, 2000). Slides were passaged through xylene and graded alcohols; 0.2 M HCl; Tris/EDTA with 3 $\mu$g/ml proteinase K/0.2% glycine/4% paraformaldehyde in phosphate-buffered saline (pH 7.4); 0.1 M triethanolamine containing 1/200 (vol/vol) acetic anhydride; and 2 × standard sodium citrate. Slides were hybridized overnight at 50°C with $^{35}$S-labeled riboprobes in the following mixture: 0.3 M NaCl/0.01 M Tris (pH 7.6)/5 mM EDTA/0.02% (wt/vol) Ficoll/0.02% (wt/vol) polyvinylpyrollidone/0.02% (wt/vol) BSA fraction V/50% formamide, 10% dextran sulfate/0.1 mg/ml yeast tRNA/0.01 M dithiothreitol. Post-hybridization washes included 2 × standard sodium citrate/50% formamide/10 mM dithiothreitol at 50°C, 4 × standard sodium citrate/10 mM Tris, 1 mM EDTA with 20 $\mu$g/ml ribonuclease at 37°C; and 2 × standard sodium citrate/50% formamide/10 mM EDTA at 65°C and 2 × standard sodium citrate. Slides were dehydrated through graded alcohols containing 0.3 M ammonium acetate, dried, coated with Kodak NTB 2 emulsion (Rochester, NY), and stored in the dark at 4°C for 2 weeks. The emulsion was developed with Kodak D19 Developer, and the slides were counterstained with hematoxylin. $^{35}$S-labeled single-stranded antisense and sense RNA probes for mouse VPF/VEGF, ang-1, -2, and tie-2 mRNA and the mouse VPF/VEGF receptors, vascular endothelial growth factor receptor 1 and 2 mRNAs, were described previously (Arbiser *et al.*, 2000; McLaughlin *et al.*, 2000).

### Adenoviral infection and *in vivo* tumorigenesis studies

The tie-2 Fc fusion construct was placed into an adenoviral cassette, and virus was prepared as described previously (Thurston *et al.*, 2000). bEnd.3 cells were infected with tie-2 Fc or Fc control adenovirus at a multiplicity of infection of 5. Twenty-four hours after infection, one million cells were injected subcutaneously into three nude mice per treatment group. Mice were monitored for the development of tumors and killed 1 month after injection. No evidence of toxicity was observed as a result of infection. For experiments utilizing tie-2 knockout bEnd.3 cells *versus* wild-type bEnd.3 cells, one million cells were injected subcutaneously as above.

### Determination of ROS production

$H_2O_2$ release was measured using the homovanillic acid assay as described previously (Martyn, 2006). Briefly, 1.5–1.75 × $0^5$ cells/well of a 12-well plate were seeded. The following day, cells were washed once with Hank's balanced salt solution and then preincubated for 15 minutes with either gentian violet (10–20 $\mu$M) or brilliant green (0.5–1.0 $\mu$M) in 1 ml of media. The cells were then washed once with Hank's balanced salt solution. Gentian violet or brilliant green was added at the same concentrations as in pretreatment to 0.5 ml of homovanillic acid assay solution (100 $\mu$M homovanillic acid assay, 4 U/ml horseradish peroxidase in Hank's balanced salt solution with $Ca^{2+}$ and $Mg^{2+}$) and incubated with the cells for 1 hour at 37°C. Cos-*phox* cells were additionally stimulated with 0.4 $\mu$g/ml phorbol 12-myristate 13-acetate. The reaction was stopped by adding 75 $\mu$l of homovanillic acid assay stop buffer (0.1 M glycine/0.1 M NaOH (pH 12) and 25 mM EDTA in phosphate-buffered saline). Fluorescence was read on a BioTek Synergy HT (BioTek Instruments Inc., Winooski, Vermont, CA) with an excitation of 320 nm and emission of 440 nm.

### Quantitative reverse transcription-PCR for ang-2 in bEnd.3 cells treated with vehicle control, brilliant green, or gentian violet

bEnd.3 cells were seeded equally into six T-75 flasks and 24 hours later were treated with 0, 1, 5, 10, 15, and 20 $\mu$M concentrations of gentian violet (Sigma-Aldrich, no. G2039) in ethanol for 6.5 hours. RNA was extracted and purified using TRI reagent (Sigma-Aldrich, T9424) and measured using spectrophotometer (Perkin-Elmer UV/VIS, Wellesley, MA). RNA (1 $\mu$g) was used for DNase Amplification (Invitrogen, no. 18068-015, Carlsbad, CA) followed by first-strand synthesis for reverse transcription-PCR (Invitrogen SuperScript, no. 12371-019). 96-well Optical Reaction Plate (ABI PRISM, no. 128, Applied Biosystems, Foster City, CA) was used for the reverse transcription-PCR reaction. A measure of 2.5 $\mu$l of template, which had been diluted 1:10 in crosslinked water, was used in each well and the experiment was performed in triplicate. Ang pt2 (Applied Biosystems, Taqman Gene Expression Assay, Mm00545822_ml) and 18S (Applied Biosystems Taqman Gene Expression Assay, Hs99999901_s1) primers were used along with crosslinked molecular grade water (Cellgro, Mediatech, Inc., Herndon, VA) and master mix (Applied Biosystems TaqMan Fast Universal PCR Master Mix (2 ×)). The reaction was run on the 7900 Applied Biosystems Reader for Absolute Quantification for 96-well plates. $C_t$ values were analyzed by $\Delta\Delta C_t$ method, and the standard error of the mean was calculated (Figure 7b). The same protocol was used for treatment with brilliant green (Sigma, no. B6756), except that the concentrations used were 0, 0.1, 0.25, 0.5, and 0.75 $\mu$M (Figure 7a).

### Treatment with vehicle control, brilliant green, or gentian violet *in vivo*

For each treatment condition, three mice were subcutaneously injected with one million bEnd.3 cells and monitored for tumor development. On day 9, tumors were measured in all nine animals, and there was no significant difference in tumor volume before the initiation of treatment. Each mouse then received intralesional injection with either 0.33 ml vehicle control, brilliant green (5 mg/kg, dissolved in 100 $\mu$l ethanol and 900 $\mu$l intralipid) or gentian violet (5 mg/kg, dissolved in 100 $\mu$l ethanol and 900 $\mu$l intralipid) on days 9 and 14. No toxicity was noted following injection. Animals were euthanized on day 20, secondary to tumor burden in the control animals. Photos represent average tumor burden in each of the three groups (Figure 8a), and tumor volume ($mm^3$) is graphically depicted (Figure 8b). Error bars represent the standard error of the mean.

### CONFLICT OF INTEREST

Ella Ioffe and George D. Yancopoulos are employees of Regeneron Pharmaceuticals, but the other authors state no conflict of interest.

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