Angiostatin Inhibits Pathological but Not Physiological Retinal Angiogenesis

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PURPOSE. Antiangiogenic treatment is a promising new therapy for angiogenesis-dependent diseases. In the current study, the biologic effects on pathologic and physiological angiogenesis in the retina of angiostatin, a very potent angiogenesis inhibitor were determined. In addition, the effects of angiostatin on the growth and development of newborn mice were examined.

METHODS. Oxygen-induced retinopathy was induced by subjecting mice postnatal day (P)7 to hyperoxic conditions (5 days) followed by normoxic conditions (relative hypoxia). Mice were treated with angiostatin (intravitreal or systemic). Retinal blood vessels were visualized by fluorescein angiography. Retinal neovascularization was assessed by counting intravitreal endothelial cell nuclei. Growth and organogenesis were determined between P0 and P14.

RESULTS. Relative hypoxia resulted in intravitreal proliferation of retinal blood vessels. However, proliferation was inhibited completely by systemic administration of angiostatin without affecting normal retinal vascularization. After intravitreal injection of angiostatin, pathologic proliferation of the retinal blood vessels was impaired by 62%. Neither systemic nor intravitreal treatment impaired the development or growth of organs throughout the body.

CONCLUSIONS. Angiostatin inhibits oxygen-induced intravitreal pathologic retinal angiogenesis without affecting the development of physiological retinal vascularization, development, and growth of newborn mice. Therefore, antiangiogenic treatment may be a useful tool in the treatment of proliferative retinopathies. (Invest Ophthalmol Vis Sci. 2001;42:3325–3330)

Retinopathy of prematurity (ROP) is a disease associated with visual impairment as a result of excessive retinal neovascularization. This pathologic retinal neovascularization is initiated when premature infants are exposed to high concentrations of oxygen. The mechanism underlying retinal neo-vascularization is considered to be mediated by hypoxic damage to immature retinal blood vessels, resulting in retinal ischemia, hypoxia, and a compensatory induction of new, leaky blood vessels, often resulting in retinal detachment and loss of vision. Several growth factors have been implicated in ischemia-induced retinal neovascularization. Vascular endothelial growth factor (VEGF), a specific endothelial cell mitogen and chemotactic factor, is upregulated by hypoxia.1–3 In a mouse model of oxygen-induced retinopathy VEGF mRNA is upregulated in the inner nuclear layer of the retina. Furthermore, it was demonstrated that VEGF acts as a survival factor for newly formed retinal blood vessels by preventing endothelial cell apoptosis. Although VEGF is an important angiogenic factor, it is likely that additional angiogenic factors play a role as well.4–8

Because of the debilitating effects of retinal neovascularization, several antiangiogenic treatment strategies have been explored in animals.3–14 Vitamin E has been evaluated in early randomized trials.15 The currently used treatment modalities in patients include laser coagulation and cryotherapy.16,17

Limited information is available on the effects of antiangiogenic agents on physiological angiogenesis. However, this is of major importance in considering the use of antiangiogenic drugs in the treatment of ROP. It has been shown that TNP-470, a potent inhibitor of angiogenesis, impairs fetal development when administered during pregnancy.18 Thalidomide is also thought to have this effect.19 Angiostatin, a novel and very potent inhibitor of angiogenesis, has predominantly been studied in murine tumor models, in which it was shown to inhibit the growth of a variety of tumors.20–23 Although its mechanism of action is presently unclear, it does not seem to involve the neutralization of a specific angiogenic factor.

In the present study the effects of angiostatin were evaluated in an oxygen-induced retinopathy model in newborn mice. The objectives of this study were to determine the effects of systemic and local treatment with angiostatin on pathologic retinal neovascularization and to evaluate the effects of angiostatin on the development of neonatal mice. In this report we provide evidence that treatment with human angiostatin completely abolished pathologic angiogenesis in the retina without affecting normal retinal vessel development and without affecting the normal growth of newborn mice.

METHODS

Purification of Human Angiostatin

Human angiostatin was generated as described by O‘Reilly et al.,20 with several modifications. Briefly, recovered outdated human plasma was diluted 2:1 with PBS, supplemented with 3 mM EDTA filtered (0.1 mm) at 37°C. The plasma was then applied to a lysine-Sepharose column (Pharmacia & Upjohn, Uppsala, Sweden) at room temperature. After washing the column with 0.5 M phosphate buffer, plasminogen was eluted with 0.2 M prewarmed (37°C) e-aminocaproic acid (e-ACA) at pH 7.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the eluant revealed one apparent band of 92 kDa, corresponding to plasminogen. The eluant was dialyzed against demiwater (molecular weight cut off [MWCO]: 6–8000; >4 × 107 dilution; 4°C) in dialysis tubing (Spectra/Por; Spectrum Europe, Breda, The Netherlands) followed by proteolytic digestion (12 hours; 37°C; 120 rpm) with porcine pancreatic elastase in a concentration of 0.8 U/mg plasminogen (Calbiochem, San Diego, CA), using a shaker (37°C; 120 rpm; overnight).
Next, the solution was applied to a lysine-Sepharose column that had been equilibrated with a salt solution (pH 7.4: 0.5 M NaCl, 0.2 M e-ACA, 0.03 M NaH₂PO₄, 0.02 M Na₂S, and 0.1% Triton X-100). The column was then re-equilibrated with 30 mM phosphate buffer at pH 7.4. Finally, angiostatin was eluted with 0.2 mM e-ACA and dialyzed against demiwater. SDS-PAGE revealed three distinct bands of approximately 40 kDa, 42 kDa, and 45 kDa, resembling the triplet first described by O’Reilly et al.²⁰

**Antiangiogenic Activity of Human Angiostatin**

To demonstrate antangiogenic activity of the purified human angiostatin in mice, the mouse cornea; neovascularization assay was used. Briefly, a pellet containing basic fibroblast growth factor was inserted into the cornea of 6- to 8-week-old BALB/c mice. Mice were treated with subcutaneous injections of increasing concentrations of angiostatin (0.5, 5, and 50 mg/kg body weight, twice daily). A dose-dependent inhibition of corneal neovascularization was observed.²⁴ A dose of 50 mg/kg body weight administered in twice-daily injections was considered an effective treatment schedule and was used in the oxygen-induced retinopathy model.

**Mouse Model of Oxygen-Induced Retinopathy**

Animal experiments were approved by and performed according to the guidelines of the Committee of Experimental Animals and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mouse model of oxygen-induced retinopathy has been described previously and has been a reproducible model of retinal angiogenesis.²⁵ Briefly, postnatal day (P)7 C57/B1 mice, obtained from breeding colonies maintained at the Common Animal Laboratory (GDL; Utrecht, The Netherlands) together with their nursing mothers were exposed to hyperoxic conditions in an incubator continuously for 5 days (75% O₂, 1.5 l/min; <300 lux of 12-hour cyclic broad-spectrum light; 23 ± 2°C). All litters and nursing mothers survived the incubation in 75% oxygen. As soon as mother and litter had returned (P12) to normoxic conditions (room air), treatment was initiated. In the systemic treatment group twice-daily subcutaneous injections with angiostatin (50 mg/kg body weight; n = 18) or saline (n = 14) were begun and continued until P17. The local-treatment groups were subjected to a single intravitreal injection (1 μl) of angiostatin (n = 6; 60 μg angiostatin/1.0 μl PBS) or PBS (1.0 μl; n = 6) at P12. Furthermore, a control group was included that received an intravitreal injection only (trauma group; n = 6) without injection of fluid. In the local-treatment groups, the transcorneal puncture (in the fourth quadrant of the cornea) was performed by a 32-gauge needle (Hamilton, Reno, NV). The needle penetrated the vitreal corpus without damaging the lens.

**Retinal Fluorescein Angiogram**

Mice were deeply anesthetized, a median laparotomy was performed, and the portal vein was exposed. One milliliter PBS containing 25 mg 2 × 10⁶ molecular weight fluorescein isothiocyanate-dextran (FD-20006S; Sigma-Aldrich, Zwijndrecht, The Netherlands) dye was injected intraperitoneally. Eyes were subsequently removed and fixed in 4% (wt/vol) formaldehyde for 24 hours. The cornea was removed, the sclera was cut sagittally (bilateral, ventral, and caudal), and the eye was exposed and mounted on a glass slide. Fluorescent micrographs were taken.

**Quantification of Neovascular Proliferative Retinopathy**

At P17 the eyes of mice were enucleated and fixed in 4% (wt/vol) formaldehyde for 24 hours and embedded in paraffin. Serial sagittal sections (6 μm) of whole eyes were stained with hematoxylin. Fifteen consecutive sections per eye (30 per mouse) were used to count endothelial cell nuclei that were located on the vitreal side of the internal limiting membrane. The mean number of nuclei per eye was used in subsequent comparisons.

**Effects of Angiostatin on Normal Neonatal Development**

To exclude inhibitory side effects of angiostatin on normal vascular development and growth, neonates not subjected to hyperoxia were subjected to systemic treatment of angiostatin (50 mg/kg body weight twice daily) from P0 to P14. All animals were monitored for gain of body weight and general health (toxicity). Tail length and organ weight (liver, kidney, spleen, heart) were used as indicators of vasculogenesis. Litters treated with saline served as the control for these experiments.

**Statistical Analysis**

Data were expressed as mean ± SEM unless otherwise stated. The significance of differences among groups was determined by the unpaired Students t-test. P < 0.05 was considered to be statistically significant.

**RESULTS**

**Effect of Angiostatin on Pathologic Retinal Neovascularization**

Inhibition of oxygen-induced pathologic retinal neovascularization by systemic administration of angiostatin and locally administered angiostatin is shown in Figure 1. In normal neonates, few intravitreal endothelial nuclei (IEN) were present (0.35 ± 0.07; Fig. 2a). In mice subjected to hyperoxic conditions, pathologic angiogenesis was represented by the penetration of newly formed endothelial nuclei (tufts) into corpus vitreous (21.9 ± 0.77 IEN; Fig. 2b). Functionality (i.e., perfusion) of these newly formed pathologic blood vessels was demonstrated by the presence of red blood cells in the lumen of the tufts (Fig. 2c). Systemic administration of angiostatin completely suppressed oxygen-induced retinal neovascularization (0.54 ± 0.06 IEN) compared with mice subjected to hyperoxic conditions only (P < 0.001). The number of IEN was not different between normal neonates and neonates subjected to hyperoxia subsequently treated with angiostatin (not significant; P = 0.06). A single intravitreal injection of angiostatin (≥60 μg/1.0 μl PBS) at P12 resulted in a 62.9% reduction of
IEN (7.65 ± 0.53) compared with control animals treated by puncture alone (trauma group; IEN count, 20.61 ± 1.06; P < 0.0001) and a 62.5% reduction compared with control animals subjected to intravitreal injection of PBS (IEN, 20.39 ± 0.89; P < 0.0001). Trauma of the vitreous body, either by puncture or by injection of PBS, did not reduce the number of endothelial nuclei when compared with mice treated with systemic PBS (Fig. 1).

**Effect of Angiostatin on the Physiological Development of the Retinal Vasculature**

To determine whether angiostatin had an effect on normal retinal vessel development, neonates were treated with twice daily subcutaneous injections of angiostatin for 14 days (P0–P14) at a dose of 100 mg/kg *d*. Vessel development was analyzed by retinal fluorescein angiograms (tortuosity, vessel dilatation, leakiness, hemorrhages). No qualitative differences were observed between angiograms of mice treated with PBS and those treated with angiostatin (Figs. 3a, 3b). Retinal cryosections stained with hematoxylin revealed no morphologic differences between either group (i.e., no IEN in either group and no cellular changes in the ganglion cell layer).

**Effect of Angiostatin on Growth and Development of Neonatal Mice**

In addition to the analysis of the retinal vasculature, other parameters of development were analyzed. Neonates were treated from P0 until P14 as described. Treatment was well tolerated and did not result in any obvious toxicity. There was no difference in weight gain (Fig. 4a). As a general marker of

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932901/)

**Figure 2.** Comparison of retinas of the normal (a), oxygen-induced retinopathy (b, c), and oxygen-induced retinopathy, angiostatin-treated (d) groups 17 days after birth. Newly formed intravitreal pathologic blood vessels, so-called tufts (b, d, arrowbeads), were in contact with preexisting normal intraretinal blood vessels. Perfusion of new blood vessels (c) is demonstrated by the presence of intraluminal erythrocytes (higher magnification of b). Hematoxylin and eosin; magnification, (a, b, d) ×100; (c) ×400.

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932901/)

**Figure 3.** Effect of angiostatin on the physiological development of the retinal vasculature. Comparison of FITC-dextran–perfused retinas of normal mice and mice supplemented with twice-daily administration of angiostatin. Normal mice (a) show large superficial radial vessels (arrows) from which collateral vessels have developed. In mice treated with angiostatin (b), no differences in development of superficial radial or collateral vessels (arrows) were observed. No differences in structure (tortuosity, vessel dilatation) between both groups were observed, and no hemorrhages were present. Original magnification, ×100.
vascular development, tail length was used. The tail development was identical for the angiostatin- and PBS-treated groups (Fig. 4b). Angiostatin treatment did not affect murine organ development, as judged by weight (Figs. 4c–e).

**DISCUSSION**

Angiostatin is one of the most promising antiangiogenic agents available. In the present study we demonstrate that systemic administration of angiostatin completely prevents retinal neovascularization in a mouse model of oxygen-induced retinopathy, without affecting physiological angiogenesis. The development of the normal retinal vasculature, tail length, and gain in body weight were not different between control and angiostatin-treated animals. In contrast, even a single intraocular injection with angiostatin had a substantial inhibitory effect on retinal neovascularization.

ROP is a debilitating disease for which no effective treatment is available. Because the disease is associated with exposure to highly concentrated oxygen, it has been suggested that reactive oxygen intermediates may play a role in its pathogenesis. We have recently shown that reactive oxygen intermediates (both hydrogen peroxide and superoxide) induce the production of VEGF in retinal pigment epithelial cells, both in vitro and in vivo. Based on randomized clinical trials, it has been advocated that vitamin E, a naturally occurring antioxidant, should be given as a prophylactic agent to a high-risk population of preterm infants.

The rapidly increasing knowledge on angiogenesis and the characterization of a large number of antiangiogenic agents has opened new avenues for the treatment of diseases associated with retinal neovascularization, such as ROP. Currently, more than 20 antiangiogenic agents are tested in clinical trials. However, with the exception of one TNP-470 phase I trial, all these are tested in adults. In contrast with growing children, the majority of endothelial cells in adults are quiescent. Because inhibitors of angiogenesis specifically inhibit proliferating endothelial cells, it could be speculated that antiangiogenic treatment may adversely affect normal vascular development as well. Contradictory to this hypothesis, we demonstrate in this study that systemic administration of angiostatin markedly suppressed excessive retinal neovascularization in newborn mice, without any apparent adverse effect on the development of...
blood vessels or organs in the newborn mice during the first 2 weeks of life.

The mechanism(s) responsible for the antiangiogenic properties of angiostatin are presently unknown. However, some investigators have hypothesized about its mechanisms. Stack et al.29 suggest that tissue plasminogen activator (t-PA), when bound to angiostatin, cannot participate in a ternary complex formation between t-PA, plasminogen, and matrix protein. This results in the inhibition of plasminogen activation and a reduced cellular migration and invasion. Moser et al.30 attribute its antiangiogenic effects to binding to the α- and β-subunits of adenosine triphosphate (ATP)-synthase on the cell surface of endothelial cells, resulting in the downregulation of endothelial cell proliferation and migration. Lucas et al.31 claim that the antiangiogenic activity of angiostatin can be ascribed to its apoptotic effect on endothelial cells. However, no consensus has been reached, so far. Obviously, the mechanism of angiostatin requires further investigation.

Initially, we intended to avoid systemic administration of angiostatin and used intravitreal injections. This approach was reported to be successful in studies in which soluble VEGF receptor chimeric proteins and VEGF antisense oligonucleotides were used.9 Although it was clearly shown that inhibition of VEGF inhibited angiogenesis over control intravitreal injections, no comparison was made with the number of retinal vessels in uninjected eyes. In our study, a single intravitreal injection with angiostatin significantly inhibited angiogenesis, thereby confirming the efficacy of such an approach. Its inhibitory effect on retinal neovascularization is not as high as that obtained with systemic administration of angiostatin: 62.9% and 96.9% inhibition, respectively. The strong inhibition of a single intravitreal injection with angiostatin may be explained by the composition of the vitreous. This may act as a slow-release compartment.

In the current investigation, we did not perform pharmacokinetic studies. This prohibits our drawing conclusions on the optimal route of administration. Control experiments demonstrated that a limited trauma in the vitreous did not contribute to the antiangiogenic effect. Ophthalmologists have used vitrectomies as a treatment modality in patients with ROP and proliferative diabetic retinopathy. This procedure temporarily improves the vascular abnormalities in the retina,32 but long-term effects are limited. The mechanism by which this manipulation of the vitreous inhibits angiogenesis has not been elucidated. However, such a procedure can hardly be called a limited trauma. Therefore, these empiric clinical data are not in contradiction to our control experiments, in which we used a single intravitreal needle puncture.

In summary, the present study suggests that angiostatin may be used safely to treat diseases associated with retinal angiogenesis. That there were no adverse effects on normal development in the newborn mice indicates that angiostatin may be used in cases of ROP. Obviously, further toxicity studies in newborn primates are required to support clinical testing.

Acknowledgments

The authors thank Colinda Aarsman for expert technical assistance.

References


