·Basic Research·

Mechanism of angiostatin induced reduction of vascular leakage in retina and iris of rats with retinopathy of prematurity

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Abstract

• AIM: To study the effect of an intravitreal injection of angiostatin on vascular leakage in the retina and iris of oxygen-induced retinopathy of prematurity (ROP).

• METHODS: Brown Norway rats at postnatal day 7 (P7) were exposed to hyperoxia (750mL/L O_2) for 5 days (P7-12) and then returned to normoxia to induce retinopathy. Angiostatin was reconstituted in sterile Phosphate Buffered Saline (PBS) and diluted to desired different concentrations. Angiostatin solution was injected into the vitreous of the right eye of the ROP rats at P14 and the age-matched normal rats through pars plana using a glass capillary, and the left eye received the same volume of sterile PBS as the control. Vascular permeability was quantified at 1, 2 and 3 days after the injection by measuring albumin leakage from blood vessels into the retina and iris using the Evans blue method and normalized by total protein concentrations. The expression of vascular endothelial growth factor (VEGF) in retina was evaluated using the Western Blot analysis and immunohistochemistry 24 hours following the injection.

• RESULTS: ROP rats showed significant increases of vascular permeability in the retina and iris ($P<0.01$). Angiostatin reduces vascular permeability in a dose-dependent manner in the retina of ROP rats. The reduction showed a time course trend. Angiostatin injection reduced retinal vascular permeability by approximately 1.5 and 2-fold at P15 ($P<0.05$) and P16 ($P<0.01$), respectively. Angiostatin injection significantly reduced VEGF levels in the retina of ROP rats but did not affect retinal VEGF levels in normal rats.

• CONCLUSION: Angiostatin significantly decreases pathological vascular permeability in the retina and iris of ROP rats but not in normal rats. Angiostatin down-regulates VEGF expression in retina of ROP rats. These results suggest that angiostatin may have a therapeutic potential in the treatment of ROP and other diseases with vascular leakage.

• KEYWORDS: angiostatin; angiogenic inhibitor; retinopathy of prematurity; permeability; vascular endothelial growth factor

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INTRODUCTION

Angiostatin is a proteolytic fragment of plasminogen (kringle 1-4). It was identified as a potent angiogenic inhibitor which blocks neovascularization of diabetic retinopathy and suppresses tumor growth and metastases[1]. The mechanism responsible for the anti-angiogenic activity of angiostatin is currently uncertain. However, angiostatin has been found to inhibit the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induced activation of the p42/p44 MAP kinase [2]. Recent study has suggested that decreased angiostatin levels in the vitreous may play a role in the development of proliferative diabetic retinopathy [3]. Moreover, recombinant angiostatin has been shown to block retinal neovascularization in a rat model of oxygen-induced retinopathy of prematurity (ROP)[4]. In this study, we have determined the effect of angiostatin on vascular permeability in oxygen-induced ROP and researched into its possible mechanism.

MATERIALS AND METHODS

Animals Time-pregnant Brown Norway rats were purchased from Harlan (Indianapolis, IN). Care, use, and treatment of all animals in this study were in strict

agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

ROP Model and Intravitreal Injection of Angiostatin ROP was induced as described by Smith method with some modifications [4]. Briefly, rats at postnatal day 7 (P7) were exposed to hyperoxia (750mL/L O_2) for 5 days (P7-12) and then returned to normoxia (room air) to induce retinopathy. Angiostatin was purchased from Angiogenesis Research Industries, Inc (Chicago, IL) and reconstituted in sterile PBS and diluted to desired concentrations. Angiostatin solution was injected into the vitreous of the right eye(3μL each eye) of the anesthetized rats at P14 through the pars plana using a glass capillary, and the left eye received the same volume of sterile PBS as the control. After the injection, the animals were kept in normoxia until they were analyzed.

Measurement of Vascular Permeability Vascular permeability was quantified by measuring albumin leakage from blood vessels into the retina and iris using the Evans blue method following a documented protocol [5] with minor modifications. After anesthetized, the rats were injected Evans blue (30mg/kg) through the femoral vein. The rats were kept on a warm pad for 2 hours to ensure the complete circulation of the Evans blue-albumin complex. Then the rats were perfused via the left ventricle with pre-warmed 4g/L paraformaldehyde in citrate buffer (pH 4.2) for 2 minutes under the physiological pressure to clear the dye from the vessel. Immediately after perfusion, the eyes were enucleated and the retina and iris were carefully dissected under an operating microscope. Evans blue dye was extracted by incubating each sample in 150μL of formamide (Sigma) for 18 hours at 70℃. The extract was centrifuged at 70 000r/min (Rotor type: TLA1003,TL;Beckman) for 20 minutes at 4℃. Absorbance was measured using 100μL of the supernatant at 620nm. The concentration of Evans blue in the extracts was calculated from a standard curve of Evans blue in formamide and normalized by the total protein concentration in the tissue. Results were expressed as micrograms of Evans blue per milligram of total protein in the tissues.

Western Blot Analysis and Immunohistochemistry of VEGF VEGF Western blot analysis was performed as described previously [6]. Immunohistochemistry was carried out following a documented protocol [7]. Briefly, retinal sections were incubated with 1∶100 dilution of the anti-VEGF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4℃. After extensive wash, the sections were incubated with biotin labeled monoclonal anti-rabbit antibody for 60 minutes at 37℃, and then developed using the ABC Complex (Vector Laboratories, Burligame, USA), with 3.3' diamino-benzidine (0.25g/L in 0.05mmol/L Tris, pH 7.4, containing 0.3mL/L hydrogen peroxide) as a chromogen.

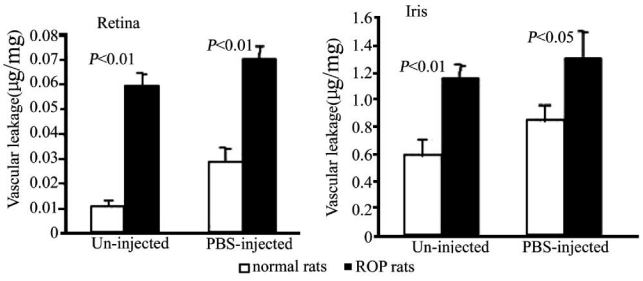

Figure 1 Vascular permeability in oxygen-induced ROP rats

Statistical Analysis Statistical analysis employed the Student's *t*-test. The paired *t*-test was used for comparison of the angiostatin-injected eye with the PBS-injected contralateral controls from the same animal, while the unpaired test was used for inter-animal comparison.

RESULTS

Oxygeninduced ROP Rats Showed Significant Increases of Vascular Permeability in Retina and Iris Vascular permeability was measured in the retina and iris of ROP rats at age P16 (4 days after the rats returned to normoxia) and compared with those in the age-matched normal rats. ROP rats showed significant increases of vascular permeability in the retina and iris (P<0.01, Figure 1).

To evaluate the influence of intravitreal injection on vascular permeability, ROP rats and age-matched normal rats received an intravitreal injection of 3μL of sterile PBS into the right eye at age P14. The retinal vascular permeability was measured at P16, 2 days following the injection. Intravitreal injection of PBS significantly increased permeability in the retina and iris over that in the un-injected contralateral eye (P<0.05, Figure 1), possibly due to injury responses. In both tissues, ROP rats with PBS injection showed significantly higher vascular permeability than those in the age-matched normal rats with the same injection, suggesting that intravitreal injection does not affect the comparison between the ROP rats and their normal controls (Figure 1).

Angiostatin Reduces Vascular Permeability in a Dose-dependent Manner in Retina of ROP Rats To determine the effect of angiostatin on vascular permeability, ROP rats (P14) received an intravitreal injection of 3μL of angiostatin with different concentrations into the right eyes, to reach doses of 1.88, 3.75 and 7.5μg each eye, and the

same volume of PBS into the left eyes for controls. Vascular permeability was measured at P16 using the Evans blue method. In the eyes injected with angiostatin, vascular permeability was reduced in an angiostatin dose-dependent manner (Figure 2). At dose of 3.75 and 7.5μg each eye, angiostatin decreased the vascular permeability, respectively (P<0.05, P<0.01, respectively), while the low dose of angiostatin (1.88μg each eye) showed no significant reduction in permeability (Figure 2). No significant reduction of vascular permeability was detected in the iris of ROP rats after the injection of angiostatin at all the doses used.

Time Course of the Angiostatin Induced Reduction of Vascular Permeability in Retina of ROP Rats At P14, the right eye of ROP rats received an intravitreal injection of angiostatin (7.5μg each eye) and left eye received PBS as the control. Vascular permeability was measured at 1, 2 and 3 days after the injection. Angiostatin injection reduced retinal vascular permeability by approximately 1.5 and 2-fold (P< 0.05, P<0.01, respectively) at P15 and P16, respectively (Figure 3). At P17, 3 days after the injection, vascular permeability returned to the level of PBS-injected contralateral control.

Angiostatin Down-regulates VEGF Expression in Retina of ROP Rats But not in Normal Rats As over-expression of VEGF is known as a major cause of vascular hyper-permeability, we have determined the effect of angiostatin on VEGF expression in ROP rats. Angiostatin (7.5μg each eye) was injected into the vitreous of the right eyes and PBS into the left eyes of ROP rats at age P14. Twenty-four hours after the injection, the retina was dissected and pooled for Western blot analysis using an antibody specific for VEGF. Angiostatin injection significantly reduced VEGF levels in the retinas of ROP but did not affect retinal VEGF levels in normal rats (Figure 4), correlating with its effect on vascular permeability.

Immunohistochemistry using the anti-VEGF antibody demonstrated that angiostatin decreased the intensity of VEGF signals in the retina, 24 hours following the injection, when compared to the PBS-injected eye. The major decrease in VEGF signal occurred in the inner retina after a single dose intravitreal injection of angiostatin (Figure 5).

DISCUSSION

Angiostatin is a potent angiogenic inhibitor. Its effect has been determined on retinal vascular leakage which is associated with diabetic macular edema, tumor growth and inflammation[4]. Increased vascular permeability in the retina of Streptozotocin-induced diabetic rats has been reported

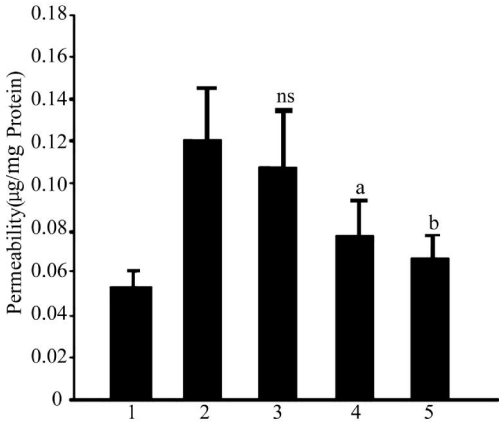

Figure 2 Angiostatin dose-dependent reduction of vascular permeability in retina of ROP rats 1:normal rats received an injection of PBS; 2:ROP rats with a PBS injection; 3, 4 and 5:ROP rats with an injection of 1.88, 3.75 and 7.5μg/eye of angiostatin, respectively. Vascular permeability in the retina was measured using the Evans blue method and normalized by total protein concentrations. Permeability was expressed as μg of Evans blue per mg of protein (mean±SD, n=4) [a] P<0.05, [b] P <0.01 *vs* Group 2

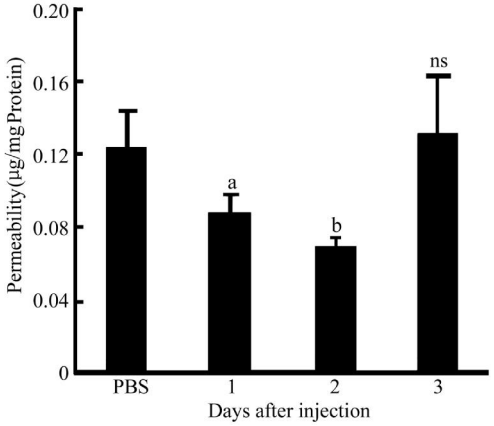

Figure 3 Time course of the angiostatin-induced reduction in vascular permeability The permeability was normalized by the total retinal protein concentrations (mean±SD, n=4) [a] P<0.05, [b] P< 0.01 *vs* PBS

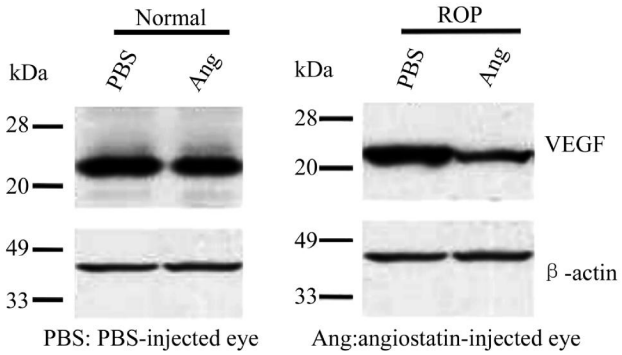

Figure 4 Angiostatin-mediated down-regulation of VEGF expression in retina of ROP rats Retinal VEGF levels were determined by Western blot analysis using an anti-VEGF antibody 1 day after the injection. The same membranes were striped and re-blotted with the anti-β-actin antibody

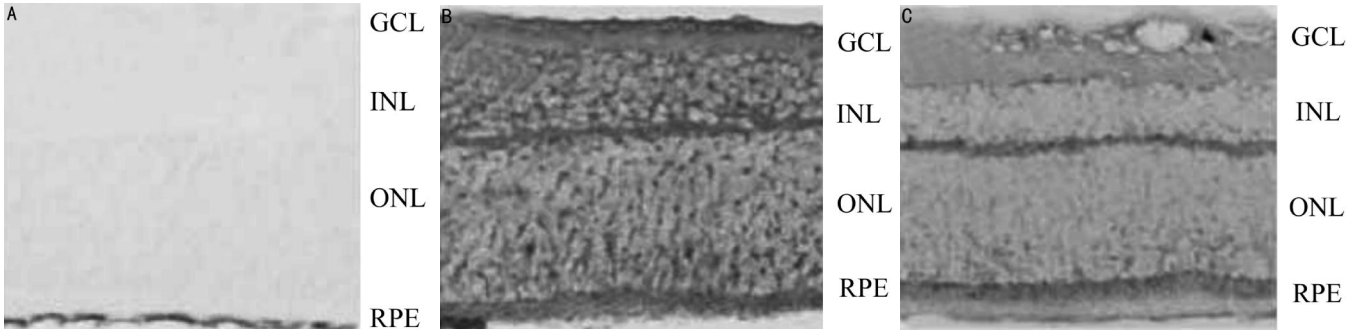

Figure 5 Immunohistochemistry of VEGF in the retina after angiostatin injection A: negative control retina from the ROP rat with PBS injection in the absence of the anti-VEGF antibody; B: retina from the ROP rat after PBS injection; C: retina from ROP rat after angiostatin injection. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. The eye was enucleated at P15 and retinal sections labeled with an anti-VEGF antibody. The signal was visualized using the ABC method. Note: VEGF signal is in brown color

previously [1]. In this present study, intravitreal injection of angiostatin reduced vascular permeability in retina of ROP rats in a dose-dependent manner. Previous studies have shown that angiostatin inhibits retinal neovascularization in the oxygen-induced retinopathy model [8]. This effect occurred at 1 and 2 days following the angiostatin intravitreal injection. This anti-angiogenic effect requires higher dose and needs several days to become detectable, however, the angiostatin-induced reduction of permeability can be detected as early as 1 day after the injection (Figure 3). Analysis of retinal vasculature showed that angiostatin injection (7.5μg each eye) did not result in any detectable decrease of retinal neovascularization 2 days after the injection when the effect on the reduction of vascular permeability reached a peak. Western blot analysis and immunohistochemistry both showed that angiostatin down-regulated retinal VEGF expression in the ROP rats but not in age-matched normal controls. These results suggest that angiostatin-induced reduction in vascular permeability is not through its inhibition of neovascularization. This reduction may be ascribed to its down-regulation of VEGF expression.

Oxygen-induced retinopathy is a widely used model of retinal neovascularization. However, vascular permeability is studied rarely in this model. The present study showed that oxygen-induced retinopathy rats also have a transient yet significant increase of vascular permeability in the retina and iris, suggesting the oxygen-induced retinopathy rat is also a model for vascular permeability studies. PBS injection significantly increased vascular permeability in the retina of normal and oxygen-induced ROP rats, compared to the eyes without injection. These increases may be due to responses to trauma from the intravitreal injection. Therefore, in the present study, all the control eyes for angiostatin received an injection of the same volume of PBS at the same time as the angiostatin injection, to exclude the interference from the trauma response.

Recent studies indicated that one of the proposed mechanisms for the pathogenesis of ROP includes overproduction of the angiogenic growth factors including VEGF [9]. VEGF is also referred as vascular permeability factor (VPF) based on its potent ability to increase vascular permeability. It has been identified as a major causative factor of retinal vascular hyper-permeability [10]. Our results are consistent with these previous findings. Another evidence has shown that angiostatin binds to integrins, predominantly αvβ3, on the surface of endothelial cells, but does not induce stress fiber formation, implying that the anti-angiogenic activity of angiostatin may be through interfering with the αvβ3-mediated signaling in endothelial cells [11]. These findings reveal therapeutic potential of angiostatin in the treatment of retinal neovascularization as well as in the treatment of cancer. This effect is mediated, at least in part, via blockage of VEGF over-expression under hypoxia.

Angiostatin blocks the over-expression of VEGF in the hypoxic retina as found in ROP but does not decrease the VEGF level in the normal retina. Correlating with this observation, angiostatin only reduces vascular permeability in ROP retina but not in the normal retina. These results suggest that the blockade of VEGF expression in the hypoxic retina is responsible for the angiostatin-induced reduction of vascular leakage in ROP rats. These studies reveal that angiostatin may have a therapeutic potential in

the treatment of ROP and other diseases with vascular leakage.

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