The effect of angiostatin on vascular leakage and VEGF expression in rat retina

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Abstract Angiostatin is a potent angiogenic inhibitor. The present study identified a new activity of angiostatin: reducing vascular leakage, which is associated with diabetic macular edema, tumor growth and inflammation. An intravitreal injection of angiostatin significantly reduced retinal vascular permeability in rats with oxygen-induced retinopathy and in those with streptozotocin-induced diabetes, but not in normal rats. Consistent with its effect on permeability, angiostatin downregulated vascular endothelial growth factor (VEGF) expression in the retina in both the rat models but not in normal controls. These results suggest that the effect of angiostatin on vascular leakage is mediated, at least in part, through blockade of VEGF overexpression.

Key words: Angiostatin; Angiogenic inhibitor; Blood-retinal barrier; Diabetic retinopathy; Permeability; Plasminogen; Macular edema; Vascular endothelial growth factor

1. Introduction

Angiostatin is a proteolytic fragment (kringle 1–4) of plasminogen [1]. It was identified as a potent angiogenic inhibitor which blocks neovascularization and suppresses tumor growth and metastases [1]. Angiostatin specifically inhibits proliferation and induces apoptosis in vascular endothelial cells [2]. Recent evidence 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 (OIR) [4]. Delivery of a recombinant virus expressing angiostatin has been found to suppress laser-induced choroidal neovascularization [5]. These findings reveal therapeutic potential of angiostatin in the treatment of retinal neovascularization as well as in the treatment of cancer.

The blood-retinal barrier (BRB) breakdown, increased vascular permeability or vascular leakage is an early complication of diabetes and a major cause of diabetic macular edema [6,7]. At early stages of diabetic retinopathy, it is found that the increase of retinal vascular permeability precedes the appearance of clinical retinopathy [6,7]. As there is no satisfactory, non-invasive therapy, diabetic macular edema is a major cause of vision loss in diabetic patients [8]. Although the pathogenic mechanism underlying the breakdown of the BRB and the increase of retinal vascular permeability is uncertain, the overproduction of vascular endothelial growth factor (VEGF) in the retina is believed to play a key role in the development of vascular hyperpermeability in diabetes [9,10].

In this study, we have determined the effect of angiostatin on vascular permeability in OIR and streptozotocin (STZ)-induced diabetic models and studied its possible mechanism.

2. Materials and methods

2.1. Animals

Brown Norway rats were purchased from Harlan (Indianapolis, IN, USA). 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.

2.2. Rat models of OIR and diabetes

OIR was induced by exposing newborn rats to 75% O_2 as described by Smith et al. [11] with some modifications [12]. Diabetes was induced in adult rats (8 weeks of age) by an intravenous injection of STZ (50 mg/kg) after overnight fasting. Rats with glucose levels higher than 350 mg/dl were considered diabetic.

2.3. Intravitreal injection of angiostatin

Angiostatin (Angiogenesis Research Industries, Inc., Chicago, IL, USA) was reconstituted in sterile phosphate-buffered saline (PBS) and injected into the vitreous of the right eye (3 μl/eye) of the anesthetized rats through the pars plana using a glass capillary. The left eye received the same volume of sterile PBS as the control.

2.4. 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 [13] with minor modifications [14].

2.5. Western blot analysis and immunohistochemistry of VEGF

VEGF Western blot analysis was performed using an antibody specific for VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as described previously [15]. Immunohistochemistry was carried out following a documented protocol [16].

2.6. Statistical analysis

Statistical analysis employed 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 interanimal comparison.

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Abbreviations: BRB, blood-retinal barrier; OIR, oxygen-induced retinopathy; STZ, streptozotocin; VEGF, vascular endothelial growth factor

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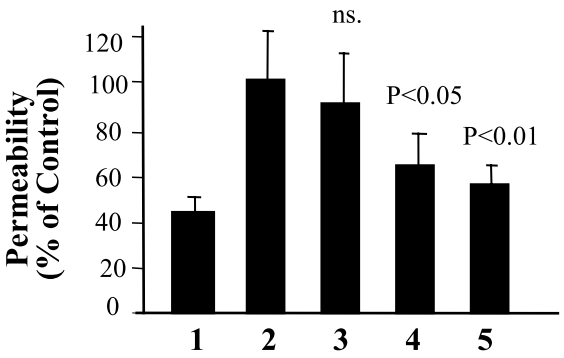

Fig. 1. Angiostatin dose-dependent reduction of vascular permeability in OIR rat retina. Rats received an intravitreal injection of angiostatin in the right eye and PBS in the left eye at age P14. Retinal vascular permeability was measured at P16 using the Evans blue method and normalized by total protein concentrations in the retina. Permeability was expressed as percentage of the contralateral control (mean ± S.D., $n = 4$). 1, age-matched normal rats with a PBS injection; 2, OIR rats with a PBS injection; 3, 4 and 5, OIR rats with an injection of 1.875, 3.75 and 7.5 μg/eye of angiostatin, respectively.

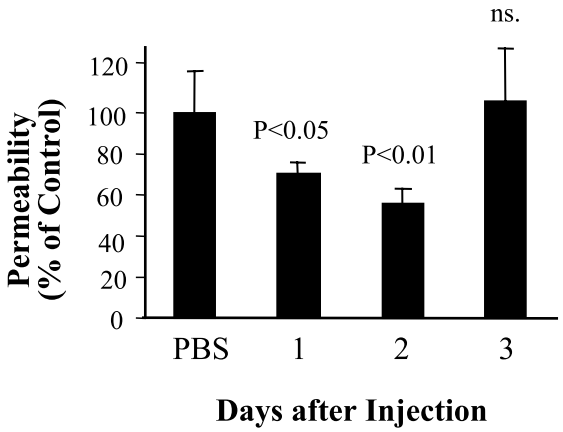

Fig. 2. Time course of the angiostatin-induced reduction of vascular permeability. At age P14, the right eye of the OIR rats received an intravitreal injection of angiostatin (7.5 μg/eye) and the left eye received PBS as the control. Vascular permeability was measured at 1, 2 and 3 days after the injection. Vascular permeability was normalized by the total protein concentrations in the retina and expressed as percentage of the contralateral control (mean ± S.D., $n = 4$). ns., not statistically significant.

3. Results

3.1. *Angiostatin reduces vascular permeability in the retina of OIR rats*

Previous studies showed that OIR rats have a transient increase of retinal vascular permeability with the peak at age P16 [17]. To determine the effect of angiostatin on vascular permeability, OIR rats (P14) received an intravitreal injection of 3 μl of angiostatin with different concentrations into the right eyes, to reach doses of 1.875, 3.75 and 7.5 μg/eye, and the same volume of PBS into the left eyes for controls.

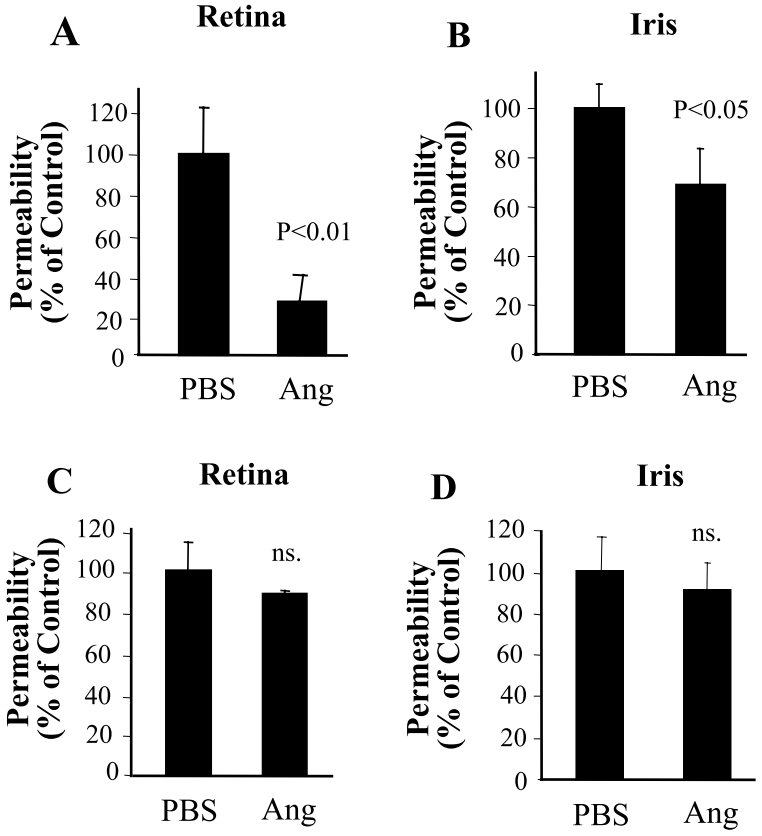

Fig. 3. Angiostatin-induced reduction of vascular permeability in STZ-diabetic rats. Angiostatin was injected into the vitreous of the right eye (7.5 μg/eye) and PBS into the left eye of the STZ-diabetic rats (A, B) and normal adult rats (C, D). Vascular permeability in the retina and iris was measured 2 days after the injection, normalized by total protein concentrations in the tissues and expressed as percentage of the contralateral control (mean ± S.D., $n = 4$). A, B: Angiostatin reduced vascular permeability in the retina and iris of STZ-diabetic rats. C, D: Angiostatin does not affect vascular permeability in normal rats. PBS, PBS-injected eye; Ang, angiostatin-injected eye.

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 (Fig. 1). At doses of 3.75 and 7.5 μg/eye, angiostatin decreased the permeability to approximately 70 and 50%, respectively, of the contralateral control with PBS injection ($P < 0.05$ and $P < 0.01$, respectively, $n = 4$), while the low dose of angiostatin (1.875 μg/eye) showed no significant reduction in permeability ($P > 0.05$, $n = 4$) (Fig. 1). No significant reduction of vascular permeability was detected in the iris of OIR rats treated with angiostatin of all the doses used (data not shown).

To determine the effect of angiostatin on retinal neovascularization, angiostatin was injected into the vitreous of the right eye (7.5 μg/eye) of OIR rats at P12 and PBS into the left eye. The retinal neovascularization was examined at P18 using both the fluorescein angiography on retinal whole mounts and by quantifying pre-retinal vascular cells as described previously [11]. Both results showed that angiostatin, at the dose used, had no detectable effect on retinal neovascularization at this early time point (data not shown), suggesting that the angiostatin-induced reduction of vascular permeability is not a consequence of inhibition of retinal neovascularization.

3.2. *Time course of the angiostatin-induced reduction of vascular permeability in OIR rat retina*

OIR rats received an intravitreal injection of 7.5 μg/eye of angiostatin at P14 in the right eye and PBS in the left eye. Vascular permeability was measured at P15, P16 and P17. Angiostatin injection reduced retinal vascular permeability to approximately 70 and 50% of the contralateral control ($P < 0.05$ and $P < 0.01$, respectively, $n = 4$) at P15 and P16, respectively (Fig. 2). At P17, 3 days after the injection, vascular permeability returned to the level of the PBS-injected contralateral control. No significant reduction of vascular permeability was observed in the iris at these time points (data not shown).

3.3. *Effect of angiostatin on retinal vascular leakage in diabetic rats*

The STZ-diabetic rats received an intravitreal injection of angiostatin (7.5 μg/eye) in the right eye and PBS in the left eye at 2 weeks following the onset of diabetes. Vascular permeability in the retina and iris was measured 2 days after the injection. Angiostatin significantly decreased vascular permeability to 30% of the control with PBS injection ($P < 0.01$, $n = 4$) in the retina and to 70% of the control in the iris ($P < 0.05$, $n = 4$) (Fig. 3A and B).

In contrast, intravitreal injection of the same dose of angiostatin did not result in any significant reduction of vascular permeability in the retina and iris of normal rats, when compared with the contralateral eye with PBS injection ($P > 0.05$, $n = 4$) (Fig. 3C and D).

3.4. *Angiostatin downregulates VEGF expression in the retina of the STZ-diabetic and OIR rats but not in normal rats*

As overexpression of VEGF is known as a major cause of vascular hyperpermeability, we have determined the effect of angiostatin on VEGF expression in OIR, STZ-diabetic and normal rats. Angiostatin was injected into the vitreous of the right eyes (7.5 μg/eye) and PBS into the left eyes of OIR rats at age P14, or into STZ-diabetic rats at 2 weeks after the onset of diabetes and age-matched normal adult rats. 1 day

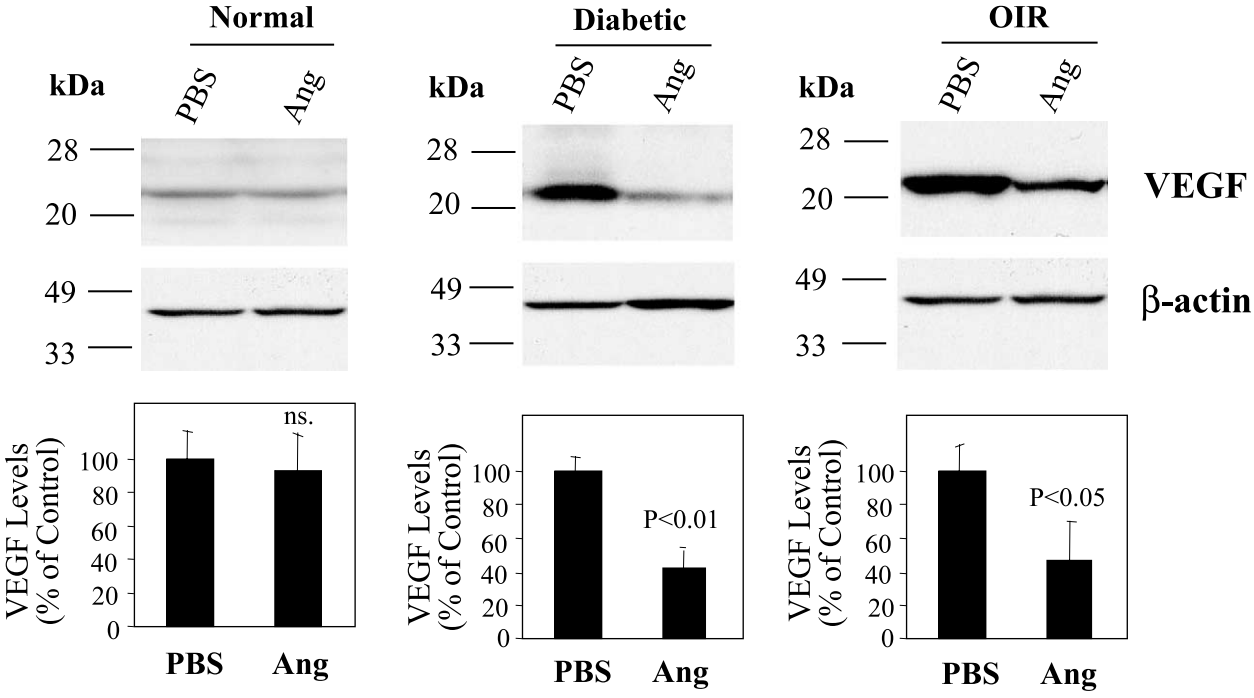

Fig. 4. Angiostatin-mediated downregulation of VEGF expression in the retinas. OIR rats (P14), STZ-diabetic rats (2 weeks of diabetes) and normal adult rats received an intravitreal injection of angiostatin (7.5 μg/eye) in the right eye and PBS in the left eye. Retinal VEGF levels were determined by 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 (upper panels). Each blot is a representative of results from three rats in each group. VEGF levels were semiquantified by densitometry, normalized by β-actin levels and expressed as percentage of control (lower panels). PBS, PBS-injected eye; Ang, angiostatin-injected eye; ns., not statistically significant.

following the injection, VEGF levels in the retina were measured by Western blot analysis using an antibody specific for VEGF. Angiostatin decreased VEGF levels by approximately 2.5- and 2-fold in the retinas of the OIR and STZ-diabetic rats, respectively, but not in normal rats (Fig. 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 of the OIR rats, 1 day following the injection, when compared to the PBS-injected contralateral eye (Fig. 5).

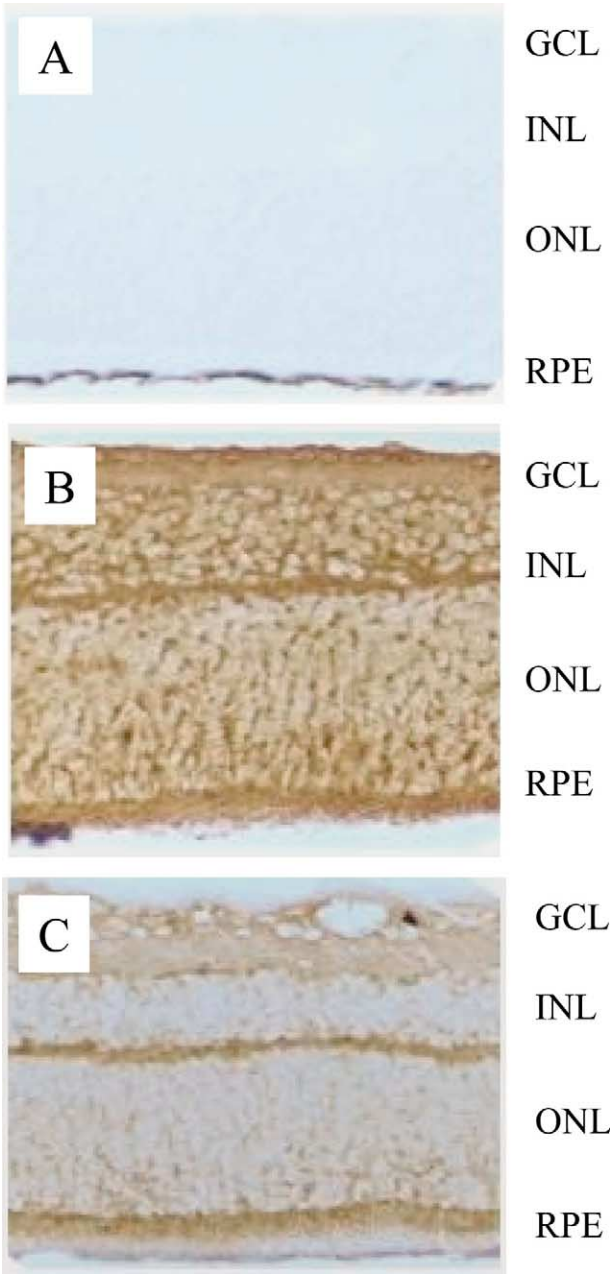

Fig. 5. Immunohistochemistry of VEGF. Rats with OIR received an intravitreal injection of 7.5 μg/eye angiostatin in the right eye and PBS in the left eye at age P14. The eye was enucleated at P15 and retinal sections labeled with an anti-VEGF antibody using the ABC method. VEGF signal is in brown color. A: Retina from the OIR rat with PBS injection stained in the absence of the anti-VEGF antibody for negative control. B: Retina from the OIR rat after PBS injection. C: Retina from the OIR rat after angiostatin injection. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

4. Discussion

Angiostatin has been shown to inhibit endothelial cells [1]. In the present study, we identified a new activity of angiostatin, i.e. reducing pathological vascular permeability in the retinas of both OIR and STZ-diabetic rat models but not in normal rats. Further, our results for the first time showed that angiostatin downregulates VEGF expression in the retinas of both the OIR and STZ diabetes models, but not in normal rat retina, correlating with its effect on vascular permeability. These findings suggest that the angiostatin-induced reduction in vascular permeability may be ascribed, at least in part, to its downregulation of VEGF expression.

Recently, we have shown that the BRB is compromised in OIR rats [17]. Therefore, we tested the effect of angiostatin on vascular permeability using both the OIR as well as STZ diabetes models. It has been shown that angiostatin-induced inhibition of neovascularization in the OIR model occurs relative late (P21) [4]. In contrast, the angiostatin-induced reduction of retinal vascular permeability in the same model can be detected as early as 1 day after the injection (Fig. 2). Analysis of retinal vasculature showed that angiostatin injection (7.5 μg/eye) did not result in any significant decrease of retinal neovascularization in the OIR model. To further confirm these findings, we also determined the effect of angiostatin on vascular permeability in STZ-induced diabetic rats which lack retinal neovascularization while showing a significant increase in vascular permeability [18]. Angiostatin also significantly reduced vascular permeability in this STZ-diabetic animal model. Taken together, these results suggest that angiostatin-induced reduction in vascular permeability is not through its inhibition of neovascularization.

Although retinal edema in diabetes is a complex disorder, several lines of evidence suggest that VEGF plays a key role in vascular leakage in diabetic retina [19–22]. In OIR and STZ diabetes models, retinal vascular leakage may be due to different structural changes in retinal capillaries. However, both the models have increased VEGF levels in the retina, which is believed to play a key role in the development of vascular abnormalities in the retina [10,22]. VEGF is also known as a vasopermeability factor [22–24] and is 50 000 times more potent than histamine in increasing dermal microvascular permeability [25]. Overexpression of VEGF is associated with vascular leakage in diabetes [26]. Angiostatin blocks the overexpression of VEGF in the hypoxic retina as found in OIR and STZ diabetes but does not decrease the VEGF level in the normal retina (Fig. 4). Correlating with this observation, angiostatin only reduces retinal vascular permeability in OIR and STZ-diabetic rats but not in normal rats.

Another evidence supporting that the angiostatin-induced reduction in vascular leakage is via blockade of VEGF production is that angiostatin did not reduce vascular hyperpermeability induced by an intravitreal injection of exogenous VEGF (data not shown). Taken together, these results suggest that the blockade of VEGF expression in hypoxic retina is responsible, at least in part, for the angiostatin-induced reduction of vascular leakage in OIR and STZ-diabetic rats.

It is unclear how angiostatin blocks VEGF expression at

the present time. However, it has been shown that angiostatin binds to integrins [27] and inhibits the activation of the p42/p44 mitogen-activated protein (MAP) kinase pathway [28]. As evidence has shown that the p42/p44 MAP kinase pathway plays a role in the regulation of VEGF expression and in angiogenesis control [29,30], the angiostatin-induced blockade of VEGF expression may be through the inhibition of MAP kinase pathway under hypoxia.

The BRB breakdown or vascular leakage is a major cause of macular edema in diabetic retinopathy and other ocular diseases such as uvietis [31–33]. Current therapies for diabetic macular edema are not satisfactory, and macular edema is still a major cause of vision loss in diabetic patients. The present study demonstrates that angiostatin can reduce vascular leakage in both diabetic and OIR rat models. Angiostatin down-regulates VEGF expression and thus blocks the major cause of vascular leakage in diabetic retinas. Therefore, the angiostatin-induced reduction of vascular leakage may have therapeutic potential in the treatment of diabetic macular edema, cystoid macular edema and other diseases with vascular leakage such as uvietis and the wet form of macular degeneration.

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References

[1] O'Reilly, M.S. et al. (1994) Cell 79, 315–328.
[2] Claesson-Welsh, L., Welsh, M., Ito, N., Anand-Apte, B., Soker, S., Zetter, B., O'Reilly, M. and Folkman, J. (1998) Proc. Natl. Acad. Sci. USA 95, 5579–5583.
[3] Spranger, J., Hammes, H.P., Preissner, K.T., Schatz, H. and Pfeiffer, A.F. (2000) Diabetologia 43, 1404–1407.
[4] Meneses, P.I., Hajjar, K.A., Berns, K.I. and Duvoisin, R.M. (2001) Gene Ther. 8, 646–648.
[5] Lai, C.C. et al. (2001) Invest. Ophthalmol. Vis. Sci. 42, 2401–2407.
[6] Cunha-Vaz, J.G., Gray, J.R., Zeimer, R.C., Mota, M.C., Ishimoto, B.M. and Leite, E. (1985) Diabetes 34, 53–59.
[7] Yoshida, A., Ishiko, S., Kojima, M. and Ogasawara, H. (1993) Br. J. Ophthalmol. 77, 158–161.
[8] Moss, S.E., Klein, R. and Klein, B.E. (1998) Ophthalmology 105, 998–1003.
[9] Murata, T., Nakagawa, K., Khalil, A., Ishibashi, T., Inomata, H. and Sueishi, K. (1996) Lab. Invest. 74, 819–825.
[10] Hammes, H.P., Lin, J., Bretzel, R.G., Brownlee, M. and Breier, G. (1998) Diabetes 47, 401–406.
[11] Smith, L.E., Wesolowski, E., McLellan, A., Kostyk, S.K., D'Amato, R., Sullivan, R. and D'Amore, P.A. (1994) Invest. Ophthalmol. Vis. Sci. 35, 101–111.
[12] Zhang, D., Kaufman, P.L., Gao, G., Saunders, R.A. and Ma, J.X. (2001) Diabetologia 44, 757–765.
[13] Xu, Q., Qaum, T. and Adamis, A.P. (2001) Invest. Ophthalmol. Vis. Sci. 42, 789–794.
[14] Gao, G., Shao, C., Zhang, S.X., Dudley, A., Fant, J. and Ma, J.-x. (2003) Diabetologia 46, 689–698.
[15] Csaky, K.G., Baffi, J., Byrnes, G. and Cousins, S.W. (2001) Invest. Ophthalmol. Vis. Sci. 42, s227.
[16] Rohrer, B. and Stell, W.K. (1995) Brain Res. 695, 110–116.
[17] Zhang, S.X., Sima, J., Shao, C., Fant, J., Chen, Y., Rohrer, B., Gao, G. and Ma, J. (2004) Diabetologia 47, 124–131.
[18] Antonetti, D.A., Barber, A.J., Khin, S., Lieth, E., Tarbell, J.M. and Gardner, T.W. (1998) Diabetes 47, 1953–1959.
[19] Adamis, A.P., Miller, J.W., Bernal, M.T., D'Amico, D.J., Folkman, J., Yeo, T.K. and Yeo, K.T. (1994) Am. J. Ophthalmol. 118, 445–450.
[20] Aiello, L.P. et al. (1994) N. Engl. J. Med. 331, 1480–1487.
[21] Pe'er, J., Shweiki, D., Itin, A., Hemo, I., Gnessin, H. and Keshet, E. (1995) Lab. Invest. 72, 638–645.
[22] Pierce, E.A., Avery, R.L., Foley, E.D., Aiello, L.P. and Smith, L.E. (1995) Proc. Natl. Acad. Sci. USA 92, 905–909.
[23] Battegay, E.J. (1995) J. Mol. Med. 73, 333–346.
[24] Dvorak, H.F., Brown, L.F., Detmar, M. and Dvorak, A.M. (1995) Am. J. Pathol. 146, 1029–1039.
[25] Senger, D.R., Connolly, D.T., Van de Water, L., Feder, J. and Dvorak, H.F. (1990) Cancer Res. 50, 1774–1778.
[26] Aiello, L.P. and Wong, J.S. (2000) Kidney Intl. Suppl. 77, S113–S119.
[27] Tarui, T., Miles, L.A. and Takada, Y. (2001) J. Biol. Chem. 276, 39562–39568.
[28] Redlitz, A., Daum, G. and Sage, E.H. (1999) J. Vasc. Res. 36, 28–34.
[29] Pages, G., Milanini, J., Richard, D.E., Berra, E., Gothie, E., Vinals, F. and Pouyssegur, J. (2000) Ann. N. Y. Acad. Sci. 902, 187–200.
[30] Milanini, J., Vinals, F., Pouyssegur, J. and Pages, G. (1998) J. Biol. Chem. 273, 18165–18172.
[31] Ciulla, T.A., Danis, R.P. and Harris, A. (1998) Surv. Ophthalmol. 43, 134–146.
[32] Bresnick, G.H. (1986) Ophthalmology 93, 989–997.
[33] Lopes de Faria, J.M., Jalkh, A.E., Trempe, C.L. and McMeel, J.W. (1999) Acta Ophthalmol. Scand. 77, 170–175.