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Unbalanced expression of VEGF and PEDF in ischemia-induced retinal neovascularization

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Abstract Retinal levels of vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), an angiogenic inhibitor, were measured and correlated with the ischemia-induced retinal neovascularization in rats. The retinas with neovascularization showed a 5-fold increase in VEGF while 2-fold decrease in PEDF, compared to the age-matched controls, resulting in an increased VEGF/PEDF ratio. The time course of the VEGF/PEDF ratio change correlated with the progression of retinal neovascularization. Changes in the VEGF and PEDF mRNAs preceded their protein level changes. These results suggest that an unbalance between angiogenic stimulators and inhibitors may contribute to retinal neovascularization.

Key words: Angiogenesis; Angiogenic inhibitor; Diabetic retinopathy; Growth factor; Pigment epithelium-derived factor; Retinal neovascularization

1. Introduction

Retinal neovascularization, abnormal formation of new vessels from preexisting capillaries in the retina, is a common complication of many ocular diseases, such as advanced diabetic retinopathy, some forms of age-related macular degeneration and retinopathy of prematurity (ROP) [1–3]. Neovascularization can lead to fibrosis and disruption of delicate tissues required for vision and hemorrhage. It is a major cause of blindness in industrialized countries and affects millions of people varying in age from infants to the elderly [2,4].

The underlying mechanism of the retinal neovascularization is not well understood. In normal ocular tissues, angiogenic homeostasis is controlled by the balance between angiogenic stimulators, such as vascular endothelial growth factor (VEGF), and angiogenic inhibitors, such as pigment epithelium-derived factor (PEDF) [2,5,6]. The endogenous angiogenic inhibitors are believed to be essential for maintaining the homeostasis of angiogenesis in the retina [7]. In previous studies, the expression and role of angiogenic stimulators in retinal neovascularization have been well studied but the role of decreased angiogenic inhibitors in pathological retinal neovascularization has not been revealed.

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Abbreviations: PEDF, pigment epithelium-derived factor; ROP, retinopathy of prematurity; VEGF, vascular endothelial growth factor

PEDF was initially identified as a potential neurotrophic factor [8]. It shares high sequence homology with other serine proteinase inhibitors (serpin) and behaves like a non-inhibitory serpin [9,10]. It is expressed in multiple tissues and is present in the interphotoreceptor matrix at a high concentration (250 nM) [11,12]. PEDF is also present in the vitreous and aqueous humors [12,13]. Recently, PEDF was shown to have potent anti-angiogenic activity as it specifically inhibited the migration of endothelial cells, an essential step in angiogenesis [6]. It was shown that PEDF contributes to most of the anti-angiogenic activity in the vitreous. Purified PEDF displayed anti-angiogenesis activity in the cornea [6]. The expression of PEDF in the retina was up-regulated by hyperoxia in the retina of a mouse model. In a cultured retinoblastoma cell line, PEDF was down-regulated by hypoxia [6]. The regulation of the PEDF expression by oxygen seemed to occur at the protein level [6]. These findings suggest that PEDF may be a key coordinator of retinal neuronal and vascular functions and may have great therapeutic potential [14]. However, the involvement of altered PEDF expression in pathological retinal neovascularization has not been demonstrated.

In the present study, we have determined the PEDF expression in an ischemia-induced retinal neovascularization model and correlated the VEGF/PEDF ratio with retinal neovascularization.

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, as well as the guidelines set forth in the Care and Use of Laboratory Animals by the Medical University of South Carolina.

2.2. Oxygen-induced retinopathy

Retinal neovascularization was induced as described by Smith et al. [15] with minor modifications. Briefly, newborn pigmented Brown Norway rats were randomly assigned to the experimental and control groups. At postnatal day 7 (P7), the rats in experimental group were exposed to hyperoxia (75% O_2) for 5 days (P7–12) and then returned to normoxia (room air) to induce retinal neovascularization. Control rats were kept at constant normoxia. At each of time points of P12, P14, P16, P18, P20, P22 and P26, the retinas of three rats were dissected and pooled for Western blot analysis, six rats for RNA preparation and Northern blot analysis, two for eye fixation and sectioning and two for fluorescein retinal angiography.

2.3. Retinal angiography with high molecular weight fluorescein

Animals were anesthetized and perfused with fluorescein via intraventricle injection of 50 mg/ml of 2×10^6 molecular weight fluorescein

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isothiocyanate-dextran (Sigma) as described by Smith et al. [15]. The animals were immediately killed. The eyes were enucleated and fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min. The retina was then isolated from the eyecup and fixed with 4% paraformaldehyde for 3 h. Several incisions were made to the retina. The retina was flat-mounted on a gelatin-coated slide. The vasculature was then examined under a fluorescent microscope (Axioplan2 Imaging, Zeiss). To confirm pathological retinal neovascularization, eyes were fixed with 10% formaldehyde, sectioned and then stained with hematoxylin and eosin. The preretinal vascular cells were examined under a light microscope.

2.4. Generation and purification of PEDF antibody

A polyclonal anti-PEDF antibody was raised in rabbits using a PEDF-specific peptide TSEFIHDIDRELKT. This peptide epitope was chosen due to its high antigenicity index when analyzed by the GCG program. The peptide was conjugated with keyhole limpet hemacyanin (KLH) protein. Two female New Zealand White rabbits were subcutaneously injected with an emulsion of 0.3 mg of the peptide-KLH and Complete Freund's Adjuvant (Gibco-BRL) and intramuscularly boosted with 0.3 mg of the same emulsion in 3-week intervals. After good immune responses were developed, the rabbits were killed and the whole serum was collected.

AminoLink Immobilization kit (Pierce) was used to purify the antibody specific to the PEDF epitope. The PEDF epitope was dissolved 1 mg/ml in coupling buffer, and added at a 1:1 ratio to the AminoLink gel slurry with the addition of 200 μl reductant. The gel slurry was rocked end-over-end overnight at 4°C and then drained. The remaining active sites were blocked by washing with the quenching buffer, and the immunized serum was loaded onto the column. The anti-PEDF antibody was eluted by eight bed-volumes of the elution buffer.

2.5. Western blot analysis

The retinas were dissected, pooled and homogenized by sonication. The insoluble pellet was removed by centrifugation, and the protein concentration of the supernatant was measured with the Bio-Rad protein assay. 100 μg of soluble proteins were resolved by SDS–PAGE (12% polyacrylamide gel for PEDF, 15% polyacrylamide gel for VEGF) and electro-transferred to Hybond ECL nitrocellulose membrane (Amersham International plc). The membrane was then blocked with 5% BLOTTO solution in TBST buffer (20 mM Tris–Cl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) with shaking for 1 h at room temperature. The primary antibody (1:500 for both a monoclonal antibody of VEGF from Santa Cruz, CA, USA, and the PEDF antibody) was added to the BLOTTO and incubated with the membrane overnight at 4°C with shaking. The membrane was washed three times with TBST at room temperature, 20 min each. An anti-rabbit or anti-mouse IgG antibody labeled with horseradish peroxidase (Vector International plc) was diluted 1:10 000 in the BLOTTO solution and incubated with the membrane for 2 h at room temperature. The membrane was washed three times with TBST. The bands were visualized using the ECL detection kit (Amersham International plc) and exposed to a Kodak X-Omat film. The same membrane was stripped and re-blotted with an antibody specific to β-actin (Sigma, 1:5000 dilution). Bands of VEGF and PEDF were semi-quantified by densitometry using the Scanimage software and normalized by β-actin levels.

2.6. Northern blot analysis

Retinas from each group were dissected and pooled for RNA isolation. Total RNA was isolated from the retinas using Trizol reagents (Gibco-BRL Life Technologies) according to the protocol recommended by the manufacturer. 15 μg of total RNA from each sample was resolved on a 1.2% agarose gel, and transferred overnight to a Nylon membrane (Amersham Life Science). After crosslinking, the blot was prehybridized with Ultrahyb solution (Ambion) for 30 min at 42°C. VEGF and PEDF cDNAs were amplified by PCR from a rat retina cDNA library and cloned. The cDNAs were labeled with [α-^{32}P]dCTP using the Nick Translation kit (Gibco-BRL). Hybridization was carried out in the Ultrahyb solution (Ambion, Inc., Austin, TX, USA) according to the manufacturer's protocol. After hybridization, the blot was washed twice for 5 min each with 2×SSC, 0.1% SDS in DEPC-H_2O, followed by two 15 min washes with 0.1×SSC, 0.1% SDS. The blot was exposed to Kodak X-Omat film and quantified by densitometry. After hybridization with the PEDF cDNA probe, the RNA blot was stripped to remove the probe and re-probed with the VEGF cDNA, and again with a labeled oligonucleotide specific to 18S RNA to normalize the loading. The RNA levels were semi-quantified by densitometry and normalized by 18S RNA levels. For each analysis, two independent experiments were performed and results averaged.

3. Results

3.1. Time course of hyperoxia-induced retinopathy in Brown Norway rats

Rats at P7 were exposed to hyperoxia for 5 days and then returned to normoxia at P12. The retinal vasculature was examined by fluorescein-dextran retinal angiography between P12 and P22. As shown in Fig. 1, the retinas from control rats between P12 and P22 developed both superficial and deep vascular layers that extended from the optic disc to the periphery. The vessels formed a fine radial branching pattern in the superficial retinal layer and a polygonal reticular pattern in the deep retinal layer (Figs. 1 and 2A). Small, non-perfused areas were observed only at an early stage (P12) (Fig. 1, C/P12). At later stages (P18–P22), the non-perfusion area disappeared and a mature capillary network formed an arborizing pattern (Fig. 1, C/P14–C/P22). This time course of retinal vascular development in Brown Norway rats was similar to that observed in the newborn mouse [15].

In hyperoxia-treated rats at P12 (0 day after the return from hyperoxia to normoxia), large areas of the central retina showed almost no capillaries, and only small perfusion areas were visible in the peripheral retina (Fig. 1, H/P12). From P14 (2 days after the return) to P22 (10 days after the return), the central hypoperfusion areas were progressively decreased and the radial vessels appeared tortuous and dilated (Fig. 1, H/P14–H/P22). The neovascular tufts, non-perfusion regions, microaneurism and hemorrhage that represent a typical pattern of pathological retinal neovascularization were observed in all of the hyperoxia-treated rats at P14–P22 (Figs. 1 and 2). These results demonstrated that retinal vasculature development was inhibited by exposure to hyperoxia and the neovascularization began at P14, 2 days after the rats were returned from hyperoxia to normoxia, and progressed from P14 to P22.

3.2. Negative correlation between PEDF levels and retinal neovascularization

To determine if endogenous angiogenic inhibitors are involved in ischemia-induced retinal neovascularization, PEDF was measured by Western blot analysis and normalized by β-actin. At P12, after exposure to 75% O_2 for 5 days, PEDF protein levels were elevated by 3.8-fold over that in age-matched controls (Fig. 3), which was consistent with the immunostaining result in the mouse model [6]. In contrast, at age P14 and P16 (2 and 4 days after the return from hyperoxia), the protein levels of PEDF decreased to 84% and 51% of respective control levels (Fig. 3). The lowest PEDF level was observed at P16 when the most aggressive progression of retinal neovascularization occurred (Fig. 1). After P18, PEDF levels began to recover gradually. When retinal neovascularization was essentially complete at P22 and P26, PEDF was brought to the control level. These results demonstrated that retinal PEDF levels are negatively correlated with the progression of neovascularization.

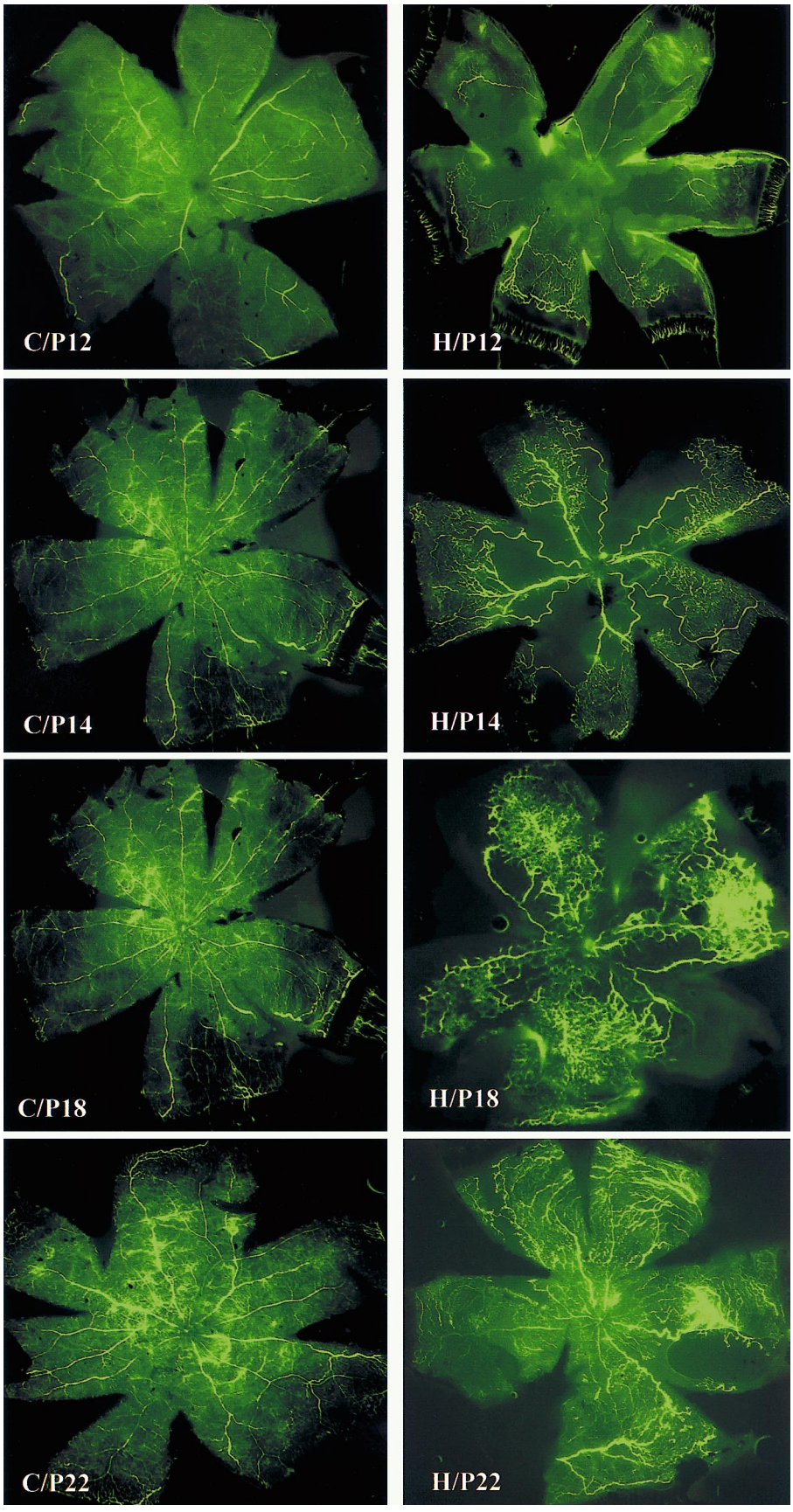
C/P12
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Fig. 1. Time course of ischemia-induced retinal neovascularization in rats. Brown Norway rats at P7 were exposed to hyperoxia for 5 days and then returned to normoxia (indicated by H). Age-matched rats kept in constant normoxia were used as normal controls (indicated by C). Fluorescein retinal angiography was performed at each of the following time points: P12, P14, P18 and P22. Note the enlarged non-perfusion areas in oxygen-treated rats at P12 and progressive retinal neovascularization from P14 to P22 (12.5×).
←

3.3. Positive correlation between VEGF levels and retinal neovascularization

At P12, after exposure to 75% O_2 for 5 days, the protein level of VEGF was reduced by 3-fold, compared to age-matched controls, in contrast to the increased PEDF levels (Fig. 4). From P14 to P16, 2 and 4 days after hyperoxia treatment, VEGF started to increase to levels higher than controls. The highest VEGF levels were found at P16 (500% of the control level), 4 days after the animals were returned to normoxia from hyperoxia. VEGF levels gradually dropped from P18 to P26 to approximate normal level. This time course of VEGF expression change was positively correlated with the onset and progression of retinal neovascularization.

3.4. Increased VEGF/PEDF ratio in the ischemia-induced retinopathy

As shown in Figs. 3 and 4, the most apparent changes in VEGF and PEDF levels occurred at the same time points but toward opposite directions. To determine the balance between VEGF and PEDF in angiogenesis regulation, the relative VEGF/PEDF ratio was calculated at each time point analyzed (Fig. 5). At P12, exposure to 5 days of hyperoxia up-regulated PEDF and down-regulated VEGF, resulting in the lowest VEGF/PEDF ratio. The lower ratio may account for the enlarged non-perfusion region in the retinas of hyperoxia-treated rats (Fig. 1). In the period from P12 to P16, the ratio increased from less than 1 up to 10 at the peak of P16. Consistently, most aggressive progression of neovascularization was observed during this period. After P18, the ratio remained higher than the control but gradually decreased to the control level at P26, when neovascularization was essentially complete.

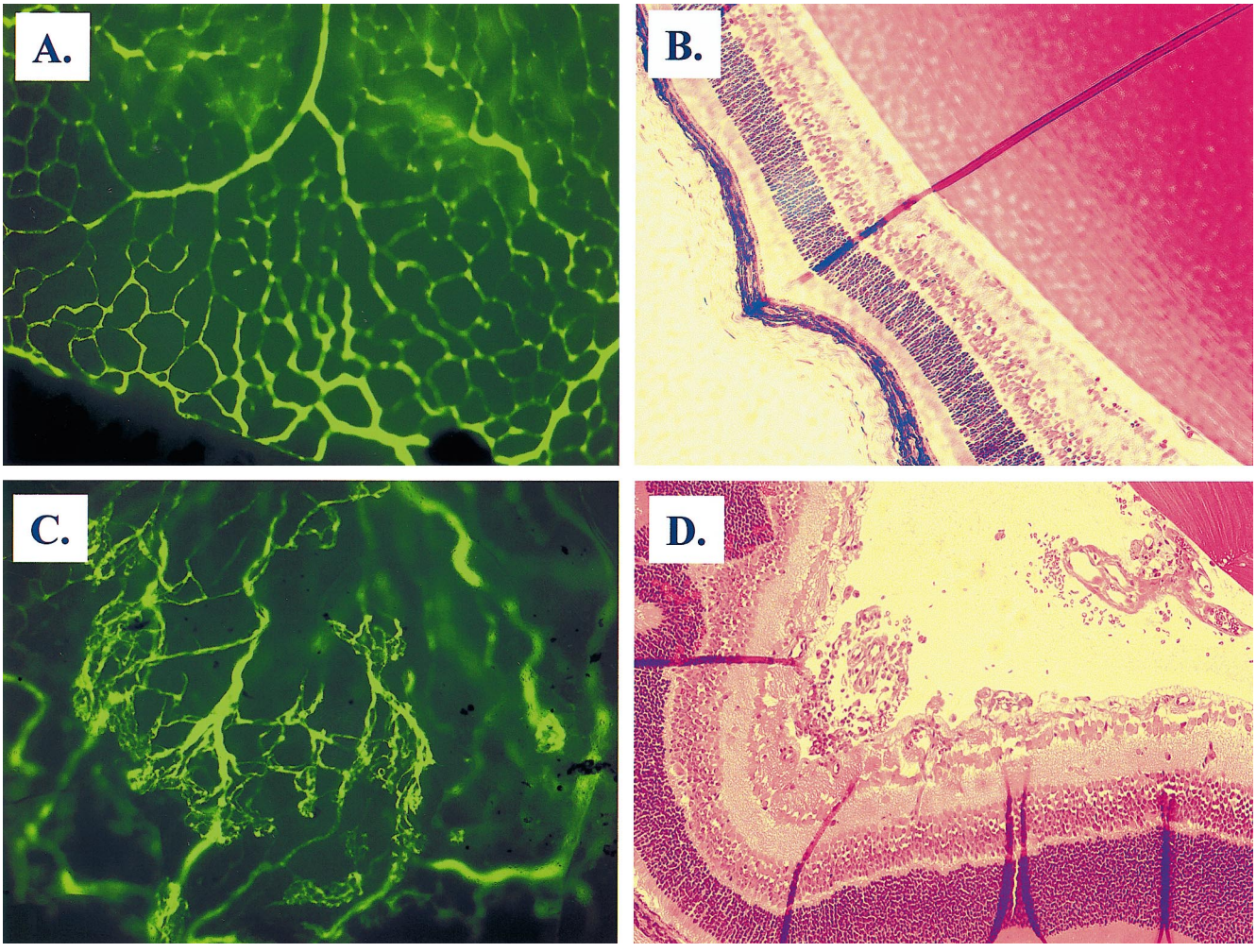

Fig. 2. Ischemia-induced retinopathy in rats. A and B: Control animals (P18) maintained in constant normoxia; C and D: newborn rats were exposed to 5 days of hyperoxia followed by 6 days of normoxia (P18). Retinas from oxygen-treated animals showed vessel dilation and neovascular tufts (C, 100×) and hemorrhage (D, 200×) which are not present in age-matched normal controls (A, 100× and B, 200×).

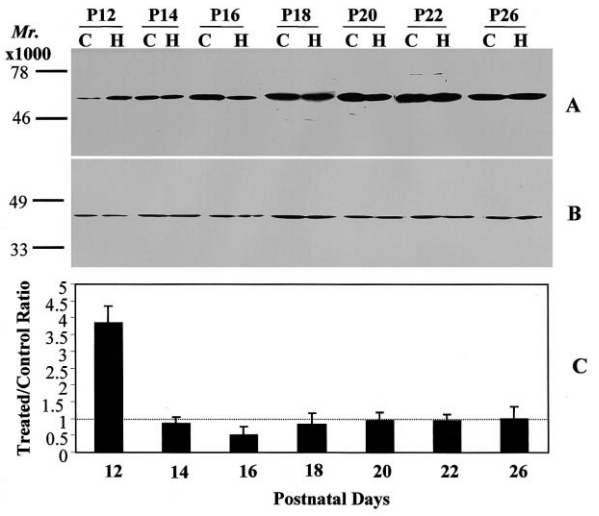

Fig. 3. PEDF levels in the retina during the development of neovascularization. (A) Western blot analysis of PEDF: retinas from three oxygen-treated (lanes indicated by H) and age-matched normal control rats (indicated by C) were dissected at each of the time points as indicated. The retinas of each group were pooled and homogenized. The same amount (100 μg) of soluble protein from each group was blotted with a specific anti-PEDF antibody. (B) The same membrane from (A) was stripped and re-blotted with an antibody specific to β-actin. The bands were semi-quantified with densitometry. PEDF protein levels were normalized by the β-actin signal at the same time points. Ratios of PEDF in hyperoxia-treated animals to the age-matched controls at each time point were calculated and plotted (mean ± S.E.M., $n = 2$) (C).

3.5. Changes of PEDF and VEGF mRNA in the retinal neovascularization model

Northern blot analysis demonstrated that the expression changes of PEDF occurred at the RNA level in the retinal neovascularization model. The changes in the PEDF mRNA level were detected prior to that of the protein level. The

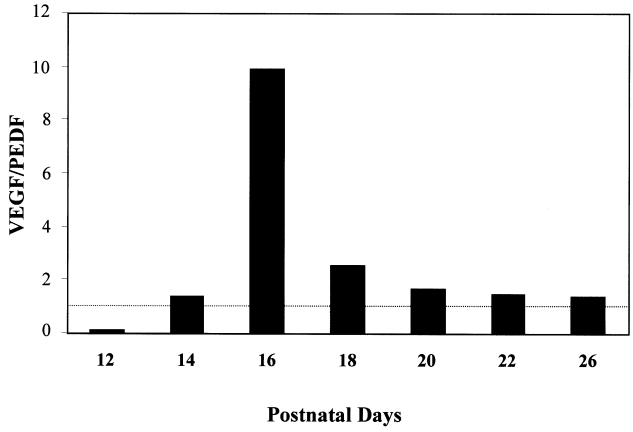

Fig. 5. Retinal VEGF/PEDF ratios in the development of neovascularization. The VEGF and PEDF levels at each time point were first normalized by β-actin to exclude artifacts. The normalized VEGF/PEDF ratio at the time point was calculated as the following: the normalized VEGF level in a hyperoxia-treated group was divided by the PEDF level in the same group to obtain the VEGF/PEDF value in the hyperoxia-treated group. Similarly, VEGF/PEDF ratio in the control group was calculated. The VEGF/PEDF ratio in the hyperoxia-treated group was divided by the VEGF/PEDF in the control group at the same time point to obtain the normalized ratio.

decreased PEDF mRNA was detected as early as P12 when the animals were just returned to normoxia. The lowest PEDF RNA level (approximately 50% of the control level) was observed at P14, 2 days before that of protein. The PEDF mRNA recovered to the control level after P16 (Fig. 6).

Consistent with previous observations in the mouse model [16], VEGF expression changes were also detected at the mRNA level in this rat model. The VEGF mRNA increase preceded that of the protein level (Fig. 7). The VEGF mRNA started to increase at P12 while the earliest increase of the

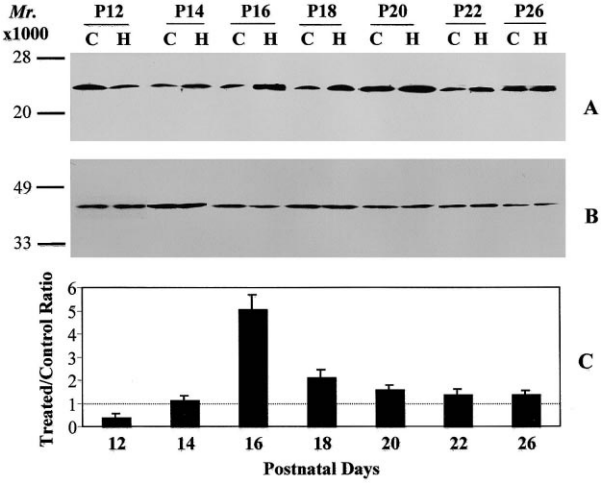

Fig. 4. VEGF levels in the retina during the development of neovascularization. (A) Western blot analysis of VEGF: retinas from the same oxygen-treated (indicated by H) and control (indicated by C) rats as those in Fig. 3 were pooled, and 100 μg of soluble proteins were blotted with an antibody specific to VEGF. (B) The same membrane was re-blotted with the anti-β-actin antibody. The bands were quantified by densitometry, normalized by β-actin. The average ratios (mean ± S.E.M., $n = 2$) of VEGF in hyperoxia-treated animals to controls were calculated at the time points as indicated (C).

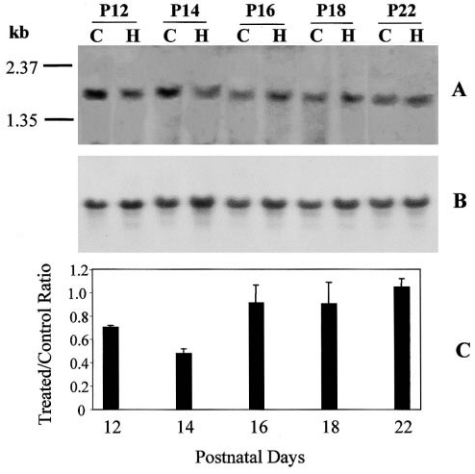

Fig. 6. PEDF mRNA levels in the retina with neovascularization. The same amounts of total RNA from hyperoxia-treated (indicated by H) and control groups (indicated by C) at various ages were used for Northern blot analysis using a PEDF cDNA probe (A) and a 18S ribosomal RNA probe (B). The levels were quantified by densitometry and normalized by 18S ribosomal RNA levels. The ratios (mean ± S.E.M., $n = 2$) of PEDF mRNA in hyperoxia-treated animals to that of respective controls were calculated at each time point (C).

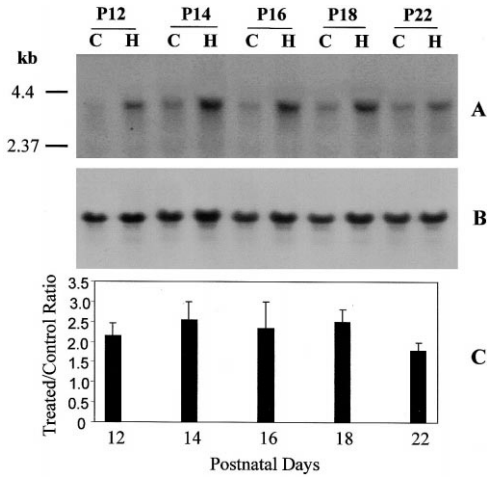

Fig. 7. VEGF mRNA levels in the retina with neovascularization. The Northern blot filter for PEDF was stripped and re-probed with the VEGF cDNA (A) and normalized by 18S ribosomal RNA (B). The ratios (mean ± S.E.M., $n = 2$) of VEGF in hyperoxia-treated animals to that in respective controls were calculated at each time point (C).

protein level was observed at P14. The peak of the mRNA level (approximately 250% of the control level) was observed at P14, 2 days before the peak of the protein (P16).

4. Discussion

PEDF is a potent angiogenic inhibitor endogenously expressed in the retina [6]. However, its role in pathological neovascularization has not been revealed previously. The present study demonstrated for the first time that the retinal PEDF levels, in contrast to that of VEGF, are negatively correlated with pathological retinal neovascularization. The decreased PEDF level may contribute to the development of retinal neovascularization in the ischemia-induced retinopathy model. This finding further confirms the function of PEDF as an angiogenic inhibitor which is involved in the pathogenesis of retinal neovascularization.

It is believed that homeostasis of angiogenesis is regulated by two counter-balancing systems: angiogenic stimulators and angiogenic inhibitors. This balance is critical for the regulation of angiogenesis [2,5]. Under pathological conditions such as diabetic retinopathy and ROP, regions of the retina become hypoxic due to blood vessel abnormalities and modification of hemoglobin. It is hypothesized that in response to hypoxia, the retina increases the production of angiogenic stimulators and reduces the production of angiogenic inhibitors, disturbing the balance between the positive and negative regulators of angiogenesis [2]. As a result, endothelial cells over-proliferate, leading to retinal neovascularization [2,6]. Our studies demonstrate an increased VEGF and decreased PEDF in the ischemia-induced retinal neovascularization rat model, resulting in a higher VEGF/PEDF ratio. The time course of the PEDF down-regulation is consistent with the increased VEGF expression, i.e. the highest VEGF levels coincide with the lowest PEDF levels (Fig. 4). The opposite changes of VEGF and PEDF magnify the change in the angiogenic stimulator/inhibitor ratio. Moreover, the time course of the VEGF/PEDF ratio change is correlated with the development and progression of retinal neovascularization. These results provide evidence supporting the hypothesis that a broken balance between angiogenic stimulators and inhibitors is a cause of pathological retinal neovascularization, at least in this animal model.

The normal vitreous body is known to contain multiple angiogenic inhibitors, including TGF-β [17], PEDF [6] and angiostatin (unpublished result). These inhibitors are believed to play a critical role in maintaining the avascular status of the vitreous. Under certain pathological conditions such as diabetic retinopathy and ROP, decreased expression of these inhibitors may weaken the inhibition of angiogenesis, resulting in over-proliferation of endothelial cells and consequently pathological neovascularization. Therefore, restoration of the balance between the angiogenic stimulators and inhibitors by provision of endogenous angiogenic inhibitors can become a potential therapeutic strategy to arrest the progression of retinal neovascularization. Identification of endogenous angiogenic inhibitors that are down-regulated in pathological retinal neovascularization may reveal the candidates for anti-angiogenic therapy using endogenous inhibitors and will contribute to the development of a new anti-angiogenic therapy.

It is well-known that regulation of VEGF by hypoxia occurs at the transcriptional level via the hypoxia-induced factor pathway [18]. The present study detected the change of VEGF mRNA in the retinal neovascularization rat model, which is consistent with previous findings in the mouse model [16]. However, the regulatory mechanism of PEDF expression is presently uncertain. It has been shown that down-regulation of PEDF expression by hypoxia occurs at the protein level in a cultured retinoblastoma cell line [6]. However, Coljee et al. reported that regulation of PEDF expression occurs at the hnRNA level in fibroblast cells [19]. Consistent with the latter report, our Northern blot analysis using whole retinal RNA detected a decreased PEDF mRNA level in the retina with pathological neovascularization. Moreover, the PEDF RNA decrease is approximately to the same extent as, but precedes the protein level change. These results seem to support a regulation at the RNA level. The disparity between our results and the previous studies by Dawson et al. [6] could be ascribed to that we used an animal model rather than a hypoxic cell culture which are in different environments. The retina consists of multiple cell types which may have a PEDF regulatory mechanism different from the transformed cell line.

Most diabetic mouse and rat models do not develop typical proliferative retinopathy [20]. The ischemia-induced retinal neovascularization model closely resembles ROP. As retinal hypoxia is the direct cause and increased VEGF is a major mediator of neovascularization, the pathological mechanism of this model is similar to that of human diabetic retinopathy. Therefore, this model is also considered an accepted model for diabetic retinopathy [16]. Previous studies have shown that compared to mice, rats are less prone to develop ischemia-induced retinal neovascularization, and require cycling between hypoxia and normoxia to develop significant retinal neovascularization [21,22]. However, most of these previous studies used Sprague Dawley rats [22,23]. For a reason yet to be revealed, we found that pigmented Brown Norway rats are much more sensitive to the hyperoxia treatment and more prone to develop retinal neovascularization, compared to Sprague Dawley rats under the same conditions (unpublished data). Similar to the mouse model, it only requires an exposure to constant hyperoxia for 5 days followed by constant

normoxia to induce significant retinal neovascularization. Moreover, the vascular abnormalities in the Brown Norway rat model are similar to those in human diabetic retinopathy and to the mouse model (Figs. 1 and 2). Because of the larger size of the rat eye compared to the mouse, the Brown Norway rat model will be convenient for intravitreal delivery of therapeutic reagents for the treatment of retinal neovascularization.

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