Placental growth factor expression is reversed by anti-vascular endothelial growth factor therapy under hypoxic conditions

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Original article

Background: **Clinical trials have revealed that the anti-vascular endothelial growth factor (VEGF) therapies are effective in retinopathy of prematurity (ROP). But the low level of VEGF was necessary as a survival signal in healthy conditions, and endogenous placental growth factor (PIGF) is redundant for development. The purpose of this study was to elucidate the PIGF expression under hypoxia as well as the influence of anti-VEGF therapy on PIGF.**

Methods: **$CoCl_2$-induced hypoxic human umbilical vein endothelial cells (HUVECs) were used for an *in vitro* study, and oxygen-induced retinopathy (OIR) mice models were used for an *in vivo* study. The expression patterns of PIGF under hypoxic conditions and the influence of anti-VEGF therapy on PIGF were evaluated by quantitative reverse transcription-polymerase chain reaction (RT-PCR). The retinal avascular areas and neovascularization (NV) areas of anti-VEGF, anti-PIGF and combination treatments were calculated. Retina PIGF concentration was evaluated by ELISA after treatment. The vasoactive effects of exogenous PIGF on HUVECs were investigated by proliferation and migration studies.**

Results: **PIGF mRNA expression was reduced by hypoxia in OIR mice, in HUVECs under hypoxia and anti-VEGF treatment. However, PIGF expression was reversed by anti-VEGF therapy in the OIR model and in HUVECs under hypoxia. Exogenous PIGF significantly inhibited HUVECs proliferation and migration under normal conditions, but it stimulated cell proliferation and migration under hypoxia. Anti-PIGF treatment was effective for neovascular tufts in OIR mice (P<0.05).**

Conclusion: **The finding that PIGF expression is iatrogenically up-regulated by anti-VEGF therapy provides a consideration to combine it with anti-PIGF therapy.**

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Key words: **human umbilical vein endothelial cells;**
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placental growth factor;
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Introduction

Retinopathy of prematurity (ROP) is a serious threat to vision in children born preterm. Improved neonatal care has resulted in increased survival of extremely immature infants at high risk for developing ROP. The suppression of normal retinal vessel development in ROP can result in ischemia-induced angiogenesis and proliferative retinopathy. Angiogenesis is a complex process that is mediated by various factors including, but not limited to, vascular endothelial growth factor (VEGF). Recently, the results of the Bevacizumab Eliminates the Angiogenic Threat of Retinopathy of Prematurity (BEAT-ROP) trial were published in the *New England Journal of Medicine*, and they demonstrated a significant benefit of bevacizumab over laser in reducing treatment-requiring recurrence.[1] However, the ocular and systemic safety profile, efficacy, mode of action, and bioactivity of bevacizumab in premature infants remain unknown, and caution should be taken before labeling intravitreal bevacizumab as the preferred mode of treatment for ROP.[2,3] VEGF blockade treatment has been proven to be effective in cancer and ocular disease. However, although concerns that systemic suppression following intravitreal injections can lead to adverse events, such as strokes, myocardial infarctions, hypertension and

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retina dystrophy, have been evaluated in numerous trials, animal experiments and meta-analyses, firm conclusions have still not been reached.[4,5]

Placental growth factor (PIGF) is an angiogenic protein belonging to the VEGF family, and it is up-regulated mainly in pathologic conditions. PIGF was discovered shortly after the identification of VEGF-A. However, the vasoactive function of PIGF is less well defined. The contribution of PIGF to angiogenesis was shown in transgenic mice, in which the overexpression of PIGF resulted in a substantial increase in vasculature, including the numbers of vessels and branching points, the size of the vessels, and increased vascular permeability.[6] PIGF and its receptor Flt-1 (also referred to as VEGFR-1) received little attention until a recent report, which demonstrated that although PIGF-deficient mice develop normally, loss of PIGF blocks pathologic angiogenesis and vascular leakage in cancer, ischemia, and wound healing.[7] PIGF induces various biological effects by acting on a wide range of different cell types. PIGF can stimulate vessel growth and maturation directly, by affecting endothelial and mural cells, and indirectly, by recruiting pro-angiogenic cell types. In ocular diseases, PIGF could play an important role in proliferative diabetic retinopathy, choroidal neovascularization and wound healing in glaucoma surgery.[8-10] In the retina, PIGF expression was not up-regulated by hypoxia, but PIGF deficiency reduced by 60% the amount of retinal neovascularization in an ROP model,[11] and PIGF had proangiogenic effects on retinal endothelial cells.[12]

PIGF can form heterodimers with VEGF, and several splice variants of PIGF exist that bind to Flt-1 and/or neuropilin-1.[13-15] When highly up-regulated PIGF transmits its signal through Flt1 by stimulating the phosphorylations of specific Flt-1 tyrosine residues, it thus induces the expression of distinct downstream target genes. PIGF also amplifies VEGF-mediated signal transduction through VEGFR-2 (also known as Flk-1) by inducing the transphosphorylations of the tyrosine residues of Flk1.[16] Furthermore, PIGF displaces VEGF-A from Flt-1, which releases VEGF-A and allows it to activate Flk-1 and to enhance VEGF-driven angiogenesis.[14] When PIGF is overexpressed in cells that also produce VEGF-A, VEGFA-PIGF heterodimers are formed, which deplete the intracellular pool of VEGF-A, thereby reducing the formation of angiogenic VEGFA-VEGFA homodimers.[17-19] Researchers[20,21] have reported that plasma PIGF levels are increased in some patients by anti-VEGF therapy. Further close investigations are required to solve the puzzle of the relationship of PIGF expression with anti-VEGF therapy in the retina under hypoxic conditions, as well as the different treatment effects of neutralization of PIGF, VEGF, or both in oxygen-induced retinopathy (OIR).

Methods

Mice and hypoxia-induced retinal vascularization

All of the animal experiments were approved by the ethics review board of the Peking University People's Hospital, and they were performed in strict adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were housed with free access to laboratory food and water and were kept in a 12:12 hour light-dark cycle. All efforts were made to minimize animal suffering and to reduce the number of animals used. OIR was induced in C57BL/6J mouse pups according to a protocol that was previously established. Briefly, at postnatal day 7 (P7), the mouse pups and their mothers were exposed to hyperoxia (75% oxygen) for 5 days, which inhibited retinal vessel growth and resulted in avascular retina. The pups were then returned to room air at P12. Then, the avascular areas of the retina became hypoxic, and the hypoxic avascular retinas triggered retinal NV, which reached its highest level at P17. At P17, the OIR mice were deeply anesthetized with sodium thiopental and were then perfused via the left ventricle with 1 mL of 50 mg/mL FITC-dextran (Sigma-Aldrich, St. Louis, MO) or 1 mL of 2% direct blue dye (Evans blue; Sigma-Aldrich, St. Louis, MO). The mice were euthanized, and retinal flat mounts were prepared after being fixed in 4% paraformaldehyde for 30 minutes. Images were obtained using a fluorescence microscope (Zeiss Axiophot, Thornwood, NY). The retinal avascular areas and NV areas were quantified using image processing and image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD), according to a previous protocol. Briefly, the avascular areas or NV areas on each retina were selected, and the numbers of pixels comprising the selected areas were counted. The relative retinal avascular areas and NV areas were calculated by comparing the number of pixels in the affected areas with the total number of pixels in the retina.

Intravitreal injections and drugs

Bevacizumab (Avastin, Genentech/Roche, San Francisco, CA) was used for anti-VEGF therapy. KH902, a patented product of Chengdu Kanghong Biotechnology Co. Ltd. (Chengdu, China), has a very high affinity for all VEGF-A isoforms, for PIGF 1 and 2, and for VEGF-B.[22] Goat anti-PIGF polyclonal antibody (M-18, Santa Cruz, CA, S C-1882p) was used as a PIGF-neutralizing antibody. Immediately after returning to 21% oxygen, P12 OIR mouse pups were anesthetized, and 1.5 μL of bevacizumab, KH902, PIGF-neutralizing antibody or nonimmune control IgG (Sigma-Aldrich) were injected intravitreally into their right eyes with a dissecting microscope using

a microinjector (Hamilton Co., Reno, NV) and a 33-gauge needle, as described previously.[23] Eyes with lens damage or vitreous hemorrhage after injection were excluded from the subsequent experiments. PIGF expression, developmental retinal vessel regrowth, and retinal NV were determined using real-time PCR and retinal flat mounts.

PIGF expression analysis

Protein expression of PIGF was detected by immunofluorescence labeling. At P17, the OIR mice were deeply anesthetized with sodium thiopental and then were perfused via the left ventricle with 1 mL of 50 mg/mL FITC-dextran. The mice were sacrificed, and retinal flat mounts were prepared after being fixed in 4% paraformaldehyde for 30 minutes; the hyaloid membrane was removed during the initial dissection, and it was then blocked in PBS with 0.5% Triton X-100 and 1% BSA for 30 minutes, followed by incubation overnight at 4 °C with goat anti-PIGF polyclonal antibody (M-18,Santa Cruz, CA, SC-1882,1:100 dilution) or mouse monoclonal anti-Flt-1 antibody (Abcam). After being washed in PBS briefly, the membranes were subsequently exposed to secondary antibodies (1:200 dilution) in the dark at 37 °C for 60 minutes. Images were obtained using a fluorescence microscope.

For each time point, the examined retinas from at least six individual mice were pooled prior to RNA extraction (Qiagen RNA extraction kit). Total RNA was reverse transcribed into cDNA. Real-time PCR products were generated in 35 cycles from cDNA in a real-time PCR platform (LightCycler 480; Roche Diagnostics, Basel, Switzerland), using a mixture containing commercial nucleic acid stain (SYBR Green) and 1 μL of 100 μmol specific primers for PIGF. The nucleic acid-stained (SYBR Green) fluorescent signals were used to generate cycle threshold values from which mRNA ratios were calculated when normalized against the β-actin housekeeping gene. The primers used for mouse and human PIGF amplification are listed in Table 1.

Retinal PIGF concentration was measured. After injection with bevacizumab, KH902 and PIGF blocking antibody for 5 days, C57BL/6J pups were sacrificed at P15, and their retinas were separated for PIGF and VEGF detection. The concentration of PIGF and VEGF (pg/mL protein) in the clarified supernatant was measured with an ELISA Kit (R&D Systems, Ltd., Oxon, UK). All of the experiments for ELISA testing were performed in 5 pups, and each experiment was repeated for three times.

Table 1. Primers for PIGF and β-actin amplification

Genes	Primer sequences	Product size	Tm
Human PIGF	GGCGATGAGAATCTGCACTGT CACCTTTCCGGCTTCATCTTC	164	56
Human β-actin	GCATCCTCACCCTGAAGTAC TTCTCCTTAATGTCACGCAC	437	57
Mouse PIGF	TGCTGGTCATGAAGCTGTTC TCTCCTCTGAGTGGCTGGTT	534	60
Mouse β-actin	AGCC ATGTACGTAGCCATCC GCTGTGGTGAAGCTGTA	222	58

PIGF: placental growth factor; Tm: temperature.

Cells and regents

HUVECs were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and were preserved in our laboratory.[24] The HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Hyclone, Grand Island, NY), in a 37°C humidified incubator and a 5% CO_2 atmosphere. Cells from passages 3 to 15 were used in experiments. Recombinant Human PIGF (rhPIGF, R&D Systems, Ltd., Oxon, UK) was used as exogenous PIGF and was dissolved with DMEM with the absence or presence of 200 μmol $CoCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) to mimic hypoxic conditions. Goat anti-PIGF polyclonal antibody (M-18, Santa Cruz, CA, SC-1882) and mouse monoclonal anti-Flt-1 antibody (Abcam) were used to label PIGF and Flt-1, respectively, in the retinal flat mounts of OIR mice. The secondary antibodies were rabbit anti-goat IgG (H+L)/TRITC and goat anti-mouse IgG (H+L)/TRITC (ZSGB-Bio, Beijing, China).

Proliferation assays

Cell Counting Kit-8 (CCK-8, Dojindo, Shanghai) assay was used to evaluate cell viability. Briefly, the HUVECs were synchronized in DMEM at a density of 1×10^4 per well in 96-well plates overnight without FBS. Seventy percent confluent cultures were starved for 12 hours and were sequentially stimulated with 5 to 50 ng/mL rhPIGF with the absence or presence of 200 μmol $CoCl_2$. The control groups were incubated in DMEM. After adding 10 μL of CCK-8 to each well, the cells were incubated at 37°C for another 30-60 minutes. Absorbance was measured with an ELISA plate reader at a wavelength of 450 nm. Each experiment was repeated in five wells and was duplicated at least three times.

Migration assay

HUVECs migration was assayed by Transwell (Corning, NY, Cat# 3422) with a pore size of 8.0 μm, as described previously.[25] Briefly, 1×10^4 cells were seeded into the upper chamber of a Transwell in 200 μL of serum-free medium. DMEM (containing 10% FBS) with 10 ng/mL of rhPIGF was placed in the bottom chamber, for a final volume of 600 μL. Then, 200 μmol $CoCl_2$ was added to the bottom medium to observe the effects of rhPIGF on

HUVECs migration under hypoxia. All of the migration assays were conducted at 37°C for 5 hours, and then the cells were fixed with 4% PFA for 30 minutes, stained with DAPI (Roche, US, 10236276001) for 15 minutes and washed in PBS three times. The cells on the upper surface that had not migrated were removed with a cotton swab, and the membrane was imaged with fluorescence microscopy. The cells from five random view fields were counted, and the average was used for statistical analysis.

Statistical analysis

Data analysis was performed using Prism 5 statistical software (GraphPad Software Inc., San Diego, CA, USA). All of the data were presented as mean±SD. Differences were evaluated with Student's *t* test or ANOVA. A $P<0.05$ was considered statistically significant.

Results

The expression pattern and distribution of PIGF in OIR

The pattern of PIGF gene expression in the retinas of OIR was compared to age-matched controls. The level of PIGF mRNA was increased under hyperoxic conditions during the period of vaso-obliteration ($P<0.01$) and returned to normal levels after 24 hours of hypoxia, after which it was decreased by hypoxia at P15. The level was highest during hyperoxia-induced vaso-obliteration (Fig. 1 A).

The distribution of Flt-1 was examined in the retinal vasculature of normal mice at P17. The results of Flt-1 immunofluorescence and FITC-dextran showed that expression of Flt-1 was restricted to the retinal vasculature. This finding was consistent with a previous research that Flt-1 is distributed to vascular endothelial cells and pericytes, whereas VEGFR-2 is localized to nonvascular cells in the mouse retina.[26] The localization of PIGF in the retinal flat mounts was also examined in the normal control and OIR mice by immunofluorescence, showing that the vascular system had intense immunoreactivity for PIGF, especially in areas of retinal NV. On examination of the retinal vasculature, PIGF and Flt-1 were localized to both the normal vessels and neovascular networks, and immunolocalization of PIGF occurred largely in the walls of the neovasculature (Fig. 1 B).

Neutralization of PIGF markedly decreased the number of neovascular tufts in OIR

To determine whether neutralization of PIGF had an anti-angiogenic effect on the OIR mouse model, bevacizumab, KH902 and PIGF blocking antibody were injected intravitreally into the right eyes of OIR mouse at P12. As shown in Fig. 2, after FITC-dextran transfusion, retinal flat mounts revealed that intravitreous injection of bevacizumab and KH902 significantly reduced the avascularized areas to 9.3.0%±4.1% and 10.3%±3.1%, which were significantly different compared to the untreated control area of 21.6%±3.4% (Fig. 2A, B). However, PIGF-blocking antibody was ineffective.

Vascular leakage is a hallmark of the pathologic neovascular tufts observed in ROP. Retinal flat mounts, after a blue dye (Evans blue) transfusion, were used to quantify the number of neovascular tufts in OIR mouse retinas. Intravitreal injection of bevacizumab, KH902 and PIGF-blocking antibody markedly decreased the number of neovascular tufts (Fig. 2C, D).

Expression of PIGF was reversed by anti-VEGF therapy in OIR

After injection with bevacizumab, KH902 and PIGF-blocking antibody for 5 days, retinal PIGF concentration measurement by ELISA showed an interesting result. After treatment with bevacizumab, PIGF levels increased from 51.6±6.7 pg/mL of retina protein in the controls to 113.2±12.1 pg/mL, but it decreased significantly in the KH902-treated group (Fig. 3A). VEGF levels in both the

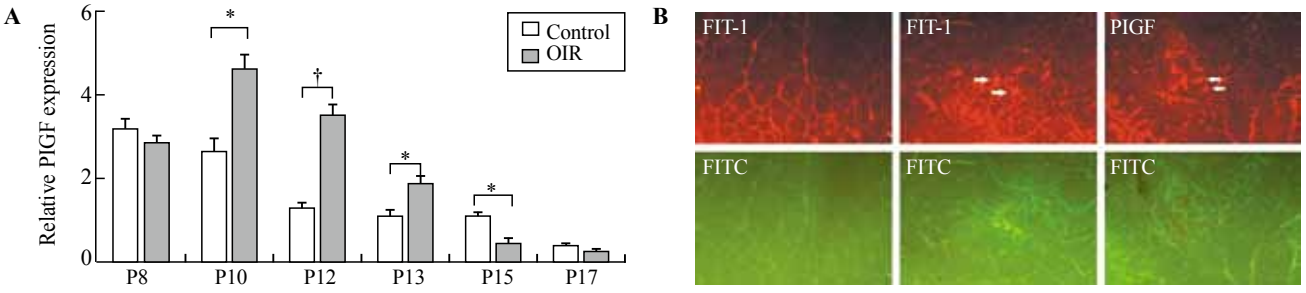

Fig. 1. PIGF levels were correlated with retinal NV. **A:** qRT-PCR analysis of the PIGF mRNA levels in control mouse retinas (75% O_2^-) and OIR mouse retinas (75% O_2^+); **B:** immunofluorescence analysis of PIGF and Flt-1 protein in control mice and OIR mice. White arrows indicate strong staining of PIGF and Flt-1. Immunofluorescence combined with FITC-dextran perfusion showed that Flt-1 was restricted in the retinal vasculature, and strong immunoreactivity of PIGF and Flt-1 was observed around retinal neovascularization. OIR: oxygen-induced retinopathy; RT-PCR: reverse transcription-polymerase chain reaction; PIGF: placental growth factor. *: $P<0.05$; †: $P<0.01$.

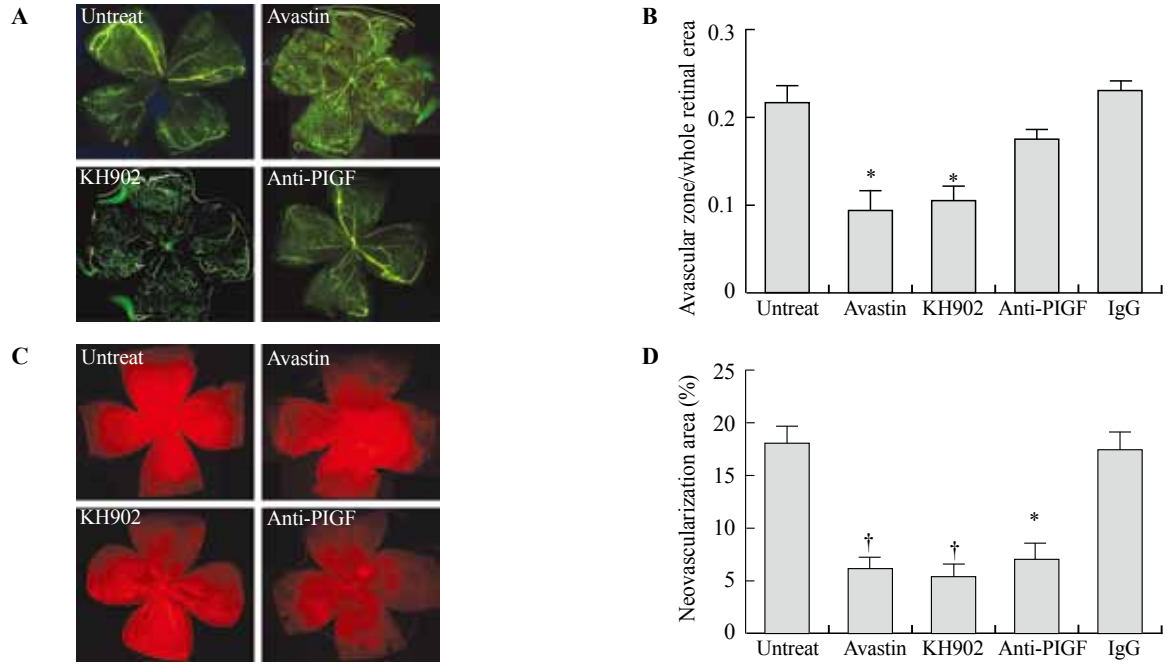

Fig. 2. The effects of each treatment in OIR mice. **A:** OIR mice, after receiving intravitreal injections, were perfused with FITC-dextran (green) at P17. Retinal flat mounts were prepared for each condition and were analyzed by fluorescence microscopy; **B:** Quantification of avascular areas in the retinas of OIR mice; **C:** At P17, OIR mice under different treatments were perfused with Evans blue. Retinal flat mounts were prepared for each condition and were analyzed by fluorescence microscopy; **D:** Quantification of the neovascular area in the retinas of OIR mice. *: $P<0.05$; †: $P<0.01$. ns: no significance, compared with untreated values; OIR: oxygen-induced retinopathy; FITC: fluorescein isothiocyanate. A minimum of six mice was tested for each treatment.

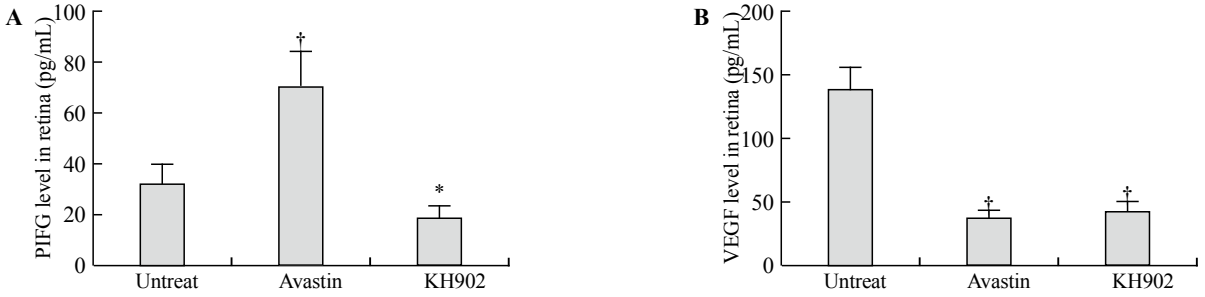

Fig. 3. Retina concentrations of PIGF and VEGF after treatment with bevacizumab and KH902. In the OIR model, the pups (10 pups in each group) were intravitreously injected at P12 (immediately after removal to normal room air). At P17, the pups were euthanized to collect their retinas to detect PIGF and VEGF by ELISA. **A:** PIGF levels of retinas in different groups (n=10); **B:** VEGF levels of retinas in different groups (n=10). *: $P<0.05$; †: $P<0.01$. PIGF: placental growth factor; VEGF: vascular endothelial growth factor; OIR: oxygen-induced retinopathy; ELISA: enzyme-linked immunosorbent assay. Each experiment was repeated at least three separate times.

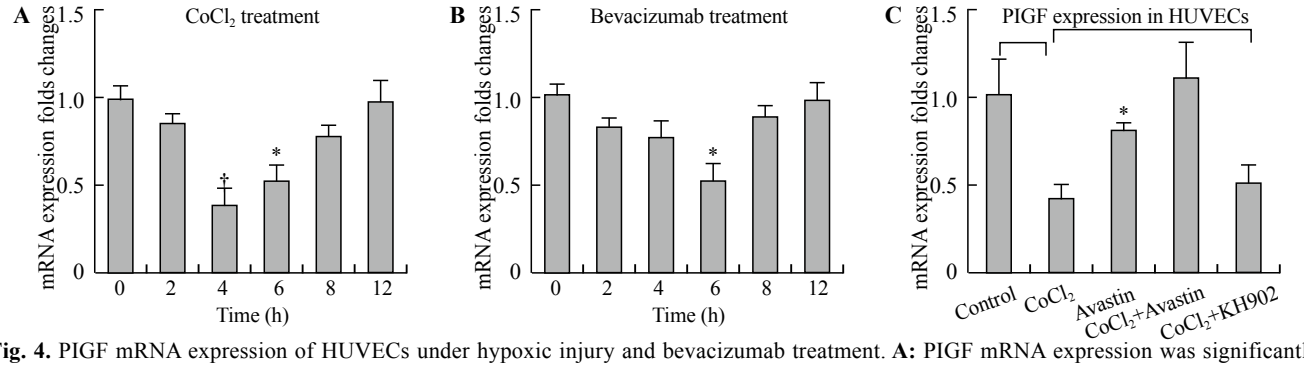

Fig. 4. PIGF mRNA expression of HUVECs under hypoxic injury and bevacizumab treatment. **A:** PIGF mRNA expression was significantly decreased at 4 and 6 hours under hypoxia. **B:** PIGF mRNA expression was significantly inhibited at 6 hours after bevacizumab treatment. **C:** The expression of PIGF was reversed by bevacizumab in hypoxia-induced HUVECs. KH902 (targeting both VEGF and PIGF) did not increase PIGF expression under hypoxia. PIGF: placental growth factor; HUVECs: human umbilical vein endothelial cells. *: $P<0.05$; †: $P<0.01$.

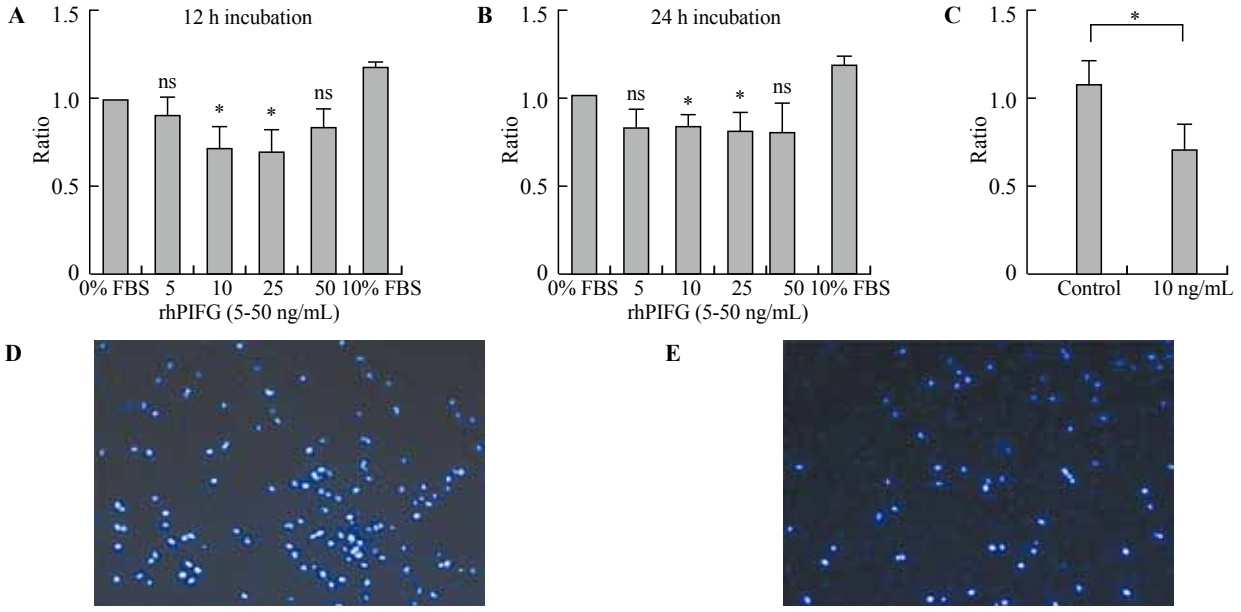

Fig. 5. HUVECs were incubated with rhPIGF (5-50 ng/mL) for 12 and 24 hours under normal conditions, while untreated cells were used as controls. Cell viability was evaluated by CCK-8 assay, and migration was assessed using Transwell cell chambers. **A&B:** The proportions of PIGF-treated cell viability compared with the control group; **C:** The transmigrated nuclei proportions of the PIGF-treated group (10 ng/ml) compared with the control group; **D:** Control group migration; **E:** PIGF-inhibited migration. The transmigrated nuclei (shown in D and E, 10×objectives) were marked with DAPI staining (blue). All of the values are expressed as mean±SD of three independent experiments. HUVECs: human umbilical vein endothelial cells; PIGF: placental growth factor; DAPI: 4',6-diamidino-2-phenylindole; ns: no significance. *: $P<0.05$.

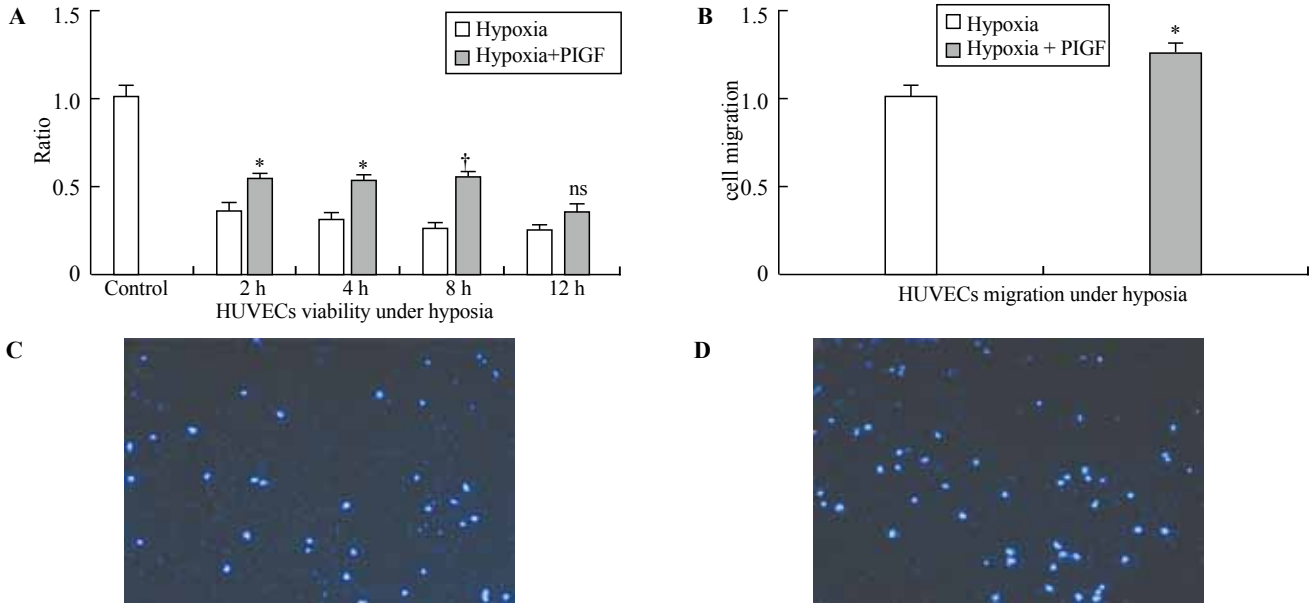

Fig. 6. HUVECs were incubated with rhPIGF (10 ng/mL) for 12 hours under hypoxia. Untreated hypoxic cells were used as controls. **A**: the viability within 2-12 hours under hypoxia; **B:** the transmigrated nuclei folds of the PIGF-treated group (10 ng/mL;) under hypoxia, compared with the hypoxia group; **C:** the hypoxia group; **D:** rhPIGF-induced migration under hypoxia. All of the values shown are mean±SD of three independent experiments. HUVECs: human umbilical vein endothelial cells; PIGF: placental growth factor. *: $P<0.05$; †: $P<0.01$.

bevacizumab- and KH902-treated groups were markedly decreased (Fig. 3B).

These results demonstrate that PIGF was closely correlated with the retinal NV process in OIR mice. Expression of PIGF was characteristically subject to altered expression during the vaso-obliterative and hypoxic phases. Expression was decreased by hypoxia but was reversed by anti-VEGF therapy.

Expression of PIGF was reversed by anti-VEGF therapy in hypoxia-induced HUVECs

Hypoxia-mimetic $CoCl_2$ treatment inhibited PIGF

expression in HUVECs. A decrease in PIGF expression was detectable within three hours, reaching a maximum value within 4 to 6 hours in HUVECs, followed by a return to basal levels after 8 hours of $CoCl_2$ treatment (Fig. 4A). PIGF expression was also decreased significantly by bevacizumab treatment at 6 hours (Fig. 4B), but the expression of PIGF was increased by bevacizumab in hypoxia-induced HUVECs (Fig. 4C).

Exogenous PIGF stimulated cell proliferation and migration in hypoxia-induced HUVECs

HUVECs were incubated for 12 and 24 hours at various concentrations (5, 10, 25 and 50 ng/mL) of rhPIGF. As shown in Fig. 5, 10 ng/mL and 25 ng/mL of rhPIGF inhibited HUVECs proliferation significantly compared to the control group. Next, 10 ng/mL rhPIGF was chosen as the incubation concentration for further treatment. Cell migration was assessed with Transwell assay. As shown in Fig. 5, the number of cells that passed through the membrane in the 10 ng/mL rhPIGF-treated HUVECs group was significantly lower than the number in the control group (Fig. 5C, $P<0.05$). To further evaluate the effects of exogenous PIGF on HUVECs in hypoxic conditions, 10 ng/mL of rhPIGF was added to the cell culture medium in the presence of 200 μmol $CoCl_2$. RhPIGF significantly enhanced the proliferation of HUVECs subjected to hypoxia at 4 , 6 and 8 hours, which was statistically different compared to the control hypoxic group (Fig. 6A, $P<0.05$). Moreover, rhPIGF-stimulated migration was also found under hypoxia (Fig. 6B, $P<0.05$).

Discussion

The pathogenesis of ROP involves two discrete phases. It is now understood that phase 1 involves relative hyperoxia and decreased VEGF levels, whereas phase 2 involves relative hypoxia and increased VEGF levels. The understanding of this relationship between oxygen and VEGF has allowed for improved strategies in managing ROP, including both prevention and early treatment or later treatment. In this study, the strong immunoreactivity of PIGF and Flt-1 around areas of retinal NV indicated that PIGF and Flt-1 were involved in OIR in mice. However, PIGF showed an expression pattern that differed from that of VEGF-A; that was not induced by hypoxia; but that was highest during hyperoxia-induced vaso-obliteration. In a previous study,[27] during retinal vascular development and hyaloid regression in the neonatal mouse, expression of PIGF was correlated with vaso-obliteration, and neutralization of this peptide significantly attenuated hyaloid degeneration. PIGF antibody treatment caused a marked persistence of the hyaloid, without significantly affecting retinal vascular development. In our study, bevacizumab and KH902 intravitreal injections markedly decreased the number of neovascular tufts and reduced the avascular retina area, thereby reducing the extent of OIR. PIGF antibody treatment significantly decreased the number of neovascular tufts and ameliorated the tortuosity of vessels in OIR. Interestingly, bevacizumab treatment reversed PIGF expression in the retina of OIR mice.

In HUVECs, hypoxia-mimetic $CoCl_2$ treatment inhibited PIGF expression levels in RNA. PIGF expression was also decreased significantly by bevacizumab treatment at 6 hours. Bevacizumab treatment itself induced hypoxia and inhibited PIGF expression in HUVECs. Under healthy conditions, VEGF is a trophic factor or survival signal that maintains the quiescent endothelial cells of healthy vessels. Emerging evidence indicates that VEGF-VEGFR inhibitors not only target proliferating endothelial cells, but they also affect quiescent endothelial cells, albeit with lower efficacy.[28] Anti-VEGF therapy itself has recently been reported to induce tissue hypoxia,[29] and the resultant hypoxia is a strong stimulus for the expression of various angiogenic cytokines. Anti-VEGF drug-induced tissue hypoxia could further alter the expression levels of other vascular modulators. The expression of PIGF was reversed by bevacizumab in hypoxia-induced HUVECs. These finding could be interpreted as indicating that endothelial cells enhance their own responsiveness to VEGF-A by producing PIGF.

Exogenous PIGF inhibits endothelial cell proliferation and migration under normal conditions. However, exogenous PIGF stimulated HUVECs proliferation and migration under hypoxia. This lack of endothelial responsiveness to exogenous PIGF might be due, at least in part, to the abundant endogenous production of PIGF by cultured endothelial cells, which can saturate Flt-1 and preclude a response to exogenous PIGF. This theory is supported by the observation that PIGF-deficient endothelial cells readily respond to exogenous PIGF.[7]

VEGF and PIGF are key angiogenic stimulators during normal development and wound healing, as well as in a variety of pathological conditions. In addition to its positive role in the regulation of pathological angiogenesis, PIGF has also been reported to be a negative regulator of angiogenesis and cell growth. This mechanism of negative regulation of angiogenesis involves the formation of VEGF-PIGF heterodimers that do not display significant angiogenic activity, compared to VEGF homodimers.[30] However, the precise role of VEGFA-PIGF heterodimers remains the subject of debate, because they have also been

suggested to be proangiogenic.[31] Studies[31,32] have shown that more modest overexpression of PIGF stimulates, but does not inhibit, tumor angiogenesis and growth, and PIGF can potentiate the proliferative and pro-permeability effects of VEGF.

We have, for the first time, demonstrated in this report that bevacizumab treatment reduced PIGF expression in HUVECs and reversed PIGF expression under chemical hypoxia. Furthermore, bevacizumab treatment reversed PIGF expression in the retina of OIR mice. The expression course of PIGF in OIR suggests that it is advised to pay greater attention to PIGF in ROP treatment. The finding that PIGF expression is iatrogenically up-regulated by anti-VEGF therapy provides a rationale to combine it with anti-PIGF therapy in late treatment of ROP. The limitation of our study is that the molecular mechanisms underlying this upregulation are not clarified, and further work is needed to reach more solid conclusions.

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