Polyethylene glycol modified pigment epithelial-derived factor: new prospects for treatment of retinal neovascularization

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ABBREVIATIONS: ROP, retinopathy of prematurity; OIR, oxygen induced retinopathy; PEDF, pigment epithelial-derived factor; VEGF, vacular endothelium growth factor; HUVEC, human umbilical vein endothelial cells; HIF-1α, hypoxia-inducible factor 1 alpha.
Abstract

Pathological retinal neovascularization and choroidal neovascularization are major causes of vision loss in a variety of clinical conditions, such as retinopathy of prematurity (ROP), age-related macular degeneration, and diabetic retinopathy. Pigment epithelial-derived factor (PEDF) has been found to be the most potent natural, endogenous inhibitor of neovascularization, but its application is restricted due to the instability and short half-life. Polyethylene glycol (PEG) has been used as a drug carrier to slow clearance rate for decades. The present study investigated PEGylated-PEDF for the first time, and evaluated its long-term effects on preventing angiogenesis \textit{in vitro} and \textit{in vivo}. PEG showed lower cytotoxicity to human umbilical vein endothelial cells (HUVECs). \textit{In vitro}, PEGylated-PEDF inhibited HUVEC proliferation, migration, tube formation, vascular endothelium growth factor (VEGF) secretion, and induced HUVEC apoptosis in a dose-dependent manner, and it showed a statistically significant difference compared to the PEDF treatment group. \textit{In vivo}, PEGylated-PEDF had a long-lasting effect, both in plasma and retinal concentrations. In an oxygen-induced retinopathy (OIR) model, one intravitreous injection of PEGylated-PEDF after mouse pups were moved into room air resulted in a significant difference in inhibition of retinal neovascularization, which decreased the non-perfusion area, compared to the PEDF treated group. Our present study demonstrated, for the first time, the long-term inhibitory effects of PEGylated-PEDF on the prevention of neovascularization \textit{in vitro} and \textit{in vivo}. These data suggest that PEGylated-PEDF could offer an innovative therapeutic strategy for preventing retinal...
neovascularization.
Introduction

Retinal neovascularization develops in various retinopathies associated with retinal ischemia, inflammatory diseases, chronic retinal detachment, etc (Gariano, 2010). These disorders include retinopathy of prematurity (ROP) (Chen and Smith, 2007), diabetic retinopathy (DR) (Cheung et al., 2010), and age-related macular degeneration (AMD) (Mousa and Mousa, 2010). Complications resulting from uncontrolled retinal angiogenesis account for the major causes of severe and irreversible loss of vision throughout the world. In the past decades, numerous studies have addressed that retinal neovascularization is driven by the production of proangiogenic growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental-like growth factor (PLGF), and transforming growth factor-b (TGFb) (Chappelow and Kaiser, 2008). Thus, anti-angiogenic drugs, which target proangiogenic growth cytokines, came into being. VEGF, which belongs to the platelet-derived growth factor (PDGF) supergene family, plays a critical role in angiogenesis. Overexpression of VEGF exacerbates neovascularization, while withdrawal of VEGF, or blocking VEGF or the receptors, causes suppression of vascular growth and regression (Mousa and Mousa, 2010).

Pigment epithelial-derived factor (PEDF) has been reported to be the most potent endogenous, natural, anti-angiogenic agent (Dawson et al., 1999). Previous research has revealed that PEDF is down regulated in ROP, and vitreous injection of exogenous PEDF reduces areas of non-vascularization, and induces apoptosis of vascular endothelial cells that have been stimulated to generate retinal
neovascularization (Tombran-Tink et al., 1991; Gao et al., 2002). However, the application of PEDF is limited, due to the route of delivery and its difficult in sustaining an active state; as such, there is great demand for maintaining PEDF tissue level for an extended period of time.

Polyethylene glycol (PEG), with the structure of HO-CH2-(CH2-O-CH2)n-CH2-OH (n=number of entities), is a polyether compound with many applications in medicine (Reddy, 2000; Pai et al., 2009). PEG has been the focus of extensive research as a carrier of therapeutic proteins, due to its longer half-life, stable plasma and tissue concentrations, superior physical and thermal stability, greater biocompatibility, and lower biodegradability (Yowell and Blackwell, 2002; Parveen and Sahoo, 2006). PEGylation, first described by Abuchowski, is the act of covalently coupling a PEG structure to another larger molecule, such as a protein (which is then referred to as PEGylated) (Abuchowski et al., 1977). In the 1990s, PEG-modified adenosine deaminase (PEG-ADA) was manufactured by Dow Chemical for pharmaceutical use (Parveen and Sahoo, 2006). Recently, PEGylated interferon alfa-2a and -2b have become commonly used injectable treatments for hepatitis C infection (Wedemeyer et al., 2002). In addition, the first FDA-approved, intravitreously injected, anti-angiogenic, anti-VEGF medication, pegaptanib (brand name Macugen), is also PEGylated (Gragoudas et al., 2004).

Because PEGylation can potentially address some of the problems associated with the delivery of PEDF, in the present study we investigated PEGylated-PEDF for the first time and evaluated its anti-angiogenic effect, both \textit{in vitro} and \textit{in vivo}. We
explored whether PEDF covalent with PEG can play a long-term inhibiting role in human umbilical vein endothelial cells (HUVECs) in vitro and prevent retinal neovascularization in oxygen-induced retinopathy (OIR) mice models in vivo. The encouraging results of our study provide an innovative strategy for the therapy of retinal neovascularization.
Methods

Construction of expression plasmid pET28a(+)-PEDF. The human PEDF encoding gene was sub-cloned into the expression vector pET28a (+) plasmid (Kit Lot No. N72770, Novagen, Germany) with a DNA Ligation Kit (Code No. D6023, Takara Bio, Japan). Competent E. coli JM109 cells (Code No.D9052, Takara) were transformed with pET28a (+)-PEDF plasmids, and positive clones were selected by the blue/white screening method and confirmed by restriction enzyme analysis with BamH I and Xho I.

Expression of rPEDF in E. coli BL21 (DE3). Purified recombinant vector pET28a (+)-PEDF plasmid was transformed into E.coli BL21 (DE3, Stratagene, US) with the aid of the inducer isopropyl-D-thiogalactopyranoside (IPTG), and the E. coli cells were harvested by centrifugation. PEDF was expressed as inclusion body protein products, and it underwent purification, renaturation, and repurification procedures.

PEGylated-PEDF preparation. Recombinant human PEDF was dissolved in 30 mM sodium acetate solution (pH 5.5), and its concentration was adjusted to 3-8 mg/ml. PEG (molecular weight 20kDa, Sigma, US) was added to the PEDF solution in a 1:2 PEG-to-PEDF molar ratio, while reducing agent CH$_3$BNNa was added in a final concentration of 20 mM. The pH was adjusted to 5.1-5.3, the reaction solution was stirred at room temperature for 4-6 hours, and it was allowed to rest at 4 °C for 10 hours.

After 20 kDa PEG was covalent to recombinant PEDF in N-terminal, and a cation
exchange column was used to separate and purify the PEGylated-PEDF. Based on the strength of charge in modified and unmodified protein, PEDF, PEGylated-PEDF was eluted. Collected proteins were detected by SDS-PAGE.

**Cells and animals.** Human umbilical vein endothelial cells (HUVECs) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and preserved in our laboratory (Yu et al., 2003). The HUVECs were cultured in DMEM with 10% fetal bovine serum (FBS; Hyclone, Grand Island, NY) in a 37°C humidified incubator with 5% CO₂ atmosphere.

Neonatal mice (C57BL/6J) and Wistar rats (200~225g) were obtained from Peking University animal center and raised in the animal room of Peking University People’s Hospital. This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was performed in accordance with the guidelines provided by the Animal Care Use Committee of Peking University (Beijing, China). The animals were housed with free access to laboratory food and water *ad libitum* and kept in a 12:12 h light-dark cycle.

**Plasma concentration detection.** Eight healthy Wistar rats were divided into two groups. A single injection of recombinant modified and unmodified PEDF was administered through the tail vein at a dose of 1 mg/kg body weight. PEDF plasma concentrations were measured 5 min, 10 min, 20 min, 40 min, 1h, 2h, 4h, 8h, 16h, 24h, 48h, 72h, 96h, 120h, 168h, 216h, and 264h after injection by detection of tail vein blood serum, using a PEDF Enzyme-Linked Immunosorbent Assay (ELISA) kit (Adlitteram Diagnostic, US, Cat# E14-090).
Retinal PEDF concentration detection. Thirty-six healthy C57 pups were injected with PEDF or PEGylated-PEDF intravitreously on postnatal day 12 (P12). All of the drugs were injected with 1.5μl, and the concentration was 5μg/μl. Three pups from each group were euthanized at 12h, 24h, 48h, 72h, 96h, and 120h after injection, and the retinas were separated for PEDF detection. The concentration of PEDF in the clarified supernatant was measured with ELISA (Adlitteram Diagnostic, US, Cat# E14-090). All of the experiments for ELISA were performed in triplicate and repeated three times.

HUVEC proliferation assays. HUVECs were used to evaluate the effects of PEGylated-PEDF on angiogenesis in vitro by using a Cell Counting Kit-8 (CCK-8, Dojindo, Shanghai) assay. Briefly, the HUVECs were synchronized in DMEM at a density of 1×10^4 per well in 96-well plates overnight without FBS. PEDF (10^{-1}~10^{-7} mg/ml), PEGylated-PEDF (10^{-1}~10^{-7} mg/ml), and PEG (1.0~1.0×10^{-2} mg/ml), diluted into a series of 1:10 concentrations, were incubated for another 24, 48, 72, 96, and 120 hours. After adding 10μl CCK-8 to each well, the cells were incubated at 37°C for another 30~60 min. Absorbance was measured with an ELISA plate reader at a wavelength of 450 nm. Each experiment was performed in five wells and was duplicated at least three times.

In order to study the angiogenic inhibitory effects of PEGylated-PEDF under the stimulation of VEGF, 20ng/ml VEGF were added to the culture medium. PEDF, PEGylated-PEDF, and PEG, in the same concentrations as tested above, were incubated for another 24, 48, and 72 hours, and CCK-8 assays were performed as
stated previously.

**HUVEC cell migration assay.** Migration was assayed by Transwell (Corning, US, Cat# 3422) with a pore size of 8.0 μm, as described previously (Huang et al., 2010). Briefly, 2×10⁴ cells were placed in the top part of a Transwell in 200 μL of serum-free medium. DMEM (containing 10% FBS) with 10⁻² mg/ml, 10⁻⁴ mg/ml, and 10⁻⁶ mg/ml PEDF or PEGylated-PEDF, and 1.0 mg/ml, 0.01 mg/ml, and 0.001 mg/ml PEG were placed in the bottom chamber, for a final volume of 600 μL. All migration assays were conducted at 37°C for six hours. At the end of the assay, the cells were fixed in 4% PFA and stained with DAPI (Roche, US, 10236276001) for 15 minutes. The cells that had not migrated were removed with a cotton swab, and the membrane was imaged. Cells from five random fields of view were counted.

**VEGF detection by Enzyme-Linked Immunosorbent Assay (ELISA).** The HUVECs were seeded in 96-well plates (1×10⁴ per well) and incubated at 37°C overnight. PEDF (10⁻¹~10⁻⁷ mg/ml) or PEGylated-PEDF (10⁻¹~10⁻⁷ mg/ml) was added to the wells after removing the DMEM medium. After 48 and 72 hours of incubation, the cell culture supernatant was harvested and centrifuged to remove cellular debris. VEGF protein secreted by HUVEC cells in the culture medium was measured by VEGF ELISA Kit (Bostar, EK0575), according to the manufacturer’s instruction.

**Flow cytometry analysis of HUVEC apoptosis.** Apoptosis was measured with a FITC Annexin V Apoptosis Detection Kit (BD Science, US, Cat# 556547), according to the manufacturer’s instructions. Briefly, HUVEC cells (1×10⁶) were
seeded in six-well plates and incubated for 24, 48, and 72h, with $10^{-2}$ mg/ml, $10^{-4}$ mg/ml, $10^{-6}$ mg/ml PEDF or PEGylated-PEDF or controls. Then, the cells were detached with EDTA, washed in cold phosphate buffered saline (PBS), and stained with Annexin-V-FITC and propidium iodide (P.I.), according to the manufacturers’ instructions. Flow cytometry analysis was immediately performed (ex/em=488/530nm). The samples were analyzed by flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ) with Cell Quest software (BD, Biosciences). Then, $10^4$ cells were collected and divided into four groups: dead cells (Annexin V-/PI+, UL), late apoptotic cells (Annexin V+/PI+, UR), viable cells (Annexin V-/PI-, LL), and early apoptotic cells (Annexin V+/PI-, LR). The apoptotic rate was calculated as the percentage of early apoptotic cells (LR) plus late apoptotic cells (UR).

**Tube formation study.** Tube formation study is a convenient and quantifiable assay to test the angiogenic/anti-angiogenic properties of compounds on vascular endothelial cells. This assay measures the ability of endothelial cells, to form capillary-like structures. Upon plating at subconfluent densities with the appropriate extracellular matrix support, endothelial cells attach and generate mechanical forces on the surrounding extracellular support matrix to create tracks or guidance pathways that facilitate cellular migration. The resulting cords of cells eventually form hollow lumens. In our study, tube formation assay was used to assess the anti-angiogenic potential of PEGylated-PEDF *in vitro*. Aliquots (150 μL) of Matrigel (BD Sciences, Cat#354234) solution were poured into 48-well plates, and then they were incubated
at 37°C for 30 min in a 5% CO₂ incubator. HUVEC cells (5×10⁴ per well) treated with 10⁻² mg/ml, 10⁻⁴ mg/ml, 10⁻⁶ mg/ml PEDF or PEGylated-PEDF, or controls, were seeded on the Matrigel and cultured in DMEM medium for 12 hours. The networks in Matrigel from five randomly chosen fields were counted and photographed under a microscope. The experiments were performed in triplicate and repeated three times.

**Induction of oxygen-induced retinopathy (OIR) mouse model and assessment of angiography.** Beginning on postnatal day 7 (P7), the C57BL/6 pups were exposed to hyperoxia (75% oxygen) for five days. On P12, the animals were brought back to normoxia (room air) to induce retinal neovascularization, until P17 (Smith et al., 1994). At P12, the OIR mice were injected intravitreally with PEDF, PEGylated-PEDF, PEG, and PBS. All of the drugs were injected with 1.5μl, and the concentration was 5μg/μl. At P18, the mice were deeply anesthetized intraperitoneally with chloral hydrate (0.2 ml/10 g body weight) and then perfused through the left ventricle with 0.5 ml of PBS containing 50mg of 2×10⁶ molecular weight fluorescein-dextran-FITC (Sigma, St. Louis, MO). The eyes were removed and fixed in 4% paraformaldehyde for 30 min, and the retinas were flat-mounted by four peripheral retinal cuts (Supplemental Figure 1). The retinas were viewed by fluorescence microscopy (Zeiss Axiophot, Thornwood, NY) and photographed. The non-perfused areas were analyzed with ImageJ software. The ratio is determined by the non-perfusion area compare to the area of the whole retina (Supplemental Figure 1). The data were analyzed by two persons, one of whom was completely blind to the study groups, and the average was considered to be the final value for statistics.
**Statistical evaluation.** Data analysis was performed using statistical software Prism 5 (GraphPad Software Inc., San Diego, CA). All data are presented as mean±SEM and have been evaluated for normality of distribution. Differences were evaluated with ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons and the Student’s $t$-test for pair-wise comparisons. For ELISA results, the data were analyzed by two-way ANOVA, followed by Bonferroni-posttests for comparisons among the two groups. A $P <0.05$ was considered to be a statistically significant difference.
Results

**PEGylated-PEDF preparation.** The recombinant vector pET28a (+)-PEDF was confirmed by restriction enzyme BamH I and Xho I digestion. Recombinant bacteria BL21 (DE3)/pET28a (+)-PEDF were cultured with the aid of IPTG. After purification, renaturation, and repurification, the purity of PEDF reached 95%, and the molecular weight was 44417.8. The PEDF protein sequence is shown in Table 1. According to the process stated in Method section, 20 kDa PEG was covalent to recombinant PEDF in N-terminal at a ratio of 70% (Figure 1). After the cation exchange column was used to separate and purify PEGylated-PEDF, collected proteins were detected by SDS-PAGE. The purification process map is shown in Figure 2.

**Long-lasting effects of PEGylated-PEDF in rat plasma concentration and mice retina.** In order to determine the *in vivo* drug metabolism rate in systemic blood circulation, plasma concentration was measured in the Wistar rats; the results are shown in Table 2. The half-life of recombinant PEDF is 4.5 hours, while the half-life of modified PEGylated-PEDF is 78 hours (Figure 3). At the same time, we detected the concentration of recombinant PEDF and PEGylated-PEDF in the retinas of healthy mouse pups. As shown in Figure 4, recombinant PEDF had a low concentration after 48h, while PEGylated-PEDF had long-lasting retinal concentration, even at 120h, at a concentration of 320 ng/ml.

**Effects of PEGylated-PEDF on HUVEC proliferation study.** A HUVEC proliferation study was used to evaluate the anti-angiogenic effect of
PEGylated-PEDF *in vitro*. HUVECs were incubated for 48, 72, 96, and 120h at various concentrations (10^{-1}~10^{-7} mg/ml rang). PEG had no effect on the proliferation of HUVECs at concentrations lower than 0.1mg/ml, compared to blank controls (DMEM+10% FBS) (p>0.05) (Figure 5). Thus, the maximum concentration of PEGylated-PEDF we chose was 10^{-1}mg/ml. As shown in Figure 5, the PEGylated-PEDF treated groups were statistically different compared to PEDF treated groups until 96h in 10^{-2}~10^{-5} mg/ml, and 120h in 10^{-3}~10^{-5} mg/ml. In order to further evaluate the inhibitory effect of PEGylated-PEDF on VEGF stimulation, we added 20ng/ml VEGF to the cell culture medium and found that PEGylated-PEDF also exhibited better inhibitory effects on HUVEC proliferation (Figure 6).

**Effects of PEGylated-PEDF on HUVEC migration study.** A HUVEC migration study was assessed using Transwell. As shown in Figure 7 and Figure 8, the number of cells that crossed the membrane in PEGylated-PEDF treated HUVEC groups was significantly lower than the number in the PEDF and DMEM+10%FBS groups in both 10^{-4} and 10^{-6} mg/ml groups (p<0.05).

**Effects of PEGylated-PEDF on HUVEC apoptosis study.** FACS was used to evaluate early and late apoptosis effects. As shown in Table 5 and Figure 9, after incubation with PEDF and PEGylated-PEDF combined with 10% FBS for 24, 48, and 72h, the early and late apoptotic HUVECs showed significant differences in the 10^{-2} and 10^{-4} mg/ml treated groups, with the percentage of apoptotic cells (UR+LR%) significantly higher than the PEDF treated cells (p<0.05).

**Effects of PEGylated-PEDF on tube formation.** Matrigel assay is one of the
most widely used methods to evaluate the reorganization of angiogenesis \textit{in vitro}. In our study, both PEDF and PEGylated-PEDF showed impaired capacity to form a regular network, as expected: HUVECs in both groups cannot form hollow lumens (Figure 10 and Figure 11). Although there was no significant difference between the PEGylated-PEDF- and PEDF- treated groups in lumen formation, the PEGylated-PEDF treated HUVECs showed statistic difference in length comparing to PEDF treated groups, and presented round morphology and no branches at all, while the PEDF treated group had a tendency to form tubes.

\textbf{Effects of PEGylated-PEDF on the prevention of VEGF secretion.} Correlated with the anti-angiogenic effect \textit{in vitro} (tube formation), PEDF and PEGylated-PEDF treated HUVECs showed a decrease in VEGF secretion levels in a time-dependent and dose-dependent manner. As shown in Figure 12, after treatment for 48h, VEGF was downregulated in the PEDF groups until $10^{-5}$ mg/ml, while the PEGylated-PEDF group inhibited VEGF secretion even in $10^{-7}$ mg/ml; the difference between the groups is significant ($p<0.01$). After treatment for 72h, the PEGylated-PEDF treated group showed good inhibitory effects on VEGF secretion, which was significantly different than the PEDF treated group ($p<0.01$).

\textbf{PEGylated-PEDF protects against OIR retinal vessel loss.} Overexpressed PEDF genes have been shown to inhibit angiogenesis and inflammation in the OIR model (Park et al., 2011). To determine whether PEGylated-PEDF had a better effect on the OIR mouse model than PEDF, PEGylated-PEDF, PEDF, and PEG were injected intravitreally into the right eyes of retinopathy rats at P12 (immediately after...
the animals were returned from hyperoxia to normoxia) and to age-matched normal rats. Consistent with other previous observations (Park et al., 2011), the PEDF injection substantially reduced the neovascularized area by 19.0±1.9%, which is significantly different compared to the untreated control results of 28.3±0.6% (p<0.0001) (Fig. 13). In the PEGylated-PEDF treated group, the neovascularization area was reduced by 12.6±1.5%, which is significantly different compared to the untreated controls (p<0.0001) and the PEDF treated group (p<0.05). Thus, these experiments demonstrated that PEGylated-PEDF protected postnatal mouse retina from hyperoxia-induced vaso-obliteration more efficiently than did PEDF.
Discussion

Antiangiogenic treatment is one of the most effective therapeutic strategies in the management of retinal neovascularization disease, including retinopathy of prematurity (ROP), age-related macular degeneration (AMD), and diabetic retinopathy (DR), which are found throughout the world and can cause irreversible blindness (Drack, 2006; Mousa and Mousa, 2010). Recently, several agents have been demonstrated to be helpful in decreasing the amount of neovascularization (Mousa and Mousa, 2010). Among these potential medications, pigment epithelium-derived factor (PEDF) is considered to be the most potent natural angiogenic inhibitor endogenously expressed in the retina (Tombran-Tink et al., 1991; Dawson et al., 1999).

PEDF, a 50kDa secreted glycoprotein that belongs to the non-inhibitory serpin family, was first discovered by Tombran-Tink and Johnson in 1991 in conditioned medium from cultured fetal retinal pigment epithelial (RPE) cells (Tombran-Tink et al., 1991). Subsequent studies revealed PEDF as a potent and versatile endogenous, anti-angiogenic, neuroprotective, antivasopermeable, glia-static, anti-tumorigenic agent (Bouck, 2002), which can inhibit the growth of new blood vessels in conditions of hypoxia/ischemia and can promote neuron growth during injury; its exact mechanisms, however, are not well known (Dawson et al., 1999; Bamstable and Tombran-Tink, 2004).

PEDF occurs naturally in the eye, is able to block multiple inducers of angiogenesis, and has been shown to be an essential contributor to the maintenance of
the avascularity status the healthy ocular tissues (Dawson et al., 1999; Barnstable and Tombran-Tink, 2004). In recent decades, several studies have shows that the inhibition effect of PEDF in angiogenesis is because of the inhibition of endothelial cell proliferation, migration, and inducing of its apoptosis (Dawson et al., 1999; Park et al., 2011). Further study has revealed that PEDF exerts its anti-angiogenic function through such activities as decreasing vascular endothelial growth factor (VEGF), downregulating hypoxia-inducible factor 1 alpha (HIF-1α), and suppressing inflammation effects etc (Tombran-Tink et al., 1991; Park et al., 2011). Although PEDF exhibits effective therapeutic potential, the application is limited by its short half-life, unstable pharmacology and administration pathway. In order to explore PEDF application, the gene therapy method has been used by many laboratories, such as AAV-mediated PEDF (Streck et al., 2005; Park et al., 2011; He et al., 2012). However, due to its the potential oncology-inducing property, immunogenicity, uncertain quantitative expression, and lower production rate, the application is limited. Other studies also have loaded the PEDF gene in poly (d, l-lactide-co-glycolide acid) (PLGA) nanoparticles for PEDF delivery, but the critical drawback of a PLGA microsphere delivery system for proteins is activity loss during formulation (Pai et al., 2009).

It is already known that the major challenge of using proteins or peptides is their poor in vivo stability, retention, and inactivation by the immune system or by the action of proteolytic enzymes (Abuchowski et al., 1977). Rapid elimination leads to frequent and excessive administration, which is both improvident and causes
non-specific toxicity. Thus, controlled release, making it accumulate to effective levels, and metabolizing with minimum toxicity and without intolerable adverse effects become particularly important. A crucial strategy for controlled release is the use of polymers (Qiu and Bae, 2006). Conjugation of proteins with polymers reduces recognition by the immune system and decreases the clearance rate (Abuchowski et al., 1977). Currently, polyethylene glycol (PEG) is one of the most widely used polymers for the modification of protein therapeutics, with many applications from industrial manufacturing to medicine (Pai et al., 2009). Because it is inert, inexpensive, low toxicity, increased solubility, PEG has been approved by the US FDA for drug modification for several years (Harris and Chess, 2003). PEGylation (i.e., the attachment of PEG to proteins, peptides, or other drugs) is an upcoming methodology for drug development that has the potential to improve the pharmacokinetic and pharmacodynamic properties of the administered drug (Parveen and Sahoo, 2006).

Until now, several PEGylated products have been available on the market, such as PEG-adenosine deaminase (Hershfield et al., 1987), PEG-L-asparaginase (Graham, 2003), PEG-Interferon-α (Heathcote et al., 2000), and PEG-human growth hormone (Drake and Trainer, 2003). In addition, pegaptanib (brand name Macugen), the first FDA-approved intravitreously used anti-VEGF medication, is also PEGylated (Gragoudas et al., 2004).

In the present study, we developed PEGylated-PEDF for the first time (Figures 1 and 2) and evaluated its anti-angiogenic effects both in vitro and in vivo. We confirmed that PEG covalent to PEDF had longer-term effects than unmodified PEDF,
both in plasma concentration (Figure 3) and retinal concentration (Figure 4). In vitro, PEGylated-PEDF showed significant proliferation and migration inhibitory effects on HUVECs compared to the controls; the inhibitory effects were dose-dependent (Figures 5 and 7), which corresponds to previous reports (Dawson et al., 1999). Even in VEGF stimulation medium, PEGylated-PEDF also showed significant proliferation inhibitory effects (Figure 6). We also evaluated the anti-angiogenic property of PEGylated-PEDF in vitro with a tube formation study. The results showed that modified PEDF interferes with network formation and could be associated with the apoptosis of vascular endothelial cells (Figure 8) and the inhibition of VEGF secretion (Figure 10). In vivo, we use oxygen-induced retinopathy (OIR) model to evaluate the anti-angiogenic effects of PEGylated-PEDF. The OIR model closely resembles retinopathy of prematurity (ROP). As retinal hypoxia is the direct cause of neovascularization, and increased VEGF is a major mediator, the pathological mechanism of this model is similar to that of ROP. In our in vivo study, PEGylated-PEDF showed significant inhibitory effects on neovascularization in the OIR model.

In summary, our study found, for the first time, that PEG covalent to PEDF could effectively inhibit the growth of neovascularization. Our data suggest that PEGylated-PEDF delivery system could be an extremely promising approach for long-term treatment of retinopathy of prematurity. The use of PEGylated-PEDF was a safe and effective drug delivery system for therapy of retinal angiogenic diseases, and it may be an innovative approach for future therapeutic strategies against other
angiogenesis as well.
Authorship contributions

Participated in research design: YJ.B, LZ.H, XX.L.
Conducted experiments: YJ.B, XL.X, W.D. AY.Z.
Contributed new reagents or analytic tools: XL.X
Performed data analysis: YJ.B, AY.Z, WZ.Y.
Wrote or contributed to the writing of the manuscript: YJ.B, LZ.H, WZ.Y.
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Footnotes

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Legends for Figures

Figure 1. PEG covalent to PEDF.
Lane 1: Marker, followed by 97, 66, 45, 35, and 20 KD; Lane 2: 20 KD single-methoxy polyethylene glycol propionaldehyde (PEG)-modified PEDF; Lane 3: Unmodified PEDF.

Figure 2. PEGylated-PEDF purification process map.
Lane 1: Marker, followed by 97, 66, 45, 35, and 20 KD; Lane 2: 20 KD single-methoxy polyethylene glycol propionaldehyde (PEG)-modified PEDF; Lane 3: penetration liquid; Lane 4: 50 mM NaCl elution fractions; Lane 5: 100 mM NaCl elution fractions; Lane 6: 150 mM NaCl elution fractions; Lane 7: 200 mM NaCl elution fractions; Lane 8: 250 mM NaCl elution fractions; Lane 9: 300 mM NaCl elution fractions; Lane 10: 500 mM NaCl elution fractions; Lane 11: 1000 mM NaCl elution fractions.

Figure 3. PEDF plasma concentration in Wistar rat.
Plasma concentration for single tail vein injection of PEDF and PEGylated-PEDF were measured by 4 animals of each group (1mg/kg body weight). After the indicated time, sera were collected, and the results were analyzed by ELISA kit.

Figure 4. PEDF retinal concentration in mice.
Retina concentration-time curve for single dose intravitreous injection of PEDF and PEGylated-PEDF (5μg/μl, 1.5μl) at indicated time by ELISA kit.

Figure 5. Effects of PEDF, PEGylated-PEDF, and PEG on the proliferation of HUVECs in general culture medium.
Cell proliferation was measured with MTT assay at 48h, 72h, 96h, and 120h. Data are presented as mean±SEM. Minimal effective concentrations (10^{-5}mg/ml) of different time-point were shown. Each experiment was repeated at least three separate times. *P<0.05; **P<0.01.

**Figure 6. Effects of PEDF, PEGylated-PEDF, and PEG on the proliferation of HUVECs under VEGF stimulation.**

Cell proliferation was measured with MTT assay at 24h, 48h, and 72h. Data are presented as mean±SEM. Minimal effective concentrations of different time-point were shown in the above figure. Each experiment was repeated at least three independent times. *P<0.05.

**Figure 7. Effects of PEDF, PEGylated-PEDF, and PEG on the migration of HUVECs.**

The migratory activities of the controls, the VEGF treated group, the PEG (10^{-2}mg/ml) treated group, the PEDF treated group (10^{-4}mg/ml), and the PEGylated-PEDF (10^{-4}mg/ml) treated group were shown in the figure. Cell nuclei were stained with DAPI which shown in blue dots.

**Figure 8. Statistic results of HUVECs migration under different treatments.**

The number of cells that had migrated through the filter of the chamber were counted. Data are presented as mean±SEM. Each experiment was repeated at least three separate times. DMEM+10%FBS control was set to 100%. *P<0.05.

**Figure 9. Effects of PEDF, PEGylated-PEDF, and PEG on HUVEC apoptosis.**

Apoptosis was quantified by flow cytometry measured by Annexin V and PI staining.
Data are presented as mean±SEM. Each experiment was repeated at least three independent times. DMEM+10%FBS control was set to 100%. *P<0.05. UR: late apoptotic cells; LR: early apoptotic cells, UR+LR: apoptotic cells.

**Figure 10. Effects of PEDF, PEGylated-PEDF, and PEG on HUVEC tube formation.**

Matrigel assay was used to evaluate the angiogenic effect in PEDF and PEGylated-PEDF treated HUVECs. VEGF, general culture medium and PEG treated HUVECs can form tube shapes *in vitro*. PEDF and PEGylated-PEDF treated HUVECs showed significantly impaired capacity to form a regular network. In particular, PEGylated-PEDF treated HUVECs presented round morphology and showed no cell branches that attempted to form networks.

**Figure 11. Statistic results of the length of tube formation under different treatments.**

The total length of tube in each groups were shown. Data are presented as mean±SEM. Each experiment was repeated at least three separate times. DMEM+10%FBS control was set to 100%. *P<0.05; **P<0.01.

**Figure 12. Effects of PEDF, PEGylated-PEDF, and PEG on VEGF secretion of HUVECs.**

VEGF released into the culture supernatant was measured by ELISA. PEGylated-PEDF treated groups showed significant suppression in the level of VEGF secretion, both at 48h and 72h (P<0.01). Data are presented as mean±SEM. Each experiment was repeated at least three separate times.
Figure 13. Effects of PEDF, PEGylated-PEDF, and PEG on OIR model.

P7 mice were put into a hyperoxia condition for five days. At P12, the pups were intravitreously injected with 5mg/ml PEG, PEDF, or PEGylated-PEDF for 1.5μl. At P18, the pups were euthanized to detect the retinal non-vascularization area. Both the PEDF and PEGylated-PEDF treated groups showed a significant difference from the OIR control groups (P<0.0001), and the PEGylated-PEDF treated group was significantly different compared to the PEDF treated group (P<0.05).
### TABLE 1. PEDF protein sequence

<table>
<thead>
<tr>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>QNPASPEEG SPDPDSTGAL VEEEDPFFKV PVNLADAAVS NFGYDLYRVR</td>
</tr>
<tr>
<td>SSMSPTTNVL LSPLSVATAL SALSLGAEQR TESIIHRALY YDLISSPDIH</td>
</tr>
<tr>
<td>GTYKELLDTV TAPQKNLKSA SRIVFEKKLR IKSSFVAPLE KSYGTRPRVL</td>
</tr>
<tr>
<td>TGNPRLDLQE INNWVQAQMK GKLARSTKEI PDEISILLLG VAHFKGQWVT</td>
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<tr>
<td>KFDSRKTSLQ DFYLDEERTV RVPMMSDPKA VLRYGLDSDL SCKIAQLPLT</td>
</tr>
<tr>
<td>GSMSIIFFLP LKVTVNLTLL EESLTSEFIH DIDRELKTVQ AVLTVPKLKL</td>
</tr>
<tr>
<td>SYEGEVTKSL QEMKLQSLFD SPDSTKTVG PIKLTQVEHR AGFEWNEKGA</td>
</tr>
<tr>
<td>GTTPSPGLQP AHLTFLDLYH LNQPFIFVLR DTDTGAFLFI GKIHPGRPG</td>
</tr>
</tbody>
</table>
TABLE 2. Plasma concentration (ng/ml) of Wistar rats.

|            | 5min | 10min | 20min | 40min | 1h   | 2h   | 4h   | 8h   | 16h  | 24h  | 48h  | 72h  | 96h  | 120h | 168h | 216h |
|------------|------|-------|-------|-------|------|------|------|------|------|------|------|------|------|------|------|
| PEDF       |      |       |       |       |      |      |      |      |      |      |      |      |      |      |      |
| MEAN       | 5168 | 4317  | 3611  | 2217  | 992  | 851  | 724  | 611  | 422  | 326  |      |      |      |      |      |      |
| SEM        | 178  | 145   | 110   | 84    | 61   | 49   | 52   | 44   | 47   | 39   |      |      |      |      |      |      |
| PEGylated-PEDF | 5476 | 4897  | 4587  | 4191  | 3438 | 2974 | 1284 | 928  | 855  | 782  | 641  | 593  | 531  | 493  | 441  | 413  |
| MEAN       |      |       |       |       |      |      |      |      |      |      |      |      |      |      |      |      |
| SEM        | 218  | 189   | 123   | 171   | 148  | 151  | 139  | 72   | 64   | 63   | 61   | 59   | 47   | 46   | 38   | 37   |
TABLE 3. Summary of proliferation effects with PEDF and PEGylated-PEDF treated HUVECs.

<table>
<thead>
<tr>
<th></th>
<th>10% FBS</th>
<th>PEG</th>
<th>PEDF</th>
<th>PEGylated-PEDF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>10⁻¹</td>
<td>10⁻²</td>
<td>10⁻¹</td>
</tr>
<tr>
<td>48h</td>
<td>MEAN</td>
<td>72.34</td>
<td>88.63</td>
<td>94.35</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>2.20</td>
<td>6.72</td>
<td>5.14</td>
</tr>
<tr>
<td>72h</td>
<td>MEAN</td>
<td>65.66</td>
<td>85.44</td>
<td>91.42</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>4.39</td>
<td>7.38</td>
<td>3.49</td>
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<tr>
<td>96h</td>
<td>MEAN</td>
<td>61.46</td>
<td>87.07</td>
<td>93.87</td>
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<tr>
<td></td>
<td>SEM</td>
<td>6.87</td>
<td>5.46</td>
<td>4.23</td>
</tr>
<tr>
<td>120h</td>
<td>MEAN</td>
<td>76.46</td>
<td>85.16</td>
<td>103.72</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>8.94</td>
<td>5.05</td>
<td>3.72</td>
</tr>
</tbody>
</table>
TABLE 4. Summary of proliferation effects with PEDF and PEGylated-PEDF combined with VEGF treated HUVECs.

<table>
<thead>
<tr>
<th></th>
<th>10% FBS (20ng/ml)</th>
<th>10^1</th>
<th>10^-1</th>
<th>10^-2</th>
<th>10^-3</th>
<th>10^-4</th>
<th>10^-5</th>
<th>10^-6</th>
<th>10^-7</th>
<th>10^-8</th>
<th>10^-9</th>
<th>10^-10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>113.91</td>
<td>75.81</td>
<td>89.72</td>
<td>99.37</td>
<td>73.62</td>
<td>69.60</td>
<td>81.11</td>
<td>83.46</td>
<td>75.98</td>
<td>75.11</td>
<td>92.68</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>1.83</td>
<td>6.00</td>
<td>7.71</td>
<td>2.98</td>
<td>9.72</td>
<td>8.03</td>
<td>4.86</td>
<td>7.22</td>
<td>9.35</td>
<td>9.27</td>
<td>5.50</td>
</tr>
<tr>
<td>24h</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>48h</td>
<td>MEAN</td>
<td>124.59</td>
<td>104.35</td>
<td>103.10</td>
<td>110.22</td>
<td>81.65</td>
<td>93.17</td>
<td>97.21</td>
<td>99.85</td>
<td>94.69</td>
<td>93.87</td>
<td>94.65</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>7.34</td>
<td>11.16</td>
<td>7.21</td>
<td>8.78</td>
<td>11.02</td>
<td>12.24</td>
<td>7.25</td>
<td>7.10</td>
<td>7.06</td>
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<tr>
<td>72h</td>
<td>MEAN</td>
<td>114.68</td>
<td>79.92</td>
<td>89.11</td>
<td>105.25</td>
<td>95.65</td>
<td>87.22</td>
<td>88.55</td>
<td>86.13</td>
<td>101.21</td>
<td>100.47</td>
<td>99.25</td>
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<tr>
<td></td>
<td>SEM</td>
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<td>8.58</td>
<td>2.75</td>
<td>5.93</td>
<td>11.74</td>
<td>1.86</td>
<td>11.59</td>
<td>7.26</td>
<td>8.51</td>
<td>9.82</td>
<td>5.08</td>
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</table>

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### TABLE 5. Summary of flow cytometry data of apoptosis measured by Annexin V and PI.

<table>
<thead>
<tr>
<th>Time point</th>
<th>% 10% FBS</th>
<th>PEG</th>
<th>PEDF</th>
<th>PEGylated-PEDF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-2</td>
<td>10-4</td>
<td>10-6</td>
<td>10-2</td>
</tr>
<tr>
<td>24h UL</td>
<td>0.94±0.13</td>
<td>2.18±0.15</td>
<td>2.18±0.07</td>
<td>2.50±0.06</td>
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<tr>
<td>UR</td>
<td>2.07±0.03</td>
<td>2.47±0.11</td>
<td>2.15±0.03</td>
<td>2.41±0.04</td>
</tr>
<tr>
<td>LL</td>
<td>94.99±0.28</td>
<td>93.26±0.36</td>
<td>93.35±0.38</td>
<td>93.40±0.32</td>
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<tr>
<td>LR</td>
<td>2.01±0.21</td>
<td>2.09±0.43</td>
<td>2.32±0.36</td>
<td>1.68±0.33</td>
</tr>
<tr>
<td>UR+LR</td>
<td>4.08±0.20</td>
<td>4.56±0.20</td>
<td>4.48±0.35</td>
<td>4.10±0.30</td>
</tr>
<tr>
<td>48h UL</td>
<td>1.43±0.13</td>
<td>1.85±0.03</td>
<td>2.31±0.08</td>
<td>3.46±0.76</td>
</tr>
<tr>
<td>UR</td>
<td>0.85±0.02</td>
<td>1.23±0.08</td>
<td>1.26±0.07</td>
<td>1.21±0.18</td>
</tr>
<tr>
<td>LL</td>
<td>95.7±0.13</td>
<td>93.17±0.32</td>
<td>93.44±0.69</td>
<td>90.40±0.23</td>
</tr>
<tr>
<td>LR</td>
<td>2.02±0.16</td>
<td>3.75±0.38</td>
<td>2.98±0.61</td>
<td>4.92±0.47</td>
</tr>
<tr>
<td>UR+LR</td>
<td>2.87±0.14</td>
<td>4.99±0.32</td>
<td>4.24±0.61</td>
<td>6.13±0.64</td>
</tr>
<tr>
<td>72h UL</td>
<td>2.08±0.30</td>
<td>1.90±0.06</td>
<td>1.86±0.09</td>
<td>1.99±0.02</td>
</tr>
<tr>
<td>UR</td>
<td>1.96±0.03</td>
<td>1.97±0.04</td>
<td>2.54±0.13</td>
<td>2.51±0.10</td>
</tr>
<tr>
<td>LL</td>
<td>92.36±0.40</td>
<td>94.29±0.25</td>
<td>91.84±0.40</td>
<td>92.56±0.43</td>
</tr>
<tr>
<td>LR</td>
<td>3.60±0.14</td>
<td>1.84±0.30</td>
<td>3.75±0.38</td>
<td>2.94±0.34</td>
</tr>
<tr>
<td>UR+LR</td>
<td>5.56±0.14</td>
<td>3.81±0.27</td>
<td>6.29±0.48</td>
<td>5.45±0.44</td>
</tr>
</tbody>
</table>
Figure 1

[Image of a gel showing bands labeled Modified PEDF and PEDF]
Figure 2

Modified PEDF

PEDF
Figure 4

retina concentration

PEGylated-PEDF

PEDF

ng/ml

- 824±37
- 831±41
- 711±27
- 590±77
- 526±50
- 405±76
- 351±63
- 320±77
- 197±15
- 96±15
- 59±17
- 36±17

12h 24h 48h 72h 96h 120h
Figure 5

proliferation analysis

Ratio (compare to 10%FBS)

PEG(0.01mg/ml)    PEDF    PEGylated-PEDF

48h    72h    96h    120h
Figure 7

<table>
<thead>
<tr>
<th>0% FBS</th>
<th>10% FBS</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>PEDF</td>
<td>PEG-PEDF</td>
</tr>
</tbody>
</table>

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Figure 8

Ratio (compared to CTL)

0% FBS  CTL  VEGF  $10^{-1}$  $10^{-2}$  $10^{-3}$  $10^{-4}$  $10^{-6}$

PEG        PEDF        PEG-PEDF

0.1±0.03  0.81±0.03  0.75±0.02  0.58±0.04
1.1±0.06  0.97±0.05  0.75±0.02  0.68±0.02
1.04±0.02 0.86±0.03  0.67±0.04  0.60±0.03
Figure 12

VEGF secretion (48h)

VEGF secretion (72h)
Figure 13

Non-Perfusion Area (NPA)

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio (NPA/whole retina)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctl</td>
<td>28.3±0.6</td>
<td>9</td>
</tr>
<tr>
<td>PEG</td>
<td>26.1±1.1</td>
<td>5</td>
</tr>
<tr>
<td>PEDF</td>
<td>19.0±1.9</td>
<td>7</td>
</tr>
<tr>
<td>PEG-PEDF</td>
<td>12.6±1.5</td>
<td>7</td>
</tr>
</tbody>
</table>

Normal

OIR Control

PEG

PEDF

PEG-PEDF