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The inhibition of retinal neovascularization by gold nanoparticles *via* suppression of VEGFR-2 activation

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ABSTRACT

The pathological angiogenesis in the retina is the major cause of vision loss at all ages. In particular, retinopathy of prematurity (ROP) is a leading cause of blindness in children. This study investigated whether gold nanoparticle (GNP) could inhibit retinal neovascularization in the animal model of ROP. Intravitreal injection of GNP significantly inhibited retinal neovascularization in the mouse model of ROP. In addition, GNP effectively suppressed VEGF-induced *in vitro* angiogenesis of retinal microvascular endothelial cells including proliferation, migration and capillary-like networks formation. GNP blocked VEGF-induced autophosphorylation of VEGFR-2 to inhibit consequently ERK 1/2 activation. GNP never affected on the cellular viability of retinal microvascular endothelial cells and induced no retinal toxicity. Our data suggest that GNP could be a potent inhibitor to retinal neovascularization without retinal toxicity. Furthermore, GNP could be extensively applied to variable vaso-proliferative retinopathies mediated by VEGF.

1. Introduction

Angiogenesis is a strictly-controlled process that plays a central role in the physiological condition of development and tissue repair which is regulated by the balance of many stimulating or inhibiting factors [1]. In the pathological condition when the balance is disrupted, angiogenic factors such as vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) lead to robust proliferation and migration of vascular endothelial cells—the pathological angiogenesis. These newly formed blood vessels are so fragile to be easily ruptured, and hemorrhagic which could result in fibrous proliferation [2]. In particular, this pathological angiogenesis also occurs in the retina, which leads to retinal edema, retinal or vitreous hemorrhage, and finally traction retinal detachment, which might result in catastrophic loss of vision [3]. The pathological angiogenesis in the retina is the major cause of vision loss at all ages including retinopathy of prematurity (ROP) in children, diabetic retinopathy (DR) in young adults, and age-related macular degeneration (AMD) in the elderly [4].

ROP is a leading cause of blindness in children [5]. Although the detail mechanism of molecular processes remains to be elucidated, ROP is known to be a biphasic vaso-proliferative retinopathy, which occurs through the pathological angiogenesis following the vaso-obliteration in developing retinal vasculature [6]. The retinal neovascularization followed by the vaso-obliteration appears to be driven by relative tissue hypoxia. The hypoxia induces VEGF production in the retina, which finally leads to pathological retinal angiogenesis [3]. The oxygen-induced retinopathy (OIR) in the mouse is an animal model of ROP, which is induced by hyperoxia-induced vaso-obliteration of capillaries in mouse pups and their subsequent return to room air. This triggers retinal neovascularization starting from the inner retina, characterized by growing into the vitreous [7]. Therefore, OIR reflects the current understanding of the pathogenesis of ROP.

VEGF, originally isolated as a vascular permeability factor, is the best known pro-angiogenic factor to involve in the initiation and development of variable retinopathies [4]. It has been known that VEGF could induce vascular abnormalities including vascular leakage and neovascularization [8]. In addition, VEGF and VEGFR system is known to play a critical role in retinal neovacularization, where VEGF interacts with the tyrosine kinase receptors, VEGFR-1 or VEGFR-2 [9]. In particular, signaling *via* VEGFR-2 is prerequisite for all processes of angiogenesis including vascular endothelial

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proliferation, migration, and tube formation. The receptor is primarily activated by VEGF-induced auto-phosphorylation of 1175-tyrosine, which consequently activates the phospholipase-Cγ and protein kinase C (PKC) pathway, and its downstream c-Raf-MEK-MAP-kinase pathway [10,11]. Therefore, many angiogenesis inhibitors have focused on the VEGF-dependent pathway [12]. During discovery of angiogenesis inhibitors, it was recently reported that gold nanoparticle (GNP) could inhibit VEGF-induced permeability and angiogenesis *in vivo* [13]. Furthermore, GNP conjugated with heparin polysaccharides reduces FGF-induced angiogenesis *in vivo*, as well [14]. Recently, it was reported that intravenously administrated GNP could pass through blood-retinal barrier and induce no retinal toxicity [15].

In the present study, we investigated whether intravitreal injection of GNP could inhibit retinal neovascularization in the animal model of ROP without retinal toxicity.

2. Materials and methods

2.1. Mouse

C57BL/6 mice were purchased from Samtako (Seoul, Korea). 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. C57BL/6 mice were kept in standard 12-hour dark–light cycles and approximately 23 °C room temperature.

2.2. Cell culture

Human retina microvascular endothelial cells (HRMECs) were purchased from the Applied Cell Biology Research Institute and grown on attachment factor-coated plates in complete medium (Cell Systems, Kirkland, WA, USA) or in M199 medium supplemented with 20% FBS, 3 ng/ml basic fibroblast growth factor (Sigma, St. Louis, MO, USA), and 10 U/ml heparin (Sigma, St. Louis, MO, USA). HRMECs used in this study were taken from passages 4 to 6. GNP (20 nm) were purchased from BB International (Cardiff, UK). GNP or VEGF (20 ng/ml, Sigma, St. Louis, MO, USA) treatment was carried out in cells cultured in serum free M199 supplemented with 1% (vol/vol) penicillin–streptomycin.

2.3. Oxygen-induced retinopathy

OIR was induced as described by Smith et al. [7] with some modifications [16]. Briefly, newborn mice were randomly assigned to experimental and control groups. At postnatal day (P) 7, pups (5–7 pups) in the experimental group were exposed to hyperoxia (75% ± 0.5% O_2) for 5 days (P 7 to P 11) and then returned to normoxia (room air) for 5 d. Neovascularization occurs upon return to normoxia and peaks at P 17. To assess the anti-angiogenic activity of GNP, the pups were injected intravitreously with 1 μM GNP (20 nm, SPI supplies Inc, West Chester, PA, USA) in 1 μl phosphate-buffered saline (PBS) on P 14, when retinal neovascularization began. These experiments were repeated at least 10 times.

2.4. Qualitative assessment of retinal neovascularization by fluorescein angiography

As our previous description [16], at P 17, deeply anesthetized mice were perfused through the tail vein with fluorescein conjugated dextran (molecular weight = 500,000; Sigma–Aldrich Ltd., St. Louis, MO, USA) dissolved in PBS. After 1 h perfusion, the eyes were enucleated and fixed in 4% paraformaldehyde for 2 h. The retinas were dissected, flat-mounted in Dako mounting medium (DakoCytomation, Glostrup, Denmark), and viewed by fluorescein microscopy (BX50, OLYMPUS, Japan) at a magnification of 4×.

2.5. Quantitative assessment of retinal neovascularization by counting vascular lumens

As our previous description [16], at P 17, the eyes were removed, fixed in 4% paraformaldehyde for 24 h, and embedded in paraffin. Sagittal sections of 5 μm, each 30 μm apart, were cut through the cornea parallel to the optic nerve. The sections were stained with hematoxylin and eosin to assess retinal vasculature *via* light microscopy (Carl Zeiss, Chester, VA, USA). Any vascular lumens on the vitreal side of the inner limiting membrane were counted in at least 10 sections from each eye by two independent observers blind to treatment (Kim JH and Kim JH). The average intravitreal vessels/section was calculated for each group. There were at least 10 animals in each group.

2.6. Cell proliferation assay on retinal microvascular endothelial cells

Cell proliferation was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as our previous description [17]. HRMECs (1×10^4 cells) were plated in 96 well plates and cultured overnight. Cells were treated with VEGF (20 ng/ml) (Sigma, St. Louis, MO, USA) or GNP (20 nm; 1 & 5 μM) (SPI supplies Inc, West Chester, PA, USA) for 48 h. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT for 4 h. After incubation, the medium was carefully removed from the plate and DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7. Wound migration assay on retinal microvascular endothelial cells

Cell migration was evaluated with wound migration assay modified from our previous description [17]. HRMECs (1×10^4 cells) were plated onto gelatin-coated culture dishes at 90% confluence, and were wounded with a razor blade. After wounding, plates were rinsed with serum-free medium. Then, the wounded monolayers were incubated with treatment of 1 μM GNP (20 nm, SPI supplies Inc, West Chester, PA, USA) or 20 ng/ml VEGF (Sigma, St. Louis, MO, USA) for 12 h. The cells were fixed with absolute methanol and stained with Giemsa's solution (BDH Labotratory Supplies, London, UK). Migration was quantified by counting the number of cells that moved beyond the reference line at a ×200 magnification (Carl Zeiss, Chester, VA, USA).

2.8. Tube formation assay on retinal microvascular endothelial cells

Tube formation was assayed as our previous description [18]. HRMECs (1×10^4 cells) were inoculated on the surface of the Matrigel, and treated with 1 μM GNP (20 nm, SPI supplies Inc, West Chester, PA, USA) or 20 ng/ml VEGF (Sigma, St. Louis, MO, USA) for 18 h. The morphological changes of the cells and tubes formed were observed under a microscope and photographed at a ×200 magnification. Tube formation was quantified by counting the number of connected cells in randomly selected fields at a ×200 magnification (Carl Zeiss, Chester, VA, USA), and dividing that number by the total number of cells in the same field.

2.9. Western blotting

Western blotting was performed using standard western blotting methods. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by electrophoresis on 5–10% SDS-PAGE and transferred electrophoretically onto nitrocellulose membrane (Amersham, Little Chalfont, UK). The membranes after blocking were incubated overnight with anti-VEGFR-2 (Cell Signaling Technology, Beverly, MA, USA), anti-phospho-VEGFR-2 (p-VEGFR-2, Cell Signaling Technology, Beverly, MA, USA), anti-ERK 1/2 (Cell Signaling Technology, Beverly, MA, USA), and anti-phospho-ERK 1/2 (p-ERK 1/2, Cell Signaling Technology, Beverly, MA, USA) at 4 °C. To ensure the equal loading of protein in each lane, the blots were stripped and reprobed with an antibody against β-actin. Intensity values were normalized relative to control values. The blots were scanned using a flatbed scanner and the band intensity analyzed using the TINA software program (Raytest, Straubenhardt, Germany).

2.10. Cell viability assay on retinal microvascular endothelial cells

Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HRMECs (1×10^4 cells) were plated in 96 well plates and cultured overnight. Cells were treated with GNP (20 nm; 0.1–10 μM) (SPI supplies Inc, West Chester, PA, USA) for 48 h. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT for 4 h. After incubation, the medium was carefully removed from the plate and DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

2.11. Histological analysis

5 μM GNP (20 nm), twice of the effective therapeutic concentration, was intravitreally injected to 8-week-old female C57BL/6J mice. The mice were sacrificed at 7 d after the injection and enucleated. The enucleated mouse eyes used for histological analysis were immersion fixed in 4% formalin and subsequently embedded in paraffin. 4 μm-thick serial sections were prepared from paraffin blocks. Sections were deparaffinized and hydrated by sequential immersion in xylene and graded alcohol solutions. The sections were mounted on glass slides and stained with hematoxlin and eosin. The slides were reviewed by two masked and independent observers (Kim JH & Yu YS) in a random order. For the evaluation of changes in the retinal layers as our previous description [19], the ratio of A (retina thickness from the internal limiting layer to the inner nuclear layer) to B (retina thickness from the internal limiting layer to the outer nuclear layer) was measured in all sections *via* light microscopy (Carl Zeiss, Chester, VA, USA).

2.12. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

5 μM GNP (20 nm), twice of the effective therapeutic concentration, was intravitreally injected to 8-week-old female C57BL/6J mice. The mice were sacrificed