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Anti-angiogenic effect of caffeic acid on retinal neovascularization

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ABSTRACT

Pathological angiogenesis is the most common cause of vision loss at all ages including retinopathy of prematurity (ROP), diabetic retinopathy, and age-related macular degeneration. ROP is a proliferative disease of the retinal vasculature in premature infants. Herein, we demonstrated caffeic acid (CA) has the anti-angiogenic activity to retinal endothelial cells and retinal neovascularization in a mouse model of ROP, which might be related to the suppression of ROS-induced VEGF expression. CA effectively inhibited VEGF-induced proliferation of retinal endothelial cells in concentration-dependent manner. In addition, VEGF-induced migration and tube formation of retinal endothelial cells were completely inhibited. This anti-angiogenic activity of CA on retinal endothelial cells was related to the anti-oxidant activity: the inhibitory activity of CA to H_2O_2-induced reactive oxygen species production and VEGF expression. Interestingly, CA significantly suppressed retinal neovascularization in oxygen-induced retinopathy as the animal model of ROP without retinal cytotoxicity. These data suggests that CA could be a potent anti-angiogenic agent for retinal neovascularization, and be applied in the treatment of other vaso-proliferative retinopathies.

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1. Introduction

Angiogenesis, the formation of the new vessel, is regulated by the balance of many stimulating or inhibiting factors (Folkman, 2006). Physiological angiogenesis is under strict control, which is activated only under strictly defined conditions such as development and tissue repair. However, disruption of this balanced functioning could lead to excessive formation of blood vessels, pathological angiogenesis. In particular, this pathological angiogenesis also exist within the eye (Gariano and Gardner, 2005). Pathological angiogenesis is the most common 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 (Aiello et al., 1994).

ROP is a leading cause of blindness in newborns (Gibson et al., 1990). ROP is a proliferative disease of the retinal vasculature in premature infants, characterized by abnormal proliferation of fibrovascular tissue at the border of vascularized and non-vascularized retina (Roth, 1977). In pathogenesis, ROP occurs through the vaso-obliteration followed by the pathologic angiogenesis in developing retinal vasculature (Chen and Smith, 2007). Oxygen-induced retinopathy (OIR) in the mouse is a highly reproducible animal model of ROP, which is based on hyperoxia-induced vaso-obliteration of capillaries in mouse pups and their subsequent return to room air (Smith et al., 1994). This triggers retinal angiogenesis starting from the inner retina, characterized by growing into the vitreous. In this oxidative stress, production of reactive oxygen species (ROS) condition increased, which directly correlates with vascular endothelial growth factor (VEGF) expression and angiogenesis and *in vivo* (Lelkes et al., 1998; Ruef et al., 1997; Shono et al., 1996). In addition, the anti-oxidant system in preterm infants is highly stressed and incompletely developed (Buhimschi et al., 2003). Therefore, ROP could be thought to be related to the action of reactive oxygen species (ROS) (Abran et al., 1997).

Caffeic acid (CA) is 3, 4-dihydroxycinnamic acid as a phenolic compound which is widely distributed in plants including vegetables, fruits, and coffee (Sondheimer, 1958). CA possesses a wide spectrum of biological activities, e.g. anti-cancer, anti-oxidant, anti-inflammatory properties (Banskota et al., 2001). Recently, CA has been reported to tumor angiogenesis by inhibiting VEGF expression *via* suppression of STAT3 phosphorylation (Jung et al., 2007). Based on the anti-oxidant and anti-angiogenic effects of CA, retinal neovascularization of ROP might be a target for the pharmacological application of CA.

Abbreviations: AMD, age-related macular degeneration; CA, caffeic acid; DCFH-DA, 2′, 7′-dichlorofluorescin diacetate; DR, diabetic retinopathy; HRMEC, human retina microvascular endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OIR, oxygen-induced retinopathy; PBS, phosphate-buffered saline; ROP, retinopathy of prematurity; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; VEGF, vascular endothelial growth factor.

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In the current study, we demonstrated CA inhibits proliferation and migration of retinal endothelial cells and *in vitro* angiogenesis of tube formation, which was related to the suppression of ROS-induced VEGF expression. Moreover, CA significantly reduced retinal neovascularization in a mouse model of ROP without cytotoxicity under therapeutic concentration. These findings suggest that CA might be pharmacologically applied to vaso-proliferative retinopathy including DR, AMD, as well as ROP.

2. Materials and methods

2.1. Animals

C57BL/6 mice were purchased from Samtako (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.

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 (bFGF, Millipore, Bedford, MA, USA), and 10 U/ml heparin (Sigma, St. Louis, MO, USA). HRMECs used in this study were taken from passages 4 to 6.

2.3. Cell proliferation assay on retinal endothelial cells

Cell proliferation was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as our previous description (Kim et al., 2008a,b,c). HRMECs (1×10^5 cells) were plated in 96 well plates and cultured overnight. Cells were treated with vascular endothelial growth factor (VEGF) (20 ng/ml) (Sigma, St. Louis, MO, USA) or CA (10–200 μM), kindly provided by Kim MY (Angiolab, Daejeon, Korea) for 24 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).

2.4. Wound migration assay on retinal endothelial cells

Cell migration was evaluated with wound migration assay modified from our previous description (Jun et al., 2007). HRMECs (1×10^5 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 100 μM CA 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.

2.5. Tube formation assay on retinal endothelial cells

Tube formation was assayed as our previous description (Min et al., 2007). HRMECs (1×10^5 cells) were inoculated on the surface of the Matrigel, and treated with 100 μM CA 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.

2.6. Intracellular ROS assay

Intracellular ROS was assayed by measuring the oxidative conversion of cell permeable 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) to fluorescent dichlorofluorescein. HRMECs (1×10^5 cells) were treated with CA (50–200 μM) an hour prior to treatment with 100 nM H_2O_2. After 12 h of CA treatment, cells were labeled with 20 μM of 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich Co., St. Louis, MO, USA) for 30 min at 37 °C. The ROS-oxidized form of DCFH-DA was measured using fluorescence microscopy (BX50, OLYMPUS, Tokyo, Japan) with excitation and emission settings of 495 and 525 nm, respectively.

2.7. Western blot analysis

HRMECs were seeded in 100 mm dish (5×10^5 cells), and 100 μM CA or 100 nM H_2O_2 was treated for 12 h. The cell lysates were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene fluoride membranes (Milipore, Bedford, MA, USA) using standard electroblotting procedures. Blots were then blocked and immunolabeled overnight at 4 °C with primary antibodies of anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-ß-actin (Upstate Biotechnology, Lake Placid, NY, USA). Immunolabeling was detected by an enhanced chemiluminescence kit (Amersham Life Science, Inc. Buckinghamshire, UK) according to the manufacturer's instructions.

2.8. Oxygen-induced retinopathy

OIR was induced as described by Smith et al. (1994) with some modifications (Kim et al., 2007, 2008b). Briefly, newborn mice were randomly assigned to experimental and control groups. At postnatal day (P) 7, pups (5 to 7 pups) in the experimental group were exposed to hyperoxia ($75\%\pm0.5\%$ O_2) for 5 days (P7 to P11) and then returned to normoxia (room air) for 5 days. Neovascularization occurs upon return to normoxia and peaks at P17. To assess the anti-angiogenic activity of CA, the pups were injected intravitreously with 100 μM CA in 1 μl phosphate-buffered saline (PBS) on P14, when retinal neovascularization began. These experiments were repeated at least three times.

2.9. Qualitative assessment of retinal neovascularization by fluorescein angiography

As our previous description (Kim et al., 2007, 2008b), at P17, 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×.

2.10. Quantitative assessment of retinal neovascularization by counting vascular lumens

As our previous description (Kim et al., 2007a; Kim et al., 2008b), at P17, 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