

Potential protective effect of angiopoietin-1 on the leakage of rat choroidal neovascularization

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ABSTRACT

الأهداف: دراسة الأثر الفعال للأنجيوبيوتين على التسرب المشيمي النوعي للفغران.

الطريقة: أجريت هذه الدراسة في قسم العيون، جامعة شandong للطب التقليدي، جنان، الصين خلال الفترة من يونيو 2012م حتى يونيو 2013م. تم تحرير 30 فأر بالليزر. كما أجري تصوير القاع للفلورسين في الجسم وتقنية المرضية لاكتشاف أثر عامل نمو الباطني الوعائي وورقان الأنجيوبيوتين داخل الجسم. كما تمت دراسة ميشيل ثيازول ومقاييسة MTT لدراسة تكاثر الخلايا الباطنية الشبكية البقرية بعد العلاج بواسطة عامل النمو الباطني الوعائي والأنجيوبيوتين. كما تم استخدام ميشيل ثيازول ومقاييسة MTT لدراسة تكاثر الخلايا الباطنية الشبكية البقرية بعد العلاج بواسطة عامل نمو الباطني الوعائي والأنجيوبيوتين، كما تم استخدام فحص انتقال الإلكترون لدراسة التغيرات التي حدثت تحت استخدام عامل نمو الباطني الوعائي والأنجيوبيوتين.

النتائج: في نموذج التسرب المشيمي النوعي للفغران ظهر تسرب أقل في مجموعة الأنجيوبيوتين من مجموعة التحكم أو مجموعة عامل نمو الباطني الوعائي. أظهرت مقاييسة MTT أن استخدام مقاييسة MTT يساعد على تكاثر الخلايا الباطنية الشبكية البقرية. كما يساعد عامل نمو الباطني الوعائي على التكاثر في تركيز أقل وفي تركيز يصل إلى 50 نانوغرام/مل. استخدام عامل نمو الباطني الوعائي مع الأنجيوبيوتين ينعد أثر الأنجيوبيوتين لوحده. أظهرت نتائج مقاييسة MTT أن موصلات داخل الخلية في مجموعة عامل نمو الباطني الوعائي أقل بالمقارنة مع مجموعة التحكم. كما أن موصلات داخل الخلية في مجموعة الأنجيوبيوتين ومجموعة عامل نمو الباطني الوعائي داخل الخلية طبيعية جمعيها.

خاتمة: يساعد الأنجيوبيوتين في تكوين موصلات داخل الخلية ويقلل من التسرب المشيمي النوعي.

Objectives: To observe the potential protective effect of angiopoietin-1 (Ang-1) on rat choroidal neovascularization (CNV) leakage.

Methods: The study was conducted at the Eye Institute of Shandong University of Traditional Chinese Medicine, Jinan, China from June 2012 to June 2013. Thirty CNV model rats were induced by laser. In vivo, fluorescein fundus angiography and pathological techniques were applied to detect the effect of vascular endothelial growth factor (VEGF) and Ang-1 intravitreous injection. In vitro, 3-(4, 5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide (MTT) assay was applied to detect the proliferation of cultured bovine retinal endothelial cells (BRECs) after treatment with VEGF and Ang-1. Transmission electron microscopy (TEM) was used to detect the morphological changes under VEGF and Ang-1.

Results: In the CNV rat model, less late leakage was found in the Ang-1 group than the vehicle control or the VEGF group. The MTT assay showed Ang-1 administration inhibited the proliferation of BRECs. The VEGF promoted proliferation at low concentrations and inhibited the proliferation when its concentration reached 50 ng/ml. The administration of VEGF+Ang-1 rescued the inhibition effect of Ang-1 alone. The TEM results showed that there were less intercellular junctions in the VEGF group compared with the vehicle control. In the VEGF + Ang-1 group, the intercellular junctions were nearly normal.

Conclusions: The Ang-1 can induce intercellular junction formation and decrease the CNV leakage.

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Choroidal neovascularization (CNV) is frequently observed in patients with high myopia, angioid streaks, inflammation, diabetic retinopathy (DR), and age-related macular degeneration (AMD). Although the molecular basis for CNV is not well understood, an imbalance between pro-angiogenic and antiangiogenic factors has been proposed in pathologic neovascularization. In previous studies, vascular endothelial growth factor (VEGF) has been identified as a validated target for several retinal vascular diseases.^{1,2} Therefore, anti-VEGF treatment has been the main method for the therapy of CNV. But, it has been shown that retinal pigment epithelium (RPE)-derived soluble VEGF has been playing an important role in maintenance of the choriocapillaris,³ and VEGF is neuroprotective in the retina. Complete blocking of VEGF could alleviate neovascularization. It could also block the positive effects of VEGF and create some side effects; for example, systemic thrombosis.⁴ Angiopoietin-1 (Ang-1) was first identified as a ligand for the Tie-2 receptor, which may be involved in endothelial developmental processes. Following the discovery of the Vascular Endothelial Growth Factor Receptor (VEGFR) system, the Tie receptors, with their corresponding angiopoietin ligands, were identified as the second endothelial cell (EC)-specific receptor Tyrosine kinase signaling system.⁵ The roles of the Ang-Tie system in controlling sprouting angiogenesis are defined by different experiments. Recent reports have shown that Ang-1 directly interacts with myocytes, ECs, and fibroblasts through integrin to mediate survival, cell adhesion, migration, vascular remodeling, and the transition from the quiescent to the activated EC phenotype.^{7,8} Angiopoietin-1 may also selectively downregulate certain factor gene expressions, thereby maintaining intravascular stability, and reducing vascular permeability. Compared with VEGF, Ang-1 has the advantages of fewer side effects and the positive role of angioplasty. The latest studies show that coexpression of Ang-1+VEGF improved blood-retinal barrier (BBB) integrity, and resulted in better neuroprotection compared with VEGF expression alone.⁹ Our study

want to know when neovascularization was induced by VEGF under some pathophysiological circumstances, angiopoietin remodeled and matures the abnormal neovessels and alleviates leakage and haemorrhage. That will be a new pathway for neovascularization treatment.

Methods. *Animals and reagents.* A selection of 6-8 week old pathogen-free female Brown Norway rats (200-300 g) were purchased from Vital River Laboratories (Beijing, China) and were housed and maintained in the animal facilities of the Eye Institute of Shandong University of Traditional Chinese Medicine (TCM), Jinan, China. Exclusion criteria included rats with other related eye diseases, as checked via a slit lamp and hand-held ophthalmoscope. All animal studies conformed to the ARVO statement for the Use of Animals in Ophthalmic and Visual Research. Institutional approval was obtained, and institutional guidelines regarding animal experiments were followed. Angiopoietin-1 and VEGF of human and rat were purchased from R&D Systems (Minneapolis, MN, USA). Our study was conducted at the Eye Institute of Shandong University of TCM, Jinan, China between June 2012 and June 2013.

Laser-induced choroidal neovascularization rat model and intravitreous cavity injection. We created the rat CNV model as previously described by Tobe et al. Dobi et al¹⁰ The rats were anesthetized with an intraperitoneal injection of ketamine (5 mg/kg). The pupils were dilated by topical administration of 1% tropicamide (Santen Pharmaceutical, Osaka, Japan). Ten laser spots concentric with the optic nerve were placed avoiding the major retinal vessels in the right eye of each rat using a diode laser (Carl Zeiss, Oberkochen, Germany). The laser parameters were spot size 100 μm, laser power 120 mw, and exposure time 0.1 seconds. The Bruch's membrane breakage was confirmed by the end point 'bubble formation'. Rats with intraocular hemorrhage on laser administration were excluded from the study.

After the laser burn, 30 rats were randomly divided into 3 groups of 10 rats each, the Ang-1 group, the VEGF group, and the control group. For these 3 groups, 50 μl Ang-1 (0.2 μg/ml), 50 μl VEGF (0.2 μg/ml), and the same volume of phosphate buffered solution (PBS) were injected into the intravitreous cavity of the right eye. The injections were performed on day (D) one and repeated on D3 after laser surgery.

Fundus photography and fluorescein fundus angiography. Fundus photography and fluorescein fundus angiography (FFA) were performed on the 3 groups of rats after laser treatment and intravitreal

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injection. The FFA was performed on D14. The rats were intraperitoneally injected with 0.5 mg/kg 10% fluorescein sodium. Photographs were taken by a fundus camera (Heidelberg, Heidelberg, Germany). Leakage of fluorescein associated with CNV lesions was monitored at several time points until 30 minutes after the dye injection including the arterial phase, the early arteriovenous phase, and the late arteriovenous phases. The FFA image output was to UTSCSA image analysis software. The background of the image of the entire FFA grayscale calibration 100 (\pm 1) grayscale. Selected a circular area, which was the optic disc as the center, 7.40 mm as the radius. Measured the gray value of CNV leakage area in the circular of the 3 groups. Repeated 3 times, and take the average.

Pathology. The neovascularization status in the eye was confirmed by histopathology. The rats were sacrificed by a sodium pentobarbital overdose, and the whole eyes were collected 14 days after laser treatment. The whole eyes were immersed for one hour in 4% phosphate buffered glutaraldehyde, and transferred to 10% phosphate buffered formaldehyde until procession. The fixed and dehydrated tissue was embedded in methacrylate and 5 μ m sections cut through the pupillary-optic nerve plane and stained with hematoxylin and eosin. Presence or absence of neovascularization was evaluated blindly by examining 6 sections cut at different levels for each eye.

Bovine retinal endothelial cells culture. Bovine retinal endothelial cells (BRECs) were obtained from harvested bovine eyes, as described by Capetandes,¹¹ and the cells were resuspended in Dulbecco's Modified Eagle Medium (DMED) supplemented with 10% (v/v) fetal bovine albumin, 100 U/ml penicillin G, and 100 mg/ml streptomycin sulphate, and plated in 25 mm² culture flasks (NEST, Suzhou, China) and then incubated at 37° under 5% CO₂. The culture medium was changed every other day. The third passage was used in the following experiments.

Endothelial cells proliferation assay. An 3-(4,5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide (MTT) assay was used to detect cell proliferation. To evaluate the bioactivity of Ang-1 and VEGF on ECs in vitro, the proliferation of BRECs was taken as an index. The cell concentration was accommodated to 1.0 \times 10⁵ cells/ml density by culture media. A 200 μ l cell suspension solution was adding to each well of a 96-well plate. We incubated the plates at 37°, 5% CO₂ overnight. The Ang-1 or VEGF was added with different concentrations or a combination of both. After 24 hours incubation, the media was replaced by

MTT containing media (5 mg/ml) in each well for an additional 4 hours. Dimethylsulfoxide (DMSO) 150 μ l/well was added after abandoning the media. The spectrophotometric absorbance was measured at 490 nm, with an automatic microplate reader Bioteck, Vermont, USA.

Transmission electron microscopy. The BRECs were grown on Thermanox cover slips to confluence. Cells were then fixed in 2.5% glutaraldehyde and washed in 0.1 M cacodylate buffer for approximately 30 minutes. For transmission electron microscopy (TEM), the fixed samples were osmicated with 1% osmium tetroxide, dehydrated in ascending ethanol concentrations (70-100% in 6 steps), and embedded in a 1/1 mixture of epoxy resin and ethanol for 2 hours. After 2 hours, fresh epoxy resin was added to the samples and they were left overnight. Then, the resin was polymerized for 2 days at 60°. Ultrathin sections were cut and contrasted with 3% uranyl acetate and lead citrate. Samples were visualized with a Zeiss EM 906 (Carl Zeiss, Oberkochen, Germany).

Statistical analysis. Data were expressed as the mean \pm SD for the results from at least 3 separate experiments. All data were analyzed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 16. One-way analysis of independent samples t-test for 2-sample comparisons was used to compare the means of 2 groups. P<0.05 was considered statistically significant.

Results. *Angiopoietin-1 counteracted the permeability-inducing effects of VEGF.* Laser burn effectively induced CNV in the Brown Norway rats. The presence of CNV was identified by FFA. For CNV, hyperfluorescence develops around the burn, which progresses to late diffuse leakage with dye pooling in the serous detachment surrounding the burning area. From fundus photography, there was fresh hemorrhage beside the laser burn sites in the control group (Figure 1A) and in the VEGF group (Figure 1B). For the Ang-1 group, there was no hemorrhage, and no exudates surrounding the laser burn (Figures 1C & 1D). Among the 30 rat models, on D14 after laser burn, the CNV occurrence rate of the control group was 65.53 \pm 3.53%, the VEGF group was 68.78 \pm 4.75%, and the Ang-1 group was 32.33 \pm 3.47%. There was late dye leakage around the laser scar in the control (Figure 2A) and VEGF groups (Figure 2B). The Ang-1 group showed late mild leakage compared with control and VEGF groups (Figures 2C & 2D).

Angiopoietin-1 stabilized and normalized laser-induced CNV. The CNV of the 3 groups was confirmed

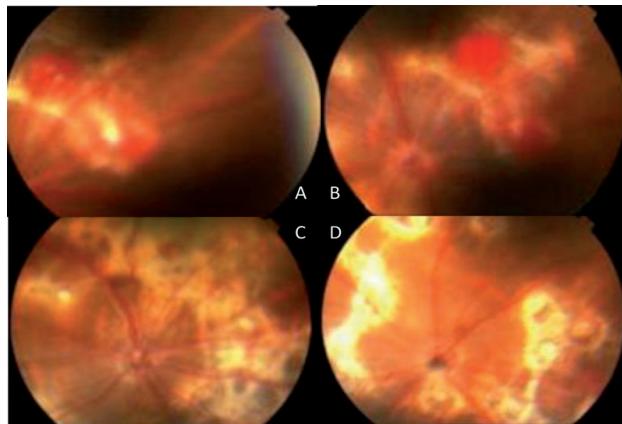


Figure 1 - Representative fundus photographs indicate Angiopoietin-1 (Ang-1) treatment significantly reduced the choroidal neovascularization leakage and hemorrhage compared with the vascular endothelial growth factor (VEGF) group and control group. There is hemorrhage around the laser scar in control group A) and VEGF group B). For the Ang-1 group, there was no hemorrhage and exudates surrounding the laser burn C, D).

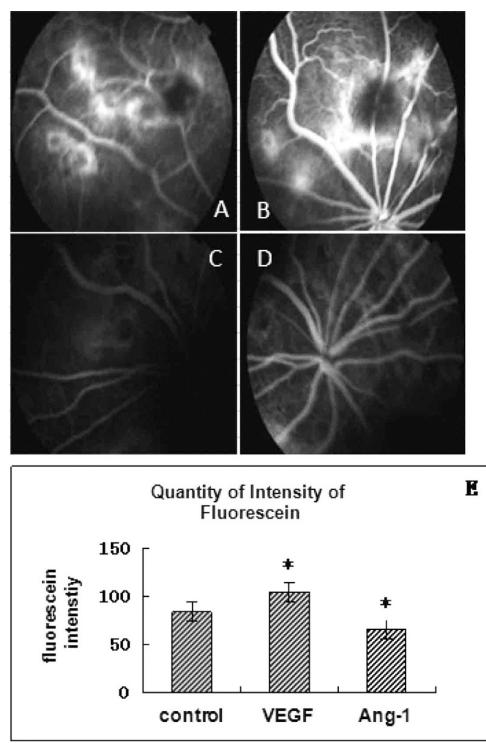


Figure 2 - Representative fluorescein fundus angiography indicates Angiopoietin-1 (Ang-1) treatment significantly reduced the choroidal neovascularization leakage compared with the vascular endothelial growth factor (VEGF) group and control group. There is late dye leakage around laser scar in the control group A) and VEGF group B). For Ang-1 group, there is no leakage surrounding the laser burn C, D). The quantity of intensity and area of fluorescein leakage in the different groups is shown in the chart E).

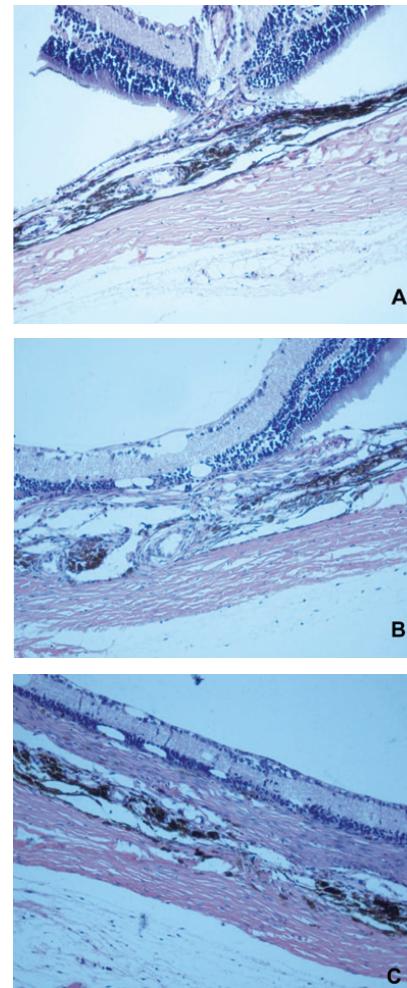


Figure 3 - The choroidal neovascularization (CNV) of the 3 groups was confirmed by pathological examination. In the control group and the group with intravitreous cavity the vascular endothelial growth factor injection, there were neovessels and hemorrhage at the choroid layer A, B). On the contrary, in the group with intravitreous cavity Angiopoietin injection, the neovessels at the choroid layer had a 'dry' and 'mature' appearance C).

by pathology. In the control and VEGF groups, there were neovessels and hemorrhage at the choroid layer (Figures 3A & 3B). On the contrary, in the Ang-1 group, the neovessels at choroid layer had a dry and mature appearance (Figure 3C).

Vascular endothelial growth factor and angiopoietin-1 affected the proliferation ability of endothelial cells. In vitro, VEGF promoted the proliferation of BRECs at a low concentration in a dose-dependent manner. However, when the VEGF concentration reached 50 ng/ml, the promotion effect switched to an inhibition effect. The proliferation

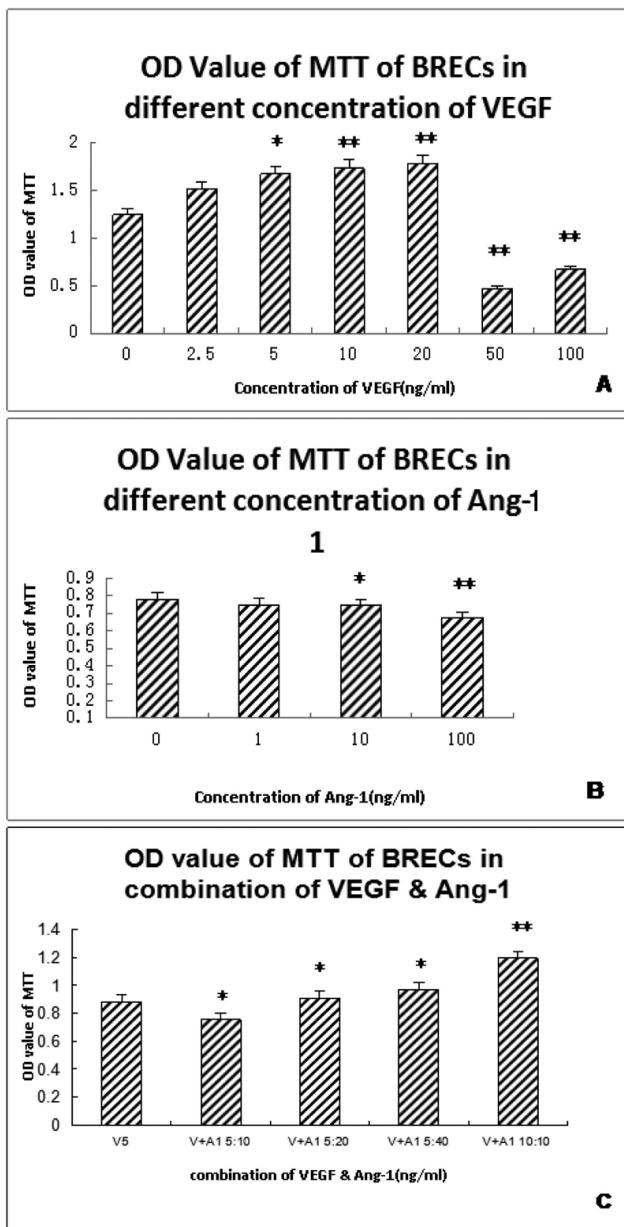


Figure 4 - In vitro, vascular endothelial growth factor (VEGF) promoted the proliferation of bovine retinal endothelial cells (BRECs) at a low concentration in a dose-dependent manner. However, when the VEGF concentration reached 50 ng/ml, the promotion effect switched to an inhibition effect. The proliferation decreased to 38% of the original value A). Angiopoietin-1 (Ang-1) inhibited proliferation of BRECs in vitro in a dose-dependent manner B). In the presence of VEGF, the inhibition effect of Ang-1 no longer existed. The proliferation of BRECs remains at a stable level even though the Ang-1 concentration increased to 40 ng/ml. In the VEGF and Ang-1 with different concentration ratios such as, 5:10, 5:20, 5:40, and 10:10, the BRECs proliferations were the same as that with 5 ng/ml VEGF C). *Comparison with the control group ($*p<0.05, **p<0.01$). OD - optical density, MTT - 3-(4, 5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide.

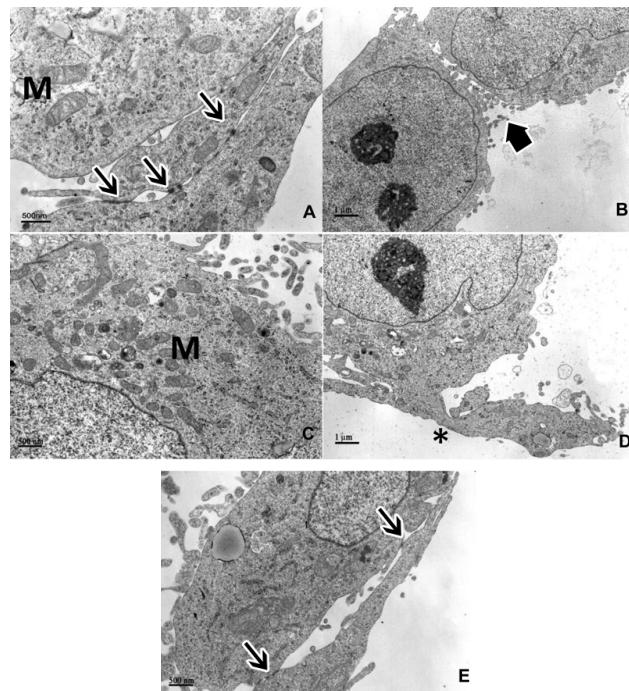


Figure 5 - Transmission electron microscopy of bovine retinal endothelial cells (BRECs). In normal endothelial cells (EC), there are intercellular junction complexes such as tight junctions, adherens junctions, and gap junctions (arrows). Double-layer membrane and crest of mitochondria were normal A). In the vascular endothelial growth factor (VEGF) group, there are more microvilli and less intercellular junctions (arrow) than the control group B). There are numerous mitochondria (M) C). There are bundle like myofilaments (star) D). In the VEGF+Angiopoietin-1 group, the intercellular junctions were nearly normal (arrows) E).

decreased to 38% of the original value (Figure 4A). Angiopoietin-1 inhibited the proliferation of BRECs in vitro in a dosage-dependent manner (Figure 4B). However, with VEGF, the inhibition effect of Ang-1 no longer existed. In both VEGF + Ang-1 with different concentration ratios such as, 5:10, 5:20, 5:40, and 10:10, the BRECs proliferations were the same as that with 5 ng/ml VEGF C). *Comparison with the control group ($*p<0.05, **p<0.01$). OD - optical density, MTT - 3-(4, 5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide.

Transmission electron microscopy. In normal ECs, there were intercellular junction complexes such as tight junctions, adherens junctions, and gap junctions. The double-layer membrane and crest of mitochondria were normal (Figure 5A). In the VEGF group, there were more microvilli and less intercellular junctions than the control group (Figure 5B). There were bundle like myofilaments and numerous mitochondria (Figures 5C & 5D). In the VEGF + Ang-1 group, the intercellular junctions were nearly normal (Figure 5E).

Discussion. Choroidal neovascularization is the result of a series of pathological events affecting the

retinal pigment epithelium, Bruch's membrane, and the choroid. The CNV model was induced in the Bruch's membrane rupture by laser blasting. The VEGF family of proteins is the most important family of angiogenic factors that controls blood vessel formation.¹¹ However, there is evidence that excessive human VEGF expression could bring about pathological and immature vessel formation and enhanced vascular wall permeability, and could lead to angioma formation.¹² Translational exploitation of the VEGF-VEGF receptor research has, in 15 years, led to the clinical implementation of antiangiogenesis as the development of a vision-enhancing treatment for AMD.

During pathological ocular angiogenesis, leakage and hemorrhage usually result from immature neovascularization. If some factors could promote maturation of neovascularization, the newly formed vessels would not leak and bleed. As we know, in normal physiological process, there are factors that guard the formation and maturation of the blood vessels. A protein that maintains stability after maturation of newly grown capillaries is Ang-1. For laser induced CNV, the Ang-1 group showed late mild leakage compared with the control and VEGF groups. Pathology examination confirmed that in the Ang-1 group, the neovessels existing at the choroid layer had a 'dry' and 'mature' appearance. Our results indicate that Ang-1 stabilized and normalized the laser-induced CNV, and Ang-1 suppressed VEGF-induced breakdown of the blood-retinal barrier. It is generally accepted that Ang1-mediated activation of Tie2 promotes vascular stabilization and quiescence,¹³ Ang-1 inhibits VEGF-mediated increases in endothelial permeability.¹⁴ Angiopoietin-1 could be produced by pericytes. Its presence in mature capillaries improves continuity of the basal membranes and the adherence of pericytes to ECs. During angiogenesis it promotes capillary growth.

The transgenic overexpression of Ang-1 decreases vessel leakage in response to permeability-inducing inflammatory agents.¹⁴ Systemic delivery of Ang-1 into adult mice has similar effects that counteract the permeability-inducing effects of VEGF administration.¹⁵ The stimulatory effects of VEGF on ECs proliferation have been well reported in vitro and in vivo.¹⁶ As our in vitro results showed, 5 ng/ml VEGF significantly promoted the proliferation of ECs. The Ang-1 inhibited proliferation of BRECs. In the presence of VEGF, the inhibition effect of Ang-1 was reversed. Both VEGF and Ang-1 are 2 important angiogenic factors. When used them together, there existed synergistic effect.

As we know, CNV and vascular leakage are the major causes of visual loss in exudative AMD. The VEGF expression is increased in membranes of AMD patients and in animal models induced by laser treatment.¹⁷⁻¹⁹ The ECs transform from the quiescent to the activated phenotype in AMD. The effect of Ang-1 on ECs is on cell permeability, but not on cell proliferation. The Ang-1 reduces the leakage of the blood vessels through Rho GTPases, and the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway and changes the balance between them.²⁰ The resting endothelial layer establishes adherens junctions and tight junctions, which regulate peri-endothelial permeability. In vitro culture, VEGF evoked proliferating ECs with ultrastructural changes features such as less intercellular junctions, more microvilli and numerous mitochondria. It promoted vascular leakage by abnormal cell structures. Ang-1-induced Tie2 signaling was directly reduced ECs permeability, it could help ECs intercellular junctions recovered normal.

Angiopoietin-1 is known to have an important function in recruiting pericyte and smooth muscle cells for maturation and maintenance of the vascular system,²¹ and it has been shown to enhance collateral vessel formation in an animal model with myocardial and hind limb ischemia.^{21,22} Angiopoietin-1 overexpression stimulates pericyte coverage, resulting in a vasculature that is more mature in appearance. The resulting vasculature seems to be normal, and therefore the process of Ang-1-mediated vessel stabilization has been named normalization.²³ In addition, recent data also suggested that altered migration may also contribute to pericyte loss in DR and that this mechanism is regulated by signaling via the Ang-1/Tie-2 pathway.²⁴

Angiopoietin-1 not only modifies the ultrastructure of ECs, but also improves EC function. Our study has shown that Ang-1 could stabilize and assist in the maturation of blood vessels. In summary, Ang-1 normalizes EC ultrastructure and alleviates leakage of CNV; these conclusions provide new research ideas for the clinical treatment of CNV and systemic vascular diseases.

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