LXHY Formula Inhibits Choroidal Neovascularization Development via Inhibiting the Recruitment and Adhesion of BMCs to the Retina

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Abstract

Objective: The objective of this study is to investigate the inhibitory effect of LXHY, a Chinese medicine compound formula, on choroidal neovascularization (CNV) and to find the possible working mechanism. Methods: CNV was induced in C57BL/6 mice by krypton laser and bone marrow-derived cells (BMCs) isolated from enhanced green fluorescent protein (EGFP) transgenic mice were injected through tail vein 0.5–1 h after the laser surgery. The BMC-treated mice were randomly divided into two groups gavaged with either distilled water (DW group) or LXHY formula solution from day 1 after laser surgery. On days 7, 14, and 28 after treatment, histopathologic examination, fundus fluorescein angiography, and choroidal flatmount assay were performed to measure the CNV severity and BMC recruitment. CXCR4 levels in peripheral blood were measured by enzyme-linked immunosorbent assay. Stromal cell-derived factor-1α (SDF-1α), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) were detected by immunofluorescent staining. Results: On days 7 and 14 after treatment, CNV lesions in the LXHY-treated mice showed less recruitment of BMCs and were smaller in size compared to DW-treated mice. Histological examination also confirmed less severe CNV lesions in the LXHY group. CXCR4 levels in peripheral blood in the LXHY group were less than that of DW group on days 7 and 14. Moreover, the expression levels of SDF-1α, ICAM-1, and VCAM-1 at the lesion sites in the LXHY group were lower compared with the DW group. Conclusion: This experiment indicated that LXHY formula could inhibit CNV formation and development, probably by inhibiting the recruitment and attachment of BMCs into CNV area.

Keywords: Age-related macular degeneration, bone marrow-derived cells, choroidal neovascularization, LXHY formula

Introduction

Choroidal neovascularization (CNV) is characterized by the formation of new blood vessels that arise from the choriocapillaris through Bruch’s membranes into the subretinal space, causing exudation of fluid and hemorrhaging. CNV is often accompanied by the atrophy and senescence of retinal pigment epithelial (RPE) cells and microfractures in Bruch’s membranes. Consequently, the overlying neurosensory retina may detach, and the ensuing damage to the retinal photoreceptors could lead to irreversible visual loss.¹ CNV is now known to be a common manifest in nearly 40 ophthalmic diseases affecting people of all ages, especially the elderly.² The most common condition associated with CNV is age-related macular degeneration (AMD), which is the primary cause of vision loss in people aged 50 and over, affecting 28% of people older than 75 years.³ Of the two forms of AMD, the dry form is by far the most common, accounting for nearly 90% of reported cases. However, 90% of all vision loss occurring from AMD results from the wet form, which is characterized by CNV.⁴ Anti-VEGF medicaments changed significantly paradigm in the treatment of AMD, enabling the essential opening of the door to a targeted therapy. At the very beginning of introduction in AMD therapy, they demonstrated clear effects as improvement of visual functions compared with other therapies and thus became the most effective treatments for AMD. The most effective treatments for AMD are based on the targeting of VEGF, which is over-produced in neovascularization and is essential for the growth and survival of choroidal neovascularization.⁵,⁶ However, recent studies have shown that VEGF is not the only mediator of choroidal neovascularization.⁷ Another major mediator is CXCR4, a receptor for SDF-1α, which is a chemokine that plays a crucial role in the development of choroidal neovascularization.⁸,⁹ Therefore, a combination of VEGF and CXCR4 inhibitors may be more effective in treating CNV.

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LXHY formula, a Chinese herbal preparation, is an herbal formula that has been used for centuries to treat several diseases, including neovascular ocular diseases such as wet diabetic retinopathy and age-related macular degeneration (AMD). The mechanism of action of LXHY formula is multifaceted and involves multiple targets.

In the present study, we tested this hypothesis in a murine model of choroidal neovascularization (CNV). Mice were treated with LXHY formula every day (30 mg/kg/d) for 4 weeks, or with a control group receiving distilled water (DW). We observed a significant reduction in choroidal neovascularization in the LXHY formula group compared to the control group.

The mechanism of action of LXHY formula on CNV involves several pathways. LXHY formula has shown to inhibit endothelial progenitor cell mobilization from the bone marrow to peripheral blood in mice with CNV induced by laser.

The whole process of bone marrow-derived cells (BMCs) contributes to CNV development includes mobilization of BMCs, recruitment and adhesion to CNV, and differentiation into vascular cells. Thus, blocking any step of the process may be a novel and potent therapeutic strategy in treating CNV formation.

Although current therapeutic strategies to treat CNV are effective for the majority of patients, we have been searching for more alternative therapies with improved efficacy, reduced cost, and less complications. A rich literature including ancient Chinese medical records and modern Chinese studies in the past decades has shown some success of traditional Chinese medicine treating neovascular ocular diseases such as wet diabetic retinopathy and AMD.

LXHY formula has been used to treat eye diseases associated with CNV in our clinic practice for decades. Our pilot clinical studies have shown that LXHY formula has good therapeutic effect on AMD and idiopathic CNV, yet the mechanism is unclear. Many researches have demonstrated that Chinese herbs can regulate whole-body homeostasis by acting on multitargets at multiple levels. As the major functional component of LXHY, Salvia miltiorrhiza Bge (Labiatae) has been widely and successfully used in Asian countries for treating various vascular disturbance-related diseases for hundreds of years. It has documented role in antioxidation, anticoagulation, and antithrombotic effect.

We have previously shown that LXHY formula could inhibit endothelial progenitor cell mobilization from the bone marrow to peripheral blood in mice with CNV induced by laser. We deduced that LXHY formula might also inhibit the recruitment and attachment of BMCs to choroid, thus preventing the initiation and development of CNV. In the present study, we tested this hypothesis in a krypton laser-induced CNV mouse model.

**Methods**

**Animals**

C57BL/6J mice (wild type, WT) were obtained from Beijing HKF Bioscience Co., Ltd. (Beijing, China) and housed in the animal care facilities of Eye Hospital, CATCM. Transgenic mice homozygous for enhanced green fluorescent protein (EGFP) were a gift from the Surgical Lab of Beijing University. Mice were handled in accordance with the institutional guidelines for the care and use of laboratory animals, and protocols were approved by the Committee on the Ethics of Animal Experiments of the Eye Hospital, China Academy of Chinese Medical Sciences.

**Laser-induced choroidal neovascularization**

Animals were anesthetized with an IP injection of 35 mg/kg ketamine HCl mixed with 5 mg/kg xylazine. The eyes were dilated with 1 drop of Compound Tropicamide Eye Drops (Santen Pharmaceutical Co., Ltd., Japan). Lesions were induced with Lumenis Zeiss Novus Varia XL Laser (Lumenis Ltd., Israel). In each animal, 10 laser lesions were induced between major retinal vessels at a distance from the optic nerve head of approximately 1.5 times the diameter of the optic disc. The laser parameters were wavelength, 532 nm; spot size, 50 μm diameter; power, 200 mW; and duration, 0.1 s. Cavitation bubbles were observed, indicating the rupture of Bruch’s membrane.

**Bone marrow-derived cell preparation and transplantation**

Bone marrow was obtained from 3 male EGFP transgenic mice. The femur and tibia were dissected and placed in phosphate-buffered saline (PBS; pH 7.2) at 4°C. Bone marrow was obtained by slowly flushing media inside the diaphyseal channel with a syringe through a 27-gauge needle and then homogenized using an 18-gauge needle and filtered through 70-μm nylon mesh (BD Biosciences; San Jose, California). Bone marrow donor cells were centrifuged and the pellets resuspended in the medium mentioned above and then adjusted to 1.0 × 108 cells/ml with PBS. 0.5–1 h after laser photoocoagulation, the wild-type C57BL/6 mice were injected with BMCs (2.0 × 107 cells/0.2 ml) through tail vein. Sixty recipient mice were randomly divided into two groups: LXHY formula group (n = 30) and DW group (n = 30).

**Choroidal flatmount**

Choroidal flatmount was performed at days 7 and 14 after receiving treatment (n = 10 mice/group) as previously described. The mice were euthanized and their eye globes were enucleated and fixed in 4% formaldehyde for 2 h and then washed in 0.01 M PBS for 30 min. The RPE–choroid–sclera complex was harvested with a sharp blade. The RPE–choroid–sclera complex was permeabilized in 0.2% Triton X-100 in Tris-buffered saline for 24 h at room temperature after being washed with 0.01 M PBS for 24 h at room temperature. The RPE–choroid–sclera complex was permeabilized in 0.2% Triton X-100 in Tris-buffered saline for 24 h at room temperature after being washed with 0.01 M PBS for 24 h at room temperature. Finally, the RPE–choroid–sclera complex was mounted on a microscope slide and examined and photographed with confocal microscopy (Olympus FV1000;
The area of CNV was analyzed with Image ProPlus 6.0 software (Kodak, USA).

**Fundus fluorescein angiography**

CNV lesions were evaluated by fundus fluorescein angiography (FFA) as previously described. On day 28 after treatment, the CNV lesions were visualized using a digital fundus camera (Topcon, Japan). The mean area of CNV was calculated using Image ProPlus 6.0 by two independent ophthalmologists blinded to the experimental design.

**Histology and immunofluorescence**

Mice were euthanized at days 7, 14, and 28 after receiving treatment. Their eye globes were enucleated and fixed in 4% formaldehyde for 2 h, then transferred to a solution of 0.1 m phosphate buffer containing 20% sucrose overnight at 4°C, then dipped in sucrose-optimal cutting temperature (OCT) solution (2:1) for 2 h, and then embedded in OCT. Serial sections (8 μm) were cut on a cryostat (Leica, Heidelberg, Nussloch, Germany) along the vertical meridian of the eye. For histology, the sections were stained with hematoxylin and eosin. Images were obtained with the light microscope.

Immunohistochemical staining was carried out as described previously. Sections were washed with PBS for 3 times, then incubated with primary antibody stromal cell-derived factor (SDF)-1α (rabbit anti-mouse, 1:100, Biosynthesis Biotechnology, Beijing, China), vascular cell adhesion molecule-1 (VCAM-1) (rabbit anti-mouse, 1:100, Biosynthesis Biotechnology, Beijing, China), intercellular adhesion molecule-1 (ICAM-1) (rabbit anti-mouse, 1:100, Biosynthesis Biotechnology, Beijing, China) overnight at 4°C, washed with PBS for 3 times, then incubated with second antibody (goat anti-rabbit Alexa Fluor 555, 1:200, Biosynthesis Biotechnology, Beijing, China) for 3 h, washed with PBS for three times, then stained with DAPI for 5 min, and washed with PBS 2 times. The slides were coverslipped with anti-fading medium and examined with the confocal microscope.

**Enzyme-linked immunosorbent assay**

The CXCR4 level in peripheral blood was measured using commercial enzyme-linked immunosorbent assay (ELISA) Quantikine kits (RandD Systems, Minneapolis, USA) according to the manufacturer’s protocol.

**Statistical analyses**

Data were analyzed using Student’s t-test and ANOVA with the aid of Prism Software (version 3.0, GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

**RESULTS**

**Analysis of choroidal neovascularization area by choroidal flatmount and fundus fluorescein angiography**

In the mice treated by LXHY formula, the number of GFP cells incorporated into CNV decreased compared with that of water-treated mice on days 7 and 14 after treatment. Accordingly, the vascular area of CNV lesions was reduced significantly in the mice treated by LXHY formula as well. On day 7 after treatment, CNV areas were 0.8 ± 0.2 mm$^2$ and 2.1 ± 0.6 mm$^2$, respectively, in the LXHY group and the DW group ($P < 0.05$). On day 14, the CNV areas were 1.0 ± 0.3 mm$^2$ and 2.2 ± 0.5 mm$^2$, respectively, in the LXHY group and the DW group ($P < 0.05$) [Figure 1]. On day 28 after treatment, the CNV areas were still less in the LXHY group evaluated by FFA as shown in Figure 2.

**Histological analysis of choroidal neovascularization**

To further determine whether LXHY could decrease neovascularization severity, we performed histologic analysis of paraffin-embedded cross-section of the mouse retinal tissues. As shown in Figure 3, the retinal and choroidal lesion was milder in the LXHY group as compared to the DW group. At day 7 after treatment, defects in Bruch’s membrane and RPE were noted at the sites of laser spots. Disorder of outer and inner nuclear layer, large amounts of fibrovascular tissue, and densely packed RPE cells were observed, while no damage was found in the ganglion cell layer. At day 14 after treatment, fibrovascular tissue, lots of microphages, and RPE hypertrophy were noticed. The lesion height and width increased. At day 28 after treatment, lesions in the retina and choroid were still observable. CNV was encysted by proliferative RPE cells,
fibroblast cells, and collagenous fiber. However, regression of fibrovascular tissue and RPE hypertrophy was noticed. As shown in Figure d-f, neovascularization was reduced in size and the retina was partially restored in the LXHY group.

**Immunofluorescence evaluation**

At day 7 posttreatment, SDF-1α-positive cells were found in CNV, RPE layer around the lesion, and choroid beneath CNV. At day 14 after treatment, the immunoreactive intensities of SDF-1α were higher than day 7. However, the intensity declined at day 28 after treatment. Compared with the DW group, SDF-1α-positive cells in the LXHY group were less at the observation time points [Figure 4].

At day 7 posttreatment, VCAM-1-positive cells were found in CNV, RPE layer around the lesion, and choroid beneath CNV. At day 14 after treatment, the immunoreactive intensities of VCAM-1 were higher than day 7. At day 28, the intensity declined slightly. As compared to the DW group, the VCAM-1-positive cells in LXHY group were less than DW group at all time points [Figure 6].

**Enzyme-linked immunosorbent assay**

At day 7 after treatment, the content of CXCR4 in peripheral blood of the mice treated by LXHY was significantly less than that of the mice in DW group and normal group ($F = 8.107$, $P < 0.05$. Compared with the normal group, the content of CXCR4 in the DW group was slightly higher ($t = 0.290$, $P > 0.05$). At day 14 after treatment, both the contents of CXCR4 in the LXHY group and DW group were higher than itself at day 7. As compared to the LXHY group and normal group, the content of CXCR4 of the DW group was significantly higher ($t = 2.747$, $P < 0.05$; $t = 2.439$, $P < 0.05$).
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**Figure 4:** Stromal cell-derived factor-1α immunofluorescence staining of choroidal neovascular lesions (×200). Representative images of lesion in the DW group at day 7 after treatment (a), day 14 after treatment (b), and day 28 after treatment (c). Representative images of lesions in the LXHY group on days 7 (d), 14 (e), and 28 (f) after treatment.

**Figure 5:** Vascular cell adhesion molecule-1 immunofluorescence staining of choroidal neovascular lesions (×200). Representative images of lesions in the DW group on days 7 (a), 14 (b), and 28 (c) after treatment. Representative images of lesions in the LXHY group on days 7 (d), 14 (e), and 28 (f) after treatment.

**Figure 6:** Intercellular adhesion molecule-1 immunofluorescence staining of choroidal neovascular lesions (×200). Representative images of lesions in the DW group on days 7 (a), 14 (b), and 28 (c) after treatment. Representative images of lesions in the LXHY group on days 7 (d), 14 (e), and 28 (f) after treatment.
There was no apparent difference between the normal group and the DW group (\( t = 0.570, P > 0.05 \)). At day 28 after treatment, CXCR4 decreased markedly in the LXHY group and DW group. There was significantly difference among the three groups (\( F = 4.643, P < 0.05 \)), while the difference was not significant between the DW group and the LXHY group (\( F = 4.643, P < 0.05 \)) [Figure 7].

**DISCUSSION**

The mouse laser-induced CNV is a classical CNV animal model and mimic important aspect of wet AMD.[26-28] It has been reported that BMCs play an important role in the development and progression of CNV as mentioned above. Our results further confirmed these reports. In the flatmount examination, we observed many green cells in CNV lesion, which indicated that BMCs circulating in peripheral blood were recruited to the sites of Bruch’s rupture and contributed to the growth of CNV. As compared to the DW group, there were less BMCs in CNV in LXHY-treated mice, and the CNV area was also smaller determined by choroidal flatmount analysis and FFA examination. The result suggested that LXHY formula could inhibit the involvement of BMCs in CNV development and thus reduce the size of CNV. The pathological results also showed that the lesion in LXHY-treated mice was milder than that of DW-treated mice. On day 7 after treatment, the outer and inner nuclear layer disappeared at the photocoagulation spot, RPE layer, and Bruch’s membrane broke, but only slight migration and proliferation of RPE cells could be noticed. On day 14 posttreatment, there was a few fibrocyte and collagenous fiber in the photocoagulation area; CNV was not as apparent as the mice in the DW group. On day 28 after treatment, slight proliferation of RPE cells was found, and the scar was smaller as compared to the DW group. This result indicated that LXHY formula could inhibit CNV formation and development.

It has been reported that SDF-1α and CXCR4 play an important role in BMC recruitment to the areas of CNV. Expression of SDF-1α was markedly upregulated in CNV lesion and surrounding RPE cells, which recruited the BMCs carrying with CXCR4 from peripheral blood to CNV area.[29] Sengupta et al. found that the number of BMCs decreased significantly in CNV area after subretinal injection of antibody against SDF-1α, and the size of CNV was smaller as well than the DW group.[30] Our study showed that CXCR4 in the peripheral blood of LXHY-treated mice was significantly less than that of normal mice and DW-treated mice; meanwhile, expression of SDF-1α decreased accordingly in the retina of LXHY-treated mice. The result suggested that LXHY formula could inhibit the recruitment of BMCs to CNV through inhibiting SDF/CXCR4-mediated migration. Involvement of the recruited BMCs to CNV lesion requires the contribution of cell adhesion molecules VCAM-1 and ICAM-1. It was reported that immunostaining for VCAM-1 and ICAM-1 was strongly upregulated in retinal blood vessels under CNV.[31] Hou et al. found nicotine administration resulted in larger diameter and surface area of CNV. Nicotine-exposed mice demonstrated increased area and density of GFP + cells and increased GFP + vascular cells area. Meanwhile, the expression of VCAM-1 in choroid beneath CNV was upregulated in nicotine-exposed mice. The results suggest that nicotine promotes recruitment and incorporation of BMCs into CNV and affects differentiation of BMCs in CNV. These effects may be partly due to indirect actions of nicotine on BMCs through VEGF or VCAM-1.[32] Our study showed that expression of VCAM-1 was less in the retina of LXHY-treated mice as compared to that of DW-treated mice, and ICAM-1 had the same trend as VCAM-1. These results indicated that LXHY formula could prevent adhesion of BMCs into CNV and thus inhibit CNV formation, and the effect might be due to downregulating the expression of VCAM-1 and ICAM-1.

**CONCLUSION**

LXHY could reduce CNV probably by inhibiting the recruitment and attachment of BMCs into CNV area. These data support LXHY as an alternative and complementary therapy for CNV-associated disorders.

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**Conflicts of interest**

There are no conflicts of interest.

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