·Basic Research·

Expression of FLK-1 in laser-induced choroidal neovascularization in mouse

Han Zhang, Zhe-Li Liu

Department of Ophthalmology, the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China

Correspondence to: Han Zhang. Department of Ophthalmology, the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China. zhanghan73@126.com
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Abstract

• AIM: To investigate the expression of vascular endothelial growth factor receptor 2 (VEGFR-2, also known as FLK-1) in laser-induced choroidal neovascularization (CNV) in mouse.

• METHODS: CNV was induced in C57BL/6 mouse eyes by krypton laser photocoagulation. Choroidal fluorescein angiography and histopathological examination were used to assess the development of experimental CNV. Cryostat sections from lesions on day 10 after laser treatment and normal eyes were prepared for immunohistochemistry for FLK-1.

• RESULTS: Laser-induced CNV developed in all lesions on day 10. The expression of FLK-1 was detected in endothelial cells, retinal pigmented epithelium (RPE)-like cells and fibroblast-like cells in neovascular lesions. In normal adult mouse retinas, FLK-1 expression was mainly observed in RPE cells, inner nuclear and ganglion cell layers.

• CONCLUSION: Our findings demonstrate that expression of FLK-1 may play a role in the formation of laser-induced CNV in mice, which suggests that FLK-1 may be a promising potential target for antiangiogenesis therapy for CNV.

• KEYWORDS: choroidal neovascularization; FLK-1; immunohistochemistry

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INTRODUCTION

Choroidal neovascularization (CNV) is associated with various disorders, often causing severe loss of vision and eventually blindness. Among these disorders, age-related macular degeneration (AMD) is the most prevalent in developed countries [1]. It has been widely known that vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen and angiogenic factor, is crucial for normal angiogenesis and also plays an important role in pathological angiogenesis[2], including CNV[3]. VEGF-mediated angiogenesis is induced by binding of VEGF to its endothelial cell receptors. Among these receptors, the vascular endothelial growth factor receptor 2 (VEGFR-2, also known as FLK-1) that binds the five isomers of murine VEGF has a more restricted expression on endothelial cells and is up-regulated once these cells proliferate during pathological angiogenesis. There is now agreement that FLK-1 is the major mediator of the mitogenic, angiogenic, permeability-enhancing effects of VEGF[2].

In this study, we produce and characterize a model of CNV in mouse and investigate the expression of FLK-1 in the development of CNV. Our results indicate the role of FLK-1 in CNV.

MATERIALS AND METHODS

Mouse Model of Laser-Induced CNV Female 7-week-old C57BL/6 mice (Japan SLC, Shizuoka, Japan) were anesthetized by intraperitoneal injection of ketamine hydrochloride (100mg/kg body weight), and the pupils were dilated with 10g/L tropicamide. Krypton laser photocoagulation (100μm spot size, 0.1 second duration, 150mW) was delivery using a slit-lamp delivery system (Nidek MC-7000) and a hand-held cover slide as a contact lens. Burns were performed in the 9, 12, 3 and 6 o'clock positions of the posterior pole of the retina. Production of a bubble at the time of laser exposure, which indicates rupture of Bruch's membrane, is an important factor in inducing CNV, and therefore only mice in which a bubble was produced for all 4 burns were included in the study.

Choroidal Fluorescein Angiography Ten days after laser treatment, 12 mice (12 eyes) used for the choroidal fluorescein angiography were anesthetized and perfused with 1mL of phosphate-buffered saline (PBS) containing 50g/L of fluorescein-labeled dextran (2×10^6 average molecular weight; Sigma, St. Louis, MO). After enucleation and fixation in 40g/L paraformaldehyde for 1-2 hours, corneas and lenses were removed and the entire retina was carefully dissected from the eyecup. Five or six radial incisions were made and the eyecup was flat-mounted with the sclera facing down and the choroid facing up. Flat mounts were examined by fluorescence microscopy and photographed.

Histopathology CNV was also confirmed by histopathological examination on day 10. Twelve mice (12 eyes) were killed and the eyes were immediately enucleated and prepared for light microscopy by immersing them in 40g/L paraformaldehyde for 24 hours. The eyes were then transferred into 70% ethanol and processed for paraffin embedding. For each burn, 6.0μm serial sections were cut through the entire lesion, stained with hematoxylin and eosin (H&E), and examined by light microscopy.

Immunohistochemistry The expression of FLK-1 in CNV and normal choroid was assessed by immunohistochemical examination. Cryostat sections (10μm thick) from 32 lesions (8 eyes) 10 days after laser treatment and 8 control eyes were fixed in 40g/L paraformaldehyde for 30 minutes at 4℃, incubated in 3mL/L H_2O_2 solution in PBS for 10 minutes and washed in PBS. Slides were incubated for 60 minutes at room temperature with rat anti-mouse FLK-1 antibody (Pharmingen, San Diego, California), and after rinsing with PBS, they were incubated with biotin-conjugated goat anti-rat Ig specific polyclonal antibody (Pharmingen) for 30 minutes at room temperature. After being washed with PBS, slides were incubated with horseradish peroxidase avidin D (Vector Laboratories, Burlingame, California) for 30 minutes at room temperature. After rinsing with PBS, slides were incubated with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories) to give a red reaction product, and counterstained in hematoxylin.

RESULTS

Mouse Model of Laser-Induced CNV Laser-induced CNV developed in all lesions on day 10. The typical appearance of experimental CNV in choroidal fluorescein angiography was shown in Figure 1. The neovascular nets had broad, flat microvessels and anastamose with uninjured choriocapillaris via small caliber vessels (Figure 1). Histopathological examination indicated the newly formed vessels with wide lumens extended from the choroid into the subretinal space where they became partially enveloped by the retinal pigmented epithelium (RPE) cells (Figure 2). Themass of blood vessels was often accompanied by an overlying serous retinal detachment.

FLK-1 Expression in Normal Retina and in Laser-induced CNV In the control retina which was not subjected to photocoagulation, the expression of FLK-1 was weakly detected in RPE cells, inner nuclear layer and ganglion cell layer (Figure 3). In laser-induced CNV 10 days after photocoagulation, strong signals for FLK-1 were observed in many cells of subretinal neovascular tissues. Under high magnification, these cells were ascertained to be endothelial cells, RPE-like cells and fibroblast-like cells. FLK-1 was also found to be expressed in the laser-induced lesions of retina (Figure 4).

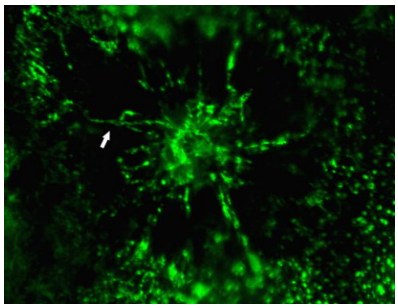

Figure 1 Representative image of CNV in choroidal fluorescein angiography on day 10 after photocoagulation The neovascular nets have broad, flat microvessels and anastamose with uninjured choriocapillaris via small caliber vessels (arrow)

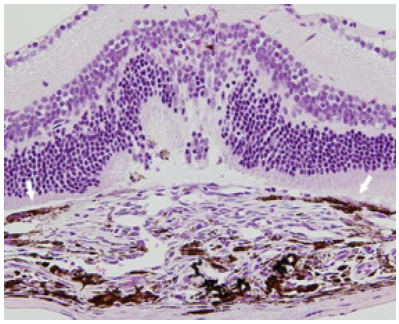

Figure 2 Histopathological appearance of CNV on day 10 after photocoagulation CNV with wide lumens extends from the choroid into the subretinal space where they become partially enveloped by RPE cells (arrows)

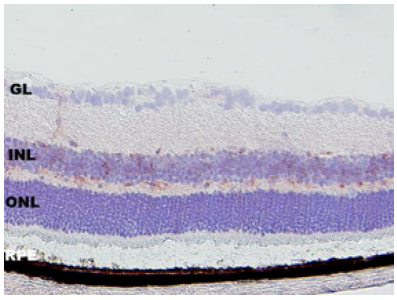

Figure 3 The expression of FLK-1 in normal retina FLK-1 protein was immunostained in normal adult mouse retina. FLK-1 expression was weakly detected in RPE cells, inner nuclear and ganglion cell layers. (GL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer, RPE: RPE cell layer)

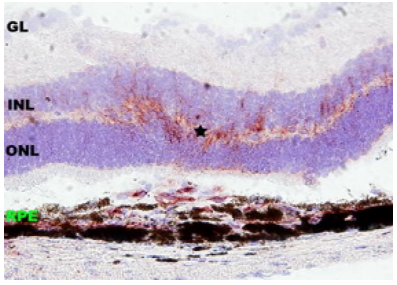

Figure 4 The expression of FLK-1 in laser-induced CNV on day 10 after laser treatment FLK-1 protein was immunostained in laser-induced CNV on day 10 after laser treatment. The expression of FLK-1 was detected in endothelial cells, RPE-like cells and fibroblast-like cells in neovascular lesions. FLK-1 is also found to be expressed in the laser-induced lesions of retina (★). (abbreviations are the same as in Figure 3)

DISCUSSION

Several models of CNV have been previously explored in several species including primates [4,5], rats [6], minpigs [7], rabbits [8] and mice [9]. Murine model has several advantages over large animal models for the scientific investigation of CNV. Because of the availability of various gene knockout mice and transgenic mice, this model can be used to investigate the impact of over-expression or under-expression of individual genes on the development of CNV. The availability of murine-specific reagents, such as antibodies and DNA/RNA probes, is another advantage for investigations of the pathogenesis and treatment of CNV in murine model. Furthermore, the procedure is rapid and the animals are low cost, making it possible to deal with a large number of animals and apply statistical analysis to the effect of interventions.

In this study, we demonstrated that FLK-1 were up-regulated during the development of CNV. Our results are consistent with recent studies suggesting the involvement of FLK-1 in the pathogenesis of subretinal neovascularization [10, 11]. The cells expressing FLK-1 in experimental CNV include vascular endothelial cells, RPE-like cells and fibroblast-like cells in our study. Other recently published data demonstrate that VEGF is also expressed in these cells in experimental CNV [12]. Based on these findings, we propose that VEGF produced by these cells acts on vascular endothelial cells, RPE-like cells and fibroblast-like cells in a paracrine and/or autocrine manner during the development of CNV.

FLK-1 is a specific receptor for VEGF, and its expression was postulated to be limited to vascular endothelial cells [13]. However, recent reports demonstrate the FLK-1 is also expressed in RPE cells [14, 15], neural progenitor cells of retina [16] and uterine smooth muscle cells [17]. Consistent with these observations, our data show that the expression of FLK-1 is detected in RPE cells, inner nuclear layer and ganglion cell layer in normal retina. Thus, the biology of VEGF and FLK-1 may be more complex than originally thought.

In conclusion, we demonstrate that expression of FLK-1 may play a role in the formation of laser-induced CNV in mice. Although angiogenic responses in the mouse may be different from those in the human eye and this model may be partly irrelevant to clinical AMD because it is induced laser treatment, it can provide some clues to understand the pathogenesis of CNV. Our results suggest that FLK-1 may be a promising potential target for antiangiogenesis therapy for CNV.

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