·Basic Research·

Expression of FLT4 in hypoxia-induced neovascular models *in vitro* and *in vivo*

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Abstract

• AIM: To investigate the expression of FLT4 in retina with oxygen induced retinopathy (OIR) and in brain endothelial cell lines (bEnd3) under hypoxia conditions in mice.

• METHODS: Fifty-two one-week-old C57BL/6J mice were divided into control group and hypoxia group. The mice of hypoxia group were exposed to 75% oxygen for 5 days and then returned to the room air to induce retinal neovascularization. Mice in control group were raised in the environment of room air at the same time. The expressions of FLT4 mRNA and protein were checked with RT-PCR and Western Blot analysis at postnatal day 14, 17 and 21 (P14, P17 and P21) respectively. 125mmol/L $CoCl_2$ were added to the culture medium of bEnd3 cell, proteins were extracted in 12, 24, 48 and 72 hours and FLT4 levels were examined by Western Blot analysis.

• RESULTS: The mRNA and protein level of FLT4 expressed in P14 and P17 OIR mice retina statistically up-regulated as compared with those in control group, but there was no statistical difference between OIR group and control group at P21. FLT4 levels increased significantly in 12, 24 and 48 hours hypoxia intervened bEnd3 cells, its levels in 72 hours raised mildly but showed no significance.

• CONCLUSION: FLT4 levels increase in OIR mice retinas and bEnd3 cells in hypoxia. It may play an important role in endothelial cells proliferation in hypoxia and retinal neovascularization in OIR mice.

• KEYWORDS: FLT4; OIR mice; retinal neovascularization

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INTRODUCTION

Retinal neovascular diseases were vision-threatening complications in many ischemic/hypoxic retinopathy. It caused by several kinds of pathogenic and dysbolismus factors resulting in the dysfunction of retinal endothelial cell permeability, damage of blood-retina inner barrier, retinal edema and vessel torture and blockings. These pathologic changes lead to inadequate blood supply and avascularity in retina, up-regulations of endothelial growth factors, and neovascularizations in retina. The growth factors have been reported to be involved in the induction of Hypoxia Induced Factorif-1α (HIF-1α), Erythropoietin (EPO) and basic Fibroblast Growth Factor (bFGF)[1-3]. VEGF is a potent pro-mitogen specific to endothelial cells and plays an important role in angiogenesis by promoting the proliferation, migration and tube formation of endothelial cells, and increasing the permeability of vessels[4,5].

VEGF family members include placenta growth factor (PlGF), VEGF-A, VEGF-B, VEGF-C and VEGF-D. In the late and adult embryogenesis, blood vessels form primarily by sprouting from pre-existing vessels. VEGF (mainly the VEGF-A) potently promotes angiogenesis and is indispensable for vascular development, and the VEGF-2 tyrosine kinase is known as the primary receptor to transmit VEGF signals in endothelial cells. Previous studies have concluded that VEGF-C and VEGF-D were mainly responsible for the growth and development of lymphatic endothelium [6]. The common receptor of VEGF-C and VEGF-D is VEGFR3 (also known as FLT4). FLT4 is present in all types of endothelia during the development, and becomes restricted to lymphatic endothelium in the adult. However, it is greatly up-regulated in the microvasculature of tumors and wounds. FLT4 knockout mice have showed marked defects in arterial-venous remodeling of the primary vascular plexus, resulting in lethality by embryonic day 10.5. The knockdown of the FLT4 homologue in zebrafish has led to defective segmental artery morphogenesis. The blocking antibodies against FLT4 inhibited angiogenesis in tumor growth, which suggested it might be a new gene therapy target in tumors.

The study of FLT4 has been focused on its role in

angiogenesis. In our study, we aimed at investigating how FLT4 was involved in the pathologic retinal angiogenesis. The expression levels of FLT4 in retinas in an oxygen induced retinopathy mice model were firstly determined, and then in the hypoxic Bend.3 cells intervened by 125mmol/L $CoCl_2$. The results indicated that FLT4 might play a role in the hypoxia induced angiogenesis.

MATERIALS AND METHODS

Cell Culture Bend.3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100g/L fetal bovine serum and 10g/L antibiotic/antimycotic solution. Cells were cultured in an incubator at 37℃ in an atmosphere of 95% O_2 and 5% CO_2. Hypoxic culture medium was prepared by adding 125mmol/L $CoCl_2$.

Animal Model The study protocol was conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The reproducible murine model of OIR has been described before [7]. C57BL/6J mice of P7 were exposed to 75±2% oxygen chamber for 5 days with the nursing mothers. On P12, the mice were removed from the chamber and maintained in room air until P17. Mice of the same strain and of the same age were kept in room air and used as control subjects.

Angiography Using Fluorescein-dextran At P19, 6 mice of each group were anesthetized with intraperitoneal injection of 100g/L chloral hydrate and then were perfused through the left ventricle with 1mL of PBS, containing 50mg fluorescein-dextran (Sigma, St. Louis, MO). Eyes were enucleated and fixed in 40g/L paraformaldehyde for 20 minutes. The retinas were dissected free of the lens and cornea and placed in 40g/L paraformaldehyde for another 5 minutes. Then the peripheral retina were cut in four pieces and flat-mounted with glycerol/PBS (50/50).

RT-PCR for Detection of FLT4 mRNA in Mice Retinas Total RNA was isolated from the P14, P17 and P21 mice retinas and 1μg templates were reverse transcribed by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The sequences of the FLT4 primers were: sense 5'-CCC GAG AGC ATC TTT GAT AAG GTG T-3' anti-sense 5'-CCA TCC CGC TGT CTG TCT GGT TAT C-3'. The sequences of the β-actin primers were: sense CAG GAG ATG GCC ACT GCC GCA; anti-sense CTC CTT CTG CAT CCT GTC AGC A. Thermal cycling conditions were as follows: initial template denaturation at 94℃ for 5 minutes followed by primer annealing for 35 cycles of amplification at 65℃ for 1 minute, extension at 72℃ for 30 seconds and melting at 95℃ for 30 seconds. PCR products were analyzed by electrophoresis in a 15g/L agarose gel and visualized by ethidium bromide staining.

Western Blot Analysis The cultured cells and murine retinas were collected and lysed in lysis buffer (150mmol/L NaCl, 50mmol/L Tris-HCl, pH 7.4, 2mmol/L EDTA, 1% NP-40) containing protease inhibitors (Boehringer Mannheim, Germany). Total protein was resolved on SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane and incubated with rat anti-mouse monoclonal antibody against FLT4 (1:100 dilution; Abcam, Calif., USA) and mouse monoclonal anti-actin antibody (1:10,000 dilution; Sigma, St. Louis, Mo., USA), followed by incubation with corresponding secondary antibodies (peroxidase conjugated). The enhanced chemiluminescence reagent was added to the membrane as instructed by the manufacturer. The membrane was exposed to an *X*-ray film for 1 minute before it was developed and fixed.

Statistical Analysis All the grayscale were analyzed by the Image J software. Results were expressed as mean±SEM. One-way ANOVA followed by the LSD *t*test was used to evaluate significant differences. A P value <0.05 was considered statistically significant.

RESULTS

Up-regulation of FLT4 Expression in OIR Mice Retina We successfully obtained the OIR murine model (Figure 1), and further detected the levels of FLT4 mRNA and protein in the retina in OIR mice of P14, P17, and P21 respectively. We found that the mRNA and protein levels of FLT4 were significantly up-regulated in retinas in P14 and P17 compared to the age-matched control. It was marginally up-regulated in P21 and did not show the statistical significance (Figures 2, 3).

Up-regulation of FLT4 Expression in Hypoxic Bend.3 Cells The FLT4 expression was significantly up-regulated at 12, 24 and 48 hours after hypoxic conditions were initiated by adding 125mmol/L $CoCl_2$ to the culture medium. Slight elevation of FLT4 was seen at 72 hours but there was no statistical significance (Figure 4).

DISCUSSION

In recent years, anti-angiogenic therapies are mainly focused on the VEGF/VEGFR2 system, which can partially suppress pathological ocular angiogenesis. Whether any other VEGF family members and receptors are involved in the ocular angiogenesis are still to be determined. FLT4 is a highly glycosylated single-stranded transmembrane protein. Its specific ligands are VEGF-C and VEGF-D. When bound to its ligand, FLT4 can mediate proliferation and differentiation of endothelial cells by inducing isogenic or allogenic dimerization of receptors [8]. It is strongly expressed in the embryonic retina development and the early postnatal period (days 1-7), then decreased and merely expressed in mature vessels at P28. Previous studies have showed that absence of

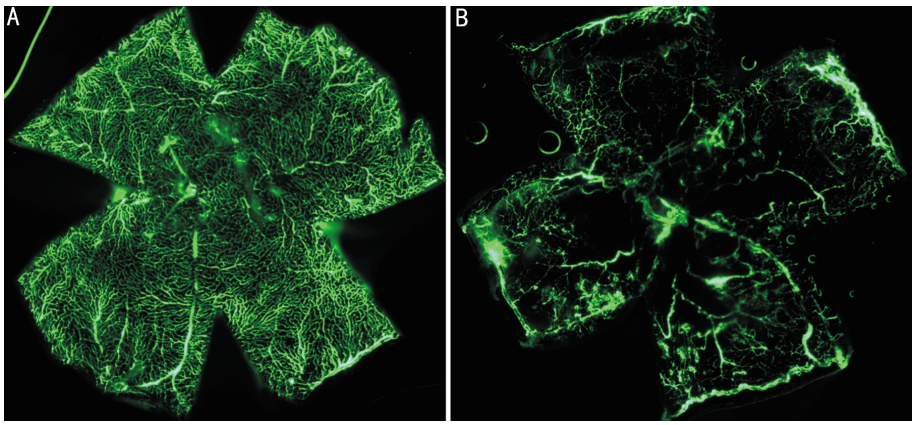

Figure 1 Fluorescein angiography of retina in normal and OIR mice A: The normal mice form a fine radial branching pattern in the superficial retinal layer and a polygonal reticular pattern in deep retinal layer; B: Neovascular tufts appear as hyperfluorescence at the junction between perfused and non-perfused area with dilation and tortuosity of radial vessels in OIR mice

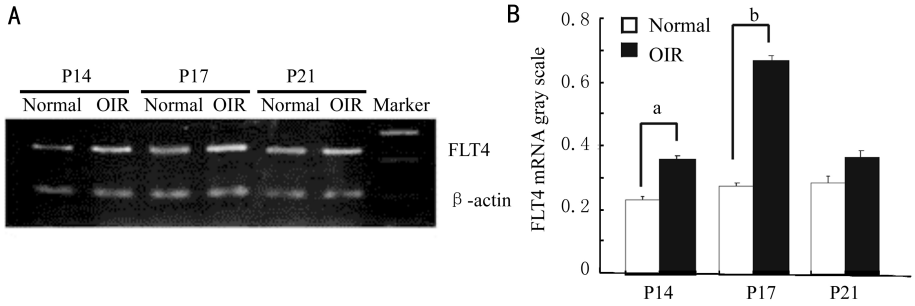

Figure 2 RT-PCR analysis of FLT4 mRNA expression in retina A: The expression of FLT4 mRNA in retina in P14, P17 and P21 mice respectively; B: Relative FLT4 mRNA quantification related to β-actin mRNA $^{a}P<0.05$, $^{b}P<0.001$ *vs* P14,P17 normal controls respectively

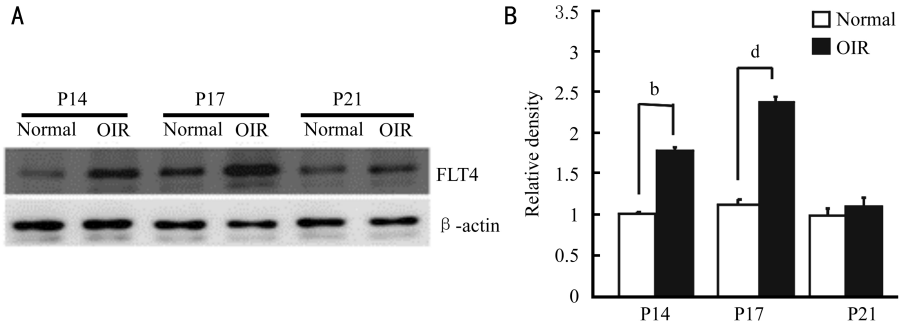

Figure 3 Expression of FLT4 in retina by Western Blot A: The expression of FLT4 in retina in P14, P17 and P21 mice respectively; B: Relative FLT4 quantification related to β-actin protein. $^{b}P<0.01$; $^{d}P<0.001$ *vs* P14,P17 normal controls respectively

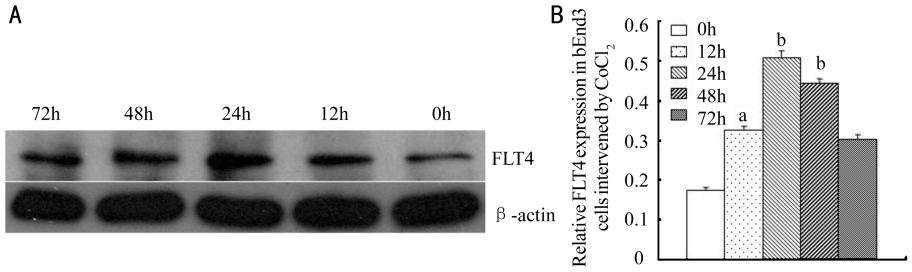

Figure 4 A: Expression of FLT4 in bEnd3 cells intervened by 125mmol/L $CoCl_2$; B Relative FLT4 quantification related to β -actin protein. FLT4 was significantly up-regulated at 12, 24 and 48 hours after being intervened by 125mmol/L $CoCl_2$. ($^{a}P<0.05$; $^{b}P<0.01$ *vs* 0h controls)

the Notch signalling component Rbpsuh (recombining binding protein suppressor of hairless) can result in excessive sprouting of segmental arteries, whereas Notch activation suppresses angiogenesis. Furthermore, they found

that FLT4 was expressed in segmental artery tip cells and became ectopically expressed throughout the sprout in the absence of Notch. Loss of FLT4 can partially restore normal endothelial cell number in Rbpsuh-deficient segmental arteries [9-11]. Our results showed that the expression level of FLT4 mRNA and protein was significantly higher in P14, P17 mice retinas than in the age-matched controls. The up-regulation of FLT4 expression was consistent with the increase of postnatal retinal neovascularization. These results suggested that the up-regulation of FLT4 might play an important role in the pathogenesis of retinal neovascularization by promoting the retinal neovascularization through a notch signaling pathway.

Mouse brain endothelial cell line (bEnd3) is a commonly used cell line to imitate a brain-blood barrier (BBB) system *in vitro*. The blood-retina barrier (BRB) is similar to the BBB system, where endothelial cells were indispensable to guarantee its integrity. Thus, we chose the cell line to generate a hypoxic model *in vitro*. In our study, the results showed that the FLT4 level was significantly up-regulated in hypoxic bEnd3 cells at 12, 24 and 48 hours. It suggested that FLT4 might regulate the proliferation or migration of the retinal endothelial cells and play a role in the pathological angiogenesis such as hypoxia induced angiogenesis in the postnatal vascular endothelial cells. FLT4 was mainly responsible for postnatal proliferation and differentiation of lymphatic endothelial cells, but is ectopically up-regulated in the vascular endothelial cells during some pathological angiogenesis, such as hypoxia and tumors by responding to its ligands [8]. FLT4 was highly expressed in angiogenic sprouts, and genetic targeting of FLT4 or blocking of its signaling can result in decreased sprouting, vascular density, vessels branching and endothelial cell proliferation in retinal angiogenesis of less than postnatal 7 days mice. Stimulation of FLT4 could augment VEGF-induced angiogenesis and sustain angiogenesis even in the presence of VEGFR2 inhibitors, whereas antibodies against FLT4 and VEGFR2 in combination resulted in additive inhibition of angiogenesis and tumor growth [8]. Our results were consistent with these studies, which suggested that FLT4 might play a role in the oxygen-induced retinal angiogenesis as well as the proliferation and differentiation of hypoxic vascular endothelial cells.

In conclusion, FLT4 is up-regulated in retinal angiogenesis of the OIR mice and in the hypoxic mouse brain endothelial cells, which suggests that FLT4 might play an important role in the pathological retinal angiogenesis. Our study might provide a new target in retinal anti-angiogenesis therapies.

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