• Basic Research •

Complement factor B knockdown by short hairpin RNA inhibits laser-induced choroidal neovascularization in rats

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Received: 2019-07-11 Accepted: 2020-01-20

Abstract

● **AIM:** To evaluate whether recombinant complement factor B (CFB) short hairpin RNA (shRNA) reduces laser-induced choroidal neovascularization (CNV) in rats.

● **METHODS:** Laser-induced rat CNV model was established, and then the animals underwent fundus fluorescence angiography (FFA) and hematoxylin and eosin (HE) staining. On day 3 and 7 after photocoagulation, the expression of CFB and membrane attack complex (MAC) was detected by immunhischemistry. A recombinant CFB-shRNA plasmid was constructed. CFB and scrambled shRNA plasmids were intravenous injected into rats *via* the tail vein on the day of laser treatment, respectively. On day 7, the incidence of CNV was determined by FFA, and the expression of CFB and vascular endothelial growth factor (VEGF) in retinal pigment epithelium (RPE)/choroidal tissues was detected by immunhischemistry, Western blot and/or semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) in CFB and scrambled shRNA groups. The possible adverse effects of CFB-shRNA injection were assessed by transmission electron microscopy and electroretinography.

● **RESULTS:** FFA and HE results indicated that a laser-induced rat CNV model was successfully established on day 7 after photocoagulation. The expression of CFB and MAC was extremely weak in normal retina and choroid, and increased on day 3 after photocoagulation. However, it started to reduce on day 7. CFB shRNA plasmid was successfully constructed and induced CFB knockdown in the retinal and choroidal tissues. FFA showed CFB knockdown significantly inhibited incidence of CNV in rats. Moreover, CFB knockdown significantly inhibited the expression of VEGF in RPE/choroidal tissues. CFB shRNA caused no obvious side effects in eyes.

● **CONCLUSION:** CFB knockdown significantly inhibits the formation and development of CNV *in vivo* through reducing the expression of VEGF, which is a potential therapy target. The alternative pathway of complement activation plays an important role in CNV formation.

● **KEYWORDS:** choroidal neovascularization; complement factor B; short hairpin RNA; membrane attack complex; vascular endothelial growth factor

DOI:10.18240/ijo.2020.03.03

Citation: Wang X, Shang QL, Ma JX, Liu SX, Wang CX, Ma C. Complement factor B knockdown by short hairpin RNA inhibits laser-induced choroidal neovascularization in rats. *Int J Ophthalmol* 2020;13(3):382-389

INTRODUCTION

Choroidal neovascularization (CNV) is a common vision-threatening complication associated with many fundus diseases[1]. In recent years, significant advances have been made in the treatment of CNV with increased understanding on the pathogenesis of CNV, especially the research and development of drugs targeting vascular endothelial growth factor (VEGF)[2-3]. Nevertheless, there are still disputes on the indications for anti-VEGF drugs, especially the formation of fibrous membrane[4-5]. Moreover, the shorter half-life of anti-VEGF drugs *in vivo* requires frequent intraocular administration, which may increase the risk of endophthalmitis[6]. Resistance to monotherapy before is a challenge in clinical practice, and there is a need for additional modifying therapies.

The complement system is an important component of the innate and adaptive immunity, which widely involved in the defense against infection and immunological regulation. The complement cascade can be activated through three distinct pathways including the classical, alternative, and lectin pathways[7]. Three activation pathways of the complement system can produce C3 convertase, C5 convertase and finally membrane attack complex (MAC). The MAC directly can lead

to cytolysis and alternatively can stimulate the production of proinflammatory molecules when inserted in cell membranes at sublytic concentrations. Activation of complement, specifically MAC formation, was essential for the development of CNV[7-8]. Complement components C3a and C5a were present in drusen of patients with age-related macular degeneration (AMD), and promoted the formation of CNV[9]. Further evidence showed that the alternative pathway activation was essential for the pathogenesis of laser-induced CNV, but not alone sufficient for injury, and initial complement activation proceeds *via* both the classical or lectin pathways[10-11]. Complement factor B (CFB) is a required positive regulator of the alternative pathway, which thus plays a critical role in the development of CNV. Moreover, the polymorphism of CFB gene was associated with AMD risk, and specific polymorphisms of CFB confer protection of AMD[12-13].

In this study, we established a laser-induced rat CNV model. Then CFB knockdown by short hairpin RNA (shRNA) was used to inhibit activation of the alternative complement pathway. We aim to investigate the roles of the alternative complement pathway in CNV *in vivo* and explore new potential therapies.

MATERIALS AND METHODS

Ethical Approval Male Brown Norway (BN) rats (8-10wk, 180-200 g) were from Vital-River Laboratory Animal Technology Co., Ltd (Beijing, China). Prior to experiments, slit-lamp and ophthalmoscopic examinations were performed to exclude ocular abnormalities. Study protocols adhere to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethical Committee of the Second Hospital of Hebei Medical University.

Construction of CFB shRNA Plasmid Small interfering RNA targeting rat CFB (NM_212466.3) and VEGF-A (NM_031836.3) gene were designed, and the selected target sequences were CFB 5'-TGTTTTCTACCAAATGATTGATGAA-3' and VEGF-A 5'-AGGGTTGTTTCTGGGATTCCTGT-3'. The shRNA sequences synthesized by Sangon Biotech Inc. (Shanghai, China) were cloned into a pRNAT-U6.1/Neo shRNA expression vector (GenScript, Piscataway, NJ, USA) digested with *BamHI* and *HindIII*. The recombinant pRNAT-U6.1/CFB-shRNA plasmid was cloned and purified with an EndoFree plasmid kit (Qiagen, Valencia, CA, USA).

Laser-induced CNV Model and Treatment For CNV model, BN rats were anesthetized with intraperitoneal injections of 10% chloral hydrate (0.35 mL/kg), and pupils were dilated with 1% tropicamide and 1% mydriacyl. Using a 532 nm laser (Coherent Novus Omni, Palo Alto, CA, USA), a slit-lamp delivery system, and a coverslip as a contact lens, five or six spots (300 mW, 200 μm, and 50ms) were placed in each eye. Lesions were 2-3 disk diameters from the optic nerve head. Development of a bubble under laser radiation confirmed rupture of Bruch's membrane. Eyes with hemorrhage were excluded. The rats were intravenously injected (at a 48h interval) with CFB or scrambled shRNA plasmid (100 μg, 3 mL) *via* the tail vein starting from day of laser treatment.

Fundus Fluorescence Angiography Fundus fluorescence angiography (FFA) was performed on day 7 after laser photocoagulation. Rats from each group received an intraperitoneal injection with 10% fluorescein sodium (0.5 mL/kg) immediately after anesthesia and mydriasis to observe the formation of CNV. The leakage of fluorescence was determined during the later vein period (8-10min after the fluorescein sodium infusion).

Electroretinography Full-field electroretinography (ERG) was performed adhering to the International Society for Clinical Electrophysiology of Vision (ISCEV) standards[14]. The animals were dark adapted overnight. The pupils were fully dilated, and the eyes were topically anesthetized. A gold active electrode was placed on the cornea, and a needle reference electrode was inserted under the scalp. A grounded stainless steel clip electrode was placed on the tail. The scotopic ERG was recorded, including an isolated rod, standard flash (maximal) response, and oscillatory potentials. After a 10-minute light adaptation, the photopic ERG was obtained including a single white flash and a 30 Hz flicker. The ERG analysis was based on the changes of a- and b-wave amplitudes in scotopic ERG and photopic ERG.

Hematoxylin and Eosin Staining The rats were executed by CO_2 inhalation, and the eyes were obtained for histology analysis about 6h after FFA. The pathological changes in the eyes were evaluated using hematoxylin and eosin (HE) staining. The eyes were fixed with 4% paraformaldehyde for 24h, and then the eye balls were cut open along the equator line, and removed of the lens and vitreous. The posterior eyecups including the optic disk and laser spot were embedded with paraffin. These sections (5 μm) were stained with HE (Sigma-Aldrich, St. Louis, MO, USA) and observed using a microscope (Carl Zeiss, Oberkochen, Germany).

Immunohistochemical Staining Immunohistochemical (IHC) was performed to determine the expression of CFB, VEGF, and MAC in CNV lesion. Sections (5 μm) were dewaxed, hydrated, and heat retrieval with microwave, and then were incubated overnight at 4℃ with primary antibodies for CFB (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), VEGF (1:100, Santa Cruz Biotechnology), and C9 (1:100, Boster, Wuhan, China), respectively. Then the sections were washed three times for 10min apiece with PBS and incubated for 1h at 37℃ with horseradish peroxidase-conjugated secondary antibodies (ZSGB-BIO, Beijing, China). The tissues were stained with diaminobenzidine (DAB) and observed with a microscope.

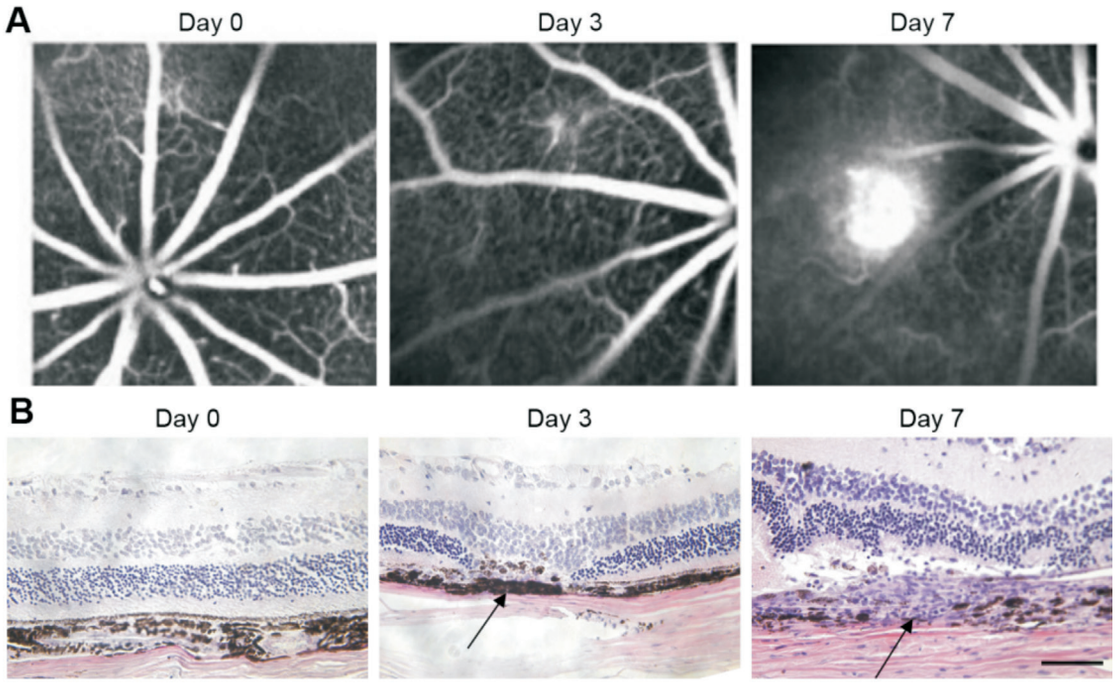

Figure 1 FFA and histological analysis of rat eyes after photocoagulation A: FFA analysis in normal eyes (day 0), and on day 3 and 7 after photocoagulation; B: HE staining of posterior segment tissues around the lesions on day 0, 3, and 7 after photocoagulation. Scale bar=50 μm.

Western Blot Analysis Retinal pigment epithelium (RPE)/choroidal tissues were triturated, lysed on ice in RIPA buffer, and boiled in SDS loading buffer. Protein extracts were subjected to 6%-12% SDS-PAGE and processed with a standard protocol. Primary antibodies for CFB (1:1000), VEGF (1:1000), and β-actin (1:4000, Abclone, Wuhan, China) were used.

Semi-quantitative Reverse Transcription-Polymerase Chain Reaction Total RNA was isolated from RPE/choroidal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Total RNA was reverse transcribed using Oligo (dT) at 42℃ for 1h. The cDNA was amplified with specific primers for CFB and VEGF. The PCR products were examined by 1.5% agarose gel electrophoresis with ethidium bromide staining. Semi-quantitative analysis was performed to determine the relative levels of gene expression. β-actin was used as endogenous control. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted using the following primers: CFB forward: 5'-GAGTACTTCGTGCTGACAGCAG-3', reverse: 5'-GCGGCTTCTCTTGTGAACAATG-3'; β-actin forward: 5'-GCCACCAGTTCGCCATGG-3', reverse: 5'-GTCAGGCAGCTCATAGCT-3'.

Transmission Electron Microscope Samples (3×3 mm^2) obtained from the tissues of the ocular posterior segment were fixed in 1% osmium tetroxide, dehydrated with gradient ethanol and acetone, and directionally embedded with epoxy resin. Semi-thin sections (1 μm) were stained with 0.5% toluidine blue and localized using the optical microscope. After staining with uranyl acetate and aluminum citrate, the ultrathin sections were observed under a transmission electron microscope (TEM; Carl Zeiss) to determine the toxic effects of CFB shRNA on the retina.

Statistical Analysis All experiments were performed at least in triplicate. SPSS 16.0 software (SPSS, Chicago, IL, USA) was used for the data analysis. Data were presented means±standard deviation (SD). χ^2 test or Student's *t*-test was used for the comparison of intergroup differences. $P<0.05$ was considered to be statistically significant.

RESULTS

Formation of Laser-induced Choroidal Neovascularization in Rats We established a laser-induced rat CNV model. FFA analysis showed on day 3 after photocoagulation, some of the laser speckle presented hypofluorescence at an early stage and cloudy hyperfluorescence at a late stage, without significant fluorescein leakage (Figure 1A). On day 7, most the laser speckle presented a clear irregular strip and granular fluorescence. Additionally, a petal-shape hyperfluorescence region with a blurred boundary was observed at the late stage (Figure 1A). HE staining showed each layer structure of the retina/choroid in normal rats appeared to be clear and integrate (Figure 1B). Macrophages together with migrated and proliferated RPE cells were observed on day 3 after photocoagulation. Meanwhile, several lesions were found in the RPE and Bruch's membrane, including rupture, deficit of the inner nuclear layer, irregular arrangement of the outer nuclear layer, injury of choriocapillary layer, and depigmentation. On day 7, endothelial cells penetrated the

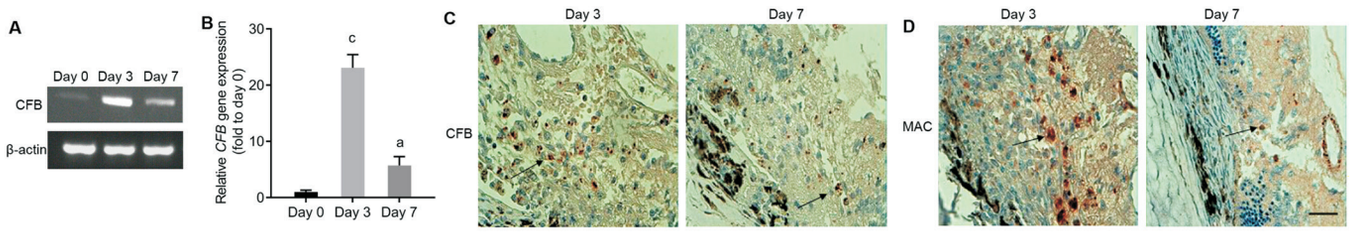

Figure 2 IHC analysis of CFB and MAC expression in laser-induced rat CNV A: Identification of CFB mRNA expression in retinal/choroidal tissues by semi-quantitative RT-PCR; B: Quantification of results in A. mRNA levels normalized against β-actin, n=3. ^{c}P<0.001 and ^{a}P<0.05 compared with baseline level (day 0); C: IHC staining of CFB in the eye of CNV rats. CFB was highly expressed in the CNV lesions on day 3 after photocoagulation. The expression of CFB in the lesions was reduced on day 7; D: IHC staining of MAC in the retina of CNV rats. MAC deposition in the CNV lesions was detected on day 3, and started to decrease on day 7. The arrows indicate positive cells. Scale bar=50 μm.

Table 1 Incidence of laser-induced CNV on day 7 after photocoagulation in groups

Groups	No. of mice	Laser spots/eye	Total spots	CNV-positive spots
Scrambled	6	5	60	54 (90%)
CFB shRNA	6	5	60	13 (22%)^{c}
VEGF-A shRNA	6	5	60	11 (18%)^{c}

CNV: Choroidal neovascularization; CFB: Complement factor B; VEGF: Vascular endothelial growth factor. ^{c}P<0.001 compared with scrambled shRNA group (χ^2 test).

broken Bruch's membrane and RPE layer and proliferated through the retina, forming new blood vessels (Figure 1B). These results indicated that a laser-induced rat CNV model was successfully established on day 7 after photocoagulation.

Increased Expression of CFB and MAC in Laser-induced CNV Semi-quantitative RT-PCR and IHC showed that the expression of CFB was very weak in the retina of normal rats. On day 3 after photocoagulation, CFB expression was up-regulated in the lesions, and its expression in the lesions proximate to the choroid was higher than that in the internal part (Figure 2A-2C). However, on day 7, the expression of CFB in the lesions was down-regulated.

IHC results showed that the expression of MAC was also very weak and was not detected in the retina of normal rats (data not shown). Similarly, deposition of MAC was displayed in the retinal and choroidal tissues on day 3 after photocoagulation, and decreased on day 7 (Figure 2D).

Inhibition of Choroidal Neovascularization Formation by CFB knockdown We constructed and purified pRNAT-U6.1/CFB-shRNA plasmid. Semi-quantitative RT-PCR and Western blot analysis showed the expression of CFB in retinal and choroidal tissues was obviously decreased on day 3 after injection of pRNAT-U6.1/CFB-shRNA plasmid *via* tail vein, indicating a successful CFB knockdown *in vivo* (Figure 3A-3D).

CNV formation was determined by FFA assay in gene knockdown and control groups. On day 7 after photocoagulation, the incidence of CNV in the CFB knockdown group was significantly reduced compared with the control group (22% *vs* 90%; Table 1). Similarly, the incidence of CNV in the VEGF knockdown group was significantly reduced compared with the control group (18% *vs* 90%; Table 1). However, no significant

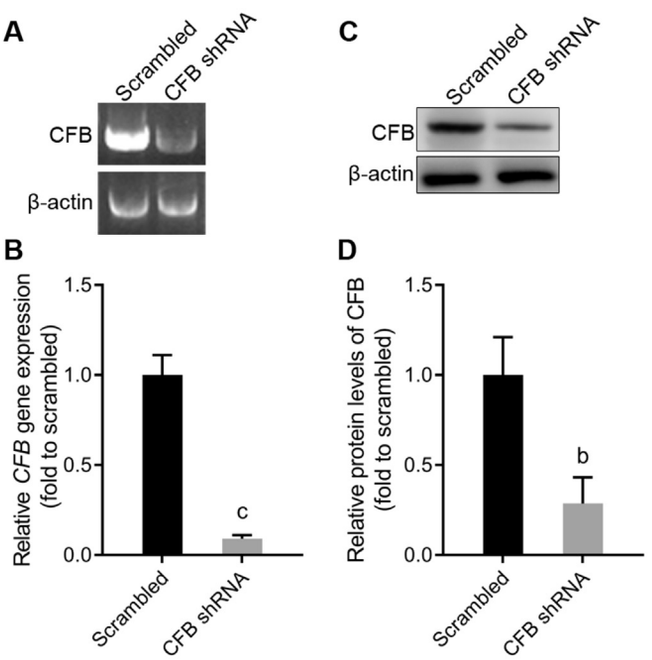

Figure 3 CFB knockdown by shRNA inhibits the formation of CNV A: The identification of CFB knockdown in retinal/choroidal tissues by semi-quantitative RT-PCR. B: Quantification of results in A. mRNA levels normalized against β-actin (n=3). ^{c}P<0.001 compared with scrambled shRNA group. C: The identification of CFB knockdown in retinal/choroidal tissues by Western blot. D: Quantification of results in C. Protein levels normalized against β-actin (n=3). ^{b}P<0.01 compared with scrambled shRNA group.

difference was found between the CFB knockdown and VEGF knockdown groups (P>0.05).

Decrease of VEGF in CNV by CFB Knockdown Western blot analysis showed on day 7 after photocoagulation, the expression of VEGF in RPE/choroidal tissues was obviously

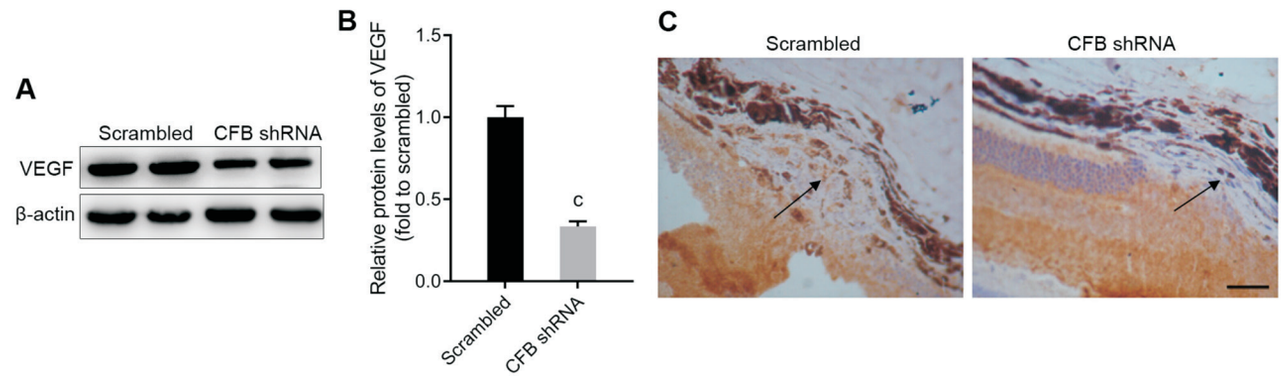

Figure 4 CFB knockdown reduces the expression of VEGF in CNV A: Western blot analysis showed CFB knockdown decreased the expression of VEGF on day 7 after photocoagulation. B: Quantification of results in A. Protein levels normalized against β-actin (n=3). ^{c}P<0.001 compared with scrambled shRNA group. C: IHC staining showed CFB knockdown reduced the expression of VEGF in the lesions on day 7. Scale bar=50 μm. The arrows indicate positive cells.

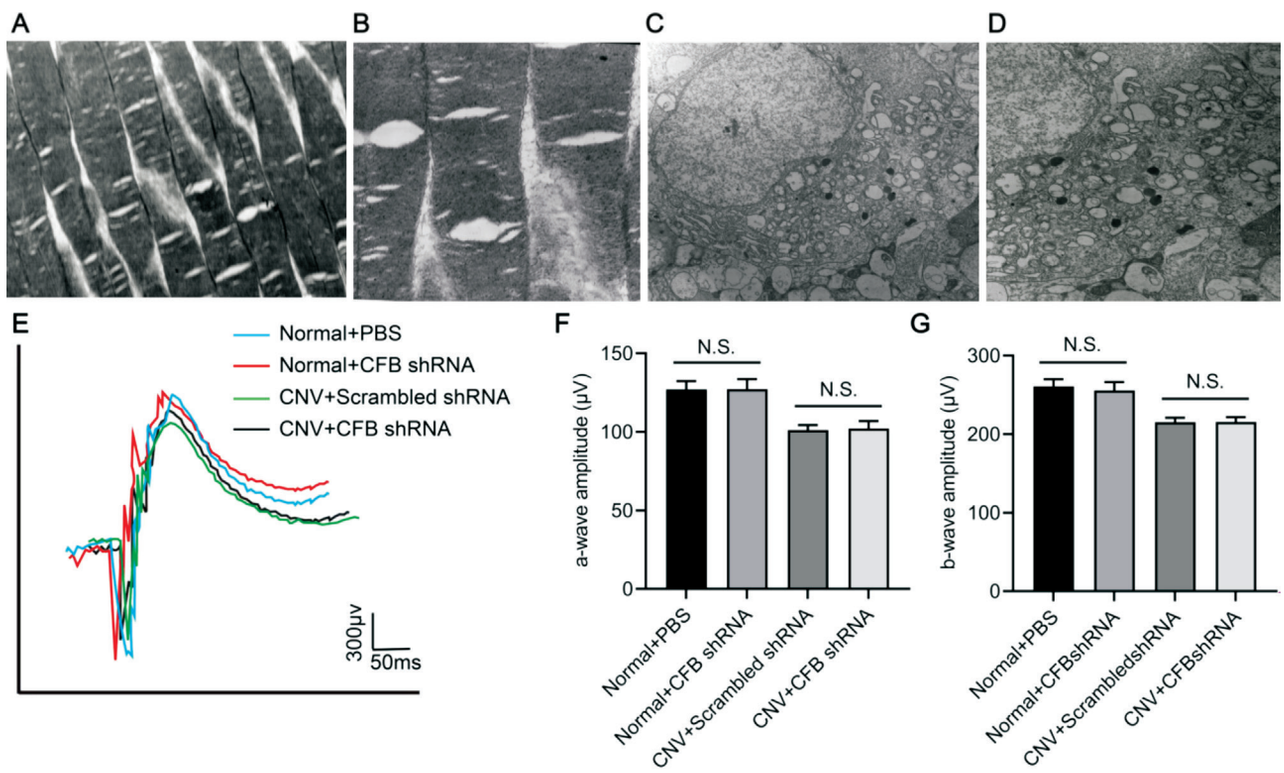

Figure 5 Analysis of the toxic effects of CFB shRNA on eyes by transmission electron microscopy and ERG A: On day 7 after CFB shRNA injection in normal eyes, the structure of rod photoreceptors and cone photoreceptors was complete; B: The structure of membranous disc had mild disorders, the gap was slightly irregular; C: The structure of mitochondria was clear; D: Endoplasmic reticulum was distended slightly. The images were observed under a magnification of 2000×; E: The representative waves of ERG in different groups; F: The a-wave amplitude in the dark adaptation electroretinogram in different groups; G: The b-wave amplitude in the dark adaptation electroretinogram in different groups. N.S.: No significance.

decreased in the CFB knockdown group compared with the control group (Figure 4A and 4B). Similarly, IHC results also conformed CFB knockdown remarkably decreased the expression of VEGF in lesions on day 7 (Figure 4C). These results suggested that CFB knockdown significantly reduce VEGF expression in CNV.

No Toxic Effects of CFB shRNA on Eyes TEM showed that on day 7 after CFB shRNA injection in normal eyes, the rod and cone cells were intact, but the structure of the membranous disk was slightly disordered. The space was slightly irregular (Figure 5A, B). Moreover, slight intercellular edema was displayed in the ganglion cell layer, together with slight edema in the nerve fiber layer, a clear structure in the mitochondrion, and slight dilation in the endoplasmic reticulum (Figure 5C, D). In addition, ERG showed that the a-waves (photoreceptor function) and b-waves (inner retinal function) from CFB-

shRNA-injected eyes were not reduced significantly compared with normal control eyes (Figure 5E-5G). These results indicated that intravenous injection of CFB shRNA reduced laser-induced CNV without causing retinal toxicity.

DISCUSSION

CNV associated with AMD is the leading cause of visual loss in individuals over age 60[15]. CNV is a complex biological process and its pathogenesis is not well defined. Several factors, including inflammation[16], ischemia[17], oxidative stress[18], and local production of angiogenic factors[19], are considered important in the pathogenesis of CNV. Moreover, increasing evidences emerged, which implicated activation of the complement cascade in CNV pathogenesis[20]. Bora *et al*[8] firstly showed $C3^{-/-}$ mice would not present CNV after laser photocoagulation, and inhibition of the *in vivo* formation of MAC markedly reduced the incidence and development of CNV. Moreover, AMD patients showed elevated levels of MAC in choroidal blood vessels and RPE[21]. In the present study, we used a rat model of laser-induced CNV[22] to investigate the roles of complement in CNV. We found that MAC deposition was displayed in the retinal and choroidal tissues on day 3 after photocoagulation in rats, and decreased on day 7. These data demonstrated that complement activation was essential for the pathogenesis of CNV.

In three pathways of complement activation, the alternative pathway provides a rapid, antibody-independent route of C activation and amplification. The alternative pathway directly activates C3 when it interacts with certain activating surfaces (*e.g.*, zymosan and lipopolysaccharides) and involves C3, factor B, factor D, and properdin[23]. Bora *et al*[10] showed inhibition of the alternative pathway resulted in decreased levels of MAC and angiogenic factors-VEGF and TGF-β2 in laser-induced CNV mice. Furthermore, a targeted inhibitor specific for the alternative pathway of complement significantly reduced CNV and the physiologic consequences of CNV on retinal function[24].

CFB is a key component of the alternative pathway. The polymorphism of *CFB* gene was associated with AMD risk, which had been confirmed in Indian, Korean, Chinese and Mexican populations[25-28]. Liver has been implicated as the primary source of plasma CFB, but less expression has been detected in retina and RPE/choroid[24]. Schnabolk *et al*[29] showed that RPE cells expressed and secreted CFB were sufficient to promote RPE damage and CNV *in vitro* and *in vivo*. Bora *et al*[10] indicated that CFB was up-regulated in complement sufficient C57BL/6 mice on day 1 post-laser and remained elevated on day 7. However, our results showed the CFB expression was increased in the lesions in BN rat on day 3 after photocoagulation, and decreased on day 7, which was consistent with changes of MAC deposition. These results demonstrated that the role of CFB and complement in CNV may be acute and dynamic. Importantly, different models and methods were utilized to further confirm previous conclusion and add understanding of CFB function in CNV.

We constructed a recombinant plasmid pRNAT-U6.1/CFB-shRNA to knock down CFB expression. A rapid intravenous injection of plasmid was established based on the fluid dynamics principles for *in vivo* gene transfection. In this method, naked purified DNA was dissolved in a relatively large quantity of buffer and then was used for a quick intravenous injection[30]. The transfection and expression efficiency are distinctive in different organs, of which liver has manifested as the highest one. Importantly, the liver is also the main organ for generation of CFB. Schnabolk *et al*[29] suggested that local production of CFB in eye is sufficient to promote CNV, but liver-derived CFB can substitute in the absence of RPE-derived CFB. Thus, global knockdown of CFB is the appropriate choice. In this study, tail vein injection of CFB shRNA was performed. Expression of CFB in liver was reduced obviously (data not shown). Semi-quantitative RT-PCR and Western blot analysis showed the expression of CFB in RPE/choroidal tissues was obviously decreased after intravenous injection of CFB-shRNA plasmid, indicating a successful CFB knockdown in eyes. Moreover, injection of CFB shRNA in rats with this method had no toxic effects on retinal structure and function.

After taking the critical role of the complement system in CNV into consideration, several trials have been initiated to investigate the effects of complement inhibitors on CNV. Our data showed that inhibition of CFB by intravenous injection of CFB-shRNA plasmid significantly reduced the incidence of laser-induced CNV in rats. Moreover, compared to VEGF-A shRNA (positive control), the effects of CFB shRNA on inhibiting CNV formation were similar. The knockdown of CFB, the positive regulator of the alternative pathway, can inhibit activation of the alternative pathway and formation and deposition of MAC. MAC has been reported to release growth factors such as β-fibroblast growth factor (β-FGF), VEGF, and platelet-derived growth factor from various nucleated cells, which may be a pathogenic mechanism in angiogenesis[8]. Thus, we detected the expression of VEGF, a key factor in the pathophysiology of CNV, which indicated that CFB knockdown could obviously decrease the mRNA and protein levels of VEGF expression in RPE/choroidal tissues. Our results clearly demonstrated that CFB-shRNA injection could significantly inhibit the formation and pathogenesis of CNV in rats, and CFB was a very effective target of gene therapy for CNV.

In summary, our data suggested that CFB knockdown could significantly inhibit the formation and pathogenesis of CNV *in vivo*. We provided a potential strategy for CNV

therapy involving in inhibition of the alternative pathway by administration of CFB shRNA.

ACKNOWLEDGEMENTS

Conflicts of Interest: Wang X, None; **Shang QL,** None; **Ma JX,** None; **Liu SX,** None; **Wang CX,** None; **Ma C,** None.

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