Experimental Eye Research 136 (2015) 78–85

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Subretinal transplantation of retinal pigment epithelium overexpressing fibulin-5 inhibits laser-induced choroidal neovascularization in rats

Fuliang Li [a, b], Yuxiao Zeng [a, b], Haiwei Xu [a, b, *], Zheng Qin Yin [a, b, *]

[a] Southwest Hospital/Southwest Eye Hospital, Third Military Medical University, Chongqing, PR China
[b] Key Lab of Visual Damage and Regeneration & Restoration of Chongqing, Chongqing, PR China

ARTICLE INFO

Article history:
Received 16 September 2014
Received in revised form
14 April 2015
Accepted in revised form 12 May 2015
Available online 14 May 2015

Keywords:
Age-related macular degeneration
Choroidal neovascularization
Fibulin-5
Retinal pigment epithelium
Transplantation

ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly. Choroidal neovascularization (CNV) is the abnormal angiogenesis that causes severe visual loss in AMD. Fibulin-5 (Fbln5), which functions as an angiogenesis inhibitor, plays an important role in the pathogenesis of AMD. Here, we investigated whether subretinal transplantation of Fbln5-overexpressing retinal pigment epithelial (RPE) cells can inhibit CNV *in vivo*. Adult Long-Evans rats were used in this study. CNV was induced by laser photocoagulation. One week after laser-induced CNV, RPE cells expressing pZlen-Fbln5-IRES-GFP or the control pZlen-IRES-GFP vectors were transplanted into the subretinal space of the right and left eyes, respectively. CNV was evaluated using fundus photography, fundus fluorescein angiography (FFA), and hematoxylin and eosin staining. We found that CNV occurred at 1 week after photocoagulation, reaching peak activity at 3 weeks and remaining at a high level at 4–5 weeks after photocoagulation. Transplanted RPE cells survived for at least 4 weeks and migrated toward the retina. Subretinal transplantation of Fbln5-overexpressing RPE cells resulted in a significant reduction in the total area of leakage and the number of leakage spots compared with transplantation of RPE cells expressing only green fluorescent protein. Our findings suggest that subretinal transplantation of Fbln5-overexpressing RPE cells inhibits laser-induced CNV in rats and thus represents a promising therapy for the treatment of AMD.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly and is characterized by a gradual loss of central vision (Pan et al. 2014; Wong et al. 2008). There are two main types of AMD: dry and wet AMD (Barak et al. 2012). In wet AMD, choroidal neovascularization (CNV) extends through Bruch's membrane into the subretinal space between the retinal pigment epithelium (RPE) and photoreceptors (Grossniklaus and Green, 2004), resulting in disruption of the macula and subsequent vision loss. CNV is induced when pathological states drive the balance of proangiogenic and antiangiogenic factors in favor of angiogenesis. Vascular endothelial growth factor (VEGF) is pivotal in the pathogenesis of CNV and plays an important role in the development of wet AMD (Bhutto et al. 2006; Churchill et al. 2006; Huang et al. 2011; Ikeda et al. 2006; Wang et al. 2011). Suppression of CNV is a useful strategy for the treatment of AMD, and anti-angiogenic agents that inhibit VEGF have been used in treating this devastating disease (Bressler, 2009).

RPE cells, located between photoreceptors and the choriocapillaries, play an important role in the maintenance of normal retinal function. Dysfunction of RPE cells has been regarded as a contributing factor in AMD, and RPE transplantation is believed to be a potential therapy for AMD (Binder et al. 2007). It has been reported that RPE cells increase the secretion of VEGF, leading to an increase in vascular permeability and inflammation (Adamis et al. 1993), and thus play an important role in the development of CNV in AMD (Gass, 1994). In addition, Bruch's membrane is believed to form a barrier against the invasion of new vessels into the retina (Chong et al. 2005). Decreased thickness and integrity of the Bruch's membrane due to disrupted assembly of elastic

* Corresponding authors. Southwest Eye Hospital, No. 30 Gaotanyan Street, Shapingba District, Chongqing, 400038, PR China.
E-mail addresses: haiweixu2001@163.com (H. Xu), qinzyin@aliyun.com (Z.Q. Yin).

http://dx.doi.org/10.1016/j.exer.2015.05.004
0014-4835/

fibers can facilitate the penetration of the newly formed blood vessels into the subretinal space, thus promoting formation and migration of CNV (Booij et al. 2010). Therefore, targeting RPE cells and Bruch's membrane may be a new strategy for the treatment of AMD.

Fibulin-5 (Fbln5) is an extracellular matrix glycoprotein that is expressed in the retina, RPE, and Bruch's membrane (Mullins et al. 2007), and missense mutations in the Fbln5 genes have been reported to be associated with increased susceptibility to AMD (Auer-Grumbach et al. 2011; Jones et al. 2010). Fbln5 is known to be essential for elastogenesis (Nakamura et al. 2002), and Fbln5 dysfunction is associated with altered assembly of elastin in Bruch's membrane (Stone et al. 2004). Therefore, Fbln5 may be important in maintaining the integrity of Bruch's membrane, thus preventing the invasion of new blood vessels into the subretinal space. In addition, Fbln5 functions as an angiogenesis inhibitor by blocking VEGF signaling and inhibiting the attachment and proliferation of endothelial cells (Albig and Schiemann, 2004; Preis et al. 2006; Sullivan et al. 2007). Furthermore, we have previously found that Fbln5 overexpression in RPE cells downregulates the expression of VEGF, chemokine receptor type 4 (CXCR4), and transforming growth factor (TGF)-β1, and inhibits cell proliferation and migration in cocultured choroidal endothelial cells (CECs) (Li et al. 2012), suggesting that Fbln5 overexpression in RPE cells may be a novel therapy for the treatment of neovascular AMD.

In the present study, we transplanted Fbln5-overexpressing RPE cells subretinally into a rat model of laser-induced CNV to investigate the effect of these cells on inhibition of CNV *in vivo*. Our experimental results suggest that subretinal transplantation of Fbln5-overexpressing PRE cells may be a novel strategy for the treatment of AMD.

2. Materials and methods

2.1. Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University. Adult Long-Evans rats (male and female, weighing 200 g) were used in this study. The animals were obtained from the Animal Care Center of the Third Military Medical University. Animals were housed individually at room temperature (25 ± 5 °C) under a 12-h light/dark cycle and were fed standard rat chow and water *ad libitum*.

2.2. Culture of RPE cells

Rat RPE cells were cultured as previously described (Li et al. 2012). Briefly, eyes were enucleated from 8 to 12-day-old Long-Evans rats and incubated at 37 °C for 45 min in phosphate-buffered saline (PBS) containing 0.1% trypsin, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 70 U/ml collagenase. After the retina was carefully removed, the RPE cells were gently scraped with a Pasteur pipette into fresh culture medium. The cells were centrifuged at 1000 rpm for 5 min, and the pellet was treated with 0.1% trypsin, resuspended in Dulbecco's Modified Eagle's Medium (DMEM)/F2 medium with 10% fetal bovine serum (FBS), and grown at 37 °C in 5% CO_2 in a fully humidified incubator. RPE cells were assessed based on morphological characteristics, the presence of pigment in the cells, and pan-cytokeratin (PCK) immunostaining. The purity of RPE cells was greater than 90%, and purified cells were used for subsequent gene transfection experiments.

Purified RPE cells were transfected with a lentivirus containing Fbln5 (pZlen-Fbln5-internal ribosome entry site [IRES]-green fluorescent protein [GFP]). The empty vector pZlen-IRES-GFP was used as a control for transfection. Twenty-four hours after transfection, cells were washed with Hanks' balanced salt solution three times and digested with 0.25% trypsin-EDTA for 5 min. The enzyme reaction was stopped with culture medium containing 10% FBS. The cell suspension was centrifuged at 1000 rpm for 5 min, and the pellet was resuspended in 1 ml serum-free DMEM/F12 medium. After centrifugation at 1000 rpm for 5 min, the cell concentration was adjusted with serum-free DMEM/F12 medium to 10^8/ml, and the cells were placed on ice for subsequent transplantation.

2.3. Laser-induced CNV in rats

Rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (3 ml/kg). Both pupils were dilated with compound Tropicamide eye drops. CNV was induced in both eyes. A glass coverslip was placed as a contact lens. The retina was viewed through a slit lamp microscope. Eight to ten laser burns were created between major retinal vessels approximately 2 papilla diameter (PD) around the optic nerve head using a Novus Varia laser photocoagulatior (Coherent, Inc., USA). A solid-state multi-wavelength laser (659 nm wavelength) was used with a laser power of 340–360 mW for 50 ms, and the spot diameter was 50 μm. The morphologic end point of the laser injury that indicated a successful photocoagulation spot was the appearance of a bubble, suggesting disruption of Bruch's membrane.

2.4. Subretinal transplantation of RPE cells overexpressing Fbln5

One week after laser-induced CNV, RPE cells expressing the pZlen-Fbln5-IRES-GFP vector or the control pZlen-IRES-GFP vector were transplanted into the subretinal space of the right and left eyes, respectively. Rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (3 ml/kg). Both pupils were dilated with compound Tropicamide eye drops. After the dorsolateral bulbar conjunctiva was cut open, the sclera were penetrated by a 29-gauge syringe needle at 3 mm posterior to the corneal limbus. The cell suspension in serum-free DMEM/F12 medium was kept on ice during surgery time. A 5-μl aliquot of the cell suspension was slowly injected into the subretinal space using a microinjector. The needle was allowed to remain in place for 30 s and then was withdrawn very slowly to minimize efflux of the transplanted cell suspension. A glass coverslip was placed as a contact lens, and the retina was viewed through a microscope. The appearance of a spherical bulge on the retina indicated a successful subretinal injection. The scleral aperture was closed. Tobramycin/dexamethasone ointment was applied to the eye, and the rats were fed with drinking water containing cyclosporine (210 mg/l) after surgery.

2.5. Visualization and quantification of CNV using fundus fluorescein angiography

The time course of laser-induced CNV was evaluated at 1, 2, 3, 4, and 5 weeks after laser photocoagulation, using fundus photography and fundus fluorescein angiography (FFA). To study the effect of subretinal transplantation of RPE cells overexpressing fibulin-5 on CNV, laser-induced CNV was evaluated before and 1, 2, 3, and 4 weeks after subretinal transplantation of RPE cells. Rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (3 ml/kg), and both pupils were dilated with Tropicamide eye drops. Rats were intraperitoneally injected with 1 mg/g of 10% sodium fluorescein (Alcon, USA). Angiograms were taken using a fundus camera (Heidelberg, Germany). Fluorescein leakage in the late-phase fluorescein angiograms (5–7 min after dye injection)