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Research paper

Notch signaling regulates M2 type macrophage polarization during the development of proliferative vitreoretinopathy

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ABSTRACT

Macrophages play an important role in the pathogenesis of proliferative vitreoretinopathy (PVR). M2 macrophages can promote tissue remodeling and repair. In this study, CD206 positive M2 type macrophages were found in preretinal fibrous membranes of the mouse model of PVR induced by the intravitreal injection of retinal pigment epithelial (RPE) cells. Notch signaling determines M2 macrophage polarization. The specific inhibition of Notch signaling pathway by the intravitreal injection of γ-secretase inhibitor DAPT attenuated RPE cells-induced PVR formation as demonstrated by the decreased expression of α-SMA, and inhibited M2 type macrophage infiltation as demonstrated by the decreased expression of Arg-1. Notch signaling may modulate PVR formation by regulating M2 type macrophage polarization.

1. Introduction

Proliferative vitreoretinopathy (PVR) is the most common cause of recurrent retinal detachment after retinal detachment repair, occurring in 5%-11% of patients [1,2]. Although the pathogenesis of PVR is not fully understood, it is generally considered a protracted wound-healing process [3]. PVR is characterized by the formation of preretinal fibrous membranes containing myofibroblasts derived from transdifferentiated retinal pigment epithelial (RPE) cells and other types of cells, such as macrophages [4]. Macrophages, one of the most important types of inflammatory cells, play an important role in the pathogenesis of PVR [5]. There are two major subsets of macrophages: classically activated M1 macrophages that secrete large amounts of pro-inflammatory mediators and alternatively activated M2 macrophages that dampen the inflammatory response and promote tissue remodeling and repair [6]. To date, there has been no report concerning whether M2 macrophages are involved in the formation of PVR and contribute to preretinal fibrous membrane development *in vivo*. Understanding the role of M2 macrophages in PVR development can give some insight into the pathogenesis of PVR.

Recent studies have reported that Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses [7]. The Notch signaling pathway is a highly conserved cell-cell interaction mechanism that regulates cell proliferation, differentiation, apoptosis or stem cell maintenance through cell physical contact in both vertebrate and invertebrate species [8]. Notch signaling is composed of Notch ligands, Notch receptors, and DNA-binding proteins. There are five transmembrane Notch ligands (Jagged1, Jagged2, Dll1, Dll3, and Dll4) and four transmembrane Notch receptor isoforms (Notch 1–4) in mammalian cells. CSL is a key DNA-binding protein in the Notch signaling pathway and is called CBF-1 or RBP-J which could recognize and bind specific DNA sequences (RTGGGAA) located in Notch-induced gene promoters. The pathway is activated through an interaction of a Notch receptor with a Jagged or Delta-like ligand leading to proteolytic cleavages of the Notch receptor at two distinct sites. The cleavage releases the Notch intracellular domain (NICD), so that it can enter the nucleus and function as a transcription activator. The second cleavage is mediated by the γ-secretase complex, and an effective inhibition of Notch activation can be achieved by pharmacological inhibition of this protelytic activity. Within the nucleus, NICD interacts with CSL (RBP-Jk/CBF-1) and activates transcription of downstream target genes, such as those of the Hes and Hey families [9].

Notch signaling is important for eye development and epithelial-to-mesenchymal transition (EMT) of RPE cells [10,11]. Elevated Notch signaling has been verified in a large range of fibrotic diseases developed in the kidney, liver, and lung, inducing the development of EMT [12–14]. Macrophages-specific disruption of

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RBP-J may result in attenuated hepatic fibrosis [15]. Little is known about whether the Notch signaling pathway is involved in the pathogenesis of PVR by regulating M2 macrophage polarization. In the present study, we investigated the expression change of M2 macrophages in PVR formation and observed the influence of Notch signaling on M2 macrophage polarization during PVR development.

2. Methods and materials

2.1. Reagents and antibodies

The primary antibodies included smooth muscle α-actin (α-SMA; ab5694, Abcam, Cambirdge, MA, USA), Hes-1 (ab71559, Abcam), the Notch-1 intracellular domain (NICD1; ab8925, Abcam), anti-mouse CD86 (B7-2) purified (GL1, eBioscience, San Diego, CA, USA), Arginase-1 (sc20150, Santa Cruze, Dallas, TX, USA), PE anti-mouse CD206 (MMR) antibody (141705, BioLegend, San Diego, CA, USA), Brilliant Violet 421™ anti-mouse F4/80 antibody (BM8, BioLegend). The secondary antibody against rabbits labeled with fluorescein isothiocyanate (FITC) was purchased from Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). Primers were purchased from the Invitrogen Corporation (Beijing, China). The γ-secretase inhibitor DAPT was purchased from Selleckehem (Houston, TX, USA).

2.2. Animal experiments

Sixty 4-week-old C57BL/6 mice were obtained from the Experimental Animal Center at the Shandong Provincial Key Laboratory of Ophthalmology of Shandong Eye Institute. All experimental procedures adhered to the ARVO statements for the Use of Animals in Ophthalmology and Vision Research, and were approved by the Institutional Review Board of the Shandong Eye Institute. All surgeries were performed on only one eye of each mouse under sodium pentobarbital anesthesia, and the mice were sacrificed with an overdose of 10% chloral hydrate.

All mice were randomly divided into three groups. Mice in the control group (group A, $n = 20$) were injected with 1 μL PBS; mice in the ARPE-19 cell group (group B, $n = 20$) received an intravitreous injection of 1 μL ARPE-19 cells (5×10^4 cells/μL) suspended in PBS; mice in the DAPT group (group C, $n = 20$) were injected with 1 μL ARPE-19 cells (5×10^4 cells/μL) suspended in PBS containing DAPT (16 ng/μL) [16]. Under an operating microscope, a 33-gauge needle attached to a 5-μL Hamilton microsyringe (Hamilton Company, Bonaduz, Switzerland) was passed through the equatorial sclera, with the tip of the needle just over the optic disc and injecting the cells, not disturbing the lens or the retina. During the injection of the RPE cells, the cells were seen flowing from the needle tip into the vitreous and dispersing in the vitreous fluid. The vitreous was observed to become cloudy immediately.

2.3. Histology, immunohistochemistry, and whole-mount staining

The experimental eyes were collected at 14 days after the intravitreal injection. The samples were fixed in 10% formalin and embedded in paraffin. Continuous 4-μm sections were stained with hematoxilin & eosin (HE staining), and fibrous proliferative membranes were observed by light microscopy. Meanwhile, paraffin sections were stained by immunohistochemistry for α-SMA to visualize fibrous proliferative membranes. The expressions of α-SMA, CD206, and F4/80 were examined by immunofluorescent staining of whole mount retinas to show fibrous proliferative membranes and macrophages [17].

2.4. Real-time PCR analysis

The total RNA was extracted with the NucleoSpin RNA II System (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol at 14 days after the intravitreal injection and reverse-transcribed using the PrimeScript RT reagent Kit (Takara Biotechnology, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Green Master Mix and specific primers. The following PCR primers from mice were used: α-SMA: forward, 5′-TGC CGA GCG TGA GAT TGT C-3′, reverse, 5′-CGT TCG TTT CCA ATG GTG ATC-3′; Hes-1: forward, 5′-TCC AAG CTA GAG AAG GCA GAC AT-3′, reverse, 5′-GGG TCA CCT CGT TCA TGC A-3′; Arginase-1 (Arg-1): forward, 5′-TGG GTG ACT CCC TGC ATA TCT-3′, reverse, 5′-TTC CAT CAC CTT GCC AAT CC-3′; iNOS: forward, 5′-TGT CTG CAG CAC TTG GAT CAG-3′, reverse, 5′-AAA CTT CGG AAG GGA GCA ATG-3′; GAPDH: forward, 5′-GAC CCC TTC ATT GA CCT CAA C-3′, reverse, 5′-CTT CTC CAT GGT GGT GAA GA-3′.

2.5. Western blot

Retinal samples were homogenized in 100 μL of ice-cold radioimmunoprecipitation assay (RIPA) buffer supplemented with a proteinase inhibitor cocktail at 14 days after the intravitreal injection. The homogenates, which contained 20 μg of proteins, were then separated by SDS-PAGE and transferred to the nitrocellulose membrane (Thermo Fisher Scientific, Beijing, China). The blots were probed with the following primary antibodies: α-SMA (ab5694, Abcam), Hes-1 (ab71559, Abcam), NICD1 (ab8925, Abcam), anti-mouse CD86 (B7-2) purified (GL1, eBioscience), and Arg-1 (sc20150, Santa Cruze). Quantification of the Western blot data was performed by measuring the intensity of the hybridization signals using Image J software.

2.6. Statistical analysis

All data of the results were expressed as the means ± standard deviations. Statistical analysis was performed using SPSS17.0. Data were analyzed using one-way analysis of independent samples *t*-test for two-sample comparisons to compare the means of two groups. A p value of less than 0.05 was considered statistically significant. The reported results were the representative of three independent experiments.

3. Results

3.1. Intravitreal injection of RPE cells for establishing a model of PVR in mice

We successfully established a mouse model to observe PVR evolution. Epiretinal fibrous membranes were observed in the vitreous cavity of all experimental eyes at 14 days after the intravitreal injection of RPE cells. These membranes were associated with tractional retinal detachment and PVR formation. Immunohistochemistry and whole-mount retinal staining analysis also showed that α-SMA positive labeled cells were involved in the PVR fibrous membrane formation (Fig. 1). After 4 weeks, five of nine mice (55.6%) developed extensive retinal detachment.

3.2. M2 type macrophage infiltration in the mouse PVR model

To observe whether M2 macrophages were involved in PVR formation, we examined CD206 expression by whole-mount retinal immunofluorescent staining. In the RPE-injected group, F4/80 positive macrophages and CD206 positive M2 type macrophages were visible in α-SMA positive labeled fibrous membranes at 14 days after the injection (Fig. 2).