·Basic Research·

Retinal stem cells transplantation combined with copolymer -1 immunization reduces interferon -gamma levels in an experimental model of glaucoma

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Abstract

• AIM: To explore the effect of immunization with copolymer-1 (COP-1) and retinal stem cells (RSCs) transplantation on interferon-gamma (IFN-γ) levels in a rat experimental glaucoma model.

• METHODS: An experimental glaucoma was induced by argon laser photocoagulation of the episcleral veins and limbal plexus in the right eye of rats. Immediately following glaucoma induction, rats were immunized with COP-1. RSCs were cultured and transplanted intravitreally into the eyes of glaucoma model animals 1 week post-laser treatment. Six experimental groups were used: COP-1/RSC, PBS/RSC, COP-1/PBS, PBS/PBS, glaucoma model group, and a normal control group. The concentration of IFN-γ in aqueous humor (AH) and serum was measured by enzyme-linked immunosorbent assay (ELISA) in each of the six groups. Retinal ganglion cell (RGC) survival was assessed by quantifying apoptosis using Hoechst staining.

• RESULTS: Concentrations of IFN-γ in AH and serum of rats that had undergone glaucoma induction were higher than those of non-induced control rats. The concentrations of IFN-γ in AH and serum of the COP-1/RSCs treated group were determined to be 2371.9ng/L and 710.9ng/L, respectively, which were significantly lower than those in the other treated groups ($P<0.05$). In fact, IFN-γ levels in the dual treated group were reduced to background levels. The COP-1/RSC group had lower number of apoptotic RGCs than the other three experimental groups ($P<0.05$).

• CONCLUSION: The reduced levels of IFN-γ in AH and serum of the COP-1/RSC group may be related to synergistic effects between RSCs transplantation and COP-1 immune modulation. It is likely that the lower levels of IFN-γ prevented RGCs glaucomatous apoptosis.

• KEYWORDS: glaucoma; interferon-gamma; RSC transplantation; COP-1 immunization
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INTRODUCTION

Glaucoma is a slow progressive neurodegenerative disorder associated with the activation of multiple mechanisms. To date, lowering of intraocular pressure (IOP) is still the first choice for treatment of glaucoma, there are no other more effective therapeutic measures to prevent glaucomatous neuropathy and loss of vision [1,2]. A growing body of research from clinical and experimental studies over the past decade strongly suggests the involvement of the immune system in the ongoing neurodegenerative process caused by glaucoma[3,4]. With the term "neuroprotection" accepted[5], there is increasing interest on cytokines and their functions in damage or protection of retinal ganglion cells [6,7,8]. The concentration of cytokines in glaucoma was associated with glaucomatous optic neuropathy and may regulate RGCs survival or death[9,10,11]. The combination of cytokines under different circumstances or their effect on different cell types such as RGC, microglial cells may result in distinct consequences [7,9]. Therefore, further detailed studies using different treatments will facilitate understanding how cytokines influence the pathogenesis of glaucoma. In this study, we combined COP-1 immunization with transplantation of RSCs into the eye of rats serving as a model for glaucoma, to reveal the change of IFN-γ concentration in the rat eye AH and serum, to further study the relationship between IFN-γ levels and RGCs survival.

MATERIALS AND METHODS

Materials The use of animals in this study was in

accordance with the Guidelines for Animal Experiments of Central South University, Changsha, Hunan, China (Permit number SCXK 2006-0002). Adult female Sprague-Dawley (SD) rats (8-12 weeks of age) were obtained from Animal Laboratory Supplies (Xiangya School of Medicine, Changsha, China). Rats were maintained on a 14:10 hour light:dark cycle and supplied with food and water daily.

Methods

Laser-induced ocular hypertension Ocular hypertension was induced using a modification of the method developed by Chiu *et al* [10]. Briefly, rats were anaesthetized with 10% chloral hydrate (0.4mL/100g; Dingguo Science & Technology Co., Ltd., Beijing, China) injected intraperitoneally and were placed in front of a slit-lamp equipped with a 532-nm diode laser that delivered 0.7-W pulses for 0.6 seconds (Carl Zeiss, Gittingen, Germany). One drop of 1% proparacaine (Xiangya-Pharm Inc., Changsha, China) was applied to each of the rat eyes as a topical anaesthetic before laser photocoagulation. Fifty to 80 laser pulses were directed to the trabecular meshwork 270° around the circumference of the aqueous out-ow area, and 50-70 laser spots on three of four episcleral AH drainage veins of the right eye. If the IOP value was not 8mmHg higher compared to the contralateral (left) eye of the rat after 1 week, treatment was repeated. The IOP of rats were measured bilaterally under anaesthesia before laser induced glaucoma and then weekly thereafter using a digital tonometer (Tonopen XL, Reichert, USA).

Active immunization with an adjuvant Rats were immunized with COP-1 (100μg/100μL; Sigma-Aldrich Inc., St. Louis, MO, USA) or PBS (100μL) emulsified in a total volume of 100μL of complete Freund's adjuvant (CFA; 2.5mg/mL; Sigma-Aldrich Inc., St. Louis, MO, USA), immediately after laser-induction. The immunization was administered subcutaneously at the right base of the tail[12].

Culture of RSCs Isolation and culture of adult SD rat RSCs were performed according to methods described previously [11,13,14]. Briefly, under anaesthesia overdose, the eyeballs of rats were excavated and the retinas were removed surgically. The tissue was minced finely with surgical scissors, and then a single-cell suspension was obtained by incubating the whole retina tissues in 0.25% trypsin (Dingguo Science & Technology Co., Ltd., Changsha, China) for 10 minutes. Dissociated cells were cultured in DMEM/F-12 medium (1:1) supplemented with B27 (Dingguo), 10ng/mL of basic fibroblast growth factor (FGF2), 20ng/mL of epidermal growth factor (EGF), and 2μg/mL insulin (all chemicals were obtained from Sigma-Aldrich Inc., St. Louis, MO, USA). Cell suspensions were cultured at 37℃ and 5% CO_2 for seven to 10 days by which time round collections of cells known as neurosphere (NS) cells had formed. The NS were dissociated and passaged to generate new spheres. For the purpose of this study, cells were transfected with GFP^+-lentivirus (green fluorescent protein; Genechemat Science & Technology Co., Ltd., Shanghai, China) and passage 2 (P2) NS (GFP^+-RSCs) were used.

RSC transplantation Cultured P2 GFP^+-RSCs were harvested with 0.25% trypsin, washed with phosphate-buffered solution (PBS; Dingguo), and suspended at a density of 20 000 cells/μL in PBS. The glaucoma model rats were anaesthetized. A 5-μL ItoMicro syringe was used to pierce the vitreous cavity through the pars plana on the superotemporal conjunctiva, guided by an operating microscope. Subsequently, 5μL of cells were injected slowly into the posterior vitreous cavity, and kept there for 3-5 minutes to reduce leakage from the injection site. An equivalent volume of 5μL PBS was injected into the right eyes of a control group (n=6)[15].

Six groups of SD rats (n=36) were treated as follows: (1) rats were vaccinated with COP-1 emulsified in CFA after laser-induced ocular hypertension[12], and received RSCs via posterior vitreous cavity injection seven days later (COP-1/RSCs); (2) rats were vaccinated with COP-1 and were injected with PBS (emulsified in CFA) seven days later (COP-1/PBS); (3) rats were injected with PBS in CFA and transplanted with RSCs seven days later (PBS/RSCs); (4) rats were injected with PBS both times (PBS/PBS); (5) glaucoma model group-- laser-induced ocular hypertension only; and (6) a normal control group-no laser-induced and any treated.

Immunohistochemistry Samples from 24 rats were used for immunohistochemistry (n=6 per group). At two weeks following RSCs injection into eye of glaucomatous model rats (three weeks after the first laser treatment), under anaesthesia the eyes of animals were perfused with 4% paraformaldehyde (PFA; Dingguo). Eyeballs were removed, and the anterior segment including the lens was excised. Posterior eyecups were postfixed by immersion in 4% PFA for 18 hours at 4℃, cryoprotected in 30% sucrose for 24 hours at 4℃, then embedded in an optimal cutting temperature compound (Sigma-Aldrich). Immunohisto-chemical analyses on frozen sections were performed according to a method described previously [14]. Rabbit anti-GFP was incubated for one hour at room temperature (Beyotime, Institute of Biotechnology, Wuhan, China). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen Inc., Carlsbad, CA, USA). The level of immunofiuorescence was analyzed using confocal microscopy and fluorescence microscopy (Zeiss).

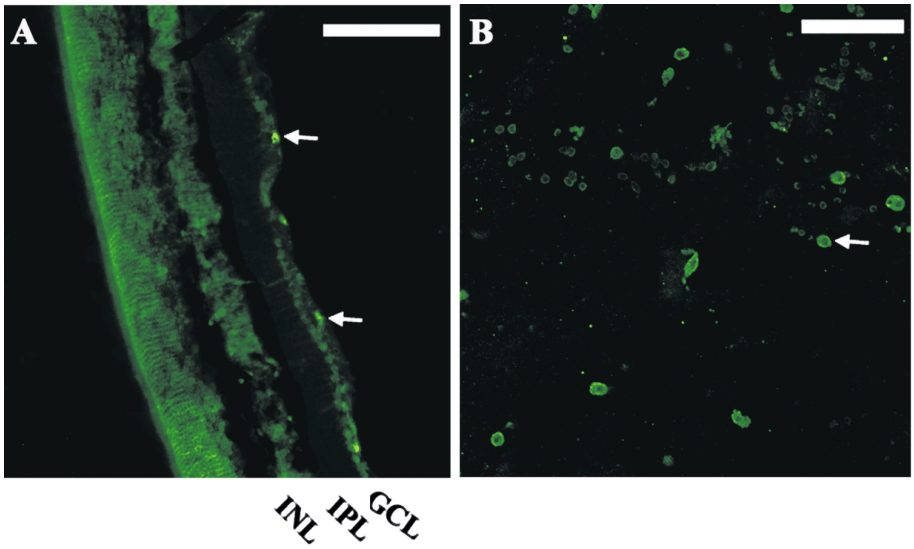

Figure 1 GFP$^+$-RSCs (GFP$^+$-lentivirus transfected retinal stem cells) in the transplanted glaucomatous retina. RSCs were transplanted in the glaucomatous rat eye one week after IOP elevation and the tissue was harvested two weeks after transplantation A: GFP$^+$-RSCs (Green) were observed to be integrated into the nerve fiber layer (NFL) and ganglion cell layer (GCL); B: The localization of GFP$^+$-RSCs in the at-mount retina (Green). INL: Inner nuclear layer; IPL: Inner plexiform layer; Scale bar: 50 μm (A), 75μm (B)

Determination of IFN-γ by ELISA To determine levels of IFN-γ in rat AH and blood, AH was obtained from the anterior and posterior chambers of each group of rats, and blood was taken from the eye artery. Following centrifugation, clear supernatants of AH and serum were used to measure the IFN-γ concentration, according to the manufacturer's instructions (Beyotime).

Determination of apoptosis in RGCs The percentage of RGCs that showed apoptosis was quantified by staining with Hoechst 33 258 dye following the manufacturer's instructions on frozen sections(Beyotime).Using confocal and fluorescence microscopy (Zeiss), cells with signs of fragmented or concentrated nuclei were counted as apoptotic.

Statistical Analysis All data are expressed as the mean ± SD. Statistical analyses were performed using SPSS software version 15.0(IBM Inc., USA).Inter-group comparisons were made using one-way analysis of variance (ANOVA) and a value of $P<0.05$ was considered significant.

RESULTS

Elevated IOP The mean IOP of glaucomatous eyes was elevated significantly compared with those of contralateral eyes (mean IOP; 31.2± 2.2 and 16.8± 1.7mmHg, respectively, $P<0.05$). There was no significant difference in the mean IOP between animals from the four groups (COP-1/RSC; COP-1/PBS, PBS/RSC; PBS/PBS) in which glaucoma had been induced ($P>0.05$; data not shown).

Histological Analysis on RSCs Transplanted Retinas Experiments that used transplantation of GFP$^+$-RSCs into rats allowed the tracking of the grafted cells two weeks after transplantation (three weeks after the onset of ocular hypertension). Analysis on frozen sections of each of the retinal layers revealed that transplanted GFP$^+$-RSCs were present and integrated into the nerve fiber layer (NFL) and retinal ganglion cell layer (GCL) (Figure 1A; $n=4$). Confocal microscopy revealed the existence of GFP$^+$-RSCs in the glaucomatous retina, and these cells were scattered throughout the whole-mount glaucomatous retina, especially in the area adjacent to the injected site (Figure 1B; $n=3$).

Levels of IFN-γ in Rat AH and Blood IFN-γ levels in blood and AH of rats in the glaucoma model group was higher than that of a normal control group ($P<0.05$; Figure 2A, B; $n=6$). IFN-γ levels in serum (Figure 2C) and AH (Figure 2D) of rats from the COP-1/RSC dual-treated group were 710.9ng/L and 2371.9ng/L, respectively, which was significantly lower than that from all other groups ($P<0.05$; Figure 2C, D; $n=6$).

Percentage of Apoptotic RGCs Decreased in Dual-treated Group Cells labelled with Hoechst dye were apparent in the GCL (Figure 3A). We counted the number of cells showing signs of apoptosis from three sections in the GCL layer (from different rats[$n=3$], and from six fields of vision in each section including two optic disc areas, two peripapillary and two peripheral areas). The COP-1/RSC group showed significantly fewer cells undergoing apoptosis compared to all other groups ($P<0.05$; Figure 3B).

DISCUSSION

Glaucoma is one of the leading causes of visual loss as a result of ongoing neurodegeneration of RGCs. Recent

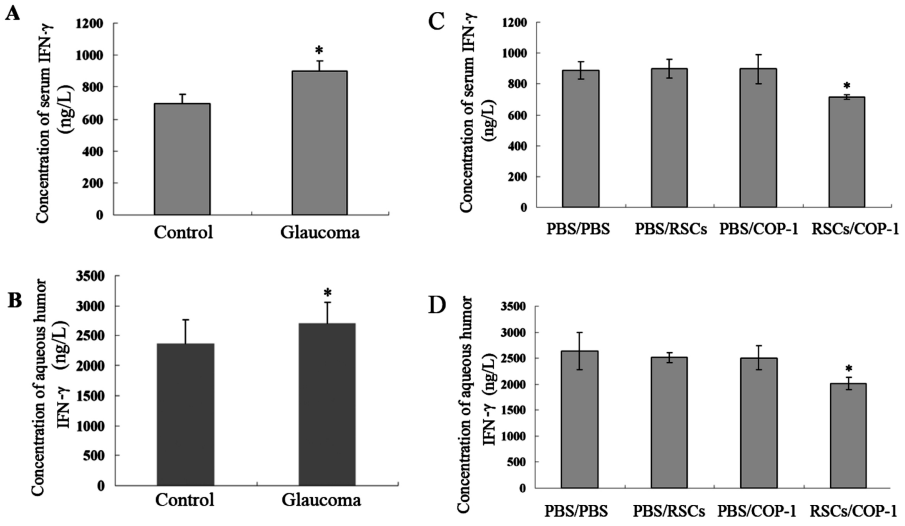

Figure 2 ELISA showing the concentration of IFN-γ in AH and serum in each experimental group and controls. Three weeks following elevation of IOP (two weeks after RSC transplantation), AH and blood samples of rats were prepared for ELISA A, B: IFN-γ levels in serum and AH of rats of the glaucoma model group was higher than that of rats from a normal non-treated control group; C, D: IFN-γ levels in serum and AH of rats from the COP-1/RSCs dual-treated group was significantly lower than that in other groups (* P<0.05)

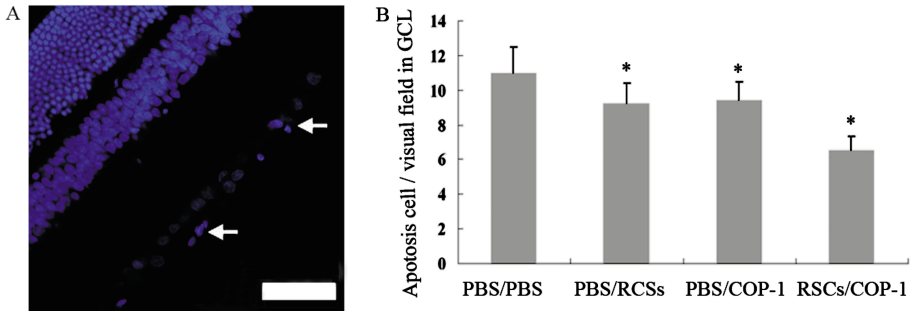

Figure 3 Cells undergoing apoptosis in retinas of animals with induced glaucoma A: Cells positive for Hoechst staining were observed in the GCL; B: Quantification of cells positive for Hoechst staining in the GCL in retinas of each experimental group

mechanistic studies have focused on immunological changes during glaucomatous pathogenesis and protective therapies[16]. Of major interest are cytokines and their function in damage or protection of RGCs. Recent advances in studies of glaucoma or RGCs revealed that cytokines are a possible factor in the pathogenesis of glaucomatous optic neuropathy and may regulate RGCs survival or death. Cytokines, such as IFN-γ, are also involved in neuron damage resulting from non-autoimmune conditions, such as ischemia [17] , and a number of neurodegenerative diseases, including Alzheimer's disease [18]. A significant number of studies on cytokine protection of neurons have been performed and their numbers are increasing year after year [7,19]. In an experimental autoimmune uveoretinitis(EAU) model, IL-27 and IFN-γ have been shown to ameliorate retina damage by EAU. This indicates that Th1 cytokines such as IFN-γ could have protective roles on neurons under certain circumstances[20].

In our study, we combined COP-1 immunization with transplantation of RSCs into glaucomatous rat eyes. We found that cytokine IFN-γ expression patterns in rat AH and serum differ between normal, non-treated rats and glaucoma model rats; in rats representing the glaucoma model IFN-γ levels were statistically significant higher than in normal rats. This result suggests that increased IFN-γ levels maybe relevant for glaucomatous neuropathy and loss of vision[21]. It led us to further examine the change of IFN-γ concentrations in AH and blood in eyes of glaucoma model rats. We found that the concentration of IFN-γ in AH and blood in COP-1/RSC dual-treated animals was significantly lower than that in the other groups with induced glaucoma, that is COP-1/PBS, PBS/RSC, PBS/PBS. In fact, IFN-γ levels in the dual treated group (AH and serum, 2371.9ng/L and 710.9ng/L, respectively)were reduced to background levels (normal control group AH and serum, 2375.3ng/L and 696.9ng/L, respectively). The number of apoptotic RGCs was also lower in the dual-treated group (COP-1/PBS) compared to the other groups, suggesting a relationship between IFN-γ levels and RGCs survival. It seems that lower levels IFN-γ and synergistic effects of

RSCs transplantation and COP-1 immune modulation benefited the survival of RGCs[22]. Butovsky[23] reported that high levels of IFN-γ up-regulated the expression of tumor necrosis factor (TNF)-α by microglia cells, which has a well-known negative effect on neuronal survival. On the other hand, extended exposure (more than 10 days) of microglia to low doses (10ng/mL) of the 'proinflammatory' T-cell derived cytokine, IFN-γ, induced expression of neuronal markers including γ-aminobutyric acid (GABA) and glutamic acid decarboxylase (GAD-67). IFN-γ activated microglia can support neurogenesis from endogenous stem cell pools [24,25]. In a mouse model of experimental autoimmune encephalomyelitis, injected neural progenitor cells (NPCs) were found to migrate to damaged sites, and local immune responses mediated by Th1-associated cytokines (such as IFN-γ) may play a role in such migration [24]. Taken together, our result and that of others revealed that low levels IFN-γ activate microglia and can support neurogenesis, which may benefit the survival of RGCs[26].

The fact that RGCs of the COP-1/RSC treated group in the present study showed lower levels of apoptosis than the groups receiving single or PBS treatments, is consistent with the notion that lower levels IFN-γ protect RGCs. Although accumulated evidence has already reveal the roles of cytokines in glaucoma, it is unclear why IFN-γ levels in AH and blood in COP-1/RSC treated group were lower than those of other groups. Further detailed studies will need to be conducted to understand the pathogenesis of glaucoma.

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