Cytotherapy, 2016; 18: 771–784

International Society for Cellular Therapy
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Neural stem cells transplanted to the subretinal space of rd1 mice delay retinal degeneration by suppressing microglia activation

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Abstract

Background aims. Retinal degeneration (RD) is an inherited eye disease characterized by irreversible photoreceptor loss. Conventionally, the activation of the resident microglia is secondary to the disease. Stem cell–based therapy has recently made rapid progress in treating RD. Although it has been demonstrated that the effect of stem cell therapy may include immunomodulation, the specific mechanisms have not been clarified. *Methods.* Immunocytochemistry, terminal deoxynucleotidyl transferase UTP nick end labelling (TUNEL) assay and Western blot were used to analyze the microglia activation and photoreceptor apoptosis in the retina of rd1 mice. GFP-C17.2 neural stem cells (NSCs) were transplanted into the subretinal space to study the immunomodulatory and neuroprotective effects. The transwell co-culture of BV2 cells with GFP-C17.2 was performed to study the proliferation, apoptosis and secretion levels of inflammatory factors. Real time-quantitative polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA) were performed to explore the gene and protein level of factors secreted by NSCs and microglia. *Results.* TUNEL-positive cells were primarily distributed in the inner nuclear layer (INL) of rd1 mice on P8d, appeared in the outer nuclear layer (ONL) on P10d and peaked on P14d. Meanwhile, microglia migrated to the ONL and reached the maximum level, accompanied by the changes in the levels of fractalkine and its unique receptor CX3CR1 protein. After transplantation of NSCs on P7d into the subretinal space of rd1 mice, the activated microglia were inhibited and the degeneration of ONL was delayed. In addition, microglia activation was suppressed by co-cultured NSCs *in vitro*. The gene and protein level of tissue inhibitor of metalloproteinase (TIMP1) in NSCs was elevated, whereas that of matrix metalloproteinase (MMP9) in BV2 microglia was markedly suppressed in this coculture system. *Conclusions.* Transplanted NSCs in the retina exerted immunomodulatory effects on microglia, thus delaying the degeneration of photoreceptors.

Key Words: *microglial cells, neural stem cells, retinal degeneration, transplantation, immunomodulation*

Introduction

Retinal degeneration (RD) is a set of inherited eye diseases that result in blindness and are pathologically characterized by retinal photoreceptor cell degeneration and apoptosis. It presents clinically and genetically as a heterogeneous condition [1–3]. The prevalence of RD is approximately 1:3000 to 1:4000.

Microglia, as the only innate immune cells in the retina, acts as active sensors of the microenvironment through their rapid transformation [4]. Increasing experimental evidence has provided a comprehensive overview of the microglia physiology and pathology in retinal degeneration [5–7]. Before retinal degeneration occurs, microglia cells are ramified and rest in the inner and outer plexiform layers. As degeneration continues, the endogenous environment induces the proliferation and migration of microglia, enhance phagocytosis and secretion of cytokines, chemokines and neurotoxins. Fractalkine, the chemokine usually expressed by apoptosis photoreceptors, may attract microglia to migrate to the outer nuclear layer (ONL) through its only receptor, CX3CR1, which was expressed by microglia [8]. Then the activated microglia cells contribute significantly to retinal tissue damage and photoreceptor apoptotic events, which in turn promote retinal degeneration [9,10].

Although not thoroughly understood, a number of therapeutic strategies have been reported for the treatment of RD. The recent progress of basic scientific research regarding stem cell therapy may soon benefit RD patients [11]. Different stem cell types have been

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(Received 19 November 2015; accepted 7 March 2016)

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http://dx.doi.org/10.1016/j.jcyt.2016.03.001

used to perform transplantation therapy, including mesenchymal stromal cells (MSCs) [12,13], neural stem cells (NSCs) [14–16] and ocular resident stem cells (RSCs) [17]. Following transplantation, either systemically or focally administered stem cells exert their beneficial effects through not only cell replacement and the release of trophic factors but also immunomodulation in the central nervous system (CNS) [18]. However, it remains unclear whether stem cell administration is also beneficial in RD therapy.

The immunosuppressive properties of stem cells have been demonstrated in a wide range of adaptive and innate immune cells, including T/B lymphocytes and microglia cells in the CNS [18,19]. The transplantation of stem cells into the subretinal space might facilitate the ability of stem cells to cross-talk with resident retinal microglia. During cross-talk, stem cells use different pathways [20], including cell-to-cell contact (juxtacrine) [16,21], the production of soluble factors gradients (paracrine) [15,22], the secretion of factors into the blood (endocrine) [23,24], and the release of extracellular membrane vesicles [25,26]. It has been reported that NSCs might modulate microglial activation through "paracrine" vascular endothelial growth factor and tissue inhibitor of metalloproteinase 1 (TIMP1) [15]. The increase of TIMP1 in the microenvironment could down-regulate the secretion of matrix metalloproteinase-9 (MMP9) by microglia because it is the only inhibitor of MMP9 [27].

In the present study, we performed subretinal space transplantation of GFP-C17.2 NSCs to investigate their immunomodulation effects on the resident retinal microglia of rd1 mice. In addition, to explore the possible immunomodulatory mechanisms of NSCs, we cocultured GFP-C17.2-NSCs or 3T3 fibroblast cells with BV2 microglial cells in an *in vitro* transwell system to mimic the microenvironment in the retina following cell transplantation.

Methods

Ethical approval

All procedures on these animals were approved by the Animal Care and Use Committee of the TMMU. All animal experimentation methodology was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the TMMU, the Guidelines on Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of Association for Research in Vision and Ophthalmology.

Animals

rd1 mice were used as an animal model for our research on retinitis degeneration. In this model, there is a mutation in the rod photoreceptor–specific cyclic guanidine monophosphate (cGMP)–phosphodiesterase 6β that causes the accumulation of cGMP, resulting in the degeneration of the rod photoreceptor. All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were housed with a 12-h light/dark cycle. rd1 mice were sacrificed with cervical dislocation at 10 AM on postnatal (P)8, 10, 12, 14, 16 and 18 d [10].

Tissue preparation

Upon sacrifice, the eyes of the mice were enucleated and fixed in 4% paraformaldehyde at room temperature for 30 min. The anterior segments were then removed using a microscope (Olympus) and fixed in 4% paraformaldehyde for another 2 h. Next, the eyes were infiltrated with 30% sucrose overnight at 4°C and embedded at the optimal cutting temperature (OCT) (Sakura) for cryosection preparation. Using a freezing microtome (Thermo), 10-μm-thick sections were cut in a sagittal plane as described previously [28].

Immunofluorescence and terminal deoxynucleotidyl transferase UTP nick end labelling Assay

Immunofluorescence staining of frozen tissue sections was performed as previously described [29]. Briefly, sections were incubated in 0.3% Triton X-100 and 3% bovine serum albumin (BSA) at room temperature for 30 min separately and incubated with anti-ionized calcium binding adaptor molecule 1 (Iba1; 1:500; Wako) in 1% BSA at 4°C overnight. For Ki67 immunostaining, cells were fixed with 4% paraformaldehyde for 30 min at 4°C, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 3% BSA for 1 h at room temperature. The cells were incubated with primary antibody anti-Ki67 overnight at 4°C. Both the tissue sections and cell slides were then incubated with cy3-conjugated secondary antibody (Jackson ImmunoResearch) for 2 h at room temperature.

After washing with phosphate-buffered saline (PBS), we counterstained cell nuclei with 40, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich). Additionally, terminal deoxynucleotidyl transferase UTP nick end labelling (TUNEL) assays were used to detect the rate of cell apoptosis, both in the tissue sections and cell slides. All of the procedures were performed according to the manufacturer's instruction using the In Situ Cell Death Detection Kit (Fluresceinor TMR, Roche Diagnostics).

All staining (both immunofluorescence and TUNEL assay) was visualized and quantified using a Leica TCS SP50 confocal microscope (Leica Microsystems). Data from three locations ranging from retinal margin to pos-

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