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Neuroprotective effects of intravitreally transplanted adipose tissue and bone marrow–derived mesenchymal stem cells in an experimental ocular hypertension model

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Abstract

Background aims. The purpose of this study was to investigate the neuroprotective effects of bone marrow bone marrow–derived and adipose tissue–derived mesenchymal stromal cells (MSCs) that were intravitreally transplanted in an experimental ocular hypertension (OHT) model. *Methods.* An OHT rat model was generated by means of intracameral injection of hyaluronic acid into the anterior chamber. MSCs labeled with green fluorescence protein were transplanted intravitreally 1 week after OHT induction. At the end of the second and fourth weeks, retinal ganglion cells were visualized with the use of a flat-mount retina method and were evaluated by means of immunofluorescence staining against green fluorescence protein, vimentin, CD105, and cytokines (interleukin [IL]-1Ra, prostaglandin E2 receptor, IL-6, transforming growth factor-β1, interferon-γ and tumor necrosis factor-α). *Results.* The retinal ganglion cell numbers per area were significantly improved in stem cell–treated OHT groups compared with that in the non-treated OHT group ($P < 0.05$). The results of immunohistochemical analyses indicated that a limited number of stem cells had integrated into the ganglion cell layer and the inner nuclear layer. The number of cells expressing proinflammatory cytokines (interferon-γ and tumor necrosis factor-α) decreased in the MSC-transferred group compared with that in the OHT group after 4 weeks ($P < 0.01$). On the other hand, IL-1Ra and prostaglandin E2 receptor expressions were increased in the rat bone marrow–derived MSC group but were more significant in the rat adipose tissue–derived MSC group ($P < 0.01$). *Conclusions.* After intravitreal transplantation, MSCs showed a neuroprotective effect in the rat OHT model. Therefore, MSCs promise an alternative therapy approach for functional recovery in the treatment of glaucoma.

Key Words: *cytokines, mesenchymal stromal cells, neuroprotection, ocular hypertension, retinal ganglion cells*

Introduction

Glaucoma is a leading cause of blindness worldwide. It is a common form of age-related optic nerve and neuropathic disease and affects approximately 60 million people around the globe, a number that is expected to rise to 80 million by 2020 [1]. Vision loss is related to the degeneration and dysfunction of retinal ganglion cells (RGCs). Retinal ganglion cells are highly susceptible to the stress caused by increased intraocular pressure (IOP) in the retina [2]. Elevated IOP is one of the most important risk factors for development of glaucoma. The underlying mechanisms associating elevated IOP with glaucomatous RGC death are not completely understood. Many factors may contribute to degeneration of RGCs, including oxidative stress [3,4], retrograde transport blockage of axoplasmic transport by trophic factors [5], ischemia due to reduced vascular perfusion [6], glutamate-mediated excitotoxicity [7,8], excessive activation of immune system components [9–11] and genetic factors. A combination of 1 or more of these causes and the apoptosis-mediated cell death that occurs as a result of these causes is the focus of current research [12–14]. These facts demonstrate the need for developing alternative treatment methods besides lowering IOP in glaucoma patients. Many researchers have been working toward the prevention of glaucoma by preventing neuronal cell loss, an intervention referred to as neuroprotection [15].

Neuroprotective treatment in glaucoma aims to prevent the death of RGCs and, if possible, other

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neuronal cells. To some extent, lowering IOP can be interpreted as neuroprotective, but the purpose of recent studies in relation to neuroprotection has been to prevent the destruction of the neurons and to stimulate cellular survival by preventing harmful events and thereby reducing the irreversible loss of ganglions. However, currently there is no preventive treatment that protects both RGCs and other neurons.

The use of stem cells in neuroprotective treatments in all areas of medicine has been under recent consideration. The retinal neuroprotective properties of embryonic and fetal stem cells have been demonstrated in various studies [16–19]. These characteristic properties of mesenchymal stem cells (MSCs) make them attractive in neuroprotective therapies. Mesenchymal stem cells secrete brain-derived neurotrophic factor (BDNF), nerve growth factor, glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF) and various cytokines and neurotrophic factors [20–25]. These factors secreted from MSCs have been shown to provide powerful neuroprotective effects on models of amyotrophic lateral sclerosis [26,27], multiple sclerosis [28,29], Parkinson disease [30,31], ischemia [32–34], spinal cord injury [35,36] and experimental glaucoma [37]. The studies involving the use of MSCs in glaucoma treatment showed that MSCs derived from bone marrow (BM) and human umbilical cord blood were effective in sustaining RGC viability in animal models [37–41]. A clinical trial using bone marrow–derived MSCs (BM-MSCs) in the treatment of glaucoma is ongoing (NCT01920867; http://clinicaltrials.gov) [42].

Adipose tissue (AT)-derived MSCs (AT-MSCs) offer a good alternative to BM-MSCs for several reasons: (i) obtaining BM-MSCs is painful, (ii) AT-MSCs are relatively easy to obtain with less morbidity, (iii) more stem cells can be obtained from adipose tissue with respect to bone marrow [43,44], (iv) AT-MSCs display similar properties to BM-MSCs and (v) AT-MSCs have better proliferation ability than BM-MSCs [45,46]. In this study, we used both rat AT-MSCs and rat BM-MSCs to compare their neuroprotective and integration abilities in an experimental ocular hypertension (OHT) model. The cells were marked with green-fluorescent protein (GFP) and transplanted intravitreally. We investigated whether transplanted stem cells prevent the destruction of RGCs and integrate into retinal layers via the vitreous humor.

Methods

In this experimental study, induction of OHT, intravitreal MSC transplantations, euthanizing of the animals and preparation of samples for immunofluorescence microscopy were performed at the Experimental Animal Centre of Kocaeli University (Kocaeli, Turkey). Immunofluorescence studies were carried out at the Kocaeli University Centre for Stem Cell and Gene Therapies Research and Practice. The study was carried out in accordance with the principles defined by the Helsinki Declaration (2008). The ethics committee of the Kocaeli University Medical Faculty approved the study (HADYEK 4/1-2010).

Animals

Forty-four adult rats (female, Wistar albino) were used in the experimental OHT study. In addition, 5 male Wistar rats were obtained for stem cell isolation from the Experimental Animal Centre of Kocaeli University. The average weight of the subjects was calculated to be 369 ± 10.7 g. The subjects were kept in standard cages in groups (4 subjects per group) and were fed *ad libitum*. The environmental temperatures were kept constant at 21 ± 2°C, and the humidity was controlled. The rooms were lit with fluorescent lights on a 12-h on-off light cycle (06:00 AM–06:00 PM). The animals were divided into 2 groups: an OHT-induced group (n = 40) and the control group (n = 4). Saline was injected with a 30-gauge needle into the anterior chambers of the rats in the control group.

The 40 OHT-induced animals were grouped as follows:

Group 1. After the induction of OHT, rBM-MSCs were intravitreally transplanted to the eyes, and the animals were euthanized after 2 weeks. This group is called the BM-MSCs second-week group (n = 8).

Group 2. After the induction of OHT, rBM-MSCs were intravitreally transplanted to the eyes, and the animals were euthanized after 4 weeks (BM-MSCs fourth-week group; n = 8).

Group 3. After the induction of OHT, rAT-MSCs were intravitreally transplanted to the eyes, and the animals were euthanized after 2 weeks (AT-MSCs second-week group; n = 8).

Group 4. After the induction of OHT, rAT-MSCs were intravitreally transplanted to the eyes, and the animals were sacrificed after 4 weeks (AT-MSCs fourth-week group; n = 8).

Group 5. After the induction of OHT, the animals were given 0.1 M phosphate-buffered saline (PBS) intravitreally and were sacrificed after 2 weeks (OHT second-week group; n = 4).

Group 6. After induction of OHT, the animals were given PBS intravitreally and were euthanized after 4 weeks (OHT fourth-week group; n = 4).

Intravitreal stem cell transplantation was not performed on the OHT second- and fourth-week groups. Instead, PBS injections were used in these

control groups following the same procedure as the stem cell injection.

Induction of ocular hypertension

Ocular hypertension was induced using hyaluronic acid (HA). For general anaesthesia, ketamine hydrochloride (25mg/kg; Ketalar, Pfizer) and xylazine hydrochloride (10 mg/kg; Rompun, Bayer) were administered intraperitoneally to the rats. Following anaesthesia, using a syringe and a 30-gauge needle, HA (Sigma) was injected into the anterior chamber of the right eye of each rat until the iris vein withered [47–49]. The eyes were focused under a surgical microscope (OMS-75; TOPCON) with coaxial light. The injections were performed every week throughout the experiment. The injections were made through the corneal limbus into the anterior chamber to create a self-sealing entry. The needle was moved in a clockwise direction, and contact of the needle tip with the iris and the lens was avoided [47]. Induction of OHT was repeated at 1-week intervals for 3 weeks [50,51].

Before and after each injection, IOP was measured with a TonoPen XL applanation tonometer (Mentor) under light anaesthesia, which was induced by 10 mg/kg ketamine hydrochloride and a drop of topically applied proparacaine HCl (Alcaine, Alcon). Injections and measurements were always performed within the same time period (between 8 AM and 10 AM).

Preparation of MSCs

Isolation and culture of rat AT-MSCs (rAT-MSCs). Rats ($n = 5$) were anaesthetized with an injection of 10 mg/kg xylazine HCl and 75 mg/kg ketamine HCl. A total of 1–2 cm^3 of peri-peritoneal AT was removed. Tissue samples were washed several times with Hanks' balanced salt solution supplemented with a 5% antibiotic–antimycotic solution (Gibco Life Technologies), and vascular structures were removed. The yellowish-white tissue was minced and enzymatically digested in an L-DMEM medium (Gibco Life Technologies) containing 0.075% collagenase 1 (Sigma) at 37°C for 60 min. The cell suspension was filtered with a 70-µm mesh nylon filter (Becton Dickinson Labware). The cells were resuspended in an L-Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; standard culture medium). After centrifugation at 1800 rpm for 10 min, the cells were cultured in a standard culture medium in 25-cm^2 culture flasks. After 5–7 days, the medium was replaced with fresh medium and was subsequently replaced twice a week. After reaching 70–80% confluence, the cells were harvested with 0.025% trypsin-EDTA for 3–4 min, collected by centrifugation and subcultured at a 1:3 ratio. The cells were counted using trypan blue (Biological Industries). The blue staining of cells after mixing (1:1) was used as an indicator of cell death.

Isolation and culture of rat BM-MSCs (rBM-MSCs). Under sterile conditions, both femur and tibiae were excised from each rat, and a 21-gauge needle was used to flush the marrow with an L-DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. The marrow plug suspension was dispersed by pipetting, filtered through a 70-µm mesh nylon filter and centrifuged at 1800 rpm for 10 min. The bone marrow was diluted to 1:3 with PBS and was layered over a Histopaque-1077 (1.077 g/mL, Sigma-Aldrich) for gradient centrifugation. The low-density mononuclear cells were collected and washed twice with PBS. The cells were seeded on plastic tissue culture flasks and incubated for 3 days under standard conditions. MSCs were isolated based on their ability to adhere to culture plates. To remove unattached cells, fresh medium was added. The cells were passaged as described as earlier. After 3 more passages, characterization studies were carried out.

Characterization of MSCs. Undifferentiated rAT-MSCs and rBM-MSCs were subjected to flow cytometry analysis. After passage 3 (P3), stem cells were harvested. Flow cytometry was performed using a FACS Calibur (BD Biosciences). Immunophenotyping analysis was performed against the following antigens: CD29, CD45, CD54, CD90, CD106 and major histocompatibility complex (MHC) class II (BD Biosciences). The data were analyzed with Cell Quest software (BD Biosciences).

For immunofluorescence staining of rAT-MSCs and rBM-MSCs, samples were rinsed briefly in PBS and fixed in ice-cold methanol for 10 min. After permeabilization with 0.025% Triton X-100 (Merck), the cells were incubated with 1.5% blocking serum (Santa Cruz Biotechnology) in PBS for 30 min at 37°C, followed by incubation overnight at 4°C with the primary antibody. After 3 PBS washes, cells were incubated with the secondary antibody for 25 min. The samples were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (Santa Cruz Biotechnology). The list of primary antibodies is given in Table I.

In vitro differentiation of MSCs. Adipogenic and osteogenic differentiations were performed *in vitro* according to a published protocol [52].

For osteogenic differentiation, the cells were incubated in standard culture medium supplemented with