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The potential neuroprotective effect of human adipose stem cells conditioned medium against light-induced retinal damage

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ABSTRACT

Human adipose-derived stem cells (hASCs) are present in adult adipose tissue and have been reported to secrete various factors that have neuroprotective effects. In the present study, we examined whether hASC-conditioned medium (hASC-CM) was effective against experimental degenerative retinal disease. Mature adipocytes (MAs) and hASCs were isolated from human subcutaneous adipose tissue. The isolated hASCs were identified based on their capacity for bone and neural differentiation. The effects of hASC-CM against tunicamycin-, H_2O_2-, and light-induced retinal photoreceptor damage were evaluated *in vitro* by measuring cell death. Moreover, we identified various factors present in hASC-CM using antibody arrays. Retinal damage induced in mice by exposure to white light was studied *in vivo*, and photoreceptor damage was evaluated according to the thickness of the outer nuclear layer and electroretinography results. In addition, the effect of hASC-CM on Akt phosphorylation at Ser473 was confirmed by western blotting. Finally, the effects of the secreted proteins identified in the hASC-CM on light-induced damage were evaluated *in vivo*. Isolated hASCs differentiated to osteocytes and neurons. hASC-CM protected against tunicamycin-, H_2O_2-, and light-induced cell death. In addition, hASC-CM inhibited photoreceptor degeneration and retinal dysfunction after exposure to light. Several proteins secreted by hASCs, such as the tissue inhibitor of metalloproteinase-1 (TIMP-1) and the secreted protein acidic and rich in cysteine (SPARC), protected against light-induced damage *in vitro* and *in vivo*. The results of the present study showed that hASC-CM has neuroprotective effects against light-induced retinal damage and suggest that hASCs have a therapeutic potential in retinal degenerative diseases via their secreted proteins, without requiring transplantation.

1. Introduction

It is well known that excessive light irradiation induces photoreceptor degeneration (Green and Robertson, 1991; Noell et al., 1966) and that the resulting retinal damage is irreversible, leading to night blindness and serious loss of visual field. Light-induced death of photoreceptor cells is mediated by various factors, such as oxidative stress (Imai et al., 2010; Wenzel et al., 2005), mitochondrial damage (Donovan et al., 2001), rhodopsin mutation (Wenzel et al., 2001), and endoplasmic reticulum (ER) stress (Yamauchi et al., 2011; Yang et al., 2008).

Adult stem cells from the bone marrow stroma, which are termed mesenchymal stem cells (MSCs), have been proposed as an alternative stem cell source of embryonic stem cells or induced pluripotent stem cells. However, the clinical use of bone marrow-derived stem cells (BMSCs) is associated with some problems that need to be addressed: the harvesting of cells is painful and the bone marrow contains few available cells. As another source of stem cells, adipose tissue has several advantages: it can be obtained in larger quantities using a less invasive method (Strioga et al., 2012). Adipose-derived stem cells (ASCs) can be obtained repeatedly in large quantities under local anesthesia. Moreover, ASCs are used in clinical applications such as breast implantation. Therefore, ASCs may have few safety issues.

Few studies have addressed the effects of ASCs on retinal damage (Yang et al., 2010; Yu et al., 2010). In contrast, BMSCs have

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been described as being useful for regenerative therapy (Inoue et al., 2007) and to have neuroprotective effects in several neurodegeneration (Parr et al., 2007; Torrente and Polli, 2008) and retinal degeneration models (Zhang and Wang, 2010). Despite the improvements observed, reports have shown that only a few cells integrate into the neural retina and that the majority of the transplanted cells survived in the vitreous cavity without engrafting because of glial reactivity (Johnson et al., 2010b). The obstacles to retinal cell therapy include the inhibitory barrier that prevents the migration of grafted cells from the transplantation site into the retina (Johnson et al., 2010b). As no engrafted cells differentiated into neural or retinal cells (Johnson et al., 2010a, 2010b; Zhang and Wang, 2010), the observed improvements may have been the result of the trophic support provided to host cells by factors released by mesenchymal stem cells.

A comparative analysis of MSCs obtained from bone marrow, adipose tissue, and umbilical cord demonstrated that ASCs are not different from these other cell types regarding morphology, immune phenotype, colony frequency, and differentiation capacity (Izadpanah et al., 2006; Kern et al., 2006). MSCs secrete a broad spectrum of cytokines, such as the vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) (Ikegame et al., 2010; Kinnaird et al., 2004). These findings indicate that the protection of the retina by stem cells depends, at least in part, on secreted cytokines. The identification of the other factors secreted by human ASCs (hASCs) that protect against retinal injury may lead to the discovery of new drug targets. Therefore, we evaluated the protective effects of the conditioned medium of hASCs (hASC-CM) against retinal degeneration *in vitro* and *in vivo*-, and identified various retinal protective factors in this medium.

2. Materials and methods

2.1. Animals

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved and monitored by the Institutional Animal Care and Use Committee of the Gifu Pharmaceutical University. Male adult ddY albino mice, aged 8 weeks, were purchased from Japan SLC (Hamamatsu, Japan) and kept under controlled lighting conditions (12/12 h light/dark cycle).

2.2. Isolation and culture of hASCs and mature adipocytes (MAs)

This study was performed according to the tenets of the Declaration of Helsinki and was conducted after receiving approval from the ethics committees of the Gifu Pharmaceutical University and Gifu University. hASCs and MAs were obtained from human fat tissue that was collected from patients after they provided written informed consent; the cells were provided by Gifu University (Department of Neurosurgery, Gifu University Graduate School of Medicine). The patients included 6 males and 4 females ranging in age from 30 to 72 years. The isolation of hASCs was performed using a technique that was described in a previous report (De Ugarte et al., 2003). The fat tissue was minced, digested with 0.075% collagenase (Wako, Osaka, Japan), and centrifuged at 1200 × *g* for 5 min. Floating adipocytes were collected and washed twice in Dulbecco's Modified Eagle's Medium (DMEM; Nacalai Tesque Inc., Kyoto, Japan). The cell pellet was re-suspended in DMEM/F-12 (Sigma–Aldrich, St. Louis, MO, USA) containing 20% fetal bovine serum (FBS) and plated in 100-mm culture dishes. The isolated hASCs were maintained in 10% FBS DMEM, 100 U/mL penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 μg/mL streptomycin (Meiji Seika) under a humidified atmosphere with 5% CO_2 at 37 °C. The cells were passaged via trypsinization every 3–4 days. Passages 3 to 8 were used in the experiments.

2.3. Osteogenic differentiation

For osteogenic differentiation, hASCs were grown to confluence prior to induction and then cultured in osteogenic medium (Kim et al., 2008) (DMEM, 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid, 100 nM dexamethasone [Sigma–Aldrich] and 10% FBS), which was changed every 3 days. After 15 days, cells were washed once with phosphate-buffered saline (PBS) carefully and fixed with phosphate-buffered formalin for 20 min. The fixed cells were washed once with distilled water and subsequently stained with 1% Alizarin Red S (Sigma–Aldrich) dissolved in distilled water for 5 min. After washing with distilled water, the cells were air dried, and images of the stained cells were captured using a light microscope (Kawazoe et al., 2008).

2.4. Neurogenic differentiation

Subconfluent hASCs were cultured for 24 h in pre-induction medium (DMEM, 20%FBS, and 1 mM β-mercaptoethanol [Wako]). The pre-induction medium was removed after 24 h, and the cells were washed with PBS and transferred to a neurogenic medium (serum-free medium containing 0.5 mM β-mercaptoethanol) for induction of neuronal differentiation. At 7 days after incubation in the neurogenic medium, the expression of the neuronal-specific nuclear protein NeuN in these cells was analyzed by immunostaining. The cultured cells were fixed in 4% fresh paraformaldehyde at room temperature, blocked using 3% goat serum and 0.2% Triton X-100 in PBS, and incubated overnight at 4 °C with the primary antibody (anti-NeuN, clone A60 [Millipore, Bedford, MA, USA]) diluted in PBS. After washing, the cells were incubated for 1 h with the secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG [Invitrogen, Carlsbad, CA, USA)], washed, and counterstained with Hoechst 33342 (Invitrogen). Images were captured using a BZ-9000 BIOREVO all-in-one fluorescence microscope (Keyence, Osaka, Japan).

2.5. Collection of hASC-CM and conditioned medium from MAs (MA-CM)

MAs and hASCs (4×10^5 cells/100 mm dish) were cultured in FBS-free DMEM. After 72 h of culture, hASC-CM and MA-CM were collected. The collected media were centrifuged at 300 × *g* for 5 min and filtered using a 0.22 μm syringe filter. MA-CM was mainly used for 2 purposes. First, this medium was used to investigate whether any cells included in adipose tissue, other than ASCs, had protective effects in retinal degenerative diseases. Second, MA-CM was used to narrow down the protective factors present in hASC-CM for the cytokine array study. Because MA-CM did not have an effect in the cell death assay, the proteins contained in MA-CM were rejected as protective factors.

2.6. Cytokine array

The RayBio® Biotin Label-based human Antibody Array I (RayBiotech, Inc., Norcross, GA, USA) was used to investigate the cytokines that were secreted by hASCs. Briefly, the membranes of the array were blocked using a blocking buffer, followed by the addition of biotin-labeled medium from hASC or MA cultures from same individuals, 69-year-old male and 50-year-old female, and incubation at room temperature for 2 h. The membranes were washed and incubated at room temperature for 2 h after the addition of horseradish peroxidase (HRP)-conjugated streptavidin.