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Protective Effect of Intravitreal Administration of Exosomes Derived from Mesenchymal Stem Cells on Retinal Ischemia

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ABSTRACT

Purpose: Exosomes derived from human mesenchymal stem cells (hMSCs) cultured under hypoxic conditions contain proteins and growth factors that promote angiogenesis. This study investigated the effect of intravitreal administration of these exosomes on retinal ischemia using a murine model.

Methods: Oxygen-induced retinopathy (OIR) was induced by exposing one-week-old male C57BL/6J mice to 5 days of 75% hyperoxic conditioning, and returning to room air. After hyperoxic conditioning, the right eye of each mouse was injected intravitreally with 1 µl saline or exosomes derived from hMSCs and compared to control mice of the same age raised in room air without OIR injected intravitreally with saline. Two weeks post-injection, fluorescein angiography (FA) and phase-variance optical coherence tomography angiography (pvOCTA) were used to assess retinal perfusion. Retinal thickness was determined by OCT. The extent of retinal neovascularization was quantitated histologically by counting vascular nuclei on the retinal surface.

Results: Among eyes with OIR, intravitreal exosome treatment partially preserved retinal vascular flow *in vivo* and reduced associated retinal thinning; retinal thickness on OCT was 111.1 ± 7.4µm with saline versus 132.1 ± 11.6µm with exosome, $p < 0.001$. Retinal neovascularization among OIR eyes was reduced with exosome treatment when compared to saline-treated eyes (7.75 ± 3.68 versus 2.68 ± 1.35 neovascular nuclei per section, $p < 0.0001$). No immunogenicity or ocular/systemic adverse effect was associated with intravitreal exosome treatment.

Conclusions: Intravitreal administration of exosomes derived from hMSCs was well tolerated without immunosuppression and decreased the severity of retinal ischemia in this murine model. This appealing novel non-cellular therapeutic approach warrants further exploration.

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Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) have tissue healing capabilities and are currently widely explored for numerous therapeutic applications. It has been demonstrated that the effects of MSCs are mediated mostly via paracrine signaling factors to surrounding endogenous cells, rather than direct cell replacement.[1–4]

Exosomes are small (50–150 nm) bi-lipid membrane intracellular vesicles, which are packaged with and transport a variety of proteins and RNAs from their cell of origin to neighboring cells.[5] Exosomes were recently characterized to provide cell-to-cell communication that mediates complex cellular processes, such as antigen cross-presentation, stem cell differentiation and angiogenesis.[6–9]

Recent studies have demonstrated that exosomes derived from MSCs have a protective effect in models of tissue ischemia and reperfusion injury.[9–16] For example, treatment with exosomes derived from MSCs significantly decreased myocardial infarction size in murine models of myocardial ischemia-reperfusion[12,13] and significantly improved blood flow recovery in hind limb ischemia models.[9] A recent comprehensive proteomic analysis of exosomes derived from human MSCs cultured under hypoxic, serum-free conditions revealed that these exosomes express a diverse profile of factors involved in angiogenesis including signaling proteins associated with nuclear factor kappa B (NFkB), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF).[17]

Studies have determined that intravitreal injection of MSCs in animal eyes with acute ischemia-reperfusion injury results in preservation of the ganglion cell layer, an effect that could be replicated by intravitreal injection of conditioned media.[18] Since some safety issues have been noted using intravitreal injection of MSCs in some animal models,[19] this study investigated the effect of intravitreal administration of exosomes derived from MSCs as a potential non-cellular therapy for retinal ischemia.

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Methods

Animals

This study protocol was approved by the Institutional Animal Care and Use Committee at the University of California Davis before initiation. The study was conducted according to an approved protocol and in accordance with AAALAC and with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Twelve C57BL/6J mice (Jackson Laboratories; strain 000664) were used in this study. All mice were male and one week of age at the initiation of the study. The mice were maintained with their nursing mothers at the vivarium for the duration of the study until 4 weeks of age. Mice were divided into three groups (see Intravitreal Injections section below), and all mice in all three groups were from the same litter.

Oxygen-induced retinopathy model

A well-established protocol for inducing oxygen-induced retinopathy (OIR) in mice was used to induce retinal ischemia simulating retinopathy of prematurity (ROP).[20] At the age of one week, eight mice were place in a closed chamber under an oxygen concentration of 75% for 5 days. The chamber remained closed during the 5-day hyperoxic exposure period. The hyperoxic condition was monitored continuously using an oxygen sensor (MiniOX I oxygen analyzer; MSA Instrument Division, Pittsburgh, PA), which ensured an oxygen concentration of 75.0%±2.0% for the entire duration. Following the hyperoxic exposure, room air becomes relatively hypoxic to the mice, and by 2 weeks, all eyes develop retinal ischemia and neovascularization.[20] An advantage of this model is that the level of ischemia is quantifiable, based on the count of neovascular cell nuclei on the vitreal surface of the retina on histology[20] (see Tissue Processing and Histology section below for the full details). The remaining four mice were kept at room air for the same 5-day period, as a control group without OIR.

Exosome isolation

Fresh bone marrow from three young non-smoking males was obtained from a commercial vendor (Lonza). MSCs were isolated as previously described and used for exosome isolation at passage 6.[17] Exosomes were isolated from media (OptiMEM) that had been conditioned by MSCs for 48 hours under 1% oxygen tension (serum-free). The conditioned media were precleared of cells and cellular debris via serial centrifugation of the supernatants: a) 500 x g for 10 minutes, b) 2000 x g for 15 minutes. Exosomes were concentrated and washed from the resulting supernatants using tangential flow filtration with a 300 kDa molecular weight cutoff polyethersulfone (PES) membrane (Pall, Port Washington, NY), using a diafiltration wash step with 500 mL of sterile PBS. This step allowed the elimination of cells, cell debris and microvesicles. The resulting exosome concentrated solution was further concentrated using a VivaSpin filtration column with a 300 kDa molecular weight cutoff PES membrane. The resulting concentrated exosome solution was aliquoted at 10 µL and kept at −80°C until used. Vesicle concentration was measured using DC assay (BioRad, Hercules, CA), and size distribution was determined by NanoSight LM10HS (Malvern, Amesbury, MA). This process has previously been described in detail.[17] This isolation protocol highly enriched the isolates for purified exosomes suspended in a PBS buffer.

Intravitreal injections

Intravitreal injections were performed after the 5 days of hyperoxic exposure, when the mice were 12 days old. An intravitreal injection was performed once in the right eye of each mouse. It was performed using a pars planar and transconjunctival approach under isoflurane (2–3% in oxygen) anesthesia. After instilling a drop of 5% betadine solution into the fornix, a sterile 33g needle attached to a Hamilton syringe was used to deliver 1 µl of isolated exosome solution or saline per eye. There were three groups of mice (n = 4 in each group), which were injected as follows: Group 1 included four mice that underwent the OIR induction and were injected with 1 µl saline; Group 2 included four mice that underwent the OIR induction and were injected with exosomes derived from human MSCs (20 µg in 1 µl), and Group 3 included four mice without OIR that were kept at room air at all times and injected with 1 µl saline. Following intravitreal injection, antibiotic eye ointment was applied to the injected eye.

Retinal imaging

Animals were imaged 2 weeks after intravitreal injection. Prior to imaging, all eyes were examined by indirect ophthalmoscopy to determine whether any ocular complications had occurred following the intravitreal injection of exosomes, such as intraocular inflammation, hemorrhage, cataract, retinal detachment or endophthalmitis. A multimodal retinal imaging system specifically designed and built for *in vivo* mouse retinal imaging was used. This system integrates multichannel scanning laser ophthalmoscopy (SLO) and optical coherence tomography (OCT) and allows simultaneous collection of complementary information from the tissue, greatly simplifying data registration and analysis. This system has been described in detail elsewhere.[21] In this study, it was used to perform simultaneous fluorescein angiography (FA) and phase variance OCT angiography (pvOCTA). The pvOCTA detects flow and perfusion in the retinal vasculature and does not visualize non-perfused vessels.

Fluorescein sodium (0.1 ml of 1%) was injected into the tail vein prior to anesthesia and imaging. The mouse retinal imaging was performed under isoflurane (2–3% in oxygen) inhalation anesthesia. A heating pad was used to maintain normal body temperature, and avoid the development of "cold cataracts" during imaging.[22] The head was held rigidly by a "bite-bar" that also served to keep its snout inside the gaseous isoflurane anesthetic delivery tube.

With its customized scanning head, the scanning field of view (FOV) can be up to 50 degrees, while software control allows limiting the scanning to any square subfield of the larger field. With a customized contact lens mounted to the scan head,

the mouse cornea was kept hydrated and clear, greatly facilitating mouse handling during a single imaging session.

Retinal thickness was measured using OCT B-scan images, which were taken at identical locations (horizontal scans through the optic disc). Retinal thickness was measured as the distance between the internal limiting membrane (ILM) and the retinal pigment epithelium (RPE), between 800 points and 1mm temporal from the optic disc margin on the OCT B-scan image. The retinal thickness values were averaged, and the means were compared using an unpaired two-tailed Student's t-test.

Tissue processing and histology

Following imaging, the mice were euthanized by asphyxiation with gaseous CO_2 in a closed chamber, and the right eyes were harvested promptly for histological analysis. The contralateral eye (untreated left eye) from the exosome-treated mice (Group 2) was harvested and fixed as well for analysis.

The eyes were enucleated, fixed in 4% paraformaldehyde, and embedded in paraffin.[23] For orientation in paraffin, the superior region of each eye was marked using tissue dye (The Davidson Marking System, Bradley Products Inc., Bloomington, MN, catalog #1003–6). Sagittal sections were cut using a Leica RM2125RT microtome (Leica, Nussloch, Germany) at 6 microns, placed on SuperFrost Plus microscope slides, and dried overnight at room temperature. Based on the previous orientation of each eye in the paraffin embedding step, a section through the optic disk represented a sagittal section.

Sections underwent standard Hematoxylin-Eosin staining. For each eye, eight sections were carefully viewed at 40X magnification, and neovascular nuclei on the retinal surface were counted. Sections used for counting included four retinal sections from either side of the optic disc, at a distance of 30 to 90 microns from the optic disc, in accordance with the OIR protocol.[20] In each section, neovascular nuclei were identified by their location on the vitreal side of the ILM on the retinal surface. Therefore, a total of eight sections (four sections from each side of the optic nerve) were counted from each eye. The means for each study group were compared using an unpaired two-tailed Student's t-test. Statistical analysis was performed using IBM SPSS Statistics version 21.0.

Slides were viewed and digitized images captured using a Nikon Eclipse E800 and QCapture software (QImaging, Surrey, Canada).

Proteomic data analysis

Proteomic data analysis was performed on data obtained from the analysis of exosomes derived from human MSCs cultured under hypoxic conditions as previously reported.[17] This is a new analysis of previously published data collected from exosomes derived from hMSC as used in this study.[17] Briefly, a multilayered analysis was employed that included clustered network analysis using CytoScape (=) and Ingenuity Pathway Analysis software (Qiagen, Redwood City, CA, USA). The Spike database was used to detect proteins for which there was experimental evidence for physical interactions (*i.e.*, yeast-2-hybrid, co-immunoprecipitation) and was accessed via CytoScape.

Results

Exosomes derived from human MSCs cultured under hypoxic and serum-free conditions were injected intravitreally into murine eyes with OIR to assess the protective effects of this therapy on ischemic retina. Table 1 summarizes the experimental design of the study arms. The eyes with OIR treated with exosomes had three different sets of controls for comparison: 1) eyes without OIR injected with saline, 2) eyes with OIR injected with saline, and 3) eyes with OIR without treatment (contralateral eye of exosome-treated eye with OIR).

In vivo *retinal imaging*

To evaluate the extent of retinal ischemia and neovascularization, the retinal perfusion of the mice was analyzed *in vivo* using simultaneous combined FA and pvOCTA imaging of the retina 2 weeks following intravitreal injection of saline or exosomes. All eyes of mice that underwent OIR induction protocol developed areas of retinal capillary non-perfusion and retinal neovascularization that were apparent on FA and pvOCTA, whereas eyes of mice grown under room air conditions (i.e., without OIR) had normal retinal vascular filling with no areas of retinal non-perfusion or neovascularization (Figure 1). These areas of retinal capillary non-perfusion and retinal neovascularization on FA and pvOCTA were more pronounced in saline-injected eyes with OIR when compared to OIR eyes treated with intravitreal exosome (Figures 1C and F).

Figure 2 illustrates B-scan cross-sectional OCT images of the retina with superimposed pvOCTA signals (red), showing the location of vascular flow relative to the retinal layers. An increased blood flow on the inner surface of the retina was noted in all eyes with OIR in groups 1 and 2 indicative of retinal neovascularization. Retinal thickness was measured using the OCT B-scan images and was found to be 111.1 ± 7.4 μm in Group 1, 132.1 ± 11.6 μm in Group 2, and 205.9 ± 18.8 μm in Group 3. Eyes with OIR (Groups 1 and 2) had significantly thinner retina in comparison with eyes without OIR of mice that had been exposed only to room air (Group 3) ($p < 0.001$ for both). Among eyes with OIR, retinal thickness in the eye treated with saline (Group 1) was thinner than in the eye treated with exosomes (Group 2) ($p < 0.001$).

Histological analysis of retinal neovascularization

To quantitate the level of retinal ischemia, histologic analysis was conducted. By counting the number of neovascular nuclei on the retinal surface in eight sections per eye taken at similar distances from the optic disc, a quantitative measure of the

Table 1. Summary of study groups.

Group	OIR	Eyes	Intravitreal injection	N	Tests performed at 2 weeks following injection
1	Yes	OD	Saline	4	FA, pvOCTA, histology
2	Yes	OD	Exosomes	4	FA, pvOCTA, histology
2	Yes	OS	No	4	Histology only
3	No (room air)	OD	Saline	4	FA, pvOCTA, histology

(OIR = oxygen induced retinopathy; FA = fluorescein angiography; pvOCTA = phase variance optical coherence tomography angiography; OD = right eye; OS = left eye).

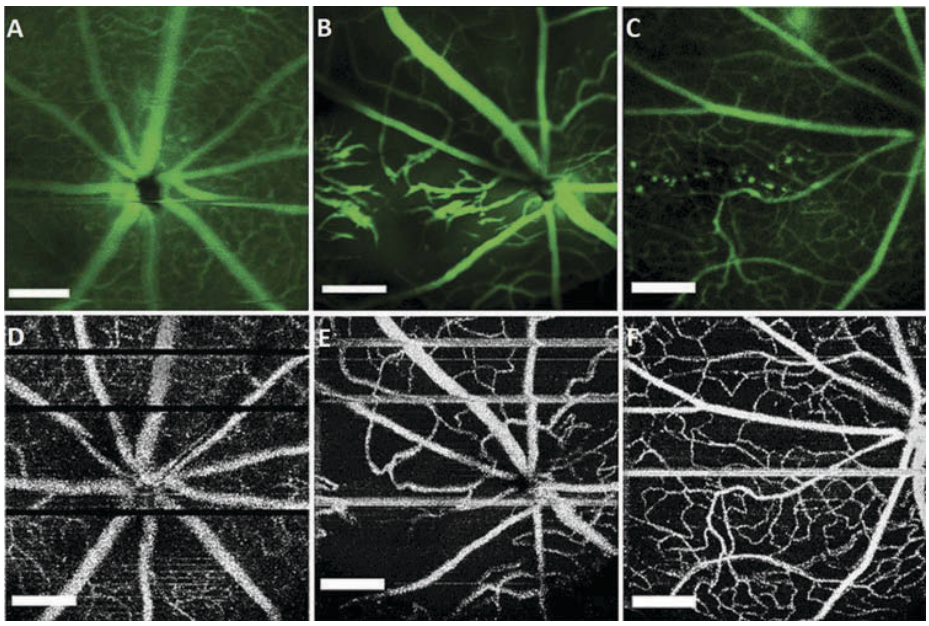

Figure 1. *In vivo* retinal vascular flow imaging of eyes with and without oxygen induced retinopathy (OIR) demonstrating protective effect of intravitreal exosome treatment on retinal ischemia. (A-C) Fluorescein angiogram. Normal retinal perfusion is demonstrated in the eye without OIR (A). In the eyes with OIR, areas of retinal ischemia and neovascularization are seen which are more pronounced in the eye that was treated with intravitreal saline (B) when compared to OIR eyes treated with intravitreal exosomes (C). (D-F) Corresponding phase variance OCT angiography maps of the retinal vascular flow of the same eyes as shown in A (D), B (E) and C (F). Normal retinal capillary perfusion is demonstrated in the eye without OIR (D). The eyes with OIR show marked retinal capillary non-perfusion which is more pronounced in the eye that was treated with intravitreal saline (E) when compared to the eye treated with intravitreal exosomes (F). The horizontal lines are motion artifacts. The length of the horizontal bar scale at the lower left corner of each image represents 0.17 mm.

degree of retinal neovascularization was obtained for each study eye. An example of retinal neovascularization on the retinal surface, that is, the vitreal side of the ILM is presented in Figure 3.

In Group 1, OIR eyes treated with saline, there was an average of 7.75 ± 3.68 neovascular nuclei per section. In Group 2, OIR eyes treated with exosomes, there was an average of 2.68 ± 1.35 neovascular nuclei per section. In the untreated fellow eyes of mice from Group 2, there was an average of 7.0 ± 2.48 neovascular nuclei per section. In eyes without OIR (Group 3), there was an average of 0.12 ± 0.33 neovascular nuclei per section. All eyes from mice exposed to OIR induction had significantly higher neovascular nuclei counts than Group 3 eyes without OIR ($p < 0.0001$ for all). There was no difference between Group 1 eyes (OIR eyes treated with saline) and the untreated fellow eyes of mice from Group 2 (untreated OIR eyes) ($p = 0.35$). In Group 2 eyes treated with exosomes, the neovascular nuclei counts were significantly lower than saline treated Group 1 eyes with OIR and the untreated fellow eyes with OIR in Group 2 ($p < 0.0001$ for both). These results are presented graphically in Figure 3D. We also note that no signs of ocular inflammation were noted on examination and histological analysis of the eyes injected with exosomes.

hMSCs-derived exosome proteomic analysis

To assess factors contained within exosomes derived from human bone marrow-derived MSCs that mediate their protective affects, we further analyzed data obtained previously using a novel, unbiased proteomics method, high-resolution isoelectric focusing liquid chromatography couple tandem mass spectrometry (HiRIEF LC-MS/MS). This new analysis was performed on previously published data collected from exosomes derived from hMSC as used in this study.[17] A total of 1927 proteins were identified in each exosome sample generated from MSCs derived from three different human donors (see Table S1, Supplemental Digital Content 1.We previously reported that the exosomes expressed 92 of the top 100 most identified exosomal marker proteins from the ExoCarta database in our exosome samples (see Table S2, Supplemental Digital Content 2, and Figure S1, Supplemental Digital Content 3). We also previously reported the vascular-protective proteins identified in MSC exosomes.[17]

Here, we present new analysis of the proteomic data demonstrating that exosomes derived from human MSCs are packaged with numerous pro-survival-associated proteins from the cAMP response element-binding protein (CREB) pathway using Ingenuity Pathway Analysis (Qiagen) (Figure 4). Clustered network analysis of protein–protein interaction networks (CytoScape, Spike database) determined clustering of proteins associated with pro-survival heat shock protein (HSP) pathways: HSPA1A, HSPA4, HSPA5, HSPA8, HSPA9, HSP90AA1, HSPB90, HSPBP1, HSPD1, HSPG2, HSPH1 (Figure 5). These data collectively demonstrate that exosomes derived from human MSCs contain numerous proteins associated with pro-survival signaling cascades. The delivery of such prosurvival proteins to retinal tissues serves as an