Delayed Administration of Bone Marrow Mesenchymal Stem Cell Conditioned Medium Significantly Improves Outcome After Retinal Ischemia in Rats

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PURPOSE. Delayed treatment after ischemia is often unsatisfactory. We hypothesized that injection of bone marrow stem cell (BMSC) conditioned medium after ischemia could rescue ischemic retina, and in this study we characterized the functional and histological outcomes and mechanisms of this neuroprotection.

METHODS. Retinal ischemia was produced in adult Wistar rats by increasing intraocular pressure for 55 minutes. Conditioned medium (CM) from rat BMSCs or unconditioned medium (uCM) was injected into the vitreous 24 hours after the end of ischemia. Recovery was assessed 7 days after ischemia using electroretinography, at which time we euthanized the animals and then prepared 4- μ m-thick paraffin-embedded retinal sections. TUNEL and Western blot were used to identify apoptotic cells and apoptosis-related gene expression 24 hours after injections; that is, 48 hours after ischemia. Protein content in CM versus uCM was studied using tandem mass spectrometry, and bioinformatics methods were used to model protein interactions.

RESULTS. Intravitreal injection of CM 24 hours after ischemia significantly improved retinal function and attenuated cell loss in the retinal ganglion cell layer. CM attenuated postischemic apoptosis and apoptosis-related gene expression. By spectral counting, 19 proteins that met stringent identification criteria were increased in the CM compared to uCM; the majority were extracellular matrix proteins that mapped into an interactional network together with other proteins involved in cell growth and adhesion.

CONCLUSIONS. By restoring retinal function, attenuating apoptosis, and preventing retinal cell loss after ischemia, CM is a robust means of delayed postischemic intervention. We identified some potential candidate proteins for this effect.

Keywords: retina, ischemia, stem cells, conditioned medium

Patients who experience ischemic events in the eye including central retinal vascular occlusion frequently sustain poor outcomes, with no satisfactory treatment.¹ Previously, it has been shown that “postischemic conditioning” (Post-C) rescued the retina from ischemic damage.² We showed the effectiveness of delayed Post-C as late as 24 hours after prolonged retinal ischemia, with improved postischemic functional recovery and decreased histological damage.³ Since patients often present late after ischemic events, delayed treatment may be feasible by harnessing endogenous neuroprotective mechanisms.

Although Post-C potentially attenuates functional and histological damage, a major disadvantage is the necessity, albeit transiently, to once again render the retina ischemic. The stimulus would also be difficult to titrate in affected patients. A potential approach to provide endogenous neuroprotection following the onset of ischemia is autologous transplantation of bone marrow mesenchymal stem cells (BMSCs). Autologous

transplant, like Post-C, uses the subject’s own tissue as a source for endogenous neuroprotection. Mouse and rat BMSCs preserved retinal structure when applied to ischemic retina.⁴ BMSCs survive in the vitreous after transplantation, although the limited retinal penetration and differentiation of the cells has disappointed investigators’ attempts to regenerate damaged retinal cells.^{5,6} Rather, BMSCs may be secreting protective factors in a paracrine-like fashion. A recent report showed that conditioned medium (CM) from human mesenchymal stem cells (MSCs) prevented cell loss in rat retinal explants and in a rat model of glaucoma.⁷ Injection of CM may thus prove a simple and safe means to rapidly provide neuroprotection directly to the retina in vivo.

In this study we theorized that delayed postischemic administration of BMSC CM attenuates the damage from retinal ischemia. We examined both the functional and histological outcomes of BMSC CM application in a well-established rat model of retinal ischemia. To study the mechanisms, we studied

anti-apoptotic mechanisms of CM and examined the secretome of CM in comparison to uCM using tandem mass spectrometry (MS/MS), followed by bioinformatic analysis of protein interactions.

MATERIALS AND METHODS

Retinal Ischemia

Procedures conformed to the ARVO Resolution on the Use of Animals in Research and were approved by our Animal Care Committee. Male Wistar rats (200–250 g; Harlan, Indianapolis, IN, USA) were maintained on a 12-hour on/12-hour off light cycle. For retinal ischemia, rats were anesthetized with chloral hydrate, 275 mg/kg intraperitoneally (i.p.), and intraocular pressure increased to 130 to 135 mm Hg for 55 minutes in the right eye using a 27-gauge one-half-inch needle inserted into the middle of the anterior chamber of the eye, avoiding contact with the lens of the eye, and connected to an elevated reservoir of ocular irrigating solution and an electronic transducer to continuously measure the intraocular pressure. Insertion of the anterior chamber cannula and the loss of retinal circulation during ischemia were visualized with an operating microscope. The left eye served as an internal control for comparison, without elevation of intraocular pressure, as previously described.⁸ The eyes were treated with topical Vigamox (0.5%; Alcon, Fort Worth, TX, USA), cyclomydril (Alcon), and proparacaine (0.5%; Bausch & Lomb, Tampa, FL, USA) prior to ischemia. Temperature was maintained at 36°C to 37°C using a servo-controlled heating blanket (Harvard Apparatus, Natick, MA, USA). Oxygen saturation was measured with a pulse oximeter (Ohmeda, Louisville, CO, USA) on the rat's tail. Supplemental oxygen, when necessary to maintain O₂ saturation > 93%, was administered via a cannula placed in front of the nares and mouth.

Electroretinography

Procedures have been described previously.⁸ Animals were dark adapted for at least 2 hours before electroretinogram (ERG) recordings. For baseline and postischemic (i.e., after 7 days) follow-up ERG, rats were injected with ketamine (35 mg/kg i.p.) and xylazine (5 mg/kg i.p.) every 20 minutes to maintain anesthesia.

The ERG was recorded at baseline (prior to the experiments) and at 7 days after ischemia. Electrode preparations were adapted from Weymouth et al.⁹ In brief, custom Ag/AgCl electrodes were fashioned from 0.010-in Teflon-coated silver wire (Grass Technologies, West Warwick, RI, USA). Approximately 10 mm of wire was exposed and fashioned into a small loop to form the corneal/positive electrodes, while ~20 mm of wire was exposed to form a hairpin loop, the sclera/negative electrodes looped around the eye. Electrodes were then attached to a 9-V battery and placed in a 1 N HCl bath for 12 seconds until coated with AgCl. All electrodes were referenced to a 12-mm × 30-gauge stainless-steel needle electrode (Grass) inserted two-thirds down the length of the tail.

Stimulus-intensity ERG recordings were obtained using a UTAS-E 4000 ERG system and a full-field Ganzfeld (LKC Technologies, Gaithersburg, MD, USA), with the rat's head centered 7 in from the stimulator. The low pass filter was 0.05 Hz and the high pass 500 Hz. Flash intensities varied electronically from $-3.39 \log \text{ cd}\cdot\text{s}/\text{m}^2$ to $1.40 \log \text{ cd}\cdot\text{s}/\text{m}^2$. Responses were averaged across 3 to 10 flashes delivered 4 to 27 seconds apart, with the number of flashes decreasing and time between them increasing with intensity. Settings were confirmed by photometry (EG & G Model 550 photometer;

Electro-Optics, Boulder, CO, USA). To prevent attenuation of dark adaptation, flash series were progressively delivered from the lowest intensity to the highest and at least 1 minute elapsed between each series of flashes for the intensity settings.

Isolation and Culture of BMSCs

BMSCs were acquired from donor anesthetized male Wistar rats by flushing the femur bone marrow with phosphate-balanced saline (PBS). The solution was centrifuged at 300g for 5 minutes. The pellet was then resuspended in 40 mL of medium (RPMI-1640, 10% FBS, 100× Pen/Strep, 100× L-glut), and 20 mL was plated into a T75 flask. The following day, nonadherent cells were washed from the flask with PBS and 15 mL of medium was added. Medium was changed every 3 to 4 days for the next 4 weeks. Starting during week 4 and continuing once every 2 weeks, cells were passaged by adding 0.25% trypsin for 2 minutes. Trypsin was neutralized with 12 mL of serum-containing medium and the cells were replated in a T25 flask. Afterward, the medium was changed every 3 to 4 days for 1 to 2 weeks. The BMSC CM, from cells passaged 10 to 25 times, was collected after 72 to 96 hours of incubation in cultured BMSCs that were initially 75% to 100% confluent in a T25 flask. Unconditioned medium (uCM) was collected from cell-free T25 flasks that were incubated for the same length of time. Four microliters of CM or uCM was injected into the vitreous of both the ischemic and nonischemic eyes at 24 hours after retinal ischemia. Therefore, the normal, nonischemic left eye was an internal control. The impact of no injections and additionally effect of injections into the vitreous prior to or after ischemia has been previously reported by our laboratory. In brief, injections of control material for our experiments into the ischemic eye did not provide any neuroprotective effect.^{3,10}

Flow Cytometry

Cells were microcentrifuged and the supernatant aspirated. The cells were then washed in 0.5% BSA/PBS. After the final supernatant was aspirated, cells were resuspended in 100 μL 0.5% BSA/PBS and the solution kept at room temperature for 10 minutes. Primary antibodies, mouse monoclonal anti-CD90-FITC and anti-CD45-FITC (BD Biosciences, San Jose, CA, USA), were added and the solution was incubated at room temperature for 60 minutes. The cells were then washed three times in 0.5% BSA/PBS, resuspended in 500 μL PBS and used for flow cytometry. Samples were analyzed using the FAC Scanto flow cytometer and FACSDiVa software (BD Biosciences, San Jose, CA). IgG2a-FITC mouse isotype control was used to examine background levels.

Histology

Eyes enucleated on the seventh day after ischemia were immediately placed in Davidson's fixative (11% glacial acetic acid, 2% neutral buffered formalin, and 32% ethanol in H₂O) for 24 hours, then transferred to 70% ethanol for 24 hours and stored in PBS at 4°C. Eyes were embedded in paraffin, sectioned to 4 μm and stained with hematoxylin and eosin. Sections were examined by light microscopy and cell counts were quantitated using ×40 optics. Specifically, the number of cells in the retinal ganglion cell (RGC) layer was counted in a standardized region in all of the retinae, centered 1280 μm distant (measured by an eyepiece reticule) from the thinning of the neurofilaments arising from the optic nerve head. The counts were made in both directions from the optic nerve head in a region spanning 128 μm . The average number of cells in the RGC layer is reported as previously described.⁸ Cell

numbers in the inner nuclear layer (INL) were determined by capturing images with Micron (Westover Scientific, Mill Creek, WA, USA). Several INL cell regions were selected (around 1280 μm from the optic nerve bundle), and the numbers of cells were manually counted and determined per area, as previously described.

Fluorescent TUNEL

We used a Fluorescein FragEL DNA Fragmentation Detection Kit (Calbiochem, La Jolla, CA, USA) on frozen retinal sections as described previously.¹¹ Frozen tissue was fixed and hydrated in 4% formaldehyde followed by Tris-buffered saline immersion. After permeabilization with proteinase K in 10 mM Tris, pH 8 (1:100), tissue was labeled using a TdT enzymatic reaction. These studies were performed on retinæ removed 24 hours after injection of either CM or uCM (i.e., 48 hours after ischemia), because unlike the histological damage, which is greater 7 days after ischemia than 1 day, peak in TUNEL has been shown to be within 24 to 48 hours.^{12,13}

Imaging

For imaging TUNEL, we utilized a fluorescence microscope (Olympus IX81 inverted microscope; Olympus, Center Valley, PA, USA), a Fast firewire Retiga EXi chilled CCD camera (Q Imaging, Surrey, British Columbia, Canada), and a $\times 40$ oil lens. Excitation/dichroic/emission settings were 530 to 550 nm—570DM-590LP for fluorescein. TUNEL cells were identified as previously reported.¹¹

Western Blotting on Retinal Homogenates

Retinas were rapidly dissected, frozen in liquid N_2 , crushed with a tissue pulverizer (Beckman, Fullerton, CA, USA) on dry ice, and solubilized in 9 M urea, 4% Nonidet P-40, and 2% 2-mercaptoethanol (pH 9.5). Protease inhibitor cocktail (P8340; Sigma, St. Louis, MO, USA) consisting of 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin, and E-64 prevented protease activity. Samples were centrifuged 10 minutes at 10,000g, the supernatant used for SDS-PAGE and the pellet discarded. Protein concentration was determined by modified Bradford assay (Bio-Rad, Hercules, CA, USA).

Equal amounts of protein per lane (40 μg) were diluted with SDS sample buffer and loaded onto gels (4%–20% or 16%; Invitrogen). Proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) with efficiency of transfer confirmed by Ponceau S red (Sigma). Nonspecific binding was blocked with 5% nonfat dry milk in Tween-Tris-buffered saline. Membranes were incubated overnight at 4°C with anti-rabbit polyclonal caspase-3 (Enzo Life Sciences, Plymouth Meeting, PA, USA) or anti-rabbit polyclonal caspase-6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies.

Anti-rabbit horseradish peroxidase (HRP)-conjugated (goat IgG; Jackson ImmunoResearch, West Grove, PA, USA) or anti-mouse HRP-conjugated (sheep IgG; Amersham, Buckinghamshire, England) secondary antibodies were applied at 1:20,000. Chemiluminescence was developed with a kit (Super Signal West Pico; Pierce, Rockford, IL, USA). Protein bands were digitally imaged with a CCD BIO 16SC Imaging System (Hitachi Genetic Systems/MiraiBio, Alameda, CA, USA) and quantitated by densitometry (Gene Snap and Gene Tools; Syngene, Frederick, MD, USA). Data for the protein expression in the ischemic retina is presented as the percentage of the opposite eye, normal nonischemic densitometry results. Equal protein loading was confirmed by immunoblotting with rabbit polyclonal anti-opsin (Santa Cruz Biotechnology).

Mass Spectrometry

Three frozen samples (~ 5 mL) of both CM and uCM were prepared by precipitation using cold acetone/trichloroacetic acid (100–150 μL per sample), then they were redissolved in 100 μL of 8 M urea. Cysteine amino acids were reduced and alkylated by incubation in 45 mM dithiothreitol at 37°C for 45 minutes, then samples were treated with 100 mM iodoacetamide in the dark at room temperature for 30 minutes. Protein samples were then digested with both Lys-C and trypsin.

For separation prior to mass spectrometry, nanoscale liquid chromatography (LC) was performed with a Dionex Ultimate 3000 LC equipped with a C18 PepMap 100 Peptide trap (Thermo Scientific, Hanover Park, IL, USA) and a Zorbax 300SB C18 NanoLC column (Agilent, Santa Clara, CA, USA). The digested sample was desalted by loading the peptide trap with mobile phase A at 50 $\mu\text{L}/\text{min}$ with 5% B to 40% B gradient for 90 minutes at 250 nL/min. For MS we used a quadrupole LTQ Orbitrap Velos Pro (Thermo Scientific) and an LTQ nanospray source (Thermo Scientific), with ion spray voltage 1.8 kV at heated capillary temperature of 275°C. The full scan mass range was 400 to 1800 Da at an Orbitrap resolution of 30,000. MS/MS data dependent acquisition with dynamic exclusion was set at 120 seconds, where the 20 most intense ions, above the minimum signal threshold with charge states greater than or equal to 2, were selected for low-energy collision-induced dissociation in the ion trap. A blank sample was run for an instrument control.

Analysis of MS/MS Data

The PARIS 2 guidelines (available in the public domain at http://www.mcponline.org/site/misc/ParisReport_Final.xhtml) were followed for analyzing and reporting MS/MS proteomics experiments.¹⁴ Raw data files were processed using the Mass Matrix Conversion tool (<http://www.massmatrix.net>) to generate Mascot generic files (MGFs) for the protein database search. Mascot (version 2.2.07; Matrix Science, London, UK) was set to search the NCBI nr_20110824 database (15,057,399 entries) with digestion enzyme trypsin. The fragment ion mass tolerance was 0.60 Da and parent ion tolerance of 10.0 ppm. Carbamido-methylation of cysteine was specified as a fixed modification. Deamidation of asparagine and glutamine, and oxidation of methionine were specified in Mascot as variable modifications (see Supplementary Data files S1–S3 for full listing of MS/MS analysis parameters).

Mascot results were imported into Scaffold 4.2.1 (<http://www.proteomesoftware.com/>; Proteome Software, Portland, OR, USA) to validate MS/MS peptide and protein identifications. The program uses rigorous statistical modeling for protein identification.¹⁵ Peptide identifications were accepted if they could be established at >90.0% probability by the Peptide Prophet algorithm¹⁶ with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at >99% probability and contained at least two identified peptides. These settings produced an estimated protein false discovery rate of 0.01%. Protein probabilities were assigned by the Protein Prophet algorithm.¹⁷ Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.¹⁸

We quantitated relative protein abundance by spectrum counting.^{19–21} Scaffold assigned unweighted spectrum counts for each identified protein; for normalization, data were expressed as percentages of the total spectra in the entire sample. The mean normalized abundance was compared between the three CM and three uCM samples.

FIGURE 1. Characterization of the BMSCs. Flow cytometry results of BMSC immune-labeled with markers CD45 (negative; *left panel*) and CD90 (positive; *right panel*); 99.9% of the cells were positive for CD90 and only 16.1% were immune-labeled with negative marker CD45. These data suggest a sufficiently pure population of BMSCs.

Bioinformatic Data Analysis and Protein Network Modeling

Interaction network analysis was performed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, <http://string-db.org/>) version 9.1.²² The analysis was set for high confidence (score 0.7). We tested the protein lists generated by Scaffold of 19 proteins identified using the spectral analysis and subsequent statistical comparison of the means of the CM and uCM; accordingly, these represent the final protein list generated by the most stringent analysis.

STRING examines databases of physical interactions and biological pathway analysis.²³ It uses a “functional association,” the specific and meaningful interaction between two proteins that jointly contribute to the same functional process, but it does not consider posttranslational modification. Prediction analysis tools in the program yield high experimental predictive value.²⁴

Data Handling and Statistical Analysis

The a-wave, b-wave, and the P2 from ischemic eyes 7 days after ischemia were expressed as intensity-response plots with stimulus intensity (log cd-s/m²) on the x-axis, and corresponding percent recovery of baseline on the y-axis. We normalized the responses by accounting for day-to-day variation in the normal eye and referencing the baseline in the ischemic eye, as previously described.²⁵ Results in the control, nonischemic eyes, which were, like the ischemic eyes, injected with CM or uCM, were compared to their baseline absolute amplitude values.

Recorded amplitude, time course, and intensity were exported and analyzed in Matlab 2011a (MathWorks, Natick, MA, USA). For each rat, the waveforms were averaged across each flash series and the a-wave, b-wave, and P2 were taken at each intensity. The a-wave values were calculated as the absolute value of the minimum amplitude following the flash stimulus, while the b-wave values were calculated as the difference between the negative a-wave value and the maximum amplitude recorded thereafter. The P2 was derived by first fitting the Hood and Birch phototransduction model to the leading edge of the a wave,⁸ as previously described, to determine the P3, with the transformation: $P3(i, t) = R_{\max}[1 - e^{-iSS(t-t_{\text{eff}})^2}]$ where $R_{\max}$ is the saturating photocurrent, i is the flash energy, S is the sensitivity parameter that scales flash energy, t is the time after flash onset, and t_{eff} is a delay approximating a number of extremely brief stages.²⁶

Data were analyzed using Stata version 10.0 (College Station, TX, USA). Comparisons between groups were per-

formed using unpaired *t*-tests, with $P < 0.05$ considered statistically significant. MS/MS total spectra data were compared using the Mann-Whitney test.

RESULTS

Characterization of BMSCs

By flow cytometry (Fig. 1), 99.9% of BMSCs were positive for CD90 and only 16.1% for the negative marker CD45. These data suggest a sufficiently pure population of BMSCs since nearly every cell was positive for the CD90 positive marker and relatively few expressed the negative marker CD45.

Functional Neuroprotection and Preservation of Histology

Injecting CM 24 hours after ischemia significantly improved recovery of the a- and b-waves and P2 (measured 7 days after ischemia, $P < 0.05$ versus uCM, Fig. 2a). In the normal nonischemic left eye, also injected with either CM or uCM, there were no changes in the wave amplitudes from baseline (Fig. 2b). Histological examination (Fig. 3) of the retinae 7 days after ischemia showed a significant difference between the number of cells in the RGC layer in CM-treated ischemic retina (9.4 ± 0.8) versus uCM (6.2 ± 0.5 ; $P = 0.02$, Table 1).

TUNEL and Caspases

CM significantly attenuated the percentage of apoptotic cells in the RGC layer in ischemic retinae at 24 hours after injection (48 hours after ischemia, $7.2 \pm 1.9\%$ versus uCM, $23.2 \pm 2.2\%$; $P = 0.01$, Fig. 4). Western blot analysis of cleaved caspase-3 protein levels, expressed as percent change of ischemic retinae as compared to the control normal retinae protein intensity values, were $137 \pm 23\%$ for CM and $121 \pm 33\%$ uCM 1 hour after intravitreal injections (25 hours after ischemia, $P = 0.70$). At 24 hours post injection (i.e., 48 hours after ischemia), the levels increased from $145 \pm 27\%$ for CM to $241 \pm 54\%$ for uCM, but these results were not statistically significant ($P = 0.30$); Fig. 5a). Cleaved caspase-6 was $146 \pm 28\%$ for CM versus $185 \pm 40\%$ for uCM 1 hour after intravitreal injection (25 hours after ischemia, not significantly altered, $P = 0.44$). At 24 hours post injection (i.e., 48 hours after ischemia), the levels significantly increased from $86 \pm 10\%$ for CM to $206 \pm 46\%$ for uCM ($P = 0.05$; Fig. 5b).

Mass Spectrometry

The complete lists of protein and peptide identification appear in Supplementary Tables S1 through S3, and representative spectra are in Supplementary Figure S4. In total, 258 proteins were identified by MS. There were 16 unique proteins in at least two CM samples and not in any of the uCM samples. Of these, 11 proteins were significantly increased by spectrum analysis and Mann-Whitney test in CM versus uCM. None of these were present in the uCM and therefore represent infinite change (Table 2). Three additional proteins present in both CM and uCM, collagen α 1(I) chain precursor, β -actin, periostin, and osteoblast-specific factor isoform-C, exhibited increased levels from CM as compared to uCM, at approximately 6000%, 800%, and 400% enrichment, respectively (Table 2).

For the 19 proteins in Table 2, STRING found 33 interactions with $P < 0.05$. The STRING interaction network model is shown in Figure 6, with the known functions of the proteins in Table 3. Nearly all these identified proteins interacted because they are primarily extracellular matrix

FIGURE 2. (A) Stimulus-intensity responses for a-wave, b-wave, and P2 from electroretinograms in animals subjected to retinal ischemia and eyes injected 24 hours later with BMSC CM ($n = 10$) or control uCM ($n = 5$). The recordings were at baseline (prior to ischemia), and 7 days later. ERG data for the waves over a range of flash intensities are shown as mean $\pm$ SEM. There was significant improvement with injection of CM 24 hours after ischemia on all the ERG waveforms. $*P < 0.05$ for CM versus uCM. (B) Stimulus-intensity responses for a-wave, b-wave, and P2 from electroretinograms from the normal nonischemic eyes (left eyes) injected with CM ($n = 10$) or uCM ($n = 5$). ERG data for the waves over a range of flash intensities are shown as mean $\pm$ SEM. There was no change on all of the ERG waveforms compared to baseline when measured 7 days later.

(ECM) and structural proteins or involved in cell growth and adhesion. The nodal interactions centered around the collagen subtype proteins, particularly Col1a1 and Col1a2, where the strongest interactions were found (darker lines in Fig. 6 indicate stronger strength of association). Four proteins shown in Table 2 were independent of the network associations (Fig. 6).

DISCUSSION

In this study, we examined the effect of delayed injection of BMSC CM on the outcome after retinal ischemia in a rat model. Our hypothesis, that BMSC CM protected against functional and histological damage in our rat model of retinal ischemia, was confirmed. Treatment with BMSC CM 24 hours after ischemia significantly protected against ischemic injury, maintaining retinal function, decreasing cell loss, and attenuating apoptosis. The functional ERG recovery 1 week after ischemia, for the ischemic retina injected with CM was profound, at $\sim 50\%$ recovery. In contrast, the percent recovery of the b-wave and P2 in the ischemic retina in this study injected with uCM ($\sim 15\%$) was comparable to the recovery

shown in our previous studies of either no injection, saline, or transfection medium injection into the vitreous ($\sim 20\%$).^{8,10} Moreover, we showed in the present study no effect of either injecting CM or uCM on the opposite, nonischemic control eyes. These results show the feasibility of delayed treatment of retinal vascular occlusion using easily obtained CM, avoiding potential adverse effects and limitations of cell-based treatments.

Some studies showed that BMSCs incorporate into the retina and they may differentiate into neuronal cells.²⁷ However, most of the cells remain in the vitreous with little retinal incorporation.^{5,6} Thus the effects of MSCs on retinal survival are more likely due to the secretion of paracrine factors by the cells, resulting in release of growth and other factors to support survival and immunomodulation.²⁸ CM derived from various types of cell cultures promoted survival of retinal cells in vitro. Growth factors including nerve growth factor, brain-derived neurotrophic factor, and platelet-derived growth factor are among the factors implicated.^{29,30} Johnson et al.,⁷ using a candidate protein approach by protein multiplexing, identified 29 specific factors in conditioned human MSCs, and showed that a cocktail of these growth factors and cytokines enhanced survival of rat retinal explants. However,

FIGURE 3. Representative histological preparations of a retina 7 days after ischemia. The ischemic retinae from the CM group showed retention of cells in the RGC layer as well as less disorganization versus uCM. Normal retinae (*top row*) and ischemic retinae (*bottom row*). Scale bar: 50 μ m.

the effect of CM on retinal neuroprotection in disease models *in vivo* has not been previously studied.

We earlier found that the attenuation of apoptosis is a mechanism for protection from retinal ischemia provided by ischemic preconditioning,³¹ postischemic conditioning,³ and others also by additional modalities, including inhaled carbon monoxide and hydrogen sulfide.^{32,33} Here we find that the mechanism of protection of the BMSC CM may be due, in part, to the reduction in apoptotic cell death in the inner retina. The 24-hour time point after injection of either CM or uCM (that is, 48 hours after ischemia) was used for TUNEL due to previous studies by our group and others showing that the peak of

apoptosis was 24 to 48 hours after ischemia.^{11,12} The timing of the apoptosis studies therefore differs from the 7-day follow-up period for examination of retinal histology, as discussed in our previous reports. The evidence of the attenuation of apoptosis by the BMSC CM, at the same postinjection time point, is further supported by the Western blot analysis of caspase cleavage.

Proteomics analysis based upon MS/MS yielded a list of candidate proteins unique to the BMSC CM. Unlike more recent studies that examined MSC CM in comparison to that of a different cell type CM⁷ we used uCM as a directly comparable control. Also, the study by Johnson et al.⁷ used a candidate

TABLE 1. Histological Results After Ischemia

No. of Cells in RGC Layer		P Value
No. of cells in the RGC layer in the ischemic retinae 7 d after ischemia in eyes injected with CM (<i>n</i> = 6) versus uCM (<i>n</i> = 5)		
24 h uCM	6.2 $\pm$ 0.5	—
24 h BMSC CM	9.4 $\pm$ 0.8	0.02
No. of INL Cells/Area		P Value
No. of INL cells/area (μ m ² ; $\times$ 100) in the ischemic retinae 7 d after ischemia in eyes injected with CM versus uCM		
24 h uCM	2.5 $\pm$ 0.2	—
24 h BMSC CM	2.7 $\pm$ 0.1	0.20
No. of ONL Cells/Area		P Value
No. of ONL cells/area (μ m ² ; $\times$ 100) in the ischemic retinae 7 d after ischemia in eyes injected with CM versus uCM		
24 h uCM	5.6 $\pm$ 0.2	—
24 h BMSC CM	5.8 $\pm$ 0.2	0.52

24 h Unconditioned Media

24 h BMSC Conditioned Media

FIGURE 4. Fluorescent TUNEL. Apoptosis was attenuated in the ischemic retinae 24 hours after CM injection ($n = 6$) as compared to uCM injection ($n = 6$); that is, at 48 hours after ischemia. *White arrows* denote TUNEL positive cells. *Brackets* denote the RGC layer. DAPI (*green*) and TUNEL positive (*red*) are depicted with false colors.

protein approach, in which the relative levels of proteins hypothesized to be present in CM were examined using protein expression multiplexing. Our approach identified differences in the proteins present in CM versus uCM by first separating the mixtures with liquid chromatography then

evaluating the content with MS spectrum analysis, which allowed for quantitation of the differences between CM and uCM. We used stringent criteria for protein identification and statistical analysis of the comparison of CM and uCM, which resulted in a relatively small number of candidate proteins.

FIGURE 5. Western blot analysis of caspase protein expression 1 hour after CM or uCM injection (i.e., 25 hours after ischemia), and 24 hours after CM or uCM injections (i.e., at 48 hours after ischemia). (A) Average percent change of ischemic retinae ($\pm$ SEM) versus paired normal retinae for 17-kD cleaved caspase-3 protein expression at 1 hour after CM ($n = 6$) or uCM ($n = 6$) and 24 hours after CM ($n = 8$) or uCM injections ($n = 6$). There was a trend toward increased expression for the uCM versus the CM at 24 hours, suggesting attenuation of the apoptotic pathway by CM. Representative Western blots are shown. (B) Average percent change of ischemic retinae ($\pm$ SEM) versus paired normal retinae for 17-kD cleaved caspase-6 protein expression at 1 and 24 hours after CM or uCM injections. There was a significant increase in the uCM versus the CM at 24 hours suggesting attenuation of the apoptotic pathway by CM. Representative Western blots are shown. (C) Representative Western blots denoting equal protein loading are shown for the groups using an antibody to opsin.

TABLE 2. Proteins Identified in Mass Spectrometry Experiments That Exhibit Increased Expression Levels From CM as Compared to uCM

Identified Proteins				CM	CM				uCM	uCM			P Value
	CM1	CM2	CM3	Mean	SEM	uCM1	uCM2	uCM3	Mean	SEM	% Change	M-W	
Unique proteins found in all 3 CM but not in uCM													
Collagen α -2(I) chain precursor	1.3	2.1	3.9	2.43	0.77	0	0	0	0.00	0.00		0.04	
Fibronectin	0.62	1.1	1.9	1.21	0.37	0	0	0	0.00	0.00		0.04	
Procollagen, type VI, α 3, isoform CRA_b	0.057	0.37	2.1	0.84	0.64	0	0	0	0.00	0.00		0.04	
Procollagen, type V, α 1	0.11	0.11	0.17	0.13	0.02	0	0	0	0.00	0.00		0.04	
Collagen α -1(III) chain precursor	0.57	0.29	0.72	0.53	0.13	0	0	0	0.00	0.00		0.04	
72 kDa type IV collagenase precursor	0.34	0.35	0.62	0.44	0.09	0	0	0	0.00	0.00		0.04	
Microfibrillar-associated protein 4	0.28	0.43	0.7	0.47	0.12	0	0	0	0.00	0.00		0.04	
Pcolce protein	0.057	0.43	0.47	0.32	0.13	0	0	0	0.00	0.00		0.04	
Fibromodulin	0.4	0.32	0.35	0.36	0.02	0	0	0	0.00	0.00		0.04	
Histone H2A type 1-B/E	0.057	0.053	0.27	0.13	0.07	0	0	0	0.00	0.00		0.04	
Secreted acidic cysteine-rich glycoprotein	0.17	0.29	0.17	0.21	0.04	0	0	0	0.00	0.00		0.04	
Unique proteins found in 2 CM but not in uCM													
Collagen, type VI, α 1	0	0.24	0.45	0.23	0.13	0	0	0	0.00	0.00		0.12	
Procollagen, type V, α 2	0	0.08	0.55	0.21	0.17	0	0	0	0.00	0.00		0.12	
Collagen, type VI, α 2	0	0.027	0.32	0.12	0.10	0	0	0	0.00	0.00		0.12	
Plasminogen activator inhibitor 1 precursor	0	0.053	0.075	0.04	0.02	0	0	0	0.00	0.00		0.12	
Transforming growth factor β induced	0	0.053	0.075	0.04	0.02	0	0	0	0.00	0.00		0.12	
Other proteins (not unique to CM)													
Collagen α -1(I) chain precursor	1.5	2.7	4.1	2.77	0.75	0	0.074	0.049	0.04	0.02	6747.97	0.05	
β -actin	0.11	0.35	0.35	0.27	0.08	0	0.025	0.074	0.03	0.02	818.18	0.05	
Periostin, osteoblast specific factor , isoform CRA_c	0.23	0.56	1	0.60	0.22	0	0.2	0.17	0.12	0.06	483.78	0.05

FIGURE 6. STRING representation of the interactions of the 19 proteins found in at least two CM and no uCM samples and proteins with significant increases in CM versus uCM. The analysis was set for high confidence with the required confidence (score) 0.700. The *P*-value was <0.05, there were 33 interactions, and the interactions expected were 2.20. The thicker the blue line, the stronger the connection between those proteins. Connections are based on seven criteria: neighborhood in the genome, gene fusions, co-occurrence across genomes, co-expression, experimental and biochemical data, association in curated databases, and co-mentioned in PubMed articles (text-mining).

TABLE 3. Key to the STRING Analysis Proteins Used in Figure 6

Protein Name		Description
COL6A1	Collagen, type VI, α 1 (1025 aa)
COL6A2	Collagen, type VI, α 2 (1025 aa)
SERPINE1	Plasminogen activator inhibitor 1 precursor. This inhibitor acts as “bait” for tissue plasminogen activator, urokinase, and protein C. Its rapid interaction with TPA may function as a major control point in the regulation of fibrinolysis (402 aa)
FMOD	Fibromodulin precursor (FM). Affects the rate of fibrils formation. May have a primary role in collagen fibrillogenesis (376 aa)
COL3A1	Collagen α -1(III) chain precursor. Collagen type III occurs in most soft connective tissues along with type I collagen (1463 aa)
COL5A2	Collagen, type V, α 2 (1467 aa)
COL1A1	Collagen α -1(I) chain Precursor (α -1 type I collagen). Type I collagen is a member of group I collagen (fibrillar forming collagen) (1453 aa)
COL5A1	Collagen α -1(V) chain precursor. Type V collagen is a member of group I collagen (fibrillar forming collagen). It is a minor connective tissue component of nearly ubiquitous distribution. Type V collagen binds to DNA, heparan sulfate, thrombospondin, heparin, and insulin (1840 aa)
HIST1H2AO	Histone H2A type 1-C. Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries that require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication, and chromosomal stability. DNA accessibility is regulated via a complex set of posttranslational modifications of histones, also called histone code, and nucleosome remodeling
COL1A2	Collagen α -2(I) chain precursor (α -2 type I collagen). Type I collagen is a member of group I collagen (fibrillar forming collagen) (1372 aa)
FN1	Fibronectin precursor (FN). Fibronectins bind cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. Fibronectins are involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape (2477 aa)
MMP2	Matrix metalloproteinase 2 fragment (72 kDa type IV collagenase) (661 aa)
COL6A3	Procollagen, type VI, α 3 (2665 aa)
TGFB1I1	Transforming growth factor β -1-induced transcript 1 protein. Functions as a molecular adapter coordinating multiple protein-protein interactions at the focal adhesion complex and in the nucleus. Links various intracellular signaling modules to plasma membrane receptors and regulates the Wnt and TGFB signaling pathways (481 aa)
ACTB	Actin, cytoplasmic 1 (β -actin). Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells (375 aa)
POSTN	Periostin, osteoblast-specific factor (838 aa)
MFAP4	Microfibrillar-associated protein 4 (281 aa)
PCOLCE	Procollagen C-endopeptidase enhancer 1 precursor. Binds to the C-terminal propeptide of type I procollagen and enhances procollagen C-proteinase activity (493 aa)
SPARC	SPARC precursor (secreted protein acidic and rich in cysteine). Appears to regulate cell growth through interactions with the extracellular matrix and cytokines. Binds calcium and copper; several types of collagen, albumin, thrombospondin, PDGF, and cell membranes. There are 2 calcium binding sites; an acidic domain that binds 5–8 Ca^{2+} with a low affinity and an EF-hand loop that binds a Ca^{2+} ion with a high affinity (302 aa)

The protein descriptions show brief protein properties as well as function and gene ontology.

The list of proteins unique to the BMSC CM secretome contained primarily structural and ECM proteins (e.g., collagen, collagen precursors, and procollagen), proteins involved in cell growth and adhesion (e.g., SPARC, TGF, FM), and a transcription factor protein, histone H2A.³⁴ The results showing primarily ECM proteins in the CM are consistent with studies of CM in other stem cell types and species.^{35,36} Among the many functions of ECM proteins, it has been found that some provided neuroprotection in stroke in animal models,^{37,38} and that they support repair and regeneration of nerve tissue.³⁹ Moreover, directly relevant to the present results, increased RPE survival was achieved on aged sub-

macular human Bruch's membrane by resurfacing with a cell-deposited ECM.⁴⁰ Furthermore, ECM proteins have been shown to be protective via attenuation of apoptosis, which thus fits in well with our findings and the protein-protein interactional analysis.^{41,42} The functional interactions of the identified proteins in our study were largely centered around several collagen subtypes. This suggests that rat BMSCs deposited a complex ECM that is critical to a supportive environment for MSC growth and renewal.

There are limitations of MS in detecting differences in the secretome of CM versus uCM, which include sample handling, instrumentation, data processing as well as database search-

es.⁴³ Also, there could be inefficient trypsin digestion of putative novel proteins. Furthermore, initial MS may not include low abundance proteins and collision-induced dissociation may render peptides too small to sequence efficiently. Novel unknown protein products may be excluded from database searches. Therefore, it is possible that our MS/MS experiments may have missed the discovery of candidate protective proteins.

We cannot exclude the possibility that the functional and histological neuroprotective effects of CM in our present study may have arisen from nonprotein factors as well. Recent studies have shown that exosomes, an important component of stem cells, may have important neuroprotective capabilities.⁴⁴ Future experiments should include the investigation of the possible neuroprotective effects of isolated exosomes from BMSC CM.

Much like our results with delayed post conditioning after retinal ischemia, our current results highlight a potent and possibly clinically important procedure by which to prevent ischemic retinal damage. The functional and histopathological protection afforded to the BMSC CM involves attenuation of apoptosis. CM itself, devoid of fat, cartilage, and bone, which are undesirable cell types, appears a safer means in future clinical study. While our experiments only addressed short-term outcomes (7 days after ischemia), longer term experiments could shed valuable light on the potential clinical safety of BMSC CM injection. These future endeavors may allow for the discovery of individual factors and/or a combination of several factors that can efficiently protect against retinal ischemic damage in a clinical trial setting.

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