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SHORT REPORT

Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival *in vitro*

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Abstract Bone marrow-derived mesenchymal stem cells (MSCs) are of therapeutic interest in a variety of neurological diseases. In this study, we wished to determine whether human MSCs secrete factors which protect cultured rodent cortical neurons from death by trophic factor withdrawal or nitric oxide (NO) exposure. Medium conditioned by MSCs attenuated neuronal death under these conditions, a process which was dependent on intact PI_3kinase/Akt pathway signaling. Trophic withdrawal and NO exposure in cultured cortical neurons led to reduction in Akt signaling pathways, whereas NO administration activated p38 MAPkinase in neuronal cultures. Addition of MSC-conditioned medium significantly activated the PI3kinase/Akt pathway and in neurons exposed to NO, MSC-conditioned medium reduced p38 signaling. We show that MSCs secrete brain-derived neurotrophic factor (BDNF) and addition of anti-BDNF neutralising antibodies to MSC-conditioned medium attenuated its neuroprotective effect. Exposure of neurons to BDNF increased activation of Akt pathways and protected neurons from trophic factor withdrawal. These observations determine the mechanisms of neuroprotection offered by MSC-derived factors and suggest an important role for BDNF in neuronal protection.

Introduction

In recent years, there has been considerable interest in the potential of stem cells as therapeutic agents in neurological diseases (Lindvall and Kokaia, 2006). Specifically, autologous human bone marrow-derived mesenchymal stem cell (MSC) transplantation offers significant potential, since it avoids problems of rejection and many ethical issues associated with other stem cell-based therapies (Rice and Scolding, 2008). In neurological diseases it has been postulated that stem cell therapies may replace lost cells by differentiating into functional neural tissue; modulate the immune system to prevent further neurodegeneration and effect repair; or

Abbreviations: BDNF, brain-derived neurotrophic factor; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagles medium; EAE, experimental autoimmune encephalomyelitis; MAPkinase, mitogen-activated protein kinase; MIN, DMEM supplemented with chemically defined medium with no serum; MNC, mononuclear cells; MSC, mesenchymal stem cell; NGF, nerve growth factor; NO, nitric oxide; PI_3kinase, phosphatidylinositol 3-kinase.

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provide a source of trophic support for the diseased nervous system. With regard to the latter possibility, neurons of the adult central nervous system require continued trophic support from surrounding cells. For instance, oligodendrocytes and the myelin they produce support axons and evidence from disease states suggests that loss of myelin leads to progressive axonal degeneration (Griffiths et al., 1998; Lappe-Siefke et al., 2003). If human MSCs are to be used as therapeutic cell replacement therapies for neurological disease, an understanding of their neurotrophic properties and mechanisms by which they may reduce neuronal death is required.

Many intracellular signaling pathways have been implicated in the process of neuronal cell death, and precise mechanisms may vary dependent on specificity of the environmental insult. For instance, toxic metabolites may induce cell death by activating different intracellular cascades to those induced by trophic factor withdrawal (Cao et al., 2004). The death of neurons induced by trophic factor withdrawal is an important process during development of the central nervous system, in which competition for neurotrophic factors shapes the integration of individual components (Raff et al., 1994). The mechanisms by which neurotrophic factors attenuate neuronal death have also attracted much attention (Goldberg and Barres, 2000). Neurotrophic factors may inhibit death-inducing pathways and also activate a variety of cell survival pathways: several are now well defined and a major role for the PI_3kinase/Akt pathway has emerged (Kaplan and Miller, 2000). This pathway may be activated by a variety of trophic factors, including brain-derived neurotrophic factor (BDNF) *in vitro* (Zheng and Quirion, 2004).

In the current study we aimed to define mechanisms of neuronal cell death under conditions of trophic deprivation and exposure to nitric oxide, and to determine the influence of factors released by human bone marrow-derived mesenchymal stem cells on neuronal death and the intracellular pathways activated by these stressors.

Results

MSC-conditioned medium attenuates trophic factor withdrawal-induced neuronal death and nitric oxide (NO)-mediated neuronal death

Cortical neurons (1.4×10^3 cells/mm^2) were maintained in B27-supplemented Dulbecco's modified Eagles medium (DMEM) for 5 days *in vitro* (DIV) before exposure to DMEM supplemented with chemically defined medium with no serum (MIN). B27-supplemented DMEM is rich in trophic support factors, whereas chemically defined medium with no serum represents culture medium lacking in trophic support

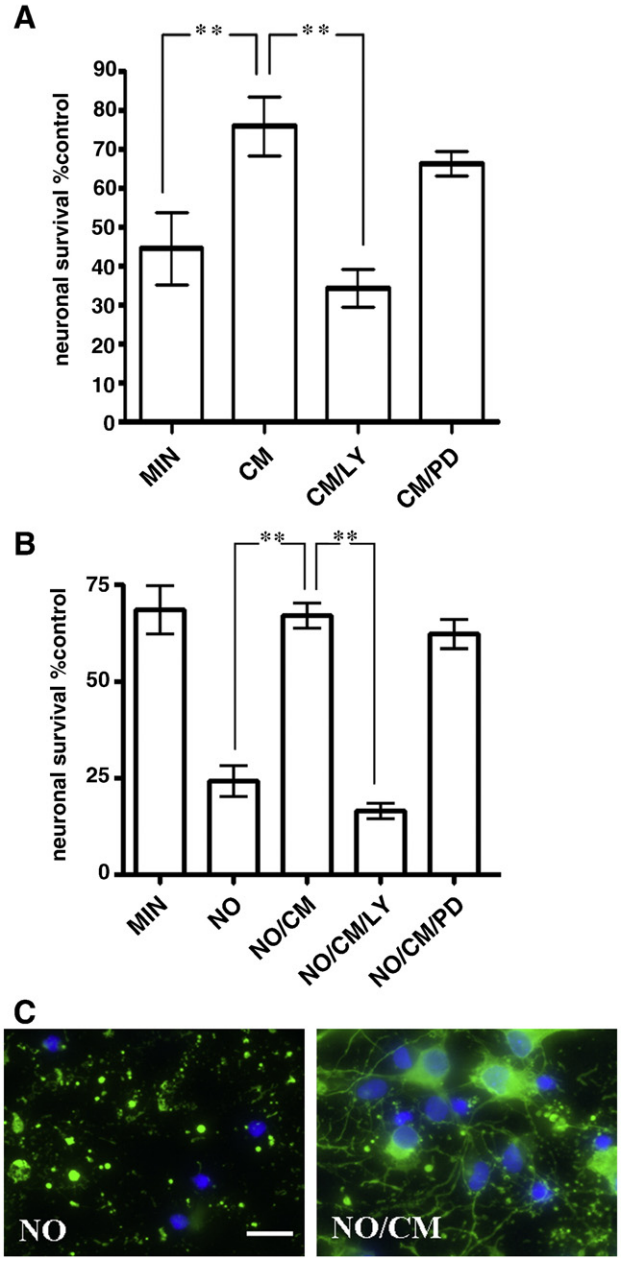


Figure 1 (A) MSC-conditioned medium increases survival of neurons exposed to trophic deprivation: Effect of chemically defined medium with no serum (MIN), MSC-conditioned medium (CM), MSC-conditioned medium plus LY290042 (10 μM; CM/LY), and MSC-conditioned medium plus PD98059 (30 μM; CM/PD) on live β-tubulin-positive cells per field. Controls were cultured in 2% B27 throughout and values expressed as a percentage of this control. Neurons were cultured for 5 DIV and exposed to test conditions for 3 DIV ($n=5$; ** $P<0.01$). (B) MSC-conditioned medium increases survival of neurons exposed to nitric oxide: Effect of chemically defined medium with no serum (MIN), DETANONOate (0.2 mM; NO), DETANONOate plus MSC-conditioned medium (NO/CM), DETANONOate plus MSC-conditioned medium plus LY290042 (10 μM; NO/CM/LY), and DETANONOate plus MSC-conditioned medium plus PD98059 (30 μM; NO/CM/PD) on live β-tubulin-positive cells per field. Controls were cultured in 2% B27 throughout and values are expressed as a percentage of this control. Neurons were cultured for 5 DIV and exposed to test conditions for 3 DIV ($n=4$; ** $P<0.01$). (C) Immunographs showing the effect of DETANONOate (NO) and DETANONOate plus MSC-conditioned medium (NO/CM) on neuronal survival (β-tubulin staining; green) in cultures (5 DIV) exposed to test conditions for 1 DIV. Nuclei stained with Hoechst (blue). Scale bar: 20 μm.

factors. After a further 3 DIV, cultures were fixed and stained for β-tubulin and the nuclear marker bisbenzamide, and neuronal survival was determined. Neurons exposed to chemically defined medium with no serum showed decreased survival compared to control (Fig. 1A). Neurons exposed to mesenchymal stem cell-conditioned medium showed increased survival compared to those exposed to chemically defined medium with no serum medium.

Cortical neurons (1.4×10^3 cells/mm^2) were also exposed to the nitric oxide donor DETANONOate (after 5 DIV maintained in B27-supplemented DMEM). After a further 1 DIV of DETATNONOate exposure, cultures were fixed and stained for β-tubulin and the nuclear marker bisbenzamide, and neuronal survival was determined. Neurons exposed to the DETANONOate showed decreased survival compared to control, a process which was attenuated by MSC-conditioned medium (Figs. 1B and 1C).

MSC-conditioned medium promotes neuronal survival via PI3kinase/Akt-dependent pathways

In order to investigate intracellular mechanisms underlying these phenomena we investigated signaling changes occurring during trophic factor withdrawal and DETANONOate exposure. Neurons were cultured as above for 5 DIV before exposure to DMEM supplemented with 2% B27, DMEM supplemented with chemically defined medium with no serum, or chemically defined medium with no serum containing DETANONOate. After 1 or 6 h of exposure, cells were washed and lysed and immunoblotting for cell signal intermediates was performed.

We determined the influence of MSC-conditioned medium on signaling changes occurring during trophic factor withdrawal or NO exposure. Exposure of neurons to MSC-conditioned medium increased activation of Akt compared to those exposed to chemically defined medium with no serum medium alone (Fig. 2A). Akt activation was also seen in neurons exposed to MSC-conditioned medium in the presence of DETANONOate, compared to neurons exposed to DETANONOate alone (Fig. 2B). Furthermore, addition of the PI3kinase/Akt inhibitor LY290042 (10 μM) inhibited MSC-conditioned medium-induced survival of cortical neurons exposed to trophic factor withdrawal or NO exposure (Figs. 1A and 1B). The MAPkinase/Erk inhibitor PD98059 (30 μM) did not inhibit the survival effect of MSC-conditioned medium (Figs. 1A and 1B). Furthermore exposure of neurons to chemically defined medium with no serum alone did not lead to activation of p38 MAPkinase pathways, which occurred on exposure to DETANONOate (Fig. 2C). MSC-conditioned medium attenuated DETANONOate-induced p38 activation within cortical neurons (Fig. 2C).

MSCs secrete BDNF

Given previous reports suggesting that MSCs secrete brain-derived neurotrophic factor, we next sought to determine whether BDNF is important in mediating, at least part, the MSC effects on neuronal survival (Arnhold et al., 2006). BDNF ELISA demonstrated significant amounts of BDNF secreted from MSCs. Chemically defined medium with no serum conditioned by MSCs (5×10^5 cells conditioned 1 ml of

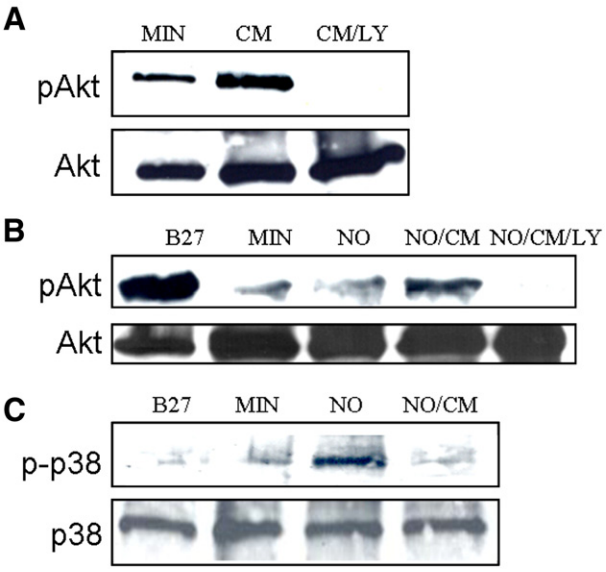
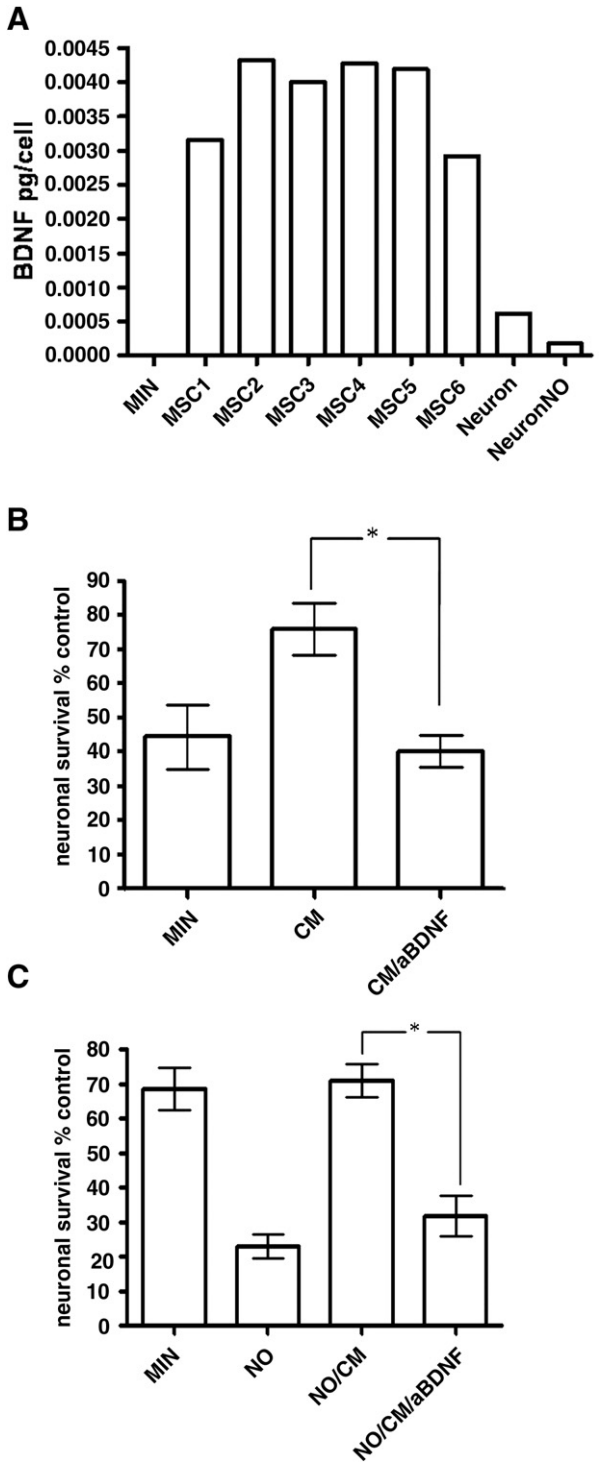


Figure 2 (A) MSC-conditioned medium activates Akt in neurons exposed to trophic deprivation: Akt phosphorylation in neurons (cultured for 5 DIV) exposed to chemically defined medium with no serum (MIN), MSC-conditioned medium (CM), or MSC-conditioned medium plus LY290042 (10 μM; CM/LY) for 1 h. Upper panel shows phospho-Akt immunoblot and lower panel total Akt of identical amounts of protein lysate. Representative blot of 3 independent experiments. (B) MSC-conditioned medium activates Akt in neurons exposed to DETANONOate: Akt phosphorylation in neurons (cultured for 5 DIV) exposed to 2% B27 (B27), chemically defined medium with no serum (MIN), DETANONOate (0.2 mM; NO), DETANONOate plus MSC-conditioned medium (NO/CM), or DETANONOate plus MSC-conditioned medium plus LY290042 (10 μM; NO/CM/LY) for 1 h. Upper panel shows phospho-Akt immunoblot and lower panel total Akt of identical amounts of protein lysate. Representative blot of 3 independent experiments. (C) MSC-conditioned medium reduces p38 activation in neurons exposed to DETANONOate: p38 phosphorylation in neurons (cultured for 5 DIV) and exposed to 2% B27 (B27), chemically defined medium with no serum (MIN), DETANONOate (0.1 mM; NO), or DETANONOate plus MSC-conditioned medium (NO/CM) for 6 h. Upper panel shows phospho-p38 immunoblot and lower panel shows total p38 of identical amounts of protein lysate. Representative blot of 3 independent experiments.

medium for 24 h) contained 190.5 pg/ml (+/− 12.56 SEM; $n=6$; Fig. 3A) of BDNF. In order to demonstrate the functional significance of BDNF within MSC-conditioned medium functional BDNF blocking antibodies were used. Addition of neutralising antibodies to BDNF (5 μg/ml; added with medium conditioned by MSCs for either 1 DIV for NO-exposed neurons or 3 DIV for trophically deprived neurons) significantly reduced MSC-conditioned medium-induced attenuation of neuronal death on trophic withdrawal and NO exposure (Figs. 3B and 3C). Control nonselective mouse IgG was also added to medium conditioned by MSCs, with no effect on cell survival (data not shown).

We next determined whether recombinant BDNF improved neuronal survival under conditions of trophic factor withdrawal. Cortical neurons (1.4×10^3 cells/mm^2) were maintained in B27-supplemented DMEM for 5 DIV before

exposure to DMEM supplemented with chemically defined medium with no serum or chemically defined medium with no serum containing recombinant BDNF. After a further 3 DIV, cultures were fixed and neuronal survival was determined. Neurons exposed to the BDNF (both 10 and 100 ng/ml) showed increased survival compared to those cultured in chemically defined medium with no serum alone (Figs. 4A and 4B). Addition of the PI_3kinase/Akt inhibitor, LY290042, significantly reduced the survival effect of BDNF. Furthermore exposure of cortical neurons to BDNF induced high levels of phosphorylated-Akt compared to neurons exposed to chemically defined medium with no serum alone (Fig. 4C).



Discussion

In this report we have shown that human bone marrow-derived mesenchymal stem cells secrete factors which protect rodent neurons from trophic deprivation and nitric oxide-induced death. We have demonstrated that medium conditioned by human MSCs influences intracellular signaling within neurons. We have also shown that human MSCs secrete brain-derived neurotrophic factor which acts as a neuroprotective agent via the PI_3kinase/Akt pathway.

The prospect of stem cell therapies for neurodegenerative disorders has created much interest (Rosser et al., 2007). Specifically, human MSC transplantation has been shown to improve the outcome in a variety of animal models of neurological disease including stroke and spinal cord injury (Zhang et al., 2004; Himes et al., 2006; Zhang et al., 2006). In experimental autoimmune encephalomyelitis (EAE), there is evidence that MSCs may improve clinical outcome via immunomodulation, reduction in central nervous system inflammation, and neuroprotection (Zappia et al., 2005; Kassis et al., 2008). Inflammatory disorders, such as EAE, are associated with elevated levels of nitric oxide within the central nervous system, combined with reductions in trophic support from surrounding glia (Kanwar et al., 2004). The current study provides a potential mechanism by which MSCs may protect neurons from NO-mediated damage or trophic deprivation and may be important in determining potential therapeutic targets for central nervous system inflammatory disorders, such as multiple sclerosis.

Although the precise mechanisms by which MSCs act has not been fully elucidated, it has been postulated that they

Figure 3 (A) Human MSCs produce BDNF: Production of BDNF (pg/cell) by different MSC populations (derived from different patients; MSC1–6). Chemically defined medium with no serum (MIN), rat cortical neurons (6 DIV; Neuron), and rat cortical neurons exposed to DETANONOate for 24 h (6 DIV; NeuronNO) are used as controls. (B) Neutralising antibodies to BDNF attenuate MSC-conditioned medium survival effects under conditions of trophic deprivation: Effect of chemically defined medium with no serum (MIN), MSC-conditioned medium (CM), and MSC-conditioned medium plus anti-BDNF neutralising antibodies (5 μg/ml CM/aBDNF) on live β-tubulin-positive cells per field. Controls were cultured in 2% B27 throughout and values expressed as a percentage of this control. Neurons were cultured for 5 DIV and exposed to test conditions for 3 DIV ($n=4$; * $P<0.05$). (C) Neutralising antibodies to BDNF attenuate MSC-conditioned medium survival effects under conditions of DETANONOate exposure: Effect of chemically defined medium with no serum (MIN), DETANONOate (0.2 mM; NO), DETANONOate plus MSC-conditioned medium (NO/CM), and DETANONOate plus MSC-conditioned medium plus anti-BDNF neutralising antibodies (5 μg/ml NO/CM/aBDNF) on live β-tubulin-positive cells per field. Controls were cultured in 2% B27 throughout and values expressed as a percentage of this control. Neurons were cultured for 5 DIV and exposed to test condition for 3 DIV ($n=3$; * $P<0.05$).

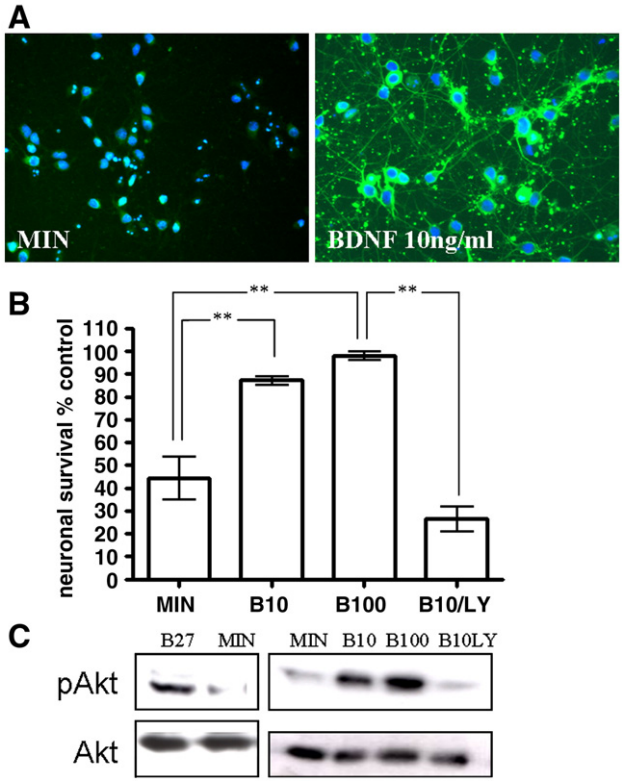


Figure 4 (A) Immunographs showing the effect of trophic deprivation (MIN) and BDNF (10 ng/ml; BDNF) on neuronal survival (β-tubulin staining; green) in cultures (5 DIV) exposed to test conditions for 3 DIV. Nuclei stained with Hoechst (blue). Scale bar: 40 μm. (B) BDNF increases neuronal survival: Effect of chemically defined medium with no serum (MIN), BDNF 10 ng/ml (BDNF10), BDNF 100 ng/ml (BDNF100), or BDNF 10 ng/ml plus LY290042 (10 μM; B10/LY) on live β-tubulin-positive cells per field. Controls were cultured in 2% B27 throughout and values are expressed as a percentage of this control. Neurons were cultured for 5 DIV and exposed to test conditions for 3 DIV ($n=4$; ** $P<0.01$). (C) BDNF phosphorylates Akt: Akt phosphorylation in neurons (cultured for 5 DIV) and exposed to 2% B27 (B27), chemically defined medium with no serum (MIN), BDNF 10 ng/ml (B10), BDNF 100 ng/ml (B100), or BDNF 10 ng/ml plus LY290042 (10 μM; B10LY) for 1 h. Upper panel shows phospho-Akt immunoblot. Lower panel shows total-Akt immunoblot of same membrane. Representative blot of 3 independent experiments.

might be effective treatments for neurological disorders by replacement of lost cells by differentiation into functional neural tissue; modulation of the immune system to prevent further neurodegeneration, cell fusion, or provision of trophic support for the diseased nervous system. Previous reports have demonstrated that bone marrow-derived stem cells are able to eloborate various potentially neuroprotective growth factors *in vitro* including BDNF (Arnhold et al., 2006; Neuhuber et al., 2005; Hokari et al., 2008). Human MSCs have also been shown to release other neurotrophic factors, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor-2, and insulin-like growth factor type 1 (Pisati et al., 2007; Boucherie et al., 2008; Crisostomo et al., 2008). There is also evidence that MSCs may alter neurotrophic factor expression dependent on the environment in which they exist (Boucherie et al., 2008; Crisostomo et al., 2008). Understanding the mechanisms and factors involved in MSC-derived neuroprotection is an important prerequisite to the design of future cell-based therapies for neurodegenerative diseases.

Intracellular signaling underlying processes of neuronal cell death is complex, but evidence points to a critical role for both MAPkinase signaling pathways and PI_3kinase/Akt signaling (Vaillant et al., 1999). Members of MAPkinase pathways are known to be activated by a variety of signals in neurons and specifically p38 has been implicated in neuronal cell death pathways (Harper and LoGrosso, 2001). Several reports have shown p38 MAPkinase activation during NO mediated neuronal death and trophic factors have been shown to inhibit p38 signaling and promote survival in this context (Lin et al., 2001; Ishikawa et al., 2003; Wilkins and Compston, 2005).

The PI_3kinase/Akt pathway is a critical cellular survival pathway (Kaplan and Miller, 2000). Akt pathways are particularly important in mediating neuronal survival, for instance, in determining the response of sympathetic neurons to depolarization and NGF exposure (Vaillant et al., 1999; Crowder and Freeman, 1998). These reports again highlight the convergence of antiapoptotic processes on Akt, since both exposure to potassium chloride, leading to depolarization of the cell, and NGF treatment of cells lead to Akt phosphorylation and a reduction in apoptosis. The PI_3kinase/Akt pathway has also been implicated in the survival of other neuronal types, such as sensory neurons, cerebellar granule cells, and retinal ganglion cells (Klesse and Parada, 1998; Diem et al., 2001; Encinas et al., 2001).

Many neurotrophic factors have been identified which activate several intracellular pathways *in vitro* and the requirement for combinations of factors is apparent for CNS neurons, with the precise nature of such signals being dependent on type, region, and age of neurons. BDNF is a member of the neurotrophin family of growth factors that is widely expressed in the adult and developing nervous system. *In vitro*, BDNF has been shown to increase survival of many neurons, including hippocampal and retinal ganglion cells (Lindholm et al., 1996; Meyer-Franke et al., 1995). BDNF activates a number of signaling pathways in neurons, but PI_3kinase/Akt pathways appear crucial in its survival-promoting effects (Yamada et al., 2001).

We have demonstrated that human MSCs are strongly neuroprotective *in vitro* and analysed some of the intracellular signaling mechanism underlying these effects. Understanding the mechanisms by which human MSCs provide protection for neurons is an important step in designing rational therapies for neurodegenerative diseases.

Materials and methods

Neuronal cell culture

Neuronal cultures were prepared from cortices of E16 rat embryos as previously described (Wilkins et al., 2001). Following enzymatic dissociation, cells were plated onto poly-L-lysine coated 13-mm coverslips and cultured in

Dulbecco's modified Eagles medium (Sigma-Aldrich, Gillingham, UK) supplemented with 2% B27 (Gibco, UK). After 6 DIV,>95% of cells were positive for the neuronal marker β-tubulin. At this point, cultures were exposed to experimental conditions. Chemically defined medium with no serum consisted of DMEM supplemented with insulin-free Sato (containing bovine serum albumin (100 μg/ml), transferrin (100 μg/ml), progesterone (0.06 μg/ml), putrescine (16 μg/ml), selenite (0.04 μg/ml), thyroxine (0.04 μg/ml), triiodothryonine (0.04 μg/ml)).

A stock solution (50 mM in 10 mM NaOH) of (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]daizen-1-ium-1,2-diolate] (DETANONOate; Alexis Biochemicals, Nottingham, UK) was prepared immediately before use. In order to confirm nitric oxide generation in cultures, a modified Greiss reaction (Sigma, UK) was performed on culture supernatants after 24 h of DETANONOate exposure. Recombinant BDNF was obtained from Sigma. The pathway inhibitor PD98059 and neutralising antibodies to BDNF were obtained from Calbiochem (USA) and LY290042 from Sigma.

MSC culture

Bone marrow samples were obtained by orthopedic surgeons at the Avon Orthopaedic Centre, Bristol, UK, with informed written consent and hospital ethics committee approval. Bone marrow was taken at the time of total hip replacement surgery from the femoral shaft and placed into a sterile 50-ml tubes containing 1000 IU heparin. Femoral shaft bone marrow donors were healthy apart from osteoarthritis (those with history of malignancy, immune disorders, or rheumatoid arthritis were excluded), and were not receiving drugs known to be associated with myelosuppression or bone-marrow failure.

Femoral shaft marrow samples were broken up with a scalpel and washed with DMEM until remaining material (bone) looked white at the bottom of the 50-ml tube. All washings were pipetted into a new 50-ml tube and kept for centrifugation. The suspension was centrifuged and resuspended in DMEM and overlaid onto an equal volume of Lymphoprep (Axis-Shield, Dundee, UK; density 1.077+/−0.001 g/ml) and centrifuged at 600 g for 35 min at room temperature to separate the mononuclear cells (MNC) from neutrophils and red cells. The MNC layer was harvested and washed twice in DMEM.

Isolated MNCs were centrifuged and resuspended in MSC medium (consisting of DMEM with 10% FCS selected for the growth of MSC (StemCell Technologies, London, UK), and 1% penicillin and streptomycin (Sigma-Aldrich). Vented flasks (25 cm^2) containing 10 ml of MSC medium were seeded with 1×10^7 cells for primary culture. Flasks were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and fed every week with MSC medium by half medium exchange to remove nonadherent hematopoietic cells until the adherent fibroblast-like MSCs reached approximately 70% confluence.

On reaching confluence the adherent cells were resuspended using 0.25% trypsin (Sigma-Aldrich) and reseeded at 2.25×10^5 cells per (75 cm^2) flask into first passage. Cultures were then incubated, fed every week with MSC medium by half medium exchange, and again trypsinized, a cell count was taken and cells were reseeded at 2.25×10^5 cells per flask (75 cm^2).

To ensure that a homogenous population of MSC had been cultured immunophenotyping of surface markers was carried out using flow cytometry according to previous reports (Pittenger et al., 1999). Cells were examined at third passage using APC-conjugated anti-CD105, APC-Cy7-conjugated anti-CD45 (eBioscience), PE– conjugated anti-CD166, FITC-conjugated anti-CD90 (BD Biosciences), and with PE-Cy7-conjugated anti-CD44 (Serotec). Mesenchymal stem cells were induced into adipogenic, osteoblastic, and chondrogenic differentiation by culturing MSC, at third passage, in NH Adipodiff medium, NH Osteodiff medium, and NH Chondrodiff medium (Miltenyi Biotec), respectively, according to the manufacturers instructions. Adipogenic, osteogenic, and chondrogenic differentiations were characterized using immunofluorescent detection by indirect labeling using a anti-lipoprotein lipase (Abcam), anti-alkaline phosphatase (Abcam), and anti-human aggrecan (4F4) (Santa Cruz Biotechnology), respectively (Gordon et al., 2008; Gordon et al., 2008).

Preparation of mesenchymal stem cell-conditioned medium

Confluent mesenchymal stem cell cultures, at third passage, were washed in DMEM and cultured for 24 h in chemically defined medium with no serum (described above). Media were then removed and stored at-20 °C prior to use in culture experiments.

Assays for cell survival and cell morphology

Evaluation of neuronal cell survival and morphology within cultures was carried out by immunocytochemistical analysis using antibodies against β-tubulin III (Sigma; 1:400). Cells were stained after fixation with 4% paraformaldehyde and treatment of cultures with 100% methanol at −20 °C for 10 min. Secondary antibodies coupled to FITC fluorochromes were used to visualise primary antibody staining. Hoechst 33258 (bisbenzamide 1:5000) was used (10 min at room temperature) for nuclear identification and morphological assessment of cell survival. Nuclear morphology was assessed for each cell analysed to determine whether it was alive or undergoing apoptotic cell death. Neuronal survival was assessed using counts of live β-tubulin-positive stained cells, taking 5 random fields per culture and at least 3 cultures per experiment. In all cases, control cultures grown throughout the experimental period in DMEM/2% B27 were also analysed and values for experimental conditions divided by this value, in order to standardize results between experiments.

Immunoblotting for cell signaling proteins

Neurons were cultured at high density (2×10^6 per 6-well plate) before exposure to test conditions. After set time points, cells were lysed and stored in loading buffer, consisting of 150 mM Tris-HCl, 8 M urea, 2.5% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, 3% w/v DTT, 0.1% bromophenol blue, pH 6.8, and stored at −20 °C until use. Continuous gradient SDS-polyacrylamide gels (5–20%) were used and equal quantities of cell lysates (protein

determination using the BCA protein assay kit, (Pierce, Cheshire, UK)) run on each lane. After transfer to nitrocellulose membrane (Hybond C-super, Amersham, UK) and blocking in 4% w/v powdered milk, membranes were incubated overnight in primary antibody at 4 °C (in TBS/5% BSA). Antibodies used were phospho-p38 (Thr180/Tyr182), total p38 antibody, phospo-Akt (ser473), and total Akt (all 1:1000, Cell Signal Technology, USA). Immunoreactivity was visualized by secondary anti-rabbit HRP-conjugated antibodies (1:2000; Dako) and enhanced chemiluminescence (Renaissance ECL reagent, NEN Life Science Products, Boston, MA).

BDNF ELISA

Mesenchymal stem cell-conditioned media (100 μl), derived from MSCs (5×10^5 cells conditioned in 1 ml of chemically defined medium with no serum for 24 h) at varying passages, were analyzed by ELISA using the RayBio Human BDNF ELISA kit (Raybiotech Inc., USA) according to the manufacturer's instructions. All samples were analyzed in triplicate.

Statistical analysis

Statistical analysis on data expressed as a percentage of control was performed on arcsine transformed values. Counting data were analysed using nonparametric tests (Kruskal-Wallis with post hoc Dunn's testing between groups). Other data were analysed using one-way ANOVA with post hoc testing for comparison of multiple sets (Neumann-Keuls). Values are expressed as the mean+/− standard error of the mean from at least 3 independent experiments, unless otherwise stated.

Acknowledgments

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