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Research Article

Intrinsic mechanisms underlying the neurotrophic activity of adipose derived stem cells

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ABSTRACT

Adipose derived stem cells (ADSC) can be differentiated into Schwann cell-like cells which enhance nerve function and regeneration. However, the signalling mechanisms underlying the neurotrophic potential of ADSC remain largely unknown. In this study, we hypothesised that ADSC, upon stimulation with a combination of growth factors, could rapidly produce brain derived neurotrophic factor (BDNF) with a similar molecular mechanism to that functioning in the nervous system. Within 48 h of stimulation, ADSC demonstrated potent neurotrophic effects on dorsal root ganglion neurons, at a magnitude equivalent to that of the longer term differentiated Schwann cell-like cells. Stimulated ADSC showed rapid up-regulation of the neuronal activity dependent promoter *BDNF* exon IV along with an augmented expression of full length protein encoding *BDNF* exon IX. BDNF protein was secreted at a concentration similar to that produced by differentiated Schwann cell-like cells. Stimulation also activated the *BDNF* expression gating transcription factor, cAMP responsive element binding (CREB) protein. However, blocking phosphorylation of CREB with the protein kinase A small molecule inhibitor H89 did not suppress secretion of BDNF protein. These results suggest rapid BDNF production in ADSC is mediated through multiple compensatory pathways independent of, or in addition to, the CREB neuronal activation cascade.

Introduction

Autologous glial cell transplantation of Schwann cells, olfactory ensheathing cells or oligodendrocytes, to treat peripheral nerve or spinal cord injuries, has shown promising effects on regeneration. The impracticality of large scale *in vitro* expansion of primary glia has however hindered their clinical applications [1,2]. Mesenchymal stem cells (MSC) have emerged as a clinically translational, alternative adult cell source for autologous cell transplantation and therapy. MSC isolated from adipose and bone marrow tissues have

Abbreviations: *ADSC*, adipose derived stem cells; *BDNF*, brain derived neurotrophic factor; *bFGF*, basic fibroblast growth factor; *CREB*, cAMP responsive element binding; *dADSC*, differentiated Schwann cell-like cells; *DMSO*, dimethyl sulfoxide; *DRG*, dorsal root ganglia; *ELISA*, enzyme linked immunosorbant assay; *GGF-2*, glial growth factor-2; *HBSS*, Hank's balanced salt solution; *MSC*, mesenchymal stem cells; *PDGF-AA*, platelet derived growth factor-AA; *RT-PCR*, reverse transcription-polymerase chain reaction

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been shown to have multi-potency with the capacity to differentiate into cells of the chrondrogenic, osteogenic and adipogenic lineages [3–5]. MSC can also be induced to adopt a glial cell phenotype by *in vitro* manipulation [6–8], or differentiate *in vivo* in a nervous tissue environment [9–11]. Nevertheless, while the potential use of adult MSC to treat neurological disorders and injuries attracts considerable clinical interest, the underlying mechanisms and rationale of using cells of the non-ectodermal or neural crest lineage for such purposes remains to be elucidated.

Robust trophic activity has been postulated as one of the mechanisms underscoring the functionality of MSC [12]. We have previously shown the phenotypic and functional differentiation of bone marrow MSC and adipose derived stem cells (ADSC) into a Schwann-cell like lineage, which was defined by the expression of glial cell markers and promotion of neurite outgrowth *in vitro* [6,7,13] and *in vivo* regeneration [14,15]. Part of the beneficial effects of induced MSC could be attributed to neurotrophin secretions, in particular, brain derived neurotrophic factor (BDNF), since axonal outgrowth could be blocked by neutralising BDNF or inhibiting TrkB signalling pathways [16,17].

BDNF is an immediate early gene in the nervous system and plays crucial roles in synaptic plasticity, neuron-glia communication and regulating neurite outgrowth [18,19]. In rodents, the *BDNF* genome constitutes of eight promoters and each bind to a common BDNF full length protein encoding exon [20]. The transcription of *BDNF* is tightly regulated by multiple transcription factors (reviewed by [21–23]). Of these, cAMP response element-binding (CREB) protein has been extensively studied since it governs the transcription of exon IV, namely the neuronal activity dependent promoter of *BDNF* [24,25]. Studies have demonstrated the role of exon IV (designated as exon III prior to Aid et al. [20]) in synaptic plasticity of neurons and cognition [26,27]. More recent studies suggest that glia selectively increase BDNF production in response to neuronal signals acting through glutamatergic receptors [28–30], purinergic receptors [19,31], or neuregulin receptors [32]. These studies support the view of glia-neuron interactions playing an important role in axonal/dendritic maintenance and synaptic plasticity. In this study we hypothesise that the factors used to modulate ADSC phenotype (neuregulin/glial growth factor, basic fibroblast growth factor, platelet derived growth factor and cAMP elevating agent forskolin), mimic the natural physiological signals and are able to trigger rapid BDNF transcription and protein secretion from the stem cells. If this is the case, it may be possible to use MSC acutely, without the need for weeks of *in vitro* stimulation with growth factors and thereby reduce the time to treatment of nerve injuries.

Materials and methods

Reagents and chemicals were purchased from Sigma Aldrich or Fisher Scientific if not otherwise specified. Animals were from Scanbur BK AB. All experiments involving the use of animals were approved by the Northern Swedish Committee for Ethics in Animal Experiment and in compliance with the European Communities Council Directives (86/609/EEC).

Adipose derived stem cell (ADSC) culture

Stem cells were isolated from adipose tissue taken from 7–8 weeks old Fischer 344 rats using methods previously reported [6]. Visceral adipose tissue of the gastrointestinal region was aseptically harvested, dissected and minced. Type I collagenase (0.15% w/v) was applied to digest the tissue for 1 h at 37 °C. The suspension of digested tissue was then filtered through a cell strainer of 70 μm pore size. The resulting cell suspension was centrifuged (10 min at 400*g*). The cell pellet was isolated, resuspended in fresh growth medium which was made of MEM-α, 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (all from Invitrogen) and plated on a 75 cm^2 tissue culture flask. Every 24 h, the cells were washed with Hank's balanced salt solution (HBSS) for 3–5 days to dislodge haematopoietic cells, followed by replacement with fresh growth medium. 5000 cells/cm^2 were passaged to a new culture flask by using trypsin/EDTA and maintained at 37 °C and 5% CO_2 until the cells reached confluence. In all experiments, cells from passage 4 to 6 were used.

Induction of the Schwann cell-like phenotype

To differentiate the ADSC into Schwann cell-like cells (dADSC) [6] the growth medium was replaced with fresh medium containing 1 mM β-mercaptoethanol and the cells cultured for 24 h. Subsequently, fresh medium containing 35 ng/ml all-trans-retinoic acid was added to the cells for the next 72 h. Finally, the cells were washed with HBSS and the medium was replaced with growth medium containing 252 ng/ml glial growth factor-2 (GGF-2, a gift from Acorda Therapeutics Inc, Ardsley, NY, USA), 10 ng/ml basic fibroblast growth factor (bFGF, Millipore), 5 ng/ml platelet derived growth factor-AA (PDGF-AA Millipore) and 14 μM forskolin. One-third of the cells were passaged once they became confluent. Cells were washed with HBSS and sub-cultured in fresh differentiation medium every 48–72 h for 2 weeks. Cells were then passaged and plated as described below. At the same time, parallel cultures of undifferentiated ADSC were plated and treated with the stimulating medium (as above) for up to 48 h.

Dorsal root ganglia (DRG) neuron culture

DRG were harvested from 7–8 weeks old adult female Sprague-Dawley rats as described previously [7]. After isolation and dissociation, neurons were suspended in modified Bottenstein and Sato's medium composed of DMEM/F12 medium containing 1 mg/mL BSA, 0.01 mM cytosine arabinoside, 10 pM insulin, 100 μM putrescine, 30 nM sodium selenite, 20 nM progesterone, plus 0.1 mg/mL transferrin. The DRG neurons were plated on sterile 12 mm circular, poly-L-lysine and laminin coated coverslips in duplicates, which were held on a 35 mm petri dish covered by 1 ml of media.

ADSC conditioned medium and DRG neurite outgrowth assay

ADSC or 2 weeks differentiated Schwann cell-like cells (dADSC) cells, at a density of 125,000 cells/ml, were seeded into six-well plates in medium containing the stimulating growth factors as described above. Conditioned media from these wells were collected at 48 h, centrifuged at 800*g* for 10 min to remove any suspended cells and then 1 ml of the supernatant was applied directly to the petri dishes containing DRG, which had been harvested 24 h previously. In some experiments anti-BDNF neutralising antibody (10 μg/ml; Millipore) was added with the