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Reduced neuroprotective potential of the mesenchymal stromal cell secretome with *ex vivo* expansion, age and progressive multiple sclerosis

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Abstract

Background. Clinical trials using *ex vivo* expansion of autologous mesenchymal stromal cells (MSCs) are in progress for several neurological diseases including multiple sclerosis (MS). Given that environment alters MSC function, we examined whether *in vitro* expansion, increasing donor age and progressive MS affect the neuroprotective properties of the MSC secretome. *Methods.* Comparative analyses of neuronal survival in the presence of MSC-conditioned medium (MSCcm) isolated from control subjects (C-MSCcm) and those with MS (MS-MSCcm) were performed following (1) trophic factor withdrawal and (2) nitric oxide–induced neurotoxicity. *Results.* Reduced neuronal survival following trophic factor withdrawal was seen in association with increasing expansion of MSCs *in vitro* and MSC donor age. Controlling for these factors, there was an independent, negative effect of progressive MS. In nitric oxide neurotoxicity, MSCcm-mediated neuroprotection was reduced when C-MSCcm was isolated from higher-passage MSCs and was negatively associated with increasing MSC passage number and donor age. Furthermore, the neuroprotective effect of MSCcm was lost when MSCs were isolated from patients with MS. *Discussion.* Our findings have significant implications for MSC-based therapy in neurodegenerative conditions, particularly for autologous MSC therapy in MS. Impaired neuroprotection mediated by the MSC secretome in progressive MS may reflect reduced reparative potential of autologous MSC-based therapy in MS and it is likely that the causes must be addressed before the full potential of MSC-based therapy is realized. Additionally, we anticipate that understanding the mechanisms responsible will contribute new insights into MS pathogenesis and may also be of wider relevance to other neurodegenerative conditions.

Key Words: *cell therapy, mesenchymal stromal cells, multiple sclerosis, neuroprotection*

Introduction

Recently, there has been increasing appreciation of the potential of cell-based therapies for treatment of neurodegenerative diseases including multiple sclerosis (MS) [1]. Multipotent mesenchymal stromal cells (MSCs) have received considerable attention given that they can be relatively easily isolated from bone marrow or other tissues and expanded *in vitro*. MSCs secrete a wide range of factors and have a multiplicity of actions in diverse processes, including immunomodulation, inflammation, apoptosis and angiogenesis. Many reparative processes are now recognized to be mediated, orchestrated or stimulated by the MSC secretome–the collective term for factors secreted as soluble molecules and/or in extracellular vesicles. With respect to inflammatory demyelination, MSCs have been shown to have anti-inflammatory as well as neuro- and glioprotective effects, and administration of MSC-conditioned medium (MSCcm) improves the outcome of the MS model experimental allergic encephalomyelitis (EAE) [2]. Such properties, combined with their favorable safety profile, have accelerated translation of MSC-based therapy, which is currently being explored in clinical trials in MS [1].

Characterization of bone marrow microenvironment and sub-populations of bone marrow–derived cells such as MSCs has been relatively limited in MS [3–8], although an increase in senescence and altered cytokine secretion have been noted [5,6]. This is of importance and potential therapeutic relevance given

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that donor factors, including age, expansion *in vitro* and disease states, have previously been reported to influence MSC properties, including T-cell immunosuppression [9], with functional effects in a disease model of MS [10]. We have recently shown that the bone marrow microenvironment is abnormal in MS and that MS MSCs have reduced proliferative potential and display signs of premature aging *in vitro* [11]. However, it is not known whether MSC function is impaired in MS.

In this study, we assessed whether MSC donor age and expansion of MSCs *in vitro* alters their support for neurons under conditions of trophic factor withdrawal and whether there were differential effects of the MSC secretome depending on whether MSCs were isolated from control subjects or those with progressive MS. Furthermore, we examined whether MSC expansion and donor age or the presence of progressive MS alters neuroprotective potential of the MSC secretome using well-characterized *in vitro* assays of MSC-mediated neuroprotection [12–15] in nitric oxide (NO)–induced toxicity, a mechanism known to be of pathophysiological relevance to inflammatory demyelinating disease.

Materials and methods

MSC isolation and culture

Bone marrow samples from control subjects who had no prior exposure to immunomodulatory drugs were obtained from the femoral shaft during total hip replacement for osteoarthritis (UK Research Ethics Committee [REC] 10/H102/69). Bone marrow from patients with progressive MS was obtained as a posterior iliac crest aspirate from participants in the trials "Assessment of Bone Marrow-Derived Cellular Therapy in Progressive Multiple Sclerosis (ACTiMuS)" (NCT01815632; REC 12/SW/0358) [16] or "Repeat Infusion of Autologous Bone Marrow Cells in MS (SIAMMS-II)" (NCT01932593; UK REC 13/SW/0255) [17].

In the full cohort, the age of control subjects (n = 9; mean age, 59.3 years) was greater than patients with MS (n = 19; mean age, 50.6 years; Student *t* test $P = 0.004$; Supplementary Table S1). There was a strong trend for duration of progressive disease to increase with age (Pearson $r = 0.364$; $P = 0.052$). Not all samples were analysed in all experiments and the number of biological replicates (n) for each experiment is presented with the results. Summary data regarding the cohort including details of exposure to disease-modifying therapies are presented as supplementary information (Supplementary Table S1). No participant with primary progressive MS (n = 8) had prior exposure to disease-modifying therapy. Of 11 participants with secondary progressive MS, five had been treated with disease-modifying therapy: two with beta-interferon, two with glatiramer and one with beta-interferon then glatiramer. No one had been exposed to disease-modifying therapy in <12 months prior to bone marrow isolation.

Control bone marrow from the femoral shaft was collected in RPMI medium (Sigma) with 1000 IU heparin. Patient samples were collected in heparin before being transported in ethylenediaminetetraacetic acid (EDTA; K2). Subsequently, marrow samples were processed identically; MSCs were isolated using a density gradient, expanded *in vitro* and demonstrated to conform to expected cell surface phenotype and mesenchymal differentiation potential [4].

Preparation of MSCcm

Culture flasks (T175 seeded with 450,000 cells) were washed twice with Dulbecco's Modified Eagle's Medium (DMEM) to remove standard MSC culture medium. Minimum medium (MIN) consisting of 50 mL DMEM, 500 µL Pen-Strep (Gibco Penicillin-Streptomycin Ref 15140-122), 500 µL Sato concentrate (containing 100 µg/mL of bovine serum albumin, 0.06 µg/mL progesterone, 16 µg/mL putrescine, 0.04 µg/mL selenite, 0.04 µg/mL thyroxine and 0.04 µg/mL triiodothyronine) [18], 500 µL holotransferrin (Sigma-Aldrich Ref T0665) and 250 µL L-glutamine (Sigma Aldrich Ref I5500) was added to flasks (22 mL per T175) and allowed to condition for 24 h. Conditioned medium was collected from cultures of control MSCs (C-MSCcm) or MSCs isolated from patients with MS (MS-MSCcm), centrifuged, filtered and stored at -20°C [14].

Cortical neuron cultures

Isolation of rodent cortical neuron cultures was undertaken as previously described [19] and 300,000 cells/well were seeded for immunocytochemistry in a 24-well plate. For a 96-well plate, 100,000 cells/well were seeded. Incubation experiments were performed at 5 days *in vitro*.

NO-induced toxicity

Cortical neurons were conditioned in MIN, C-MSCcm or MS-MSCcm for 3 h prior to exposure to NO (0.4 mmol/L DETANONOate for 24 h) as previously described [19].

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

Neuronal survival was quantified using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [20]. To correct for any