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Research Article

Undifferentiated MSCs are able to myelinate DRG neuron processes through p75

Maddalena Ravasi[1], *Arianna Scuteri*[*,1], *Silvia Pasini, Mario Bossi, Virginia Rodriguez Menendez, Daniele Maggioni, Giovanni Tredici*

Dipartimento di Chirurgia e Medicina Interdisciplinare, Università degli Studi di Milano-Bicocca, via Cadore 48, 20900 Monza, Italy

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ABSTRACT

Over the last few years the therapeutic approach to demyelinating diseases has radically changed, strategies having been developed aimed at partnering the classic symptomatic treatments with the most advanced regenerative medicine tools. At first, the transplantation of myelinogenic cells, Schwann cells or oligodendrocytes was suggested, but the considerable technical difficulties, (poor availability, difficulties in harvesting and culturing, and the problem of rejection in the event of non-autologous sources), shifted attention towards more versatile cellular types, such as Mesenchymal Stem Cells (MSCs). Recent studies have already demonstrate both in vitro and in vivo that glially-primed MSCs (through exposure to chemical cocktails) have myelogenic abilities. In spite of a large number of papers on glially-differentiated MSCs, little is known about the ability of undifferentiated MSCs to myelinate axons and processes. Here we have demonstrated that also undifferentiated MSCs have the ability to myelinate, since they induce the myelination of rat DRG neuron processes after direct co-culturing. In this process a pivotal role is performed by the p75 receptor.

Introduction

Demyelination is a common feature of several neurological diseases of the central and peripheral nervous systems, and it may also be a feature of several neurodegenerative diseases [48,37,43,22,2,21,9]. Currently there are only rather ineffective palliative treatments able to restore myelin into demyelination sites [34,42,32]. A promising alternative for the treatment of demyelination is the use of cellular replacement techniques, which make use of myelinogenic cells, i.e. Schwann cells for repairing the peripheral nervous system, and precursors of oligodendrocytes (OPCs) for repairing the central nervous system. However, these cells present some limitations: (i) they are difficult to harvest and to expand in vitro; (ii) they do not always reach the lesion sites when systemically administered [45,16]; (iii) in the central nervous system they may require additional factors to promote remyelination, e.g. oligodendrocytes can remyelinate chronic plaques only in presence of an inflammatory reaction [4]; (iv) in the event of autologous use, the transplanted cells could have the same defects present in the degenerating cells, or be the

Abbreviations: MSCs, Mesenchymal Stem Cells; DRG, Dorsal Root Ganglia; NGF, Nerve Growth Factor; BDNF, Brain Derived Neurotrophic Factor; FUDR, Fluorodeoxyuridine; GFAP, Glial Fibrillary Acidic Protein; MBP, Myelin Basic Protein; MAG, Myelin Associated Glicoprotein; PI, Propidium Iodide

*Corresponding author. Fax: +39 02 64488250.
E-mail address: arianna.scuteri@unimib.it (A. Scuteri).
[1] These authors contributed equally to this work.

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target of an altered immune system action [25]. Schwann cells have some advantages with respect to OPCs since they can both remyelinate axons in vivo [3,4,13,14,30] and can be derived from an autologous peripheral nerve biopsy. However, they are difficult to obtain and to expand, and have a limited migration capacity [38]. More recently, the use of Mesenchymal Stem Cells (MSCs) has been proposed as, in theory, they may be able to overcome the myelinogenic cells' limitations because of their particular properties, i.e. their self-renewing capacity, their multipotency, their easy harvesting and expandibility, and their immunomodulatory action [30]. Moreover, when systemically administered, they can reach the damaged area [30]. MSCs, by using trophic factors, can be induced to transdifferentiate into Schwann-like cells and, subsequently, to remyelinate axons [38,23] as reported both in vivo [11,40] and in vitro [44,20,26].

It has been demonstrated in vivo that undifferentiated MSCs injected into demyelinated spinal cord express the myelin proteins P0 and MBP and promote remyelination [1]. However, the in vitro myelinogenic activity of undifferentiated MSCs has been studied in a very limited number of papers, and with less than satisfactory results [20,44], with the achievement of only no compact myelin sheaths, ascribed to a glial transdifferentiation process.

Here we have demonstrated the ability of undifferentiated MSCs to form compact myelin sheaths along the processes of rat DRG neurons after direct and long-lasting co-culturing, and we have studied the possible molecular mechanisms underlying this effect. Since we had previously demonstrated the need for cellular contact between neurons and MSCs for long-lasting neuronal survival and maturation [36,35], we focused our attention on the molecules involved both in cellular interaction and in myelination. Among the proteins present on the neuronal membrane, neurotrophin receptors may act both as positive and negative regulators of the process [29]. In particular, the interaction between p75, the low affinity neurotrophin receptor, and BDNF (Brain Derived Neurotrophic Factor) leads cells to start the myelination process [46,41,17].

Material and methods

All the procedures on animals were carried out under anesthesia in accordance with the European Communities Council Directive 86/609/EEC.

All the experiments were repeated at least three times to validate the results.

DRG neuron primary cultures

Dorsal Root Ganglia (DRG,) from 15-day-old embryonic Sprague-Dawley rats (Harlan Italy, Udine, Italy) were aseptically removed, pelleted and dissociated with trypsin. Neuronal cells, which are postmitotic neurons, were plated onto rat tail collagen-coated 35 mm dishes in AN_2 medium made up of MEM (Invitrogen, Carlsbad, CA), plus 15% Calf Bovine Serum (Hyclone, Logan, UT, USA), 50 μg/ml ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA), 1.4 mM L-glutamine (Invitrogen), 0.6% glucose (Sigma Chemical Co.) and supplemented with 5 ng/ml NGF (Invitrogen). After 24 h, neurons were treated for 5 days with AN_2 medium to which were added 5 ng/ml NGF and 2′-Deoxy-5-fluorouridine (FUDR 10^{-5} M Sigma Chemical Co.) to remove satellite cells. After FUDR treatment neurons were incubated with AN_2 medium plus 5 ng/ml NGF.

For BDNF treatment, BDNF (Invitrogen) was dissolved in bidistilled water and added to AN_2 medium (plus NGF) 24 h after FUDR withdrawal at different concentrations (100 ng/ml, 50 ng/ml and 100 pg/ml). The medium was changed once a week. Since we did not observe any differences in terms of myelination ability among the different BDNF concentrations, we performed the experiments with BDNF 100 ng/ml.

For the experiments with p75-neutralizing antibody, 24 h after FUDR withdrawal the cultures were treated with AN_2 medium (plus NGF) supplemented with various dilutions of p75-neutralizing antibody (1:500; 1:1000; Millipore Co., Billerica, MA, USA).

MSC cultures

MSCs were obtained from the bone marrow of 10-week-old female Sprague-Dawley rats by flushing the femur and tibia diaphisis with 2 ml/bone of α-MEM to which was added 2 mM L-glutamine and antibiotics. MSCs were expanded in α-MEM medium (Lonza Group Ltd Switzerland) plus 20% ES cell screened Fetal Bovine Serum (FBS, Hyclone) [12].

For direct co-cultures, MSCs were added to neurons at a density of 100,000 cells/dish 24 hours after FUDR withdrawal. Co-cultures were maintained in AN_2 medium with 5 ng/ml NGF. The medium was changed once a week.

Sudan Black B staining

The cultures were washed with PBS and fixed overnight in 4% paraformaldehyde and 2% glutaraldehyde at 4 °C. After 24 h the cells were washed with cacodylate buffer, postfixed in 2% osmium tetroxide for 1 h, dehydrated in increasing ethanol concentration solution, stained with 1% Sudan black in 70% ethanol for 1 h and washed with bidistilled water. The cells were then mounted and observed at light microscope.

Electron microscopy

The cultures were fixed for 30 min in 4% paraformaldehyde and 2% glutaraldehyde in 0.12 M phosphate buffer and then post-fixed in 1% OsO_4 in cacodylate buffer for 30 min, dehydrated in ethanol and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed with a Philips CM10 transmission electron microscope (Philips Medical Systems S.p.A., Monza, Italy).

Immunofluorescence

An immunofluorescence study was performed as previously described [35]. Briefly, the cultures were washed with PBS and then fixed in 4% paraformaldehyde. Immunostaining was performed according to the manufacturer's instructions. Fluorescence studies were performed using anti-NeuN (Chemicon Int., Temecula, CA, USA, 1:50), anti-MAP2 (Chemicon Int, 1:50), anti-GFAP (Dako Corp., 1:200), anti-S-100 (Chemicon Int., 1:50), anti-MBP (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:50), anti-Connexin 32 (Abcam Ltd, Cambridge, MA, USA, 1:50), anti-p75 (Upstate Biotechnology Inc, Lake Placid, NY, USA, 1:50), anti-TrkA (Calbiochem, San Diego, CA,