Molecular and Cellular Neuroscience 86 (2018) 16–24

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Comparing the different response of PNS and CNS injured neurons to mesenchymal stem cell treatment

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ARTICLE INFO

Keywords:
Mesenchymal stem cells
Sensory neurons
Cortical neurons
Neurotoxicity
Neuroprotection

ABSTRACT

Mesenchymal stem cells (MSCs) are adult bone marrow-derived stem cells actually proposed indifferently for the therapy of neurological diseases of both the Central (CNS) and the Peripheral Nervous System (PNS), as a panacea able to treat so many different diseases by their immunomodulatory ability and supportive action on neuronal survival. However, the identification of the exact mechanism of MSC action in the different diseases, although mandatory to define their real and concrete utility, is still lacking. Moreover, CNS and PNS neurons present many different biological properties, and it is still unclear if they respond in the same manner not only to MSC treatment, but also to injuries. For these reasons, in this study we compared the susceptibility of cortical and sensory neurons both to toxic drug exposure and to MSC action, in order to verify if these two neuronal populations can respond differently.

Our results demonstrated that Cisplatin (CDDP), Glutamate, and Paclitaxel-treated sensory neurons were protected by the co-culture with MSCs, in different manners: through direct contact able to block apoptosis for CDDP- and Glutamate-treated neurons, and by the release of trophic factors for Paclitaxel-treated ones. A possible key soluble factor for MSC protection was Glutathione, spontaneously released by these cells. On the contrary, cortical neurons resulted more sensitive than sensory ones to the toxic action of the drugs, and overall MSCs failed to protect them. All these data identified for the first time a different susceptibility of cortical and sensory neurons, and demonstrated a protective action of MSCs only against drugs in peripheral neurotoxicity.

1. Introduction

Mesenchymal stem cells (MSCs) are adult bone marrow-derived stem cells which have been suggested as a cell-based therapeutic approach for the treatment of several neurodegenerative diseases of both the Central (CNS) and Peripheral Nervous System (PNS), based on their several peculiar properties (Cova et al., 2012; Glavaski-Joksimovic and Bohn, 2013; Oliveira et al., 2013; Pluchino et al., 2009). So far, a plethora of molecular mechanisms have been suggested to mediate the positive effect of MSCs, ranging from neuronal replacement through differentiation to the release of trophic factors and immunomodulation (Scuteri et al., 2011; Scuteri, 2012; Slavin et al., 2008), but generally, the mechanisms by which MSCs support the neuronal survival are still controversial. Moreover, it is plausible that such different diseases require and would profit from different and distinct mechanisms, overall when PNS or CNS neurons are involved. Therefore, it is pivotal to shed light on which mechanism can be useful for each disease, in order to identify and maximize the most appropriate MSC use and a "disease-designed" therapy.

It has already been demonstrated that MSCs are able to support the survival and maturation of untreated Dorsal Root Ganglia (DRG) sensory neurons (Crigler et al., 2006; Scuteri et al., 2006a; Scuteri, 2012). However, it is still unknown if such supportive and protective effect can counteract the damage induced by exposure to toxic stimuli, as well as if MSCs can exert their beneficial effect also on CNS neurons. Here, to clarify on this important aspect, the rescue effect of MSCs on CNS and PNS neurons exposed to different toxic stimuli was evaluated, focusing on the supportive action, rather than on the MSC-immunomodulatory effect, which has already extensively investigated by other authors (Uccelli and Kerlero de Rosbo, 2015; Zappia et al., 2005).

In particular, the different susceptibility of PNS and CNS neurons has been investigated on different disease models: on neurons treated with Cisplatin (CDDP), with Paclitaxel (Pacli), and with Glutamate (Glut). CDDP and Pacli are two chemotherapeutic drugs that induce a

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https://doi.org/10.1016/j.mcn.2017.11.004
Received 25 July 2017; Received in revised form 3 October 2017; Accepted 5 November 2017
Available online 06 November 2017

peripheral neuropathy, which is their main dose-limiting factor (Cavaletti et al., 2000; Cavaletti et al., 1992), while Glut mimics the excitotoxic effect present in Multiple Sclerosis (MS) (Kostic et al., 2013; Sulkowski et al., 2013), a disease causing axonal and neuronal degeneration which is still absent from effective therapies.

In addition to this, direct and indirect co-cultures with MSCs were set up to investigate the effect of MSCs on the different neurons and elucidate their molecular mechanisms of action. Also, to verify the action of soluble molecules released my MSCs, the effect of their Conditioned Medium (CM) has been studied.

2. Materials and methods

All the procedures on animals were carried out under anaesthesia in accordance with ARRIVE guidelines and with national (D. L.vo 26/2014) and international law (EEC Directive 2010/63).

2.1. DRG neuron primary cultures

Dorsal Root Ganglia (DRG) from E15 (15-day-old) embryonic Sprague-Dawley rats (Envigo, Udine, Italy) were aseptically removed, pelleted and dissociated with trypsin. Post mitotic neurons were plated onto 35 mm rat tail collagen-coated dishes and cultured for 5 days 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.) supplemented with 5 ng/ml NGF (Invitrogen). To obtain enriched neuronal cultures, after 24 h neurons were treated for 5 days with AN_2 medium added with 5 ng/ml NGF and 10^{-5} M 2′-Deoxy-5-fluorouridine (FUDR, Sigma Chemical Co.) to remove satellite cells, which remain as contaminants in a percentage lower than 5% at the end of the treatment (Scuteri et al., 2009). Neurons were then incubated with AN_2 medium with 5 ng/ml NGF for 24 h, and the next day were exposed to different drugs.

2.2. Cortical neurons primary cultures

Cortical neurons were obtained as previously reported (Maggioni et al., 2015) from dissociation of the cortex of E18 Sprague-Dawley rat embryos (Envigo). Briefly, cortices were aseptically removed, pelleted and dissociated with 0.25% trypsin plus 1% DNAse for 20 min at 37 °C. Then trypsin was inactivated with FBS and cells were pelleted and resuspended in NeuroBasal medium-A (NB-A, Life Technologies Italia, Monza, Italy) supplemented with 2% B27, 1% L-glutamine and 1% penicillin/streptomycin and 5% Horse serum (Euroclone SpA, Milan, Italy). The cells were then filtered through a 70 μm cell strainer, and neurons were plated on poly-L Lysine coated 35 mm dishes at the density of 500,000 cells/dish. After 2 days, neurons were treated for 2 days with 10 μM Arabinofuranosyl Cytidine (Ara C, Sigma Aldrich) to remove glial cells from primary cultures. After Ara C withdrawal, the neurons were exposed to different drugs. During the culture, half-medium changes were performed twice a week.

2.3. Drug treatments

CDDP (Sigma Aldrich Co.) was dissolved in physiological solution to make a stock solution of 1 mg/ml and then diluted with medium to obtain working concentrations (6 μM or 3 μM).

Glutamate (Sigma Aldrich Co.) was dissolved in sterile water to make a stock solution of 1 M and then diluted with medium to obtain working concentrations (20 mM or 10 mM).

Paclitaxel (LC Laboratories, Woburn, MA, USA) was dissolved in Ethanol 100% to make a stock solution of 1 mM and then diluted with medium to obtain working concentrations (1 μM or 100 nM).

Glutathione (GHS, Sigma Aldrich Co.) was dissolved in sterile water to make a stock solution of 10 mM and then diluted with culture medium to obtain working concentration 10 μM.

N-acetyl cysteine (NAC, Sigma Aldrich Co.) was dissolved in culture medium to obtain working concentration 100 μM.

α-Tocopherol (α-Toco, Sigma Aldrich Co.) was dissolved in ethanol to make a stock solution of 1 mM and then diluted with culture medium to obtain working concentration 1 μM.

2.4. MSC cultures

MSCs were obtained from the bone marrow of 10 week-old female Sprague-Dawley rats by flushing the femur and tibia diaphysis with 2 ml/bone of α-MEM (Euroclone) supplemented with 2 mM L-glutamine and antibiotics. MSCs were plated in α-MEM medium (Euroclone) plus 20% Defined Fetal Bovine Serum (FBS, Hyclone, Logan, UT, USA) (Donzelli et al., 2007). Medium was changed twice a week.

2.5. Direct and indirect MSC co-cultures

At the end of the drugs' treatments, direct and indirect MSCs co-cultures were set up. Direct co-cultures were obtained by adding MSCs on neurons at the density of 100,000 cells/dish. Indirect co-cultures were established with neurons seeded on the upper surface of a Poly-L Lysine or collagen coated cover-glass resting on a single layer of MSCs plated at a density of 100,000 cells/dish. Co-cultures were maintained in complete AN_2 medium plus NGF (sensory neurons) or complete NBA medium (cortical neurons) at 37 °C and 5% CO_2 for 5 days.

2.6. MSC conditioned medium (CM)

MSCs were plated at a density of 100,000 cells/dish (35 mm). After 24 h the MSC medium was collected and used as CM in 1:1 ratio with fresh AN_2 medium plus NGF (sensory neurons) or complete NBA medium (cortical neurons)

2.7. Neuronal survival evaluation

Toxicity was evaluated by a neuronal count of viable cells, characterized by the presence of a birefringent outline which is on the contrary absent both in dead neurons and in glial cells (Scuteri et al., 2006b). The number of neurons of the same microscopic fields were counted before drug treatment, and then after 24, 48 h and 5 days. The percentage of neuronal survival was calculated as viable neurons over total neurons ∗ 100. The same results have been obtained by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich Co.), as previously described (Aras et al., 2008) and according to manufacturer's instructions.

2.8. Immunofluorescences

An immunofluorescence study was performed as previously described (Scuteri et al., 2006a). Briefly, the cultures were washed with PBS and then fixed in 4% paraformaldehyde. Immunostaining was performed according to manufacturer instructions. Fluorescence studies were performed using anti-MAP2 (Chemicon International, Temecula, CA, USA Inc., 1:50), anti-active Caspase 3 (Cell Signaling Technologies, Beverly, MA, USA, 1:500), anti-active Caspase 7 (Cell Signaling Technologies, 1:50) as primary antibodies. Non-specific binding was blocked with 3% BSA in PBS for 1 h, then cells were incubated overnight at 4 °C with the primary antibodies. After washing with PBS, the secondary antibodies were incubated for 1 h at room temperature. Then cells were washed and cover slips were mounted using mounting medium with DABCO (Sigma Chemicals Co.). Cells were then examined using confocal laser microscopy, carried out with a Radiance 2100 confocal microscope equipped with a krypton/argon laser (BioRad, Milan, Italy). Noise reduction was achieved by Kalman filtering during acquisition.