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Research paper

Unveiling the effects of the secretome of mesenchymal progenitors from the umbilical cord in different neuronal cell populations

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ABSTRACT

It has been previously shown that the secretome of Human Umbilical Cord Perivascular Cells (HUCPVCs), known for their mesenchymal like stem cell character, is able to increase the metabolic viability and hippocampal neuronal cell densities. However, due to the different micro-environments of the distinct brain regions it is important to study if neurons isolated from different areas have similar, or opposite, reactions when in the presence of HUCPVCs secretome (in the form of conditioned media-CM). In this work we: 1) studied how cortical and cerebellar neuronal primary cultures behaved when incubated with HUCPVCs CM and 2) characterized the differences between CM collected at two different conditioning time points. Primary cultures of cerebellar and cortical neurons were incubated with HUCPVCs CM (obtained 24 and 96 h after three days of culturing). HUCPVCs CM had a higher impact on the metabolic viability and proliferation of cortical cultures, than the cerebellar ones. Regarding neuronal cell densities it was observed that with 24 h CM condition there were higher number MAP-2 positive cells, a marker for fully differentiated neurons; this was, once again, more evident in cortical cultures. In an attempt to characterize the differences between the two conditioning time points a proteomics approach was followed, based on 2D Gel analysis followed by the identification of selected spots by tandem mass spectrometry. Results revealed important differences in proteins that have been previously related with phenomena such as neurl cell viability, proliferation and differentiation, namely 14-3-3, UCHL1, hsp70 and peroxiredoxin-6. In summary, we demonstrated differences on how neurons isolated from different brain regions react to HUCPVCs secretome and we have identified different proteins (14-3-3 and hsp70) in HUCPVCs CM that may explain the above-referred results.

1. Introduction

Mesenchymal stem/stromal cells (MSCs) have emerged in the last decade as potential tools/vehicles for regenerative medicine purposes [1,2]. These cells are characterized for: 1) their adherence to plastic in standard culture conditions; positive expression for specific markers like CD73, CD90, CD105 and negative expression for hematopoietic markers like CD34, CD45, HLA-DR, CD14 or CD11B, CD79α or CD19 and 2) *in vitro* differentiation into at least osteoblasts, adipocytes and chondroblasts [3]. In recent years it has been increasingly accepted that their regenerative effects are mainly mediated by their secretome [4–6]. The secretome, which comprises the proteins released by cells, tissues or organisms, has been shown to be crucial to the regulation of different cell processes [7].

Due to its low regenerative potential the Central Nervous System (CNS) has been one of the main targets of the regenerative potential of MSCs and their secretome. Initial *in vitro* studies revealed that the latter was able to promote neuronal and glial survival [8–10], neuritogenesis [8] and neural/glial differentiation [11]. These effects were then related, by different authors, with the expression of growth factors such as brain derived neurotrophic

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factor (BDNF), nerve growth factor (NGF), insulin growth factor 1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast derived growth factor 2 (FGF-2), stem cells factor (SCF) and glial derived neurotrophic factor (GDNF), as reviewed by Teixeira et al. [12]. Similar phenomena were also reported *in vivo* [13–16]. In fact, Munoz et al. [13] reported that the injection of bone marrow MSCs (BM-MSCs) in the mice hippocampus led to an increased neuronal differentiation, mediated by neurotrophic factors. Cova et al. [14] and Weiss et al. [15] also reported that BM-MSCs and MSCs isolated from the umbilical cord Wharton Jelly (WJ-MSCs) were able to ameliorate the condition of 6-hydroxydopamine injected Parkinsonian rats through the active secretion of growth factors. While for BM-MSCs this was attributed to the expression of epidermal growth factor (EGF), neurotrophin 3 (NT3), FGF-2, HGF and BDNF, for WJ-MSCs this was attributed to GDNF and FGF-2. Other studies have also shown that similar effects could be observed in *in vivo* models of Spinal Cord Injury (SCI) and brain ischemia [17–19].

Despite the increased knowledge on this topic, there are still a number of questions that remain to be answered. For instance, so far it was not described if neuronal cell populations isolated from different areas of the brain have the same or different response profiles, when exposed to MSCs secretome. This is particularly important as the CNS possesses niches with different neuroregulatory needs. Thus, different brain areas may have a different response to the secretome, a fact that can impact the range of therapeutic applications of the latter. Another important topic that should be addressed is the characterization of the secretome itself. Although important progress has been made, it remains likely that other molecules as well as vesicles in the MSCs secretome are related with the phenomena that have been described to date. An example of this is the work described by Lai et al., where the presence of exosomes in the secretome of MSCs derived from human embryonic stem cells (hESCs), was related to their cardioprotective effects [20]. Exosomes are formed from multivesicular bodies with a bilipid membrane. They have a diameter of 40–100 nm and are known to be secreted by different cell types [20].

Herein we have focused on determining the effects of conditioned media (CM) of Mesenchymal Progenitors isolated from the Wharton Jelly of the umbilical cord (HUCPVCs) on post-natal populations of cortical and cerebellar neurons along with its proteomic characterization. Results revealed that the secretome of HUCPVCs increased cell viability, proliferation and neuronal cell densities in both cortical and cerebellar neuronal cultures, while exhibiting proteins with possible neuroprotective character, which had different expression profiles.

2. Materials and methods

2.1. Cell culture

2.1.1. Human umbilical cord perivascular cells

HUCPVCs were isolated from umbilical cords from consenting full-term caesarean section patients. Ethical approval had been previously obtained from Hospital de S. Marcos, Braga. All human studies were conducted in accordance with the Helsinki accords. All subjects signed an informed consent document prior to their donation of tissue and participation.

They were isolated according to the procedure originally described by Sarugaser et al. [21]. Pieces of cord, 4–5 cm long, were dissected by first removing the epithelium of the UC section along its length to expose the underlying WJ. Each vessel, with its surrounding WJ matrix was then pulled away, after which the ends of each dissected vessel were tied together with a suture creating "loops" that were placed into a 50-ml tube containing a solution of 0.5–0.75 mg/ml collagenase (Sigma, USA) with phosphate buffered saline (PBS, Gibco, USA). After 18 h, the loops were removed from the suspension, which was then diluted with PBS to reduce the viscosity of the suspension and centrifuged. Following the removal of the supernatant, cells were resuspended in culture media, α-MEM (Gibco) supplemented with 10%FBS (Gibco) and 1% antibiotic/antimycotic (Sigma), counted using a hemocytometer and platted in T75 flasks at a density of 4000 cells/cm^2. The culture medium was changed every 2/3 days. Upon confluence cells were trypsinized and passaged to new T75 flasks.

2.1.2. Primary cultures of cerebellar and cortical neurons

Cortical and Cerebellar neuronal cultures were prepared from P4 Wistar Rats [9]. Briefly, and upon dissection, brain tissue was submitted to a trypsin based enzymatic digestion followed by mechanical dissociation. Isolated cells were then plated on coverslips previously coated with Poly-D-Lysine (Sigma) at a density of 40,000 cells/cm^2. Characterization of the cultures by immunocytochemistry (microtubule associated protein (MAP-2)-neurons) revealed that they possessed approximately 45–50% of mature neurons.

2.1.3. Conditioned medium collection and experiments

Conditioned media (CM) were collected from P4 HUCPVCs, as previously described [9]. For this purpose cells were plated out at a density of 4000 cells/cm^2 and allowed to grow for 3 days. Following this, culture medium was renewed and CM collected 24 and 96 h thereafter (cell culture media was not renewed or added during this time period). Upon collection CM were frozen, being later on thawed on the day of the experiments. For CM collection Neurobasal-A medium supplemented with kanamycin (Gibco, 0.1 mg/ml) was the chosen medium. Experiments with the neuronal cultures were done as follows: upon isolation cortical and cerebellar neurons were plated out at the densities referred above and incubated from *T*0 with the previously collected CM ($n = 3$/CM time point) for 7 days (with half of the volume of CM being renewed at day 4 of culture), after which cell densities, viability and proliferation were assessed (see below). Besides kanamycin and glutamax, no further supplements were added to the HUCPVCs CM. As the objective of these experiments was to assess if the secretome alone could induce higher levels of neuronal survival, thus without the presence of any additional factors, control cultures were kept in Neurobasal-A media supplemented with kanamycin and glutamax.

2.2. Cell viability assessment

Cell viability was assessed by the MTS test. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2*H* tetrazolium) (Promega, USA) test is an assay in which the substrate – MTS – is bioreduced into a brown formazan product by NADPH or NADP produced by mitochondrial enzymes, which are active in living cells. Cell culture coverslips ($n = 3$) were placed in culture medium containing MTS in a 5:1 ratio and incubated in a humidified atmosphere at 37 °C and 5% CO_2. After 3 h of incubation 100 μl of solution from each sample were transferred to 96 well plates and the optical density was determined at 490 nm ($n = 3$/CM time point ± SD). Results are shown as a ratio between CM incubated cultures and controls ($n = 3$/CM time point ± SD).

2.3. Cell proliferation

Cell proliferation was determined by a colorimetric assay based on 5-bromo-2′-deoxyuridine (BrdU) incorporation (Roche, Germany). Primary cortical and cerebellar neuronal cultures incubated with CM, and respective controls were incubated with BrdU on day

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