Acta Biomaterialia xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Neuroprotective effect of human mesenchymal stem cells in a compartmentalized neuronal membrane system

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ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form 4 June 2015
Accepted 9 June 2015
Available online xxxx

Keywords:
Hippocampal neurons
Mesenchymal stem cells
Ischemic insult
Neuroprotection
Membrane system

ABSTRACT

In this work, we describe the development of a compartmentalized membrane system using neonatal rodent hippocampal cells and human mesenchymal stem cells (hMSCs) to investigate the neuroprotective effects of hMSCs. To elucidate this interaction an *in vitro* oxygen-glucose deprivation (OGD) model was used that mimics central nervous system insults *in vivo*. Cells were cultured in a membrane system with a sandwich configuration in which the hippocampal cells were seeded on a fluorocarbon (FC) membrane, and were separated by hMSCs through a semipermeable polyethersulfone (PES) membrane that ensures the transport of molecules and paracrine factors, but prevents cell-to-cell contact. This system was used to simulate a cerebral ischemic damage by inducing OGD for 120 min. The core contribution of the work highlights the neuroprotective effects of hMSCs on hippocampal cells in a membrane system for the first time. The novel results show that hMSC secretome factors protect hippocampal cells against OGD insults as indicated by the conservation of specific structural and functional cell features together with the development of a highly branched neural network after the damage. Moreover, neuronal cells co-cultured with hMSCs before OGD insult were able to maintain BDNF production and O_2 consumption and did not express the apoptotic markers that were expressed in similarly insulted neuronal cells that had not been co-cultured to with hMSCs. This compartmentalized membrane system appears to be a very useful and reliable system for studying the neuroprotective effects of hMSC and identifying secreted factors that may be involved.

Statement of Significance

This paper is based on a combined synergism of biomaterials technology and stem cell approach, focusing on the development of a compartmentalized membrane system that serves as an innovative tool for highlighting the role of hMSCs on hippocampal neurons upon damage. The membrane system consists of two different flat sheet membranes, giving rise to double and separated cell membrane compartments that prevent cell-to-cell contact but allow the transport of paracrine factors. This system strongly corroborates the paracrine mediated neuroprotection of hMSCs on ischemic damaged neurons. The challenging and pioneeristic approach by using biomaterials allowed to perform a stepwise analysis of the phenomena, providing new insights into the field of MSC therapy.

1. Introduction

Mesenchymal stem cells (MSCs) are an inviting resource for regenerative therapies due to their neuroprotective potential. It is known that MSCs can protect and stimulate neural tissue regeneration through the secretion of cytokines (i.e., tumor necrosis factor-alpha, interferon-gamma, transforming growth factor-beta, interleukin-6, TSG-6), growth factors and neuroregulatory molecules (i.e., nerve growth factor, vascular endothelial growth factor, glial-derived neurotrophic factor, brain-derived neurotrophic factor) [1,2]. Several studies have shown that hMSCs promote neuronal recovery for *in vitro* models of cerebral ischemia, in animal

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http://dx.doi.org/10.1016/j.actbio.2015.06.013
1742-7061/

stroke, brain and spinal cord injuries, multiple sclerosis and Parkinson's disease animal models [3–7]. However, the role of hMSCs in moderating neuronal responses to ischemic injury is currently unclear. A wide investigation of different aspects of the neuronal outcome could help to clarify the cross-talking value between hMSCs and neurons, and above all, on its influence in preventing neuronal damage during ischemic conditions. The strategy for obtaining the most effective neuroprotection remains to be determined. Identifying the time window of therapeutic efficacy and dose/response characteristics of the neuroprotective response will be important both for clarifying the cellular mechanisms involved and assessing the therapeutic potential of hMSCs for ischemic insults. To this purpose, our strategy was aimed at developing a compartmentalized membrane system able to promote the communication between neurons and hMSCs through their paracrine factors and to prevent their direct contact. This system may prove to be very useful for gaining indications regarding the recovery of neuronal structural and functional properties after ischemic injury when previously exposed to the effect of hMSCs. In order to elucidate these neuroprotective effects of hMSCs on neurons, a cerebral ischemia condition was reproduced by using an *in vitro* oxygen-glucose deprivation (OGD) model to mimic central nervous system insults *in vivo*. This compartmentalized membrane system consists of two different flat sheet membranes, giving rise to double and separated cell membrane compartments that prevent cell-to-cell contact but allow the transport of paracrine factors. The choice of different membranes employed in the system was related not only to their ability to induce specific cellular response but also to different functions that they have to accomplish in this system. Both cell types were cultured directly in contact with the membranes so that membranes with adequate surface and structural properties were required. Previous studies in our group showed that neuronal cells respond to different membrane surfaces by changing their morphology and neurite outgrowths. In particular, it was shown that the preparation of fluorocarbon membranes (FC) as smooth membranes can favor the development of a well-polarized and highly branched neuronal network [8]. Taking into account these findings, the FC membrane was used for culturing the hippocampal cells. MSCs were cultured on a microporous polyethersulfone membrane (PES) that is a suitable substrate for cell culture [9,10]. This membrane, thanks to its structural, physico-chemical and transport properties, acts as a semipermeable barrier that prevents direct contact between neuronal cells and hMSCs as well as ensuring the permeation of the entire pool of paracrine factors that eventually exert neuroprotective activities. The final goal was achieved through the development of this compartmentalized membrane system for culturing hippocampal neurons and hMSCs that could serve as an *in vitro* platform to elucidate the role of hMSC on damaged neurons, which may prove to be useful for future clinical perspectives. The neuroprotective properties of hMSCs were determined via the evaluation of a wide range of hippocampal neuronal morphological plus functional features.

2. Material and methods

2.1. Membranes and compartmentalized membrane system

Two different flat sheet membranes, microporous membranes of PES (Pall, USA) and dense gas permeable membranes of FC (In Vitro Systems & Services, Germany), were used for the culture of hMSCs and hippocampal cells, respectively. FC membranes were coated with poly-L-lysine (PLL, MW 30,000–70,000) [40 μg/cm^2] to improve neuronal cell adhesion. In order to evaluate their morphological properties, both membranes were characterized in terms of mean pore size, pore size distribution and thickness, by scanning electron microscope (Quanta 200F ESEM, FEI, USA).

The wettability of the membranes was specified by water dynamic contact angle (DCA) measurements, which were performed with a CAM 200 contact angle meter (KSV Instruments, Ltd., Helsinki, Finland). The hydraulic permeance of the membranes was evaluated by pure water flux measurements in the absence of solutes and at different transmembrane pressures (ΔP^{TM}). The hydraulic permeance *Lp*, was measured by the following equation:

$$L_P = \left(\frac{J_{Solvent}}{\Delta P^{TM}}\right)_{\Delta c=0}$$

This equation assumes a linear correlation between water flux and the convective driving force.

The compartmentalized membrane system presents a kind of sandwich configuration in which cells are layered in two different compartments: neuronal cells at the bottom, seeded on FC membranes, and the hMSCs, seeded on PES membrane, above it. Each cell compartment had a surface area of 2.54 cm^2 and cell-seeded membranes were separated by a space of 2.5 mm. PES membrane separates hMSC compartment from neuronal compartment. This arrangement recreates a well-defined and controlled environment useful for the screening of factors and processes involved in MSC mediated neuroprotection while keeping a physical separation between the cell types (Fig. 1).

2.2. Cell culture

Neuronal hippocampal cells were isolated from the brain of postnatal days 1–3 hamsters according to the protocol previously developed [8]. Isolated neurons were then seeded on PLL-coated FC membranes. Cultures were fed every 4 days replacing half of the medium.

Poietics™ hMSC derived from bone marrow of a single 22-year-old female donor (Lonza) were thawed and centrifuged at 500 g for 5 min and then suspended in a minimum amount of culture media in order to count the total number of viable cells. The cells were seeded at the density of 5000 cell/cm^2 in Mesenchymal Stem Cell Basal Medium supplemented with hMSC Growth Supplement, L-Glutamine and GA 100, and then incubated at 37 °C, and 5% CO_2 and 90% humidity. hMSCs were fed every 3–4 days until they reached a confluency of 90%, at this point they were passaged and plated on PES membranes at the density of 10,000 cell/cm^2.

2.3. Experimental paradigm

Neuronal cultures were kept in the FC membrane system for a day in vitro (DIV) 8, which is the temporary frame in which neuronal cells reach their maturation leading to the formation of a well-defined neuro-cytoarchitecture as evidenced by processes outgrowth in terms of axons and neurite sprouting. At this point in time hMSCs, seeded on the semi-permeable PES membrane, were layered on top of the neuronal cells in the compartmentalized membrane system and kept in this rearrangement for 24 and 48 h. Then hMSCs were removed from the system and neuronal cells were insulted simulating a model of cerebral ischemia through simultaneous oxygen glucose deprivation (OGD).

For the OGD, only neuronal cells in the compartmentalized membrane system were kept on DIV 10 at 0% O_2 with a gas mixture of 95% N_2/5% CO_2, and the medium was replaced with glucose free Neurobasal medium for 120 min [11,12].

Afterward, normal media was replaced and the culture was returned to normoxic condition for at least 72 h (DIV13).

Please cite this article in press as: A. Piscioneri et al., Neuroprotective effect of human mesenchymal stem cells in a compartmentalized neuronal membrane system, Acta Biomater. (2015), http://dx.doi.org/10.1016/j.actbio.2015.06.013