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Neurotrophic factor mediated neuronal differentiation of human cord blood mesenchymal stem cells and their applicability to assess the developmental neurotoxicity

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ABSTRACT

Plasticity and developmental capacity of stem cells have now been established as a promising tool to restore the degenerative disorders. The linearity differentiation of human mesenchymal stem cells (hMSCs) into adipogenic, chondrogenic, osteogenic and even in neuronal subtypes has been demonstrated. The number of xenobiotics such as dexamethasone, insulin, isobutyl 1-methyle xanthine and retinoic acid has been reported for the potential to differentiate hMSCs into neuronal subtypes. But, the applicability of indigenous neurotrophic factor-nerve growth factor (NGF) has not been explored for the purpose. Thus, the present investigations were carried out to study the NGF induced neuronal differentiation of hMSCs. Following the isolation, purification and characterization of hMSCs were allowed to differentiate into neuronal subtypes under the influence of NGF (50 ng/mL). At various concentrations of NGF, the neuronal makers were analysed at both mRNA and protein levels. Cells, exposed with NGF were showing the significant and gradual increase in the neuronal markers in differentiating cells. The magnitude of expression of markers was maximum at day 4 of differentiation. NGF at 50 ng/mL concentration was found to induce neuronal differentiation of hMSCs into neuronal subtypes.

1. Introduction

Neurogenesis is defined as a process of generating functional neurons from their precursors [1]. Adult neurogenesis is a dynamic, fine-tuned process and subjected to modulation by various physiological, pathological and pharmacological stimuli. Earlier it was believed that damaged neurons could not be restored, although recently many developmental studies has built the way for restoration of neurogenesis [2]. Functional integration of new neurons in the adult central nervous system (CNS) was first shown in songbirds [1]. Multipotent neural stem cells were later derived from adult mammalian brain [3] which subsequently found to play the role of raw material for neurogenesis. In the mammalian brain, there are various growth factors/neurotrophins required for neurogenesis. Among them, nerve growth factor (NGF) is the leading growth factor which regulates neurogenesis process up to a great extent. NGF was discovered almost 5 decades back, the exact role of NGF was not clear but with the availability of tools that allow sensitive and specific measurements of RNA and protein levels for NGF, it has become evident that the role of NGF is beyond the cellular development and nerve cells regulation [4]. NGF has been found to indulge in the cellular activities as rescue from cell injury and promoting cell repair systems [5]. NGF has been employed to convert many candidate cells as PC12 [6], SH-SY5Y [7], rat neural stem cell [8] into mature neurons under *in vitro* conditions. The

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ability of NGF for neuronal development can be a boon in the restoration of neurodegenerative diseases.

Researchers are using many biological systems to study the developmental neuroprotection and neuro restoration. Mesenchymal stem cells (MSCs) comprise of a rare population of multipotent progenitors having ability of haematopoiesis as well as differentiation into various cell subtypes [9]. MSCs can be isolated from number of sources. The sources can be an adult tissue as bone marrow (BM), peripheral blood (PB) and adipose tissue (AT) or neonatal birth-associated tissues as placenta (PL), umbilical cord (UC) and cord blood (CB) [10]. Besides MSCs, other stem/progenitor cell populations from cord blood include hematopoietic stem cells (HSCs) and two endothelial populations such as endothelial progenitor cells (EPCs) and endothelial colony-forming cells (ECFCs). The amounts of MSCs which can be obtained by these isolations vary enormously. Pittenger et al. isolated MSCs from bone marrow by density gradient centrifugation to eliminate unwanted cell types and only 0.001–0.01% of the cells isolated from the density interface were MSCs [11]. These cells show positive expression of CD105 (SH2), CD73 (SH3), CD44 and CD90 and negative expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR on their surface [12].

As we have mentioned, MSCs can be differentiated into various lineages as osteogenic, adipogenic, chondrogenic and neuronal subtypes. These MSCs, for their qualities like multipotency, plasticity and easy accessibility, have attracted various cell based therapies. These therapies may be associated with either direct replacement of damaged cells by exogenously implantation of MSCs or endogenous regeneration. Countless data signified successful use of MSCs in haematology, cancer therapy and numerous acquired or inherited genetic disorders. The therapeutic potential of these cells have been displayed in experimental treatment of various neurological diseases and neural tissue injuries [13]. Researchers have identified the neurogenic potential of MSCs by using many chemicals and various growth factors [14]. But endogenous factors, which are present in brain were not studied to check neuronal induction capacity of MSCs. Since, neurogenesis process is supported by neurotrophin family in brain. So for restoration purpose, endogenous factors should be studied. Therefore, in the current study, we explored the neurogenic potential of human umbilical cord blood derived MSCs by using NGF.

2. Materials and methods

2.1. Reagents and consumables

All the chemicals, reagents, and kits used in this study were purchased from Stem Cell Technologies and Sigma, unless otherwise stated. Recombinant human basic nerve growth factor (rhbFGF) was purchased from PeproTech. Alpha minimal essential medium (α MEM), MSC qualified FBS, antibiotic solution (100X), GlutaMAX™-I (100X), Sodium bicarbonate (7.5%), fluorescent antibodies, and D-PBS were purchased from Gibco (Invitrogen, Grand Island, NY, USA). Antibodies were purchased from Millipore (USA). Culture wares and plastic wares were procured from Nunc and Corning Inc. Autoclaved Milli-Q water was used in all the experiments.

2.2. Ethical clearance for collection and transportation of human tissues

The entire study was carried out following the protocols and procedures approved by the Institutional Human Ethics Committees of CSIR-Indian Institute of Toxicology Research (CSIR-IITR) and CSM Medical University, Lucknow, India. The informed consent of parents was obtained before collecting blood from umbilical cord.

2.3. Processing of human umbilical cord blood

Mothers enrolled in the study were of age, ranging from 24.5 to 30 years. They fulfilled the entire inclusion criteria and were free from malignancy or any other systemic disorder. Total, 50 blood samples (40 mL/cord) were collected from the cord vein in a sterile container having anticoagulant citrate dextrose (ACD) buffer and immediately transported to the CSIR-IITR, Lucknow for further processing. A brief description of the protocol used to isolate the mononuclear cells from umbilical cord blood (UCB) is as under. Blood was diluted in the ratio of 1:1 with Dulbecco's-Phosphate Buffered Saline (D-PBS) without Ca^{2+} and Mg^{2+}, pH 7.5. Cells were isolated by density gradient centrifugation. The diluted blood samples were transferred to centrifuge tubes (50 mL) containing Ficoll-Paque solution and submitted to centrifugation at 2000 rpm for 30 min in order to isolate low-density mononuclear cells. Mononuclear cells were transferred to a new tube and washed twice with culture medium through centrifugation at 2000 rpm for 10 min. Cells were transferred in 25 cm^2 flask for further proliferation.

2.4. Proliferation of mesenchymal stem cells (MSCs)

Expansion of MSCs was carried out following the method described with some modifications pertaining to growth factors and cytokine concentrations [15]. In brief, freshly isolated MSCs were cultured in plastic 25 cm^2 ultralow attachment culture flask (Nunc Incorporated, NY) at a density of 1×10^5 cells/mL in 5 mL of Alpha MEM supplemented with MSC qualified FBS (20%) and Glutamax (1%) (Invitrogen, Life technologies, USA), Penicillin 100 unit/mL, Streptomycin 0.1 mg/mL and Sodium Bicarbonate (Sigma Aldrich). Cells were maintained as adherent culture in humidified chamber at 37° C and 5% CO_2. After 24 h of culture, the medium changes were carried out twice weekly. The cells were monitored under inverted phase contrast microscope (Leica, Germany) every day.

Cells were passaged after reaching 85–90% confluence in cell culture flasks. The cells were subjected to 0.25% Trypsin/EDTA to detach them from culture flask. Then centrifuged at 800 rpm for 6 min and pellet was dissolved to subsequently seed in 25 cm^2 at a density of 1×10^6 nucleated cells/cm^2. These cells were nurtured with culture medium as mentioned above. Cultures were incubated at 37 °C in humidified atmosphere containing 5% CO_2.

2.5. NGF induced neuronal differentiation

MSCs were plated into 25 cm^2 flask at a density of 1×10^6 per flask and cultured for 24 h. Prior to exposure of NGF to the cells, they were incubated with incomplete medium for 24 h. Thereafter, cells were exposed with different concentration of NGF. Exposure was given up to for 4 days and exposure was changed at every alternate day.

2.6. Immunocytochemical study

For immunocytochemical study, following the incubation with primary antibodies, cells were washed three times with 1X PBS for 5 min each to remove the unbound antibodies. Then, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (1:500) and rhodamine-conjugated mouse anti rabbit (1:500) antibodies were added to each well and kept on a rocker shaker in dark for 2 h at room temperature. Cells were then washed with PBS three times for 5 min each. Thereafter, the cells were visualized under an

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