Direct pre-differentiation of rat mesenchymal stem cells into dopaminergic cells

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ABSTRACT

The use of stem cells has been proposed as an alternative treatment for certain neurodegenerative disorders. It has also been suggested that in the pre-differentiated state, stem cells might provide a better therapeutic option than cells that are undifferentiated or fully differentiated. The purpose of this study was to develop a protocol aimed at reducing the incubation time required to induce the conversion of rat mesenchymal stem cells into immature dopaminergic neurons. Stem cells obtained from rat bone marrow were incubated in a control or induction media for 2 - 24 h. Cells incubated for 24 h in induction medium demonstrated an increase on the levels of the neuronal protein markers nestin, glial fibrillary acid protein, and β-tubulin III, as well as increases in the expression of Pax3, EN1, Thy1.1, and GEF10 genes. This manuscript presents evidence that adult mesenchymal cells are capable to respond, in a short time period, to a neuroinduction medium, and give raise to pre-differentiated neuron like cells representing an alternative for Parkinson disease cell therapy transplantation.

Keywords: Cell Differentiation; Dopamine Producing Cells; Neuro-Differentiation; Parkinson’s Disease; Stem Cells

1. INTRODUCTION

Parkinson disease is a major neurodegenerative disease of the nervous system. It is characterized by motor disability as a consequence of nigral neuronal death [1]. Pharmacological correction of the brain dopamine deficit constitutes the first line of treatment for affected patients. Unfortunately, this therapy does not stop the progression of the disease. Other treatment options include deep surgical brain stimulation [2]. Cell therapy has been proposed as an alternative for treatment of this neurodegenerative disorder due to the fact that significant clinical signs are caused by specific and selective loss of nigrostriatal dopaminergic neurons. The possibility of replacing those cells has been explored through the transplantation of embryonic stem cells (ESCs), fetal stem cells, induced pluripotential stem cells (iPSCs), and mesenchymal stem cells (MSCs) in animal models. Of these, ESCs proved to be capable to differentiate into dopaminergic neurons, after transplantation into the striatum in Parkinson disease rat models [3-6]. Human clinical trials reported variable responses; in patients implanted with ESCs into the putamen, significant clinical improvements were observed based on standardized tests for Parkinson disease [7]. Improvements have been assessed in human subjects by either evaluations in their motor function, or by the reduction of daily L-dopa dosing [8-9]. However, this approach has not been widely accepted. Besides ethical concerns, a large amount of tissue is needed for each patient, because only 3% - 20% of transplanted cells survive [10-12].

Reprogramming somatic cells to pluripotency gives the opportunity to obtain iPSCs as a viable alternative for cell therapy in patients with Parkinson disease [13-15]. Although important results have been obtained with ESCs, fetal stem cells and iPSCs, these cells present ethical and genetic problems that need to be fully addressed before they could be considered for clinical application.

On the other hand, MSCs do not present any of those ethical concerns. To prove its possible efficacy as an alternative treatment for Parkinson disease, several studies have been performed in an animal model. Results showed that grafted undifferentiated human MSCs exerted neuroprotective effects against nigrostriatal degener-
eration induced by 6-hydroxydopaminehydrobromide (6-OHDA), but did not transform into functional dopaminergic neurons [16,17]. Although MSCs do not present ethical concern, it has been reported that prolonged in vitro culture could increased genetic and epigenetic abnormalities, therefore, it is important to consider their use on the first passages [18]. The general aim of this study was to develop a protocol to induce rat bone marrow MSCs conversion into immature neural dopaminergic-like cells in short time periods.

2. METHODS

2.1. Ethics Statement

Animal studies were performed after approved by the Animal Ethics Committee from the Medical School at the “Instituto Tecnológico y de Estudios Superiores de Monterrey” (ITESM), Reg. 2009-Re-001. All surgeries performed in the animals were under anesthesia and all efforts were made to minimize animal suffering. Animals were housed five per cage at 20°C - 22°C on a 12/12 h light/dark cycle, with food and water ad libitum.

2.2. Mesenchymal Cell Isolation and Culture

MSCs were obtained from adult male Wistar rats, weighing ~220 g. Hank’s balanced salt solution (Gibco, Grand Island, NY) was used to extrude the bone marrow from femurs and the suspension was filtered using a 70 μm pore size cell strainer (BD Falcon, Bedford, MA). After centrifugation, cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM-F12; Gibco) containing 20% fetal bovine serum (FBS; Gibco) and 1% antibiotics (streptomycin-penicillin) (Gibco). Cells were seeded in 100 mm culture dishes (Corning Inc.) for immunocytochemistry and cells were cultured at a density of 1 × 10^5 cells/well for 2 or 24 h. Total RNA was isolated from undifferentiated mesenchymal cells and cells incubated in induction media using a binding silica column kit (GeneElute Mammalian Total RNA; Sigma-Aldrich). The amount and quality of RNA was determined using a GeneQuant pro spectrophotometer (Amersham Biosciences, Cambridge, UK). RT-PCR was performed using a PX2 Thermo thermal cycler (Thermo Fisher Scientific), one-step reactions (Qiagen, Crawley, UK). All primers were obtained from MWG-Biotech, Huntsville, AL, USA, and are described on Table 1.

2.3. Control Medium (CM)

Before induction, cells on their fourth passage were placed in DMEM-F12 containing 5% FBS and 1% antibiotics for 24 h, which was used as the CM. Cells were then washed with PBS (pH 7.4) and the culture medium was replaced with DMEM-F12 containing 10% FBS and 1% antibiotics.

2.4. Induction Medium (IM)

IM consisted of CM plus 0.1 μM RA (Sigma-Aldrich, St. Louis, MO), 1 mM β-mercaptoethanol (Sigma-Aldrich), 2 mM glutamine (Invitrogen, Grand Island, NY), 10 ng/mL fibroblast growth factor 8 (FGF8) (Sigma-Aldrich), 0.2% dimethyl sulfoxide (Sigma-Aldrich), 40 ng/mL of epithelial growth factor (Invitrogen), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 5 μg/mL insulin-transferrin-selenium (Gibco), 2 μg/mL heparin (Pisa, Guadalajara, Jal, Mexico), 10 ng/mL brain-derived neurotrophic factor (Invitrogen), 4.5 mg/mL glucose (Sigma-Aldrich) and 10 ng/mL of sonic hedgehog (SHH) protein (R&D Systems, Minneapolis, MN).

2.5. Immunocytochemistry

Circular glass slides treated with poly-L-lysine (Sigma-Aldrich) were placed in 24-well microplates (Corning Inc.) for immunocytochemistry and cells were cultured at a density of 1 × 10^5 cells/well for 2 or 24 h. Cells were fixed in 4% paraformaldehyde for 10 min and were washed three times with PBS (pH 7.4). Cells were permeabilized using 0.3% Triton X-100 in PBS for 5 min. Non-specific antibody reactions were blocked with 5% BSA in PBS for 1 h. Next, cells were incubated overnight at 4°C with primary mouse monoclonal antibodies diluted in PBS containing 1% BSA. The used monoclonal mouse antibodies and dilutions were anti-nestin (10 μg/mL; R&D Systems), anti-glial fibrillary acid protein (GFAP) (5 μg/mL; Chemicon International Inc., Billerica, MA), anti-conjugated dopamine (1:5000; Se-rotec, Kidlington, Oxford, UK) and anti-β-tubulin III (1:2000; Promega, Madison, WI). Cells were washed three times with PBS and incubated with secondary goat anti-mouse Fc-fluorescein isothiocyanate (1:400; Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL) for 2 h in the dark. Cells were incubated for 1 min in propidium iodide (0.01 mg/mL; Fluka, Toluca, Mexico) to counterstain the nuclei. Cells were analyzed using a fluorescence microscope (Imager Z1; Zeiss, New York, NY) coupled to the microscope.
Table 1. Primers used for RT-PCR gene detection.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>GGTGAAAGGTCGCGTGTA</td>
<td>CATGAGCCCTCTCCACGA</td>
</tr>
<tr>
<td>En1</td>
<td>TCATAACTGACTGCCAGCA</td>
<td>ACTCCCCTTGAGTTCTGCA</td>
</tr>
<tr>
<td>Pax3</td>
<td>GCCTCAGACCGACTATGCTC</td>
<td>CCACGGCTAATTTGTCCAT</td>
</tr>
<tr>
<td>Thy1.1</td>
<td>CAGGGCTGCTGCTCTTCTAC</td>
<td>GGGCTGAGTACGTCATT</td>
</tr>
<tr>
<td>AADC</td>
<td>GGATTCAGGGCTTATCACTAC</td>
<td>TTCATTCACTTTGTTGGAACCTCTT</td>
</tr>
<tr>
<td>Aldh1</td>
<td>TGTTAGCTGATGCCGACTTG</td>
<td>TTCTAGCCGCTCAACCT</td>
</tr>
<tr>
<td>GEF10</td>
<td>CAGGGCTGCTGCTCTTCTAC</td>
<td>GGGCTGAGTACGTCATT</td>
</tr>
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The \textit{GAPDH} gene served as an internal control. RT-PCR reactions were performed in a final volume of 50 μL using 1 μg of total RNA, according to the Qiagen One-Step RT-PCR protocol. Reverse transcription was performed at 50°C for 30 min. PCR conditions were: activation at 95°C for 1 min, 35 cycles of amplification (1 min at 94°C, 1 min at 60°C (54.6°C for GAPDH) and 1 min at 72°C) and 10 min at 72°C for final extension. PCR reactions were resolved on 2% agarose gels. The bands were observed under ultraviolet (UV) light and photographed in a UVP High Performance UV Transilluminator (DigiDocIt, Cambridge, UK).

3. RESULTS

3.1. Immunocytochemical Detection of Neural Proteins

Immunohistochemistry was performed to determine whether an association existed between the morphological changes observed in cells, and the expression of proteins related to neural lineages. Fluorescence microscopy analysis showed a slight positive nestin signal around the nuclei of MSCs cultured in CM (Figure 1(A)). Some MSCs incubated with IM showed a significant increase in nestin after 2 h of induction (Figure 1(B)). After 24 h of incubation in IM, most of the MSCs showed positive expression for nestin in the cytoplasm (Figure 1(C)). Immunocytochemistry for β-tubulin III showed a similar distribution: some of the MSCs incubated in CM showed a thin fibrous pattern surrounding the nucleus (Figure 1(D)). After 2 h in IM, most of the MSCs became positive (Figure 1(E)) and those incubated in IM for 24 h showed a marked increase in the fibrous pattern throughout the cytoplasm (Figure 1(F)). Immunohistochemistry for GFAP showed an abundant expression of the protein, even in MSCs incubated in CM (Figure 1(G)). After 2 h, this staining became more abundant (Figure 1(H)) and after 24 h of incubation in IM, abundant GFAP distribution throughout the cytoplasm was observed, allowing us to detect dendritic extensions on the cell surface as well as cell interconnections (Figure 1(I)). Dopamine detection was negative on MSCs cultured in CM as well as in MSCs incubated in IM for 2 h; however, after 24 h, the neurotransmitter was detected inside small cytoplasm vesicles (Figure 2).

3.2. Expression of Neural Genes

We evaluated the expression of genes for the following neural markers: \textit{Pax3}, \textit{En1}, \textit{GEF10} and \textit{Aldh1}. Results showed positive expression for \textit{Pax3} and \textit{En1} in cells cultured in CM, as well as in IM, without apparent increases in the expression of those genes as a result of incubation in either medium. \textit{GEF10} gene expression was not detected from cells cultured in CM or IM after 2 h; however, after culture in IM for 24 h, expression was detected. Expression of the gene for \textit{Aldh1} was not detected with any medium on cells. The cells were also ana-
Figure 2. Immunofluorescence detection of dopamine in MSCs. (A) MSCs after 2 h of culture in induction medium. No dopamine was detected. Propidium iodide was used to counterstain nuclei (orange); (B) Representative images showing the presence of conjugated dopamine (green) in MSCs cultured in experimental media to induce neurodifferentiation; dopamine immunostaining appears as small vesicles (arrows) in the cytoplasm. Scale bar = 20 μm.

lyzed for AADC and Thy1.1 gene expression to determine whether they belonged to a dopaminergic lineage. Gel analysis showed positive expression levels for Thy1.1 in cells incubated in IM for 24 h. AADC gene expression was negative for all media (Figure 3).

4. DISCUSSION

Previous studies with adult hMSCs have demonstrated that these cells express neural genes at low, nonfunctional basal levels, suggesting that they have a neural predisposition [19]. Several culture media have been proposed to induce MSCs into neuron-like cells. Those media have been supplemented with chemicals and biological growth factors, such as DMSO, SHH, FGF8 and RA. Media containing chemicals such as DMSO have raised some questions about the effective differentiation observed in terms of cell morphology. Authors claim that these morphological changes could be artifacts induced by the direct effect of this compound on the cytoskeleton [20]. Regardless of this presumption, it has been demonstrated that such changes in the cytoskeleton can stimulate the expression of neural genes [21-24]. Nevertheless, in order to avoid these possible toxic and artificial effects, DMSO concentrations included in the IM were reduced by one order of magnitude from that previously reported to induce morphological changes [20] in the present study. The IM used in our protocol also contained SHH and FGF8. It has been shown that during mammalian embryogenesis, an interaction between SHH and FGF8 induces the development of the most important dopamine-synthesizing areas: the substantia nigra and the ventral tegmental area (VTA) [25,26]. SHH signaling also plays a role in axonal projection of dopaminergic neurons from the substantia nigra and the VTA to rostral target tissue, which plays an essential role in mammalian motor neurons [27]. Another factor added to our IM was BDNF, which plays a role in phenotypic maturation and increases the survival of dopaminergic neurons in the developing substantia nigra [28,29]. This evidence infers that the presence of BDNF, SHH, and FGF8 in IM may have enhanced the expression of dopaminergic neural markers.

After 2 h of incubation in IM, there were some morphological changes and an increased concentration of neural markers such as GFAP, β-tubulin III, and nestin. After 24 h of cultivation with IM, not only those proteins showed a notable concentration increase but the expression of the genes encoding TH, GEF10 and Pax3 were also detected. We looked for the presence of these proteins for their known participation during neuron differentiation.

Pax3 plays an important role in pattern formation in the vertebrate central nervous system and in the morphological differentiation of neural cells [30,31], as well as in the production of tyrosine hydroxylase. Tyrosine hydroxylase is the rate-limiting enzyme for dopamine synthesis in mature dopaminergic neurons [32]. GEF10 belongs to the guanine nucleotide exchange factor family and plays a role in the connectivity and morphogenesis of neural cells, including axonal growth, dendrite elaboration, plasticity, and synapse formation [33,34]. En1 maintains the survival of mature midbrain dopaminergic neurons in a terminal differentiation state during neurogenesis [35]. In this study, cells were positive for the intermediate filament, nestin, which is expressed during the early developmental stage by neural progenitor cells and downregulated upon differentiation [30,35]. This
could reflect an ongoing stage of maturity of cells cultured in IM for 24 h.

The presence of all of those markers indicates an immature neuronal state, which is desirable for transplantation programs, for it has been reported that immature cells could be a better therapeutic option than mature cells in allowing engraftment [36,37]. It is noteworthy that cells cultured for 24 h in IM showed conjugated dopamine distributed in smaller cytoplasmic inclusions detected by immunocytochemistry and this was consistent with an elevated expression of Thy-1.1, according to our RT-PCR analysis. However, further research using specific markers of dopamine vesicles such as vesicular monoamine transporter 2 is required to verify whether the visualized deposits are presynaptic vesicles.

More detailed experiments are required to elucidate the pathway by which dopamine is synthesized in induced MSCs.

Aldh1 is an enzyme related to RA synthesis and is a marker of midbrain dopaminergic neurons in the late stages of fetal development and adulthood [36]. This marker was not detected in MSCs or in pre-differentiated MSCs, suggesting that these MSC-derived dopaminergic neurons did not exhibit a complete midbrain identity. However, neurogenesis in adult progenitors cannot be fully compared with developmental neurogenesis for the former is solely under environmental control whereas the latter is coordinated by morphogenetic signaling molecules produced by localized signaling centers [25].

Methods that combine chemicals with neurotrophic factors have been reported to result in the expression of neural markers, such as Tuj-1, GFAP, NSE, nestin, and NF [38]. However, these treatments require long culture times (3-14 d) or multistep differentiation protocols. In the present study, cells incubated in our medium allowed the preparation of the manuscript. No additional external funding was received for this study. The authors express their appreciation to Rosa Maria de la Rosa for technical assistance.

REFERENCES


