EXPERIMENTAL CELL RESEARCH 317 (2011) 1649–1662

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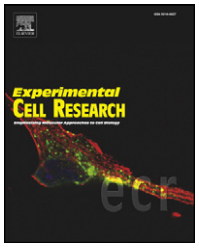

Research Article

Enhanced proliferation and dopaminergic differentiation of ventral mesencephalic precursor cells by synergistic effect of FGF2 and reduced oxygen tension

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ARTICLE INFORMATION

Article Chronology:
Received 13 August 2010
Revised version received 19 April 2011
Accepted 26 April 2011
Available online 3 May 2011

Keywords:
Dopamine
Tyrosine hydroxylase
Progenitor cell
FGF8
Parkinson's disease

ABSTRACT

Effective numerical expansion of dopaminergic precursors might overcome the limited availability of transplantable cells in replacement strategies for Parkinson's disease. Here we investigated the effect of fibroblast growth factor-2 (FGF2) and FGF8 on expansion and dopaminergic differentiation of rat embryonic ventral mesencephalic neuroblasts cultured at high (20%) and low (3%) oxygen tension.

More cells incorporated bromodeoxyuridine in cultures expanded at low as compared to high oxygen tension, and after 6 days of differentiation there were significantly more neuronal cells in low than in high oxygen cultures. Low oxygen during FGF2-mediated expansion resulted also in a significant increase in tyrosine hydroxylase-immunoreactive (TH-ir) dopaminergic neurons as compared to high oxygen tension, but no corresponding effect was observed for dopamine release into the culture medium. However, switching FGF2-expanded cultures from low to high oxygen tension during the last two days of differentiation significantly enhanced dopamine release and intracellular dopamine levels as compared to all other treatment groups. In addition, the short-term exposure to high oxygen enhanced *in situ* assessed TH enzyme activity, which may explain the elevated dopamine levels.

Our findings demonstrate that modulation of oxygen tension is a recognizable factor for *in vitro* expansion and dopaminergic differentiation of rat embryonic midbrain precursor cells.

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Abbreviations: BrdU, 5-Bromo-2-deoxyuridine; DA, Dopamine; DAB, 3.3′-diaminobenzidine; DAPI, 4′,6-diamidino-2 phenylindole; DIV, Days *in vitro*; ED, Embryonic day; FBS, Fetal bovine serum; FGF2, Fibroblast growth factor 2; FGF8, Fibroblast growth factor 8; HPLC, High performance liquid chromatography; HRP, Horseradish peroxidase; L-DOPA, 3,4-dihydroxyphenylalanine; MAP2, microtubule-associated protein; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NSC, Neural stem cell; PD, Parkinson's disease; PFA, Paraformaldehyde; SEM, Standard error of mean; TH, Tyrosine hydroxylase; VM, Ventral mesencephalic

0014-4827/$ – see front matter
doi:10.1016/j.yexcr.2011.04.012

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder mainly characterized by a progressive loss of dopaminergic neurons in substantia nigra pars compacta and subsequent depletion of striatal dopamine (DA).

Intrastriatal transplantation of fetal human ventral mesencephalic (VM) dopaminergic neurons has been used successfully as an experimental therapy for patients with severe PD. With sufficient numbers of dopaminergic neurons in the grafts, significant long-term functional improvements have been achieved in several patients [1–4]. However, a more widespread clinical application is hampered by ethical concerns, suboptimal survival and functional integration of grafted dopaminergic neurons, development of postgrafting dyskinesias in some patients, and the logistics related to the collection, pretreatment and storage of the donor tissue [5–7].

Multipotent neural stem cells (NSCs) constitute a potential alternative source of cells for transplantation in neurodegenerative diseases. NSCs can be isolated and propagated both from the developing brain [8] and certain areas of the adult brain [9–11]. It has, however, proven difficult to control their differentiation into dopaminergic neurons. Dopaminergic precursor cells or phenotypically committed neuroblasts from the developing VM represent an alternative to NSCs, given that effective methods for cell isolation and propagation can be established [12–15].

Studer et al. [16] have thus shown that embryonic day (ED) 12 rat VM cells can maintain their dopaminergic characteristics after short-term expansion in fibroblast growth factor-2 (FGF2) and epidermal growth factor (EGF) and display significant functional effects after grafting into a rat model of PD [16]. However, further validation and optimization of such *in vitro* expansion protocols as well as the subsequent postgrafting cell survival and functional integration are needed.

Oxygen levels have important effects on cell proliferation, differentiation and survival. Almost all cells, including those of the CNS, can sense and respond to changes in oxygen tension. Fine-tuning of oxygenation is of particular interest for cell viability and function, whereas both hyperoxia and hypoxia are able to trigger cell death programs [17]. The supply of oxygen to the brain depends on arterial oxygen tension as well as the blood flow and furthermore, the tissue oxygen concentration depends on cellular oxygen consumption rates. In the developing and adult brain, oxygen levels range from approximately 1–5% with variations within different brain regions [18]. Despite of this, most CNS precursor cells are cultured at non-physiologically high, atmospheric oxygen tension. Beneficial effects of low, physiological oxygen tensions on CNS precursor cells *in vitro* from both rat [19], mouse [20] and human [21,22] have been shown. Exposure to low oxygen levels during both propagation and differentiation has moreover been found to enhance the dopaminergic differentiation [19,20,22–24].

We have previously shown that both FGF2 and FGF8 stimulate proliferation of ED 12 rat VM precursor cells *in vitro*[25] and that these cells survive intrastriatal transplantation and induce functional recovery in aged hemiparkinsonian rats, with FGF2-expanded cells providing the best functional outcome [26]. Here we show that the combination of FGF2 treatment at low oxygen tension and short-term exposure to high oxygen tension during differentiation provides the most effective numerical expansion of fetal mesencephalic dopaminergic neurons. Furthermore, this short-term exposure to high oxygen tension is shown to increase the DA synthesis per dopaminergic neuron by increasing TH enzyme activity.

Materials and methods

Preparation, expansion and differentiation of ventral mesencephalic (VM) precursor cells

All animal experiments were carried out according to local and national animal ethics regulations. Rat embryonic day 12 VM precursor cells were prepared as previously described [25]. Cells were seeded in 24-well plates at a density of 1/5 VM per well (approximately 35,000 cells/well). The cultures were randomly divided into the experimental groups and treated with 20 ng/ml FGF2 or 20 ng/ml FGF8 (both R&D Systems). Untreated cultures served as controls. Cells were expanded for 4 or 8 days *in vitro* (DIV).

In order to label dividing cells, selected cultures, expanded for 4 or 8 DIV, were exposed to 30 μg/ml bromodeoxyuridine (BrdU; Sigma) for 6 hours (h) just prior to fixation. Some cell cultures grown on glass coverslips were also exposed to BrdU for 6 h at 2 DIV or 4 DIV followed by differentiation for 6 DIV (as described below). Cultures exposed to BrdU at 2 DIV were followed by expansion for 2 DIV before differentiation for 6 DIV.

At DIV 4 and 8, cell differentiation was induced by withdrawal of FGFs and addition of 0.5% (v/v) fetal bovine serum (FBS; Gibco). Half of the medium was replaced by fresh medium every second day until fixation and histological processing.

At DIV 10 or 14, cells were fixed for 20 min at room temperature in 4% paraformaldehyde (PFA; Fluka, Switzerland) in 0.15 M phosphate buffer, pH 7.4 (containing KH_2PO_4 and Na_2HPO_4, $2H_2O$). Before fixation conditioned culture medium was collected for HPLC analysis of dopamine (DA) and lactate dehydrogenase (LDH) measurements (see later).

Oxygen tensions treatment protocols

Cultures were grown in an incubator at 36 °C with 5% CO_2 and 95% air (20% O_2) or 5% CO_2, 92% N_2, and 3% O_2 monitored by an O_2-sensitive alarm system (Forma Scientific Inc., OH, USA). Some cell cultures were both expanded and differentiated at low (3%) oxygen tension (Low–Low group) or high (20%) oxygen tension (High–High group), while others were either expanded at low oxygen followed by differentiation at high (20%) oxygen tension (Low–High group) or *vice versa* (High–Low group).

Immunocytochemistry

Fixed cells were rinsed with 0.05 M Tris-buffered saline, pH 7.4 (TBS) containing 0.1% Triton-X-100 (Sigma), pre-incubated with TBS containing 10% donkey or sheep serum (Gibco) according to the host of the secondary antibodies, and incubated ON at 4 °C with primary antibodies diluted in TBS/10% donkey or sheep serum. Antibodies were used in the following concentrations: rabbit anti-tyrosine hydroxylase (TH) antibody at 1:1200 (Chemicon), mouse anti β-III-tubulin antibody at 1:2000 (Sigma), mouse anti-microtubule-associated protein ab (MAP2ab) at 1:2000 (Sigma), mouse