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Highly efficient generation of glutamatergic/cholinergic NT2-derived postmitotic human neurons by short-term treatment with the nucleoside analogue cytosine β-D-arabinofuranoside

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ABSTRACT

The human NTERA2/D1 (NT2) cells generate postmitotic neurons (NT2N cells) upon retinoic acid (RA) treatment and are functionally integrated in the host tissue following grafting into the rodent and human brain, thus representing a promising source for neuronal replacement therapy. Yet the major limitations of this model are the lengthy differentiation procedure and its low efficiency, although recent studies suggest that the differentiation process can be shortened to less than 1 week using nucleoside analogues. To explore whether short-term exposure of NT2 cells to the nucleoside analogue cytosine β-D-arabinofuranoside (AraC) could be a suitable method to efficiently generate mature neurons, we conducted a neurochemical and morphometric characterization of AraC-differentiated NT2N (AraC/NT2N) neurons and improved the differentiation efficiency by modifying the cell culture schedule. Moreover, we analyzed the neurotransmitter phenotypes of AraC/NT2N neurons. Cultures obtained by treatment with AraC were highly enriched in postmitotic neurons and essentially composed of dual glutamatergic/cholinergic neurons, which contrasts with the preferential GABAergic phenotype that we found after RA differentiation.

Taken together, our results further reinforce the notion NT2 cells are a versatile source of neuronal phenotypes and provide a new encouraging platform for studying mechanisms of neuronal differentiation and for exploring neuronal replacement strategies.

1. Introduction

The pluripotent NTERA2/D1 (NT2) cell line, derived from human testicular embryonic carcinoma cells (Andrews, 1984), represents a committed neuronal precursor cell that can be induced to differentiate *in vitro* into postmitotic cells, known as NT2N (or hNT) neurons. Moreover, NT2N cells have the ability to establish functional synapses and to develop neuronal electrophysiological properties, including generation of action potentials and the presence of spontaneous excitatory and inhibitory postsynaptic currents (Hartley et al., 1999a; Podrygajlo et al., 2010; Öz et al., 2013). Consequently, the NT2N cell type is now widely accepted as a suitable model for the study of neuronal networks and differentiation and synaptogenesis processes.

In addition, the NT2 clonal line represents a promising cell source for neuronal replacement therapy applicable to the treatment of some neurological conditions, including neurodegenerative disorders such as Parkinson's, Huntington's, and Alzheimer's diseases and acute brain injury such as stroke and head trauma. To this end, grafts or cells in suspension from embryonic ventral mesencephalic tissue, adrenal medullary cells, other neuronal and non-neuronal cells, and even genetically modified cells have produced encouraging results, but none of them are fully satisfactory (Martínez-Morales et al., 2013). As a consequence, researchers have focused their efforts on either trying to increase the efficacy of existing tissue and cell candidates or searching for alternative sources of donor cells. In this sense, NT2N neurons maintain their neuronal phenotype after transplantation into the rodent brain (Trojanowski et al., 1993; Kleppner et al., 1995; Baker and

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Mendez, 2005) and spinal cord (Hartley et al., 1999b; Lee et al., 2000). Furthermore, NT2N grafts have been shown to improve motor deficits in animal models of stroke (Borlongan et al., 1998a,b; Saporta et al., 1999) and Huntington's disease (Hurlbert et al., 1999), making NT2N neurons excellent candidates for cell replacement therapy in neurological disorders (Trojanowski et al., 1997; Lee et al., 2000; Nelson et al., 2002; Borlongan et al., 2006; Hara et al., 2008). Indeed, in a phase I clinical trial on 12 patients with basal ganglia stroke and stable motor deficits, NT2N cells were seen to integrate with the host brain tissue for long periods and with no detectable tumor formation (Kondziolka et al., 2000; Meltzer et al., 2001; Nelson et al., 2002), leading to a Phase II study in 18 patients with basal ganglia infarct (Kondziolka et al., 2005). Although statistical significance could not be reached due to the small population size analyzed, both studies showed an apparent trend towards functional improvement (Banerjee et al., 2010, for review).

Progress in the field of neuronal replacement using NT2 committed to differentiate with retinoic acid (RA) is limited by the long time frame necessary to generate postmitotic NT2N neurons and also by the low proportion of terminally differentiated neurons. Thus, neuronal differentiation of NT2 progenitors, first described by Andrews (1984) and later on improved by Pleasure et al. (1992), involves treatment of NT2 progenitors with RA during 4 weeks followed by mitotic inhibitors for 7–10 days, with only about 5% of the initial population of NT2 cells becoming differentiated. Recently, using nucleoside analogues, Musch et al. (2010) achieved successful differentiation of NT2 cells in less than 1 week, thus offering a rapid protocol to obtain postmitotic neurons, which could be potentially useful for neuronal replacement therapy. However, whereas NT2N neurons obtained by treatment with RA have been shown to express a variety of classical neurotransmitters, neurotransmitter-related enzymes and transporters (Podrygajlo et al., 2009; Coyle et al., 2011), there are no published data about neuronal phenotypes resulting from nucleoside-induced differentiation of NT2 cells.

In this study, using neuron-specific and neurotransmitter phenotype markers, high-resolution fluorescence microscopy, and western blot analysis, we demonstrate that NT2 progenitors can be committed to differentiate into NT2N neurons with high efficiency by short-term (6 days) exposure to the nucleoside analogue cytosine β-D-arabinofuranoside (AraC). AraC-differentiated neurons are irreversibly postmitotic, express late neuronal markers, are morphometrically identifiable and display a dual glutamatergic/cholinergic neurotransmitter phenotype, which differs from that observed in RA-differentiated NT2N neurons. Our data, further confirm that NT2 progenitors constitute a versatile source of neurons with a potential use in cell-replacement therapy for multiple degenerative disorders.

2. Materials and methods

2.1. Cell culture and differentiation

Human teratocarcinoma NTERA2-D1 (hereafter referred to as NT2) cells from the American Type Culture Collection (ATCC®, CRL-1973™) were maintained in complete medium (Dulbecco's Modified Eagle medium (DMEM®), ATCC 30-2002™- supplemented with 10% fetal bovine serum (FBS), Sigma-Aldrich, St Louis, MO, USA; and antibiotics, 100 U/mL penicillin and 100 μg/mL streptomycin, Gibco, Life Technologies S.A., Madrid, Spain) at 37 °C under a humidified atmosphere containing 5% CO_2. For differentiation of NT2 progenitors with all-trans retinoic acid (RA, Sigma-Aldrich), cells were treated as previously described by Pleasure et al. (1992), thus obtaining RA-differentiated NT2N cells, which will be referred to as RA/NT2N cells in this paper (see supplementary material and methods for details).

The induction of neuronal differentiation using 2′-deoxy-5-azacytidine (DAC) or cytosine β-D-arabinofuranoside (AraC) was performed based on a previous report showing that exposure of NT2 cells for only 6 days leads to neuronal differentiation (Musch et al., 2010). For preliminary assays, progenitor NT2 cells were exposed to equitoxic concentrations of DAC or AraC (Tan et al., 2007; Musch et al., 2010). Briefly, NT2 cells at 80–90% confluence were treated with 1 μM DAC or 20 μM AraC-containing medium for 24 h, 48 h, 72 h, or 6 days. Medium from cultures treated for more than 48 h was replaced with fresh complete medium supplemented with the corresponding nucleoside analogue every 2 days. Results of preliminary experiments led us to choose AraC to induce neuronal differentiation (see results below), and therefore, this compound was used for subsequent experiments. Because low cell density can compromise neuronal survival and differentiation, we tested whether increased confluence improves the yield of terminally AraC-differentiated neurons (hereafter referred to as AraC/NT2N cells). The protocol used for this purpose is described in detail in supplementary material and methods.

2.2. Western blot

Cell pellets were collected by centrifugation at different times of differentiation treatment as indicated for each experiment and lysed in homogenization buffer (10 mM Tris–HCl buffer, pH 7.4, 2 mM $MgCl_2$, and 0.32 M sucrose) containing protease inhibitors (0.5 mM iodoacetamide and 1 mM phenylmethylsulfonyl fluoride, PMSF). Depending on the antigen to be analyzed, different amounts of denatured lysates (5–30 μg) were loaded, resolved by SDS–polyacrylamide (SDS–PAGE) gels and transferred to polyvinylidene fluoride membranes (Bio-Rad, Madrid, Spain), and specific protein bands were detected using conventional immunoblot protocols. Primary and secondary antibodies are described in Table 1 and Table S1, respectively (further information about immunoblot analysis is provided as supplementary material).

2.3. Immunofluorescence

Cells were fixed in buffered 4% paraformaldehyde for 4 min, both at 20°–25 °C. After blocking for 1 h with gelatin-histology buffer (0.1 M PBS, pH 7.4, containing 0.22% gelatin (Panreac, Barcelona, Spain), 0.05% saponin (Sigma-Aldrich), and 1% serum albumin bovine (BSA, Sigma-Aldrich), cells were incubated with primary antibodies (Table 1, for details) at 4 °C overnight. Then, the appropriate fluorescent dye-conjugated secondary antibodies (Table S1 for details) were applied and nuclei were counterstained with Hoechst 33,342 (Sigma-Aldrich, diluted to 0.1 μg/mL in gelatin histology buffer) (see supplementary material and methods for further details).

2.4. BrdU labeling

Undifferentiated NT2 progenitors and AraC-treated NT2 cultures were labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU; ThermoFisher Scientific, Barcelona, Spain) at various times after initiation of AraC treatment (0, 2, 4, 8, 20 and 68 h) and fixed for immunofluorescence labeling 4 h later. Additionally, to test whether differentiated AraC/NT2N neurons were irreversibly postmitotic, after the 6-day differentiation process, AraC-containing medium was replaced with AraC-free complete medium, and cells were treated with BrdU for either 4 or 24 h and analyzed at survival times of 7, 9, and 11 days. Antigen retrieval for BrdU staining was performed as described by Solovei et al. (2006). Briefly, coverslips containing the paraformaldehyde-fixed cells were treated with PBS (0.1 M, pH 7.4) containing 0.1% (v/v) TX-100 (PBS-T), treated with HCl 0.1 M for 10 min, and then equilibrated in Saline Sodium Citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate) at room temperature (21–23 °C). Thereafter, cells were incubated in 50% formamide in SSC during 15 min at room temperature, followed by treatment