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Optimisation of culture conditions for differentiation of C17.2 neural stem cells to be used for in vitro toxicity tests

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ABSTRACT

Here we present a multipotent neuronal progenitor cell line for toxicity testing as an alternative to primary cultures of mixed cell types from brain tissue. The v-myc immortalised C17.2 cell line, originally cloned from mouse cerebellar neural stem cells, were maintained as monolayer in cell culture dishes in DMEM supplemented with fetal calf serum, horse serum and antibiotics. Different media and exposure scenarios were used to induce differentiation. The optimal condition which generated mixed cultures of neurons and astrocytes included serum-free DMEM:F12 medium with N2 supplements, brain-derived neurotrophic factor and nerve growth factor. The medium was changed every 3rd or 4th day to fresh N2 medium with supplements. After 7 days, the culture contained two different morphological cell types, assumed to be neurons and glia cells. The presence of astrocytes and neurons in the culture was confirmed by RT-PCR and Western blot analyses, indicating increased mRNA and protein levels of the specific biomarkers glial fibrillary acidic protein (GFAP) and βIII-tubulin, respectively. Concomitantly, the expression of the neural progenitor cell marker nestin was down-regulated.

1. Introduction

Neurotoxicity studies using alternative methods to animal models are usually performed on established cell lines, primary cultures or non-mammalian cell models (Aschner et al., 2011; Bal-Price et al., 2008; Costa et al., 2011; Llorens et al., 2012; Peterson et al., 2008; Smith, 2009). However, primary brain tissue cultures of mixed cell types should be the most physiological in vitro cell model for estimation of neurotoxicity. Indeed, glia cells have been shown to modulate sensitivity of neurons to chemical insult (Eskes et al., 1999; Morken et al., 2005; Zurich et al., 2004). The complexity of the brain structure and cell–cell communication is difficult to mimic with the cloned cell line approach (Forsby et al., 2009). Nevertheless, for animal welfare reasons and for simplicity of toxicity test strategies, the usage of primary cultures should be minimised. Still, complex systems must be available for toxicity tests. Here we present a multipotent neural progenitor cell line, C17.2, as an alternative to the primary brain tissue cultures. The C17.2 cell line originates from neural stem cells of the external germinal layer of mouse cerebellum, which were immortalised by v-myc transfection (Snyder et al., 1992).

All-trans retinoic acid (RA) is known to induce differentiation in embryonic stem cells (Kim et al., 2009) and in several cell lines (Pahlman et al., 1984, 1990). Previous results show that RA seems to promote astrocyte differentiation rather than neuronal development in C17.2 cells (Asano et al., 2009; Bajinskis et al., 2011). In order to obtain mixed cultures with more equal distribution of neurons and astrocytes, three types of cell culture media for the C17.2 cells were tested: (1) Dulbecco's modified essential medium (DMEM) with horse serum and fetal calf serum (HS and FCS, respectively), (2) FCS-deprived DMEM, supplemented with nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) and (3) serum-free DMEM:F12 medium with N2 supplements, NGF and BDNF. The media were either not changed during the differentiation period (autocrine-conditioned medium) or changed every 3rd to 4th day to fresh medium. The autocrine-conditioned media were either supplemented with extra NGF and BDNF every 3rd to 4th day or left without extra additions. Concomitantly with morphological studies, expression of the cell-specific biomarkers nestin (a type-IV intermediate filament identifying neural progenitor cells) (Frederiksen and McKay, 1988; Lendahl and McKay, 1990), βIII-tubulin (part of the microtubular complex identifying neurons) (Roskams et al., 1998) and glial fibrillary acidic protein (GFAP, a type-III intermediate filament identifying astrocytes) (Eng et al., 1971) were used to validate the different cell types in the cultures.

Abbreviations: BDNF, brain-derived neurotrophic factor; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HS, horse serum; NGF, nerve growth factor; RA, all-trans retinoic acid; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulphate.

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2. Methods

2.1. Cell lines and culture medium

The C17.2 cells are mouse-derived multipotent neural stem cells isolated from cerebellum, which were immortalised by avian myelocytomatosis viral-related oncogene (v-myc) transfection (Snyder et al., 1992). The cells were a generous gift from Professor Sandra Ceccatelli (Karolinska Institute, Stockholm, Sweden), with permission of Prof. Evan Snyder (Harvard Medical School, Boston, USA).

The C17.2 cells were grown in cell culture dishes (Corning Inc., Corning NY) in DMEM supplemented with 5% HS, 10% FCS, 2 mM L-glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml (all from Life Technologies, Gibco, Invitrogen), referred to as complete DMEM, in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells were detached every 3rd to 4th day using 0.05/0.02% trypsin/EDTA and reseeded in 55 cm^2 cell culture dishes at a density of 1.5×10^5 cells/dish in 10 ml complete DMEM. For differentiation experiments, cells were seeded in complete DMEM which was changed to differentiation medium one day post seeding. Three different differentiation medium compositions were used; (1) complete DMEM, (2) complete DMEM without FCS but supplemented with NGF and BDNF [10 ng/ml of each neurotrophic factor], and (3) DMEM:F12 medium with N2 supplements (Bottenstein and Sato, 1979) together with NGF and BDNF (RnD systems Inc.). Along with the three different media, three different exposure conditions were studied; conditioned medium (no change of differentiation medium for 7 days), exchange of the differentiation medium every 3rd day and conditioned differentiation medium with addition of NGF and BDNF to the media every 3rd day. The differentiation conditions are summarised in Table 1.

2.2. Morphology study

To morphologically characterise the differentiation process, 2.15×10^3 cells were seeded in a 8 cm^2 cell culture plate in complete DMEM one day prior medium change. The cells undergoing differentiation were treated for 7 days. Native neural stem cells kept in complete DMEM for 3 days were used as control cells. In addition to the nine exposure scenarios described above and in Table 1 for 7 days, the morphological differentiation process was followed in more detail at day 3, 7 and 10 by culturing the cells in DMEM:F12 medium with N2 supplements, NGF and BDNF [10 ng/ml] with a change of the medium every 3rd day.

Table 1
Cell culture conditions for differentiation of C17.2 All media were supplemented with L-glutamine, penicillin and streptomycin, see Methods.

Complete DMEM with 5% HS and 10% FCS	DMEM with 5% HS, NGF and BDNF [10 ng/ml][a]	DMEM:F12 medium with N2 supplements, NGF and BDNF [10 ng/ml][a]
(1) Control cells cultured for 3 days	(4) Conditioned medium, no medium change for 7 days	(7) Conditioned medium, no medium change for 7 days
(2) Conditioned medium, no medium change for 8 days	(5), Cells in differentiation medium for 7 days, medium change after 3 days	(8) Cells in differentiation medium for 7 days, medium change after 3 days
(3) Cells in culture for 8 days, medium change after 4 days	(6) Conditioned medium, extra addition of NGF and BDNF after 3 days	(9) Conditioned medium, extra addition of NGF and BDNF after 3 days

[a] Cells were plated in complete DMEM the day before the change to differentiation medium.

2.3. RT-PCR

For analysis with reverse transcriptase (RT)-polymerase chain reaction (PCR), 1.9×10^4 cells were seeded in an 8 cm^2 cell culture plate in complete DMEM one day prior medium was changed to the differentiation media. Cells were lysed and mRNA was isolated using the Qiagen RNeasy kit (Fermenta) after 7 days of exposure for the differentiation conditions (Table 1). Native cells kept in complete DMEM medium for three days were used as the neural stem cell control. Two μg of RNA was reversed transcribed to yield cDNA by the use of specific primers. The following primer sequences were used; nestin 5'-GGAGGGCAGAGAAGACAGTG-3' and 5'-TGAC ATCCTGGACCTTGACA-3', βIII-tubulin 5'-GAATGACCTGGTGTCCG AGT-3' and 5'-CAGAGCCAAGTGGACTCACA-3' and GFAP 5'-CAC GAACGAGTCCCTAGAGC-3' and 5'-TCACATCACCACGTCCTTGT-3' and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference; 5'-GGCATTGCTCTCAATGACAA-3' and 5'-TGTGAGGGAGATGCTCAGTG-3'. The mRNA levels of nestin, βIII-tubulin and GFAP were analysed after 22–26 PCR cycles. The PCR products were analysed on 1.5% agarose gels and visualised with ethidium bromide and UV radiation. The intensity of the bands was quantified with the Image Gauge 3.46 program (Fujifilm Co. Ltd.).

2.4. Protein levels and western blot

Based on the results from the morphological evaluation and mRNA expression, the protein expression levels after differentiation were studied. 1.9×10^4 cells were seeded in a 55 cm^2 cell culture plate in complete DMEM one day prior differentiation. Differentiation proceeded in DMEM:F12 with N2 supplements, NGF and BDNF [10 ng/ml of each neurotrophic factor] (treatment 8 in Table 1) followed by Western blot analysis. The cells were lysed in a hypotonic buffer containing NP-40. Twenty μg of total protein (determined with the DC Protein Assay, BioRad) were separated in 10% SDS- poly-acrylamide gels. The proteins were subsequently transferred to nitrocellulose membranes and hybridised with primary antibodies diluted accordingly: βIII-tubulin (ab18207) 1:5000, nestin (ab6142) 1:200 and GFAP (ab7260) 1:1000 (all from Abcam) and β-actin (sc-1616) 1:5000 (Santa Cruz). Horse radish peroxidase-conjugated anti-rabbit IgG (NA934 V) 1:3000 and anti-mouse IgG (NA931 V) 1:3000 (Amersham) and anti-goat IgG (sc-2020) 1:3000 (Santa Cruz) were used as secondary antibodies. Densitometric analysis of visual blots was performed using Image Gauge 3.46 program (Fujifilm Co. Ltd.).

3. Statistical analyses

The data were analysed using one-way ANOVA followed by Tukey's Multiple Comparison Test (Fig. 2a–c) or by Student's *t*-test (Fig. 3) (GraphPad Prism 5.0).

4. Results

4.1. Morphology of differentiated C17.2 cells

Cells grown in complete DMEM for 3 days (treatment 1 in Table 1) remained their native, neural stem cell state. Only one morphological phenotype with no visual outgrowth of neurites was observed in the cultures (Fig. 1a). For cells grown in conditioned complete DMEM for 8 days (treatment 2 in Table 1) or with medium change after day 4 (treatment 3 in Table 1) no morphological signs of neuronal differentiation were observed (not shown). Cells cultured for 7 days in complete DMEM without FCS but with neurotrophic factors added (treatment 4–6 in Table 1) displayed