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A human pluripotent carcinoma stem cell-based model for in vitro developmental neurotoxicity testing: Effects of methylmercury, lead and aluminum evaluated by gene expression studies

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ABSTRACT

The major advantage of the neuronal cell culture models derived from human stem cells is their ability to replicate the crucial stages of neurodevelopment such as the commitment of human stem cells to the neuronal lineage and their subsequent stages of differentiation into neuronal and glial-like cell. In these studies we used mixed neuronal/glial culture derived from the NTERA-2 (NT-2) cell line, which has been established from human pluripotent testicular embryonal carcinoma cells. After characterization of the different stages of cell differentiation into neuronal- and glial-like phenotype toxicity studies were performed to evaluate whether this model would be suitable for developmental neurotoxicity studies. The cells were exposed during the differentiation process to non-cytotoxic concentrations of methylmercury chloride, lead chloride and aluminum nitrate for two weeks. The toxicity was then evaluated by measuring the mRNA levels of cell specific markers (neuronal and glial). The results obtained suggest that lead chloride and aluminum nitrate at low concentrations were toxic primarily to astrocytes and at the higher concentrations it also induced neurotoxicity. In contrast, MetHgCl was toxic for both cell types, neuronal and glial, as mRNA specific for astrocytes and neuronal markers were affected. The results obtained suggest that a neuronal mixed culture derived from human NT2 precursor cells is a suitable model for developmental neurotoxicity studies and gene expression could be used as a sensitive endpoint for initial screening of potential neurotoxic compounds.

1. Introduction

Detection of developmental neurotoxicity (DNT) induced by chemicals represents a major challenge due to the extraordinary complexity of human brain development that originates from cellular-molecular mechanisms involved in various processes such as cell proliferation, migration and cell differentiation. Moreover, neuronal differentiation is a complex process that involves commitment of neural stem cells, proliferation of neural progenitor cells, cell migration, neuronal and glial cell differentiation, synaptogenesis, cell death, development of neuronal neurotransmitters and receptors, synaptic connections, myelination and development of the blood-brain barrier. These processes occur at different developmental windows and therefore may differ in their susceptibility to environmental chemicals induced toxicity (Bal-Price et al., 2012; Grandjean and Landrigan, 2006).

In vitro models which mimic to some extent the developmental process are numerous and cover three species: mouse, rat and human (Coecke et al., 2007; Bal-Price et al., 2010). Rat cells are commonly used as cell lines or as primary cultures. One of the most extensively studied is a rat pheochromocytoma cell line that serves as a model for the evaluation of neurite outgrowth (Harrill and Mundy, 2011; Radio et al., 2008; Radio, 2012). Primary cultures of rat cortex (Berry et al., 2012) or cerebellar granule cells (Hogberg et al., 2009, 2010) are widely used in vitro models for mechanistic toxicity studies. From the mouse, there are several neuroblastoma cell lines available such as N2a cells (Amano et al., 1972) or N1E-115 cells (Richelson, 1973).

Mouse neural progenitor cells (mNPCs) isolated from embryonic or postnatal tissue of the brains of different ages are also used. Such progenitors grow as neurospheres and are especially valuable for obtaining genetically manipulated primary cells (Gassmann et al., 2010). However, all these different cell models from various species raise question about the predictivity of human toxicity.

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Numerous human models are currently available ranging from neuroblastoma lines to stem cell-derived systems (Coecke et al., 2007). Multiple human neuroblastoma cell lines such as SH-SY5Y or IMR-32 cells (Kohl et al., 1980; Reynolds and Pérez-Polo, 1975) have been applied, however, they have a disadvantage as the expression profiles in these cells contain many tumor growth genes. There are also cell lines that are not tumor-derived, but instead are immortalized cells of primary origin such as LUHMES (Scholz et al., 2011) and ReNcell CX cell lines (Donato et al., 2007; Breier et al., 2008). However, this also limits their application in predicting the effects of chemicals on neural cell proliferation, but might allow the study of other endpoints.

In the context of the 21st century approach in toxicity testing human in vitro models and batteries of assays based on the mechanisms/pathways of toxicity are recommended as they are believed to give a better prediction of human toxicity (NRC, 2007). Therefore, recently, there is an intense focus on the various sources of human stem cells such as somatic (Buzanska et al., 2009), embryonic (Fritsche et al., 2011; Schreiber et al., 2010) and induced pluripotent stem cells (iPSCs) (Pistollato et al., 2012) since these models allow us to follow up the entire process of human cell differentiation, starting from the early progenitor cells, neural committed precursor cells that can differentiate into fully mature neuron- and glial-like cells. A number of specific cell based methods have been subject to more in-depth investigations for suitability in neurodevelopmental toxicity testing. These include a neural stem cell line generated from human umbilical cord blood stem cells (Buzanska et al., 2009) and human neural progenitor cells grown as neurospheres (Gassmann et al., 2010). Recently, human embryonic stem cells derived for in vitro models were also differentiated for the purpose of DNT testing (Ylä-Outinen et al., 2010).

In these studies we used the well-established NTERA-2 (NT2) cell line, which has been derived from human pluripotent testicular embryonal carcinoma (Przyborski et al., 2004) since it is easy to use (does not require any feeder layer) and reduces ethical concerns over the use of human or animal embryos (Stewart et al., 2003). NT-2 cells can be harvested at different stages of differentiation ranging from non-differentiated stem cells, committed neural progenitors to differentiated neuronal and glial cells (Sandhu et al., 2002; Ozdener, 2007). In the presence of retinoic acid (RA) the process of neuronal differentiation is triggered and the NT-2 neural precursor cells can be differentiated into post-mitotic neurons expressing MAP2, NF200, neuronal class Tub-βIII, MAPT-Tau, synaptophysin and other proteins that can be used as markers for the various stages of cell differentiation (Pleasure et al., 1992). Under specific culture conditions neurons can form functional synapses (Hartley et al., 1999) and express a variety of synapse formation markers and neurotransmitters phenotypes (Serra et al., 2007). Consequently, the NT2 cell line has become a widely accepted model of human neuronal and glial cells, although further in depth characterization is still lacking.

The first goal of this work was to characterize the process of neuronal differentiation by studying mRNA and protein expression of neuronal (MAP2, NF-200, Tub-βIII, MAPT-Tau and synaptophysin) and astrocytes (GFAP) markers at different developmental stages. Furthermore the evaluation of neuronal differentiation was accompanied by electrical activity measurements using Microelectrode Array (MEA chips) technology to determine whether neurons have the ability to generate spontaneous activity. Measurement of the electrical activity seems to be one of the most sensitive, functional endpoints suitable for developmental neurotoxicity studies (Hogberg et al., 2011; Robinette et al., 2011).

Our second goal was to evaluate whether NT2 derived mixed neuronal/glial-like culture would be a suitable model for in vitro neurodevelopmental toxicity testing. Therefore during the process of differentiation the cells were exposed to a range of non-cytotoxic or IC10/20 concentrations of methylmercury chloride, lead chloride or aluminum nitrate (well known neurotoxicants) and the expression of earlier characterized neuronal and glial markers was studied. Furthermore, the results obtained were compared against the data published in our previous studies using rat primary neuronal culture exposed to the same chemicals (Hogberg et al., 2010) to determine whether human cells are more (or less) sensitive towards the toxicity induced by the studied neurotoxicants.

2. Material and methods

2.1. NT2 cells maintenance and differentiation

NTERA-2/cl.D1 cells were differentiated into neuronal/glial mixed population as described previously in Pleasure et al., 1992. Initially, undifferentiated NT2 cells (from ATCC) were maintained in Opti-MEM I (Gibco, Carlsbad, CA) supplemented with 5% foetal bovine serum (FBS) (HyClone, Logan, UT), 100 U/ml penicillin (P) and 100 μg/ml streptomycin (S) (Gibco) for 24 h. The differentiation process was started by culturing the cells in Dulbecco's modified Eagle's medium (DMEM)-High Glucose (HG) (Gibco) supplemented with 10% FBS, P/S, and 10 μM retinoic acid (RA, Sigma, St. Louis, MO) for 5 weeks (until 37 DIV, Fig. 7A). After this period, the resulting multi-layered blanket of cells was trypsinized and split 1:2 in standard medium (DMEM-HG with 5% FBS, P/S) supplemented with the mitosis inhibitors 1 μM cytosine arabinoside (Sigma), 10 μM fluorodeoxyuridine (Sigma), and 10 μM uridine (Sigma) for further 8 weeks (up 56 DIV) at 37 °C in a humidified atmosphere of 5% CO_2. The cells were plated in 6 well plates coated with 10 μg/ml poly-D-lysine (Sigma) and 0.26 mg/ml Matrigel at a density of 200.000 cells/cm^2 for the morphological characterization and the gene expression studies.

2.2. Exposure of NT2 cells to tested chemicals during differentiation

The culture was exposed for two weeks to methylmercury chloride, lead chloride and aluminium nitrate after removing RA from the medium (Fig. 7A). All chemicals were purchased from Sigma–Aldrich (Milan, Italy) and the stock solutions were prepared in water. Initially, the range of various concentrations of the compounds was evaluated for cell viability using the Alamar Blue (AB) (resazurin, Sigma, Milan, Italy) assay. Based on these results the concentrations of each compound were selected for further gene and protein expression experiments.

Additionally, the cells were exposed for two weeks to the non-neurotoxic drug, paracetamol as a negative control up to the concentration of 10 μM. The toxicity was evaluated by the AB assay and gene expression studies.

2.3. Assessment of cell viability using Alamar Blue assay

After exposure to the chemicals cell viability was determined using the AB (resazurine) assay (O'Brien et al., 2000). The blue coloured indicator dye resazurin is reduced into fluorescent resorufin by red-ox reactions in viable cells. Resazurin (10 μl of 100 μM stock) in Hank's Buffered Salt Solution was added directly to the 96-well plates, without removing the medium (100 μl). The plates were then incubated for 6 h at 37 °C, 5% CO_2 and the fluorescence of the resazurin metabolite, resorufin was measured at 530 nm/590 nm (excitation/emission) in a multiwell fluorometric reader (Tecan i-control). The results were expressed as a percentage of the mean value for the untreated cultures (control).

2.4. mRNA purification, reverse transcription and quantitative real-time PCR

The analysis of mRNA expression was performed in the cell samples of the control and treated cultures after lysing them using lysis buffer RLT from the RNeasy Mini Kit) (Qiagen, Milan, Italy). The total RNA extraction was performed according to the manufacturer's protocol of RNeasy Mini Kit. Any DNA contamination was removed by digestion process using an RNase-free DNase kit (Qiagen). RNA concentration and protein contamination were assessed spectrophotometrically (Biophotometer; Eppendorf, Milan, Italy).

Reverse transcription was performed as follows: 500 ng RNA was incubated with 2.5 mM PCR Nucleotide Mix (Promega, Milan Andorra, Italy) and 12.5 μg/ml random primers (Promega) for 5 min at 65 °C using a PerkinElmer Geneamp PCR system 9600. Subsequently 2 U/μl RnaseOut inhibitor (Invitrogen), 10 U/μl M-MLV virus reverse transcriptase (Promega) and the samples were incubated for 10 min at 25 °C for annealing, 60 min at 37 °C for cDNA synthesis and 15 min at 70 °C for inactivation of enzymes. An AbiPrism 7000 sequence detector system in conjunction with TaqMan® Universal PCR master Mix and TaqMan® Real-Time PCR Assay-on-Demand (Applera Italia, Monza, Italy) were used for investigating the gene expression and the house keeping gene (beta actin) according to the manufacturer's protocol. The primers used were as follows: microtubule-associated 2 (MAP2, Hs01103234_g1), glial fibrillary acidic protein (GFAP, Hs00909238_g1), neurofilament heavy polypeptide 200 kDa (NEFh, Hs00606024_m1), microtubule-associated

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