Toxicology in Vitro 29 (2015) 449–457

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Distinct gene expression responses of two anticonvulsant drugs in a novel human embryonic stem cell based neural differentiation assay protocol

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ARTICLE INFO

Article history:
Received 17 July 2014
Accepted 3 December 2014
Available online 15 December 2014

Keywords:
Human embryonic stem cells
Neural differentiation
Neurodevelopmental toxicity
Valproic acid
Carbamazepine

ABSTRACT

Hazard assessment of chemicals and pharmaceuticals is increasingly gaining from knowledge about molecular mechanisms of toxic action acquired in dedicated *in vitro* assays. We have developed an efficient human embryonic stem cell neural differentiation test (hESTn) that allows the study of the molecular interaction of compounds with the neural differentiation process. Within the 11-day differentiation protocol of the assay, embryonic stem cells lost their pluripotency, evidenced by the reduced expression of stem cell markers Pou5F1 and Nanog. Moreover, stem cells differentiated into neural cells, with morphologically visible neural structures together with increased expression of neural differentiation-related genes such as βIII-tubulin, Map2, Neurogin1, Mapt and Reelin. Valproic acid (VPA) and carbamazepine (CBZ) exposure during hESTn differentiation led to concentration-dependent reduced expression of βIII-tubulin, Neurogin1 and Reelin. In parallel VPA caused an increased gene expression of Map2 and Mapt which is possibly related to the neural protective effect of VPA. These findings illustrate the added value of gene expression analysis for detecting compound specific effects in hESTn. Our findings were in line with and could explain effects observed in animal studies. This study demonstrates the potential of this assay protocol for mechanistic analysis of specific compound-induced inhibition of human neural cell differentiation.

1. Introduction

Humans are exposed daily to chemicals that lack information concerning toxicity and their potential health hazards. For instance, under the REACH legislation for chemical safety in Europe, reproductive and developmental toxicity testing is only mandatory at high tonnage production levels. These tests are costly and require an estimated 65% of all animal use under REACH (der Jagt et al., 2004). There is a high need for efficient predictive alternative test methods, in order to inform about reproductive and developmental toxicity at lower production levels, and to reduce animal use in toxicological hazard assessment. During the last decades, much research has been performed toward the development of *in vitro* methods, which can contribute to the reduction of animal use in toxicological testing. For developmental toxicity testing several *in vitro* systems have been developed, varying from whole embryo cultures to assays based on cell lines (Spielmann, 2009).

The use of embryonic stem cells as a corollary of cell differentiation in the embryo is practical since they are relatively easy to culture, have a self-renewal capacity and can be cultured in undifferentiated state. Furthermore, they can differentiate into cell lineages originating from all three germ layers (Thomson et al., 1998), which make them suitable to study early developmental processes at the cellular level. In the embryonic stem cell test (EST), developed in 1993 by Heuer et al. (1993), Scholz et al. (1999), ES-D3 mouse embryonic stem cells differentiate into contracting cardiomyocytes. Embryotoxicants exert an inhibitory effect on the differentiation process, resulting in a concentration dependent inhibition of contracting cardiomyocyte foci formation.

Abbreviations: CBZ, carbamazepine; CM, culture medium; EST, embryonic stem cell test; FBS, Fetal bovine serum; hESC, human embryonic stem cells; hESTn, human neural embryonic stem cell test; ITS, insulin transferrin selenium; KOSR, knockout serum replacement; MEFs, mouse embryonic fibroblasts; mESC, mouse embryonic stem cells; mESTn, mouse neural embryonic stem cell test; MMC, Mitomycin C; PDL, Poly-D-Lysine; VPA, valproic acid.

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http://dx.doi.org/10.1016/j.tiv.2014.12.001
0887-2333/

More recent studies have enhanced the mechanistic readout of EST by incorporation of transcriptomics (van Dartel et al., 2010; van Dartel and Piersma, 2011) and by the establishment of differentiation culture protocols for several other differentiation routes, e.g. neural and osteoblast differentiation (de Jong et al., 2012; Theunissen et al., 2010). Compound exposure affected gene expression already after 24 h of exposure and resulted in compound- and concentration dependent responses (van Dartel et al., 2009). Gene expression analysis enhanced mechanistic insight into differentiation inhibition caused by compound exposure (van Dartel et al., 2009, 2011a,b).

The classical EST assays are based on murine embryonic stem cells, which are not completely representative for human cells. Current trends in toxicological hazard assessment move toward human based test systems (Mori and Hara, 2013; Liu et al., 2013; Buzanska et al., 2009; Krug et al., 2013) to facilitate human hazard and risk assessment. The application of established human embryonic stem cell lines can be instrumental in avoiding interspecies extrapolation and would result in reduced animal use in hazard assessment. Several human stem cell based differentiation assays have been developed, either for regeneration and replacement therapies (Iacovitti et al., 2007; Schulz et al., 2003), to study mechanisms involved in neurogenesis (Iacovitti et al., 2007; Fathi et al., 2011; Talens-Visconti et al., 2011; Ebert et al., 2013; Liu et al., 2013), or for neurodevelopmental toxicity testing (Buzanska et al., 2009; Schulz et al., 2003; Adler et al., 2008a; Colleoni et al., 2011; Hoelting et al., 2013). The assays differed widely in terms of culture method, with single- and multiple step replating approaches, and including rosette, neurosphere or embryoid body formation. Differentiation time differed between tests from a few days to several weeks, depending on the end-points assessed. In addition, different culture well coatings, additives and culture conditions have been employed. In the present study we developed a straightforward and relatively fast differentiation method in which pluripotent WA09 (H9) human embryonic stem cells differentiate into neural cells. In this method hESC differentiated through a minimal number of culture steps and few culture medium additives. Thus, a more spontaneous differentiation was achieved. Differentiation was studied using immunostaining, in which stem cells were stained with anti-SSEA4 and neural differentiation was evidenced by neuron specific anti βIII-tubulin staining. RT-PCR analysis was used to study the expression of mRNA transcripts associated with stem cell renewal and maintenance of pluripotency using Pou5F1 and Nanog. Gene expression involved in neurogenesis was studied with Neurogin1 and Reelin and neurons were evidenced by the expression of βIII-tubulin, MAP2 and MAPt.

To study the effectiveness of this model as an *in vitro* method to evaluate neurodevelopmental toxicity, the effects of valproic acid (VPA) and carbamazepine (CBZ) on neural differentiation were studied. Both VPA, an anticonvulsant and therapeutic drug for bipolar disorder (BPD) Gurvich and Klein (2002) and CBZ, an anticonvulsant drug (Kou et al., 2011), are known to cause neurodevelopmental toxicity *in vivo* (Jentink et al., 2010; Ornoy, 2006), VPA exposure during pregnancy can cause neural tube defects, spina bifida aperta, cleft palate and limb defects (Gurvich and Klein, 2002; Robert and Guibaud, 1982). CBZ can cause spina bifida, cardiovascular anomalies, cleft palate, skeletal- and brain anomalies (Ornoy and Cohen, 1996; Jones et al., 1989; Rosa, 1991). Earlier whole genome array gene expression studies in mouse EST have shown abundant gene expression responses of VPA with many thousands of genes responding, whereas CBZ, given at equipotent concentrations as to morphological cell differentiation inhibition, showed a relatively limited gene expression response (Theunissen et al., 2012a,b). These results already indicated that differential gene expression analysis may reveal compound-specific effects at concentrations showing similar morphological differentiation inhibition. In this study first the effects on cell viability were determined with a resazurin cytotoxicity assay. Subsequently, differential gene expression responses of selected genes were studied in non cytotoxic concentration ranges of VPA and CBZ.

2. Methods

2.1. Human embryonic stem cell culture

Human embryonic stem cells (hESC) (WA09-DL11, WiCell, Madison, Wisconsin) were cultured in 6-well plates (Corning, NY, Cat#3516) in hESC culture medium (CM), containing: DMEM-F12 (Gibco, Gaithersburg, MD, Cat#31330-038) supplemented with 20% Knock Out Serum Replacement (KOSR) (Gibco, Gaithersburg, MD, Cat#10828), 1 mM L-Glutamine (Gibco, Gaithersburg, Cat#25030-024), 0.5% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin (Gibco, Gaithersburg, MD, Cat#15070), 1% non-essential amino acids, (Gibco, Gaithersburg, MD, Cat#11140-035), 0.1 mM β-Mercaptoethanol (Sigma–Aldrich, Zwijndrecht, Cat#31350-01) and 0.2 μg/ml fibroblast growth factor-basic(bFGF) (Gibco, Gaithersburg, MD, Cat#13256-029). To maintain pluripotency, the hESCs were cultured on inactivated mouse embryonic fibroblasts. hESC culture medium was refreshed every day.

2.2. Mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) (ATCC, Wesel, Germany CF-1 SCRC-1040) were cultured in T175 culture flasks in MEF culture medium (MM), containing: DMEM (Gibco, Gaithersburg, MD, Cat#11960-044) supplemented with 15% Fetal bovine serum (FBS) Hyclone, Logan, UT, Cat#SH30070.03), 1% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin (Gibco, Gaithersburg, MD, Cat#15070), 1% 100 mM Sodium Pyruvate (Gibco, Gaithersburg, MD, Cat#11360-039) and 2 mM L-Glutamine (Gibco, Gaithersburg, MD, Cat#25030-024). When 90% confluent, the cells were incubated with 10 μg/ml Mitomycin C (MMC) (M0503, Sigma–Aldrich, Zwijndrecht, The Netherlands) for 3 h at 37 °C to mitotically inactivate the cells. Subsequently, the MMC solution was removed and the cells were washed with MM followed by Dulbecco's Phosphate-Buffered Salin D-PBS with Ca^{2+} and Mg^{2+} (PBS +/+) (Gibco, Gaithersburg, MD, Cat#14040-174). To detach the cells they were incubated with Trypsin-EDTA (Gibco, Gaithersburg, MD, Cat#25200-056) for 1–2 min. Trypsin was inactivated by adding MM at twice the volume of trypsin, and a single cell suspension was produced by gently pipetting the suspension up and down. After the cells were transferred to 15 ml tubes, they were centrifuged 6 min at 300 rpm at 37 °C. The cells were either stored in liquid nitrogen at $1.2 \cdot 10^6$ cells/ml per vial or directly used as feeder cells at $2 \cdot 10^5$ cells/ well. Inactivated MEFs were seeded into wells and incubated for 24 h in a humidified atmosphere (37 °C 5%CO2) to attach and were used at maximum up to two weeks after seeding.

2.3. hESC culture

hESC were routinely cultured on a layer of inactivated MEFs and passaged between 1 and 3 times per week, depending on growth speed and morphological quality. Since the cell number cannot be controlled, and hESC cells were passaged in fragments, the cell clusters transferred to new inactivated MEF coated dishes differed in size (Fig. 2A). After most of the clusters achieved sufficient size, differentiated clusters and areas were manually removed during the culture period, the optimal clusters were passaged again. Culture medium was refreshed every day. Based on the morphological