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A novel embryotoxic estimation method of VPA using ES cells differentiation system

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Abstract

Valproic acid (VPA), which has a wide range of therapeutic applications, is known as a potent teratogen that induces neural tube defects in vertebrates. Here, we have characterized the tissue-specific, embryotoxic effects of VPA on developmental processes using a novel system with differentiating mouse ES cells. Under our cultivating condition, ES cells differentiated into cardiomyocytes, although various cell types can be differentiated. VPA affected cell viability and differentiation from undifferentiated ES cells to cardiomyocytes in a dose-dependent manner. The analysis of tissue-specific markers also revealed that VPA potently inhibited mesodermal and endodermal development but promoted neuronal differentiation in a lineage-specific manner. Taking the *in vivo* teratogenicity of VPA into account, this assay system could be useful in predicting the degree of embryotoxicity of VPA. We, thus, propose that the *in vivo* embryotoxic effects of various medicines can be estimated fast and accurately using this *in vitro* cell differentiation system.

Keywords: ES cell; Valproic acid; Embryotoxicity

Valproic acid (VPA) is a short-chained fatty acid with a broad spectrum of antiepileptic activities and it is also used in migraine prophylaxis and the treatment of bipolar disorders, neuropathic pain [1,2]. It is also being tried as an anticancer agent. VPA, which has a wide range of therapeutic applications, is also a potent teratogen and is associated with elevated risks for neural, craniofacial, cardiovascular, and skeletal birth defects [3,4]. The predominant VPA-induced teratogenic effects are due to failure of the neural tube to close (neural tube defects, NTDs), leading to conditions such as spina-bifida-aperta, anencephaly, and exencephaly in humans, mice, and other vertebrates [3,5–7].

In this study, we attempted to characterize the tissue-specific embryotoxicity of VPA using a system with mouse embryonic stem (ES) cell differentiation. This *in vitro* embryotoxicity assay requires a simple procedure that can be accomplished in a shorter time (10 days) [8] than that required by other assays used in developmental toxicological studies with experimental animals. Since ES cells are undifferentiated pluripotent embryo-derived stem cell lines and are capable to develop into differentiated cell types representing endodermal, ectodermal, and mesodermal lineages, ES cells lines are very suitable to analyze the mutagenic, cytotoxic, and embryotoxic effects of chemical compounds *in vitro* [9]. By analyzing the expression of tissue-specific genes and conducting histological and immunocytochemical studies, we have demonstrated that VPA inhibits the differentiation of mesodermal and endodermal lineages. On the other hand, VPA can induce neural differentiation in a lineage-specific manner. It is conceivable that these abnormalities of tissue developments in ES cells reflect the embryotoxic effects of VPA *in vivo*. Using this *in vitro* embryotoxicity estimation system, it would be possible to predict the effects of chemicals on developmental processes *in vivo* in a short time.

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Materials and methods

Materials. The following antibodies were purchased: anti-troponin I from Chemicon (Temecula, CA), anti-albumin from Bethyl Laboratory (Montgomery, TX), and anti-neurofilament 200 from Sigma–Aldrich (St. Louis, MO).

ES cell culture. Mouse ES cells (R1) were maintained and used for differentiation as previously described [10]. ES cells were grown on 0.1% gelatin-coated tissue culture dishes in a standard ES-cell culture medium-containing D-MEM supplemented with 10% FCS, 2 mM glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 1000 U/ml LIF (Chemicon, Temecula, CA), 50 U/ml penicillin G, and 50 μg/ml streptomycin. For the differentiation of R1 ES cells, we followed the method of the embryonic stem cell test (EST) [8]. In brief, ES cells were suspended in an ES-differentiation medium-containing D-MEM supplemented with 20% FCS, 2 mM glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 50 U/ml penicillin G, and 50 μg/ml streptomycin and cultivated in hanging drops ($n = 500$) as aggregates (called embryoid bodies (EBs)) for 3 days [10]. The EBs were transferred to suspension culture dishes (Sumitomo Bakelite, Tokyo, Japan) and cultured for 2 days. They ($n = 1$/well) were plated onto a 24-well tissue culture plate on day 5 and incubated for 5 additional days. To estimate the differentiating efficiencies from ES cells into cardiomyocytes, the distinctive beating movements of differentiated cardiomyocytes were analyzed under an inverted phase-contrast microscope (Nikon, Tokyo, Japan).

Cytotoxicity assay. For the cytotoxicity assay, we followed the method of the embryonic stem cell test (EST) [8]. ES cells and NIH-3T3 fibroblasts (1×10^4 cells/ml) were seeded in a volume of 50 μl/well into a 96-well flat-bottomed tissue culture microtiter plate and incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 2 h. After incubation, 150 μl of a culture medium-containing the appropriate dilution of test chemicals was added. On day 3 and day 5, the culture medium was removed, and, subsequently, 200 μl of the same concentration of the test substance used on day 1 was added to the microtiter plates. On day 10, the methylthiazolyldiphenyl-tetrazolium bromide (MTT) cytotoxicity assay [11] was carried out [8]. A volume of 20 μl of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, MTT (Sigma–Aldrich, St. Louis, MO), in PBS was added into each microtiter well and incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 2 h. After removal of the supernatant, cells were incubated with 130 μl of a desorb-mix solution-containing 3.49% (v/v) of a 20% SDS aqueous solution and 96.51% (v/v) 2-propanol. After agitation for 15 min on an N-704 microtiter plate shaker (Nissin, Tokyo, Japan), the plates were transferred to a Wallac 1420 multilabel counter (Perkin-Elmer, Wellesley, MA), and the absorbance of each well at a wavelength of 520 nm and a reference wavelength of 630 nm was examined. Representative results from three separate experiments are shown in the figure. For morphological observations, samples on day 5 were examined using a Hoffman differential interference contrast microscope.

Immunocytochemistry. On day 3 of the differentiation assay, several EBs were cultured on 0.1% gelatin-coated glass-based 60 mm dishes. After changing the media on day 5, the cells of day 10 were fixed with 4% paraformaldehyde for 20 min at room temperature. After rinsing with PBS twice, samples were pretreated with PBS-containing 0.2% Triton X-100 in PBS for 30 s. After washing with PBS twice, the samples were incubated with PBS-containing 0.1 M glycine, pH 3.5, for 30 min, rinsed with PBS twice, and blocked in PBS-containing 1% BSA, 0.1% gelatin, and 0.1% Tween 20 for 1 h at room temperature. They were incubated at 4 °C overnight with the following antibodies: anti-troponin I (mouse IgG, 1:500), anti-albumin (goat IgG, 1:500), and anti-neurofilament 200 (rabbit IgG, 1:500). After washing with PBS three times, the cells were incubated for 1 h at room temperature with secondary antibodies conjugated with Alexa 488 (Invitrogen, Carlsbad, CA). After washing with PBS three times, the samples were mounted with the Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and examined with an FV500 confocal laser scanning microscope (Olympus, Tokyo, Japan). To estimate the differentiating efficiencies from ES cells into neuronal cells, NF-positive cells (neurons) were counted against DAPI-positive cells (~200 cells) under an inverted phase-contrast microscope (Nikon, Tokyo, Japan). Representative results from three separate experiments are shown in the figure.

RNA isolation, cDNA synthesis, and RT-PCR. Total RNA was extracted from samples on day 10 of the differentiation assay using the RNA extraction kit ISOGEN (Nippon Gene, Tokyo, Japan) and digested with deoxyribonuclease (RT Grade) (Nippon Gene, Tokyo, Japan) to remove any contaminating genomic DNA. The concentration of RNA was measured with a BioSpec-mini DNA/RNA/protein analyzer (Shimazu, Kyoto, Japan). cDNA was synthesized with the use of 3 μg of RNA, 500 ng of oligo-dT12-18 primers, and 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). To analyze the relative expression of different mRNAs, the amount of cDNA was normalized on the basis of the signals from ubiquitously expressed β-actin mRNA. PCR was carried out using an Ex-Taq kit (Takara Bio, Shiga, Japan) according to the manufacturer's standard protocol in a final volume of 25 μl. The primer sequences are summarized in Table 1.

Table 1
PCR primers for the detection of tissue-specific marker gene expressions used in this study

Gene	Sequences (5′–3′)		Product length (bp)
	Forward	Reverse	
Oct4	GGTGGAGGAAGCCGACAAC	TTCGGGCACTTCAGAAACATG	141
Sox2	AGATGCACAACTCGGAGATCAG	CCGCGGCCGGTATTTATAAT	146
BMP4	CTGCCGTCGCCATTCACTAT	TGGCATGGTTGGTTGAGTTG	146
Nkx2.5	CCAAGTGCTCTCCTGCTTTCC	CCATCCGTCTCGGCTTTGT	148
MLC-2v	GCTTCATCGACAAGAATGAC	GAATGCGTTGAGAATGGTCT	185
ANF	CGGTGTCCAACACAGATCTG	TCTCTCAGAGGTGGGTTGAC	187
MyoD	ACGGCTCTCTCTGCTCCTTTG	CGTGCTCCTCCGGTTTCA	138
GATA6	CGGTCATTACCTGTGCAATG	GCATTTCTACGCCATAAGGTA	159
TTR	GTCCTCTGATGGTCAAAGTC	TCCAGTTCTACTCTGTACAC	193
HNF1	AGCCGCAGAACCTTATCATG	GGTTGGTGTCTGTGATCAAC	390
AFP	ACTCACCCCAACCTTCCTGTC	CAGCAGTGGCTGATACCAGAG	422
ALB	GGAACTTGCCAAGTACATGTGTGA	CAGCAATGGCAGGCAGATC	146
Nestin	TGCATTTCCTTGGGATACCAG	CTTCAGAAAGGCTGTCACAGGAG	122
Synaptophysin	GTGGAGTGTGCCAACAAGAC	ATTCAGCCGAGGAGGAGTAG	158
NFH	AGGACCGTCATCAGGCAGACATTGC	GACCAAAGCCAATCCGACACTCTTC	201
GFAP	TGCCACGCTTCTCCTTGTCT	GCTAGCAAAGCGGTCATTGAG	146
Olig2	TGCGCCTGAAGATCAACAG	CATCTCCTCCAGCGAGTTG	182
DM20	TGAAGCTCTTCACTGGTACAG	GTCTTGTAGTCGCCAAAGAT	207
β-Actin	GCTCTGGCTCCTAGCACCAT	GGGCCGGACTCATCGTACT	146

Quantitative RT-PCR. For quantitative RT-PCR, gene expression was assessed by real-time PCR with the use of a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The reaction mixtures contained 1 μl of template cDNA with 100 nM of forward and reverse primers and 10 μl of SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) in a total volume of 20 μl. Duplicate assays were run for each sample, and each included a standard curve and a negative control. Specific oligonucleotide primers were designed to produce 122- to 422-bp products. The amplification protocol consisted of 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative quantitative expressions of tissue-specific markers, after normalization with GAPDH as a housekeeping gene, were calculated. The primer sequences are summarized in Table 1. The primers for GAPDH were purchased from Applied Biosystems (Foster City, CA).

Results

The cell viability assay (MTT assay) was used to study the cytotoxic effect of VPA with ES cells, representing embryonic tissues, and NIH-3T3 fibroblasts, representing adult tissues. In both cell lines, VPA inhibited the survival of cells in a dose-dependent manner (Fig. 1). However, there was a significant difference in cytotoxic sensitivities against VPA between ES cells and NIH-3T3 fibroblasts. The IC_{50} values, the inhibitory concentration of 50% cell viability, were calculated at 3.25 and 0.56 mM for NIH-3T3 fibroblasts and ES cells, respectively. The therapeutic range of VPA is 0.30–0.70 mM in serum. The IC_{50} value of NIH-3T3 fibroblasts, 3.25 mM, was approximately 5–11 times the therapeutic concentration. In contrast, the IC_{50} of ES cells, 0.56 mM, was within the therapeutic range of VPA. Since ES cells are more significantly affected by the therapeutic range of VPA than NIH-3T3 fibroblasts, developing cells during embryogenesis could be seriously damaged by the VPA therapeutic concentration. To observe the cytotoxic and morphological effects of VPA on ES cells and NIH-3T3 fibroblasts, ES cells and NIH-3T3 fibroblasts colored with the MTT were observed on day 5 of the cytotoxicity assay (Fig. 1). In both cell lines, the cell densities were gradually reduced in a concentration-dependent manner. In a high-medication group, NIH-3T3 fibroblasts showed strong indications of shrinking. However, in ES cells, many small cells, considered as undifferentiated cells, showed strong signs of inhibition to differentiation.

To characterize the tissue-specific effects of VPA on the ES differentiation system at the molecular levels, the expression levels of typical tissue-specific genes were examined using RT-PCR analysis with samples on day 10 of the differentiation assay (Supplemental Fig.1). In this culture condition-containing 20% FCS, ES cells were mainly differentiated into endodermal and mesodermal lineages, such as cardiomyocytes, but not into ectodermal lineages, such as neural cells. This is in agreement with the fact that some growth factors involved in FCS are known to inhibit the neural induction of various kinds of adult neural stem cells. Oct4, a representative undifferentiated marker, was highly detected in all samples of high VPA concentrations. Other primitive markers, such as BMP4 (a mesodermal marker), GATA6 (an endodermal marker), and Nestin (an ectodermal, neural marker), were also strongly detected in the samples with high VPA concentrations. In contrast, VPA reduced the expression levels of late-stage markers, such as Nkx2.5 and MLC-2v (cardiomyocyte markers) and TTR, HNF1, AFP, and albumin (ALB) (endodermal markers), whereas Synaptophysin (Syn), a typical neural marker, was increased in a VPA concentration-dependent manner. No expression of MyoD (a muscle marker) and GFAP (a glial marker) was detected. These results suggest a tendency for VPA to inhibit the differentiation into

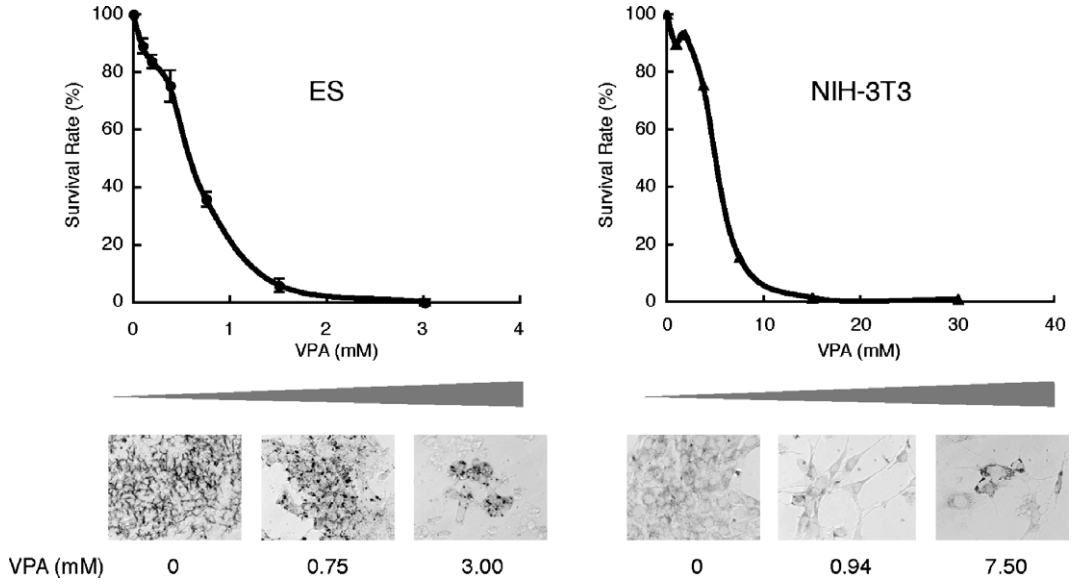

Fig. 1. Cytotoxicity assay for ES cells and NIH-3T3 fibroblasts. Cells on day 10 of the assay were stained with the MTT and solubilized. The activity of the mitochondrial enzyme of living cells was examined. The violet color of the MTT formazan, which is the enzyme product, was measured with the absorbance of 520 nm. On day 5, cells were stained with the MTT and examined with a Hoffman differential interference contrast microscope.

cardiomyocytes and endodermal lineages and to adversely induce differentiation into neural lineages. Undifferentiated ES cells also remained under a condition of high VPA concentration.

To characterize the VPA effects more closely, we performed quantitative expression analysis of tissue-specific genes and immunocytochemical or morphological analysis with samples on day 10 of the differentiation assay (Figs. 2 and 3). It was demonstrated that the expression levels of Oct4 and Sox2 (undifferentiated markers) were high under the condition of high VPA concentration (Fig. 2A-(1)). For mesodermal lineages, the expressions of BMP4, Nkx2.5, MLC-2v, and ANF were reduced, and MyoD was not detected (Fig. 2A-(2)). On day 10 of the differentiation assay, cardiomyocytes were normally differentiated (Fig. 2C, left). The number of cells stained with an antibody against troponin I (a typical cardiomyocyte-specific marker) was decreased notably under the conditions of high VPA concentration (data not shown). The ratio of induced cardiomyocytes was determined from their autonomous

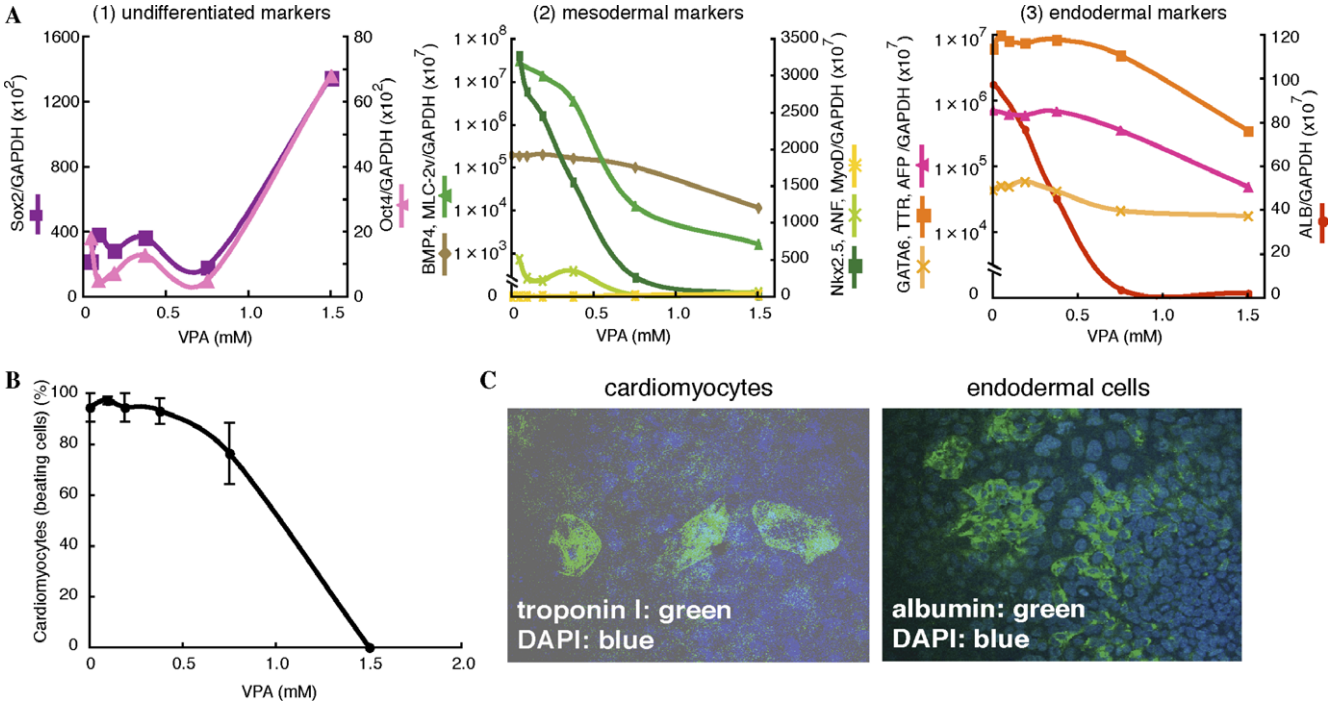

Fig. 2. ES differentiation assay 1: the undifferentiated state and mesodermal and endodermal differentiation. (A) The expression levels of typical undifferentiated markers, Oct4 and Sox2 (1), mesodermal markers, BMP4, MLC-2v, Nkx2.5, ANF, and MyoD (2), and endodermal markers, GATA6, TTR, AFP, and ALB (3), were quantified at each concentration of VPA with real-time RT-PCR. (B) Cardiomyocytes derived from ES cells were analyzed by observing their distinctive beating movements at each concentration of VPA. (C) (left) Cardiomyocytes were immunostained with an anti-troponin I antibody. (right) Endodermal cells derived from ES cells were immunostained with an anti-albumin antibody.

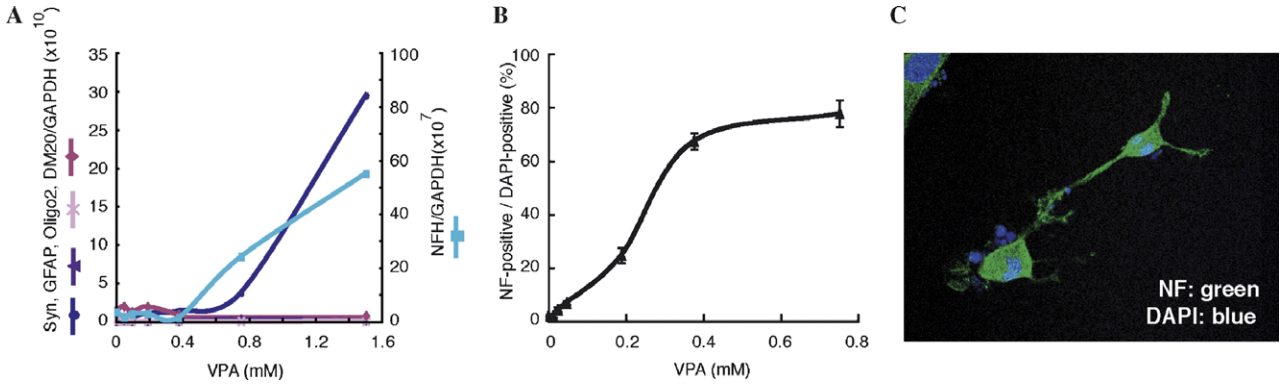

Fig. 3. ES differentiation assay 2: ectodermal differentiation. (A) The expression levels of typical ectodermal markers, Synaptophysin, NFH, GFAP, and Oligo2, were quantified with real-time RT-PCR. (B) The efficiency of neuronal differentiation from ES cells was estimated with anti-neurofilament antibody-stained cells (NF-positive) against total cells (DAPI-positive). (C) Neuronal cells derived from ES cells (VPA 0.38 mM) were immunostained with an anti-neurofilament (NF) 200 antibody.

contractile motions and was shown to decrease in a concentration-dependent manner (Fig. 2B). It was also confirmed that VPA inhibited the differentiation into cardiomyocytes at the molecular and morphological levels. On day 10 of the differentiation assay, a cellular population expressed a definitive endodermal marker, ALB was detected (Fig. 2C, right). The expression levels of GATA6, TTR, AFP, and ALB were decreased in a concentration-dependent manner (Fig. 2A-(3)), suggesting that VPA suppressed the differentiation into endodermal lineages. For ectodermal lineages, the expression levels of neuron-specific markers, such as Synaptophysin (Syn) and NFH, were increased in a concentration-dependent manner. Glial markers, such as GFAP, which is a representative astrocyte-specific marker, and Oligo2 and DM20, which are oligodendrocyte-specific markers, were not induced (Fig. 3A). With a dose of 0.38 mM VPA, many positive cells to neurofilament 200, which is a neuron-specific marker, were detected in samples on day 10 of the differentiation assay (Fig. 3C), and the ratio of anti-neurofilament 200-positive cells was increased in a concentration-dependent manner (Fig. 3B). On the other hand, cells stained with the anti-GFAP antibody were not observed (data not shown). Taken together, these results suggest that VPA promotes ES cells to differentiate into neurons but not glial cells.

Discussion

In the cell viability assay, there was a significant difference in the sensitivities against VPA between ES cells as an embryonic tissue cell model and NIH-3T3 fibroblasts as an adult tissue cell model. VPA has been in clinical use as a safe and effective antiepileptic drug (AED) in a wide range of epileptic conditions in children and adults [12]. Although VPA has been shown to have very few side effects, it is a potent teratogen and produces several malformations in embryos [5,13,14]. Thus, VPA could affect embryonic tissue cells more than adult tissue cells and, consequently, might cause damage to developing cells and, specifically, to embryos. In fact, VPA caused triploblastic differentiations of ES cells in the study herein reported.

Here, we showed that VPA stimulated the differentiation of ES cells into neuronal cells in a lineage-specific manner but attenuated the differentiation of ES cells into endodermal or mesodermal cells. Similarly to this finding in ES cells, VPA is known to promote neuronal fate and to inhibit glial fate simultaneously in multiple adult neural progenitor cells [15]. Whereas VPA can promote the differentiation of the adult neural progenitor cell by induction of neurogenic transcription factors such as NeuroD [15], it remains to be determined what mechanisms of the neuronal differential promotion are involved in ES cells. Nevertheless, it seemed that the altered development of neural cells or tissues driven by VPA could result in NTDs [4], which are predominant VPA-induced teratogenic malformations.

VPA has been reported to inhibit histone deacetylases (HDACs) in a therapeutic range (0.30–0.70 mM) and cause the hyperacetylation of histones in HeLa, F9 teratocarcinoma, and Neuro2A neuroblastoma cells [16,17]. In general, increased levels of histone acetylation are associated with increased transcriptional activity, whereas decreased levels of acetylation are associated with the repression of gene expression [18,19]. A number of specific and potent inhibitors of HDACs, such as trichostatin A (TSA) and VPA, prevent tumorigenesis in rodent and human models and have potential therapeutic roles in the treatment of malignant diseases [20,21]. Furthermore, VPA has been reported to arrest the cell cycle at a G0/1 phase, inhibit proliferation, and induce apoptosis in multiple myeloma [22,23] *in vitro*. Thus, VPA could also inhibit cell proliferation in ES cells by a similar mechanism. In addition, it is also accepted that HDACs play an important role during the embryogenesis of numerous organisms, and interfering with their functions using HDAC inhibitors, such as VPA, could result in some developmental abnormalities, such as teratogenicity [24]. These findings suggest that the effects of VPA on cell growth and neurons might be via the inhibition of HDACs.

Recently, stem cells have become important new tools for the development of *in vitro* model systems to test drugs and chemicals and have shown potential to predict or estimate toxicity [25]. Among various stem cells, ES cells are some of the most valuable cells to develop *in vitro* model systems because they are capable of self-renewing and differentiating into every cell type of the mammalian organism and therefore have higher plasticity than adult stem cells. Since mouse ES cells are prone to differentiate into cardiomyocytes, drug toxicity, such as cardiotoxicity, can be assessed using ES cells [26–30]. The embryonic stem cell test (EST) is an *in vitro* embryotoxicity assay that assesses the ability of chemical compounds to inhibit the differentiation of ES cells into cardiomyocytes [31,32]. In comparison to *in vivo* studies, EST is easy and highly accurate in predicting cellular toxicity, outperforming classical assays, such as fetal limb micromass and post-implantation whole-rat embryo cultures [32]. Thus, EST is a simple and accurate test for toxicity; however, it is not sufficient for evaluating all chemicals because only two parameters of cytotoxicity and morphological change are assessed in the reported EST [8]. Therefore, we attempted to characterize the tissue-specific embryotoxicity of VPA by analyzing the gene expression of the tissue-specific markers as well as by conducting a histological and immunocytochemical study and examining the established two parameters in the mouse embryonic stem (ES) cell differentiation system. Using this system, we demonstrated that VPA is highly cytotoxic and potent to inhibit differentiation into cardiomyocytes in a cytotoxicity and morphological study as well as stimulates the differentiation of the neuronal lineage and inhibits the differentiation of mesodermal and endodermal lineages. Taking the *in vivo* embryotoxicity of VPA into account, the system presented in this study could be useful for predicting the degree of the abnormal neural development of VPA *in vivo*.

In conclusion, this *in vitro* ES cell system allows estimating and characterizing the embryotoxic effects of various chemicals. Further research on this innovative method may help to establish high-throughput screening analysis of drugs to reduce the number of experimental animals and save time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.189.

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