*Full Paper***Detection of Thalidomide Embryotoxicity by In Vitro Embryotoxicity Testing Based on Human iPS Cells**Nobuo Aikawa¹, Atsushi Kunisato², Kenji Nagao², Hideaki Kusaka¹, Katsumi Takaba¹, and Kinya Ohgami¹¹*Drug Discovery Research Laboratories, Fuji Research Park, Research Division, Kyowa Hakko Kirin Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731, Japan*²*Biologics Research Laboratories, Tokyo Research Park, Research Division, Kyowa Hakko Kirin Co., Ltd., 3-6-6 Asahi-machi, Machida, Tokyo 194-8533, Japan**Received September 6, 2013; Accepted December 5, 2013*

Abstract. The mouse embryonic stem cell test (mEST) is used to assess the embryotoxicity of drug candidates by evaluating the effects on the cardiac differentiation of stem cells. However, thalidomide embryotoxicity has not yet been reported using the mEST. To detect the effects of thalidomide, we used human induced pluripotent stem cells (hiPSCs) instead of mouse embryonic stem cells, and assessed three endpoints: the inhibition of cardiac differentiation, the cytotoxicity to hiPSCs, and the cytotoxicity to human dermal fibroblasts, according to the mEST. From these data (IC₅₀ values), the embryotoxicity was classified into one of three different classes based on the mEST and our criteria. Valproate was used as a positive control and ascorbic acid was used as a negative control, and their effects were assessed. Similar to valproate, thalidomide was classified as a Class 2 agent, with weak embryotoxicity, by the mEST criteria, and was classified as Category 3 embryotoxic based on our criteria. Ascorbic acid was classified as a Class 1 / Category 1, non-embryotoxic agent, based on both criteria. Thalidomide embryotoxicity was detected in the embryonic stem cell test based on hiPSCs. This test system is thus considered to have a much greater predictive ability than the mEST.

Keywords: embryonic stem cell test, iPS cell, thalidomide, embryotoxicity, teratogenicity

Introduction

Developmental toxicity, including embryotoxicity and teratogenicity (an irreversible malformation), is a crucial type of toxicity that affects offspring and needs to be assessed during the nonclinical drug development process. However, in vivo reproductive and developmental studies to assess the developmental toxicity of drug candidates is rarely carried out before clinical trials of the drugs due to the use of a large number of animals, the long experimental period, and the high cost, so that pregnant females and females who may become pregnant are excluded from phase 1 clinical trials.

Three in vitro developmental toxicity tests are currently used to assess the embryotoxicity of drug

candidates and chemicals. They include the whole-embryo culture test, rat limb bud micromass test, and the mouse embryonic stem cell test (mEST), and these tests have already been validated by the European Center for the Validation of Alternative Methods (ECVAM) (1). In particular, the mEST, which uses cardiac differentiation as an indicator of the evaluation, is easy to perform in comparison to the in vivo nonclinical studies and has the advantage of not requiring the use of experimental pregnant/non-pregnant animals. The mEST has been adopted by a number of pharmaceutical companies as a tool to assess the embryotoxicity of drug candidates at an early stage in drug development.

Thalidomide is known to have adverse teratogenic effects on human fetuses (2, 3). However, the adverse effects of thalidomide are difficult to detect in in vivo reproductive and developmental studies in rodents (4, 5). This may be due to species barrier-related differences between rodents and humans. The effects of thalidomide

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Published online in J-STAGE on January 23, 2014
doi: 10.1254/jphs.13162FP

have not been reported using mEST. In view of the species differences, it is not surprising that there have been no reports regarding positive teratogenic results using murine embryonic stem (ES) cells. It is thought that if thalidomide is tested using mEST, the validity (e.g., agreement between the results of in vivo animal tests and actions in humans) of the mEST system will be spoiled, regardless of the test results obtained using murine ES cells. In the mEST system, if human ES cells are used instead of murine ES cells, it is supposed that disagreement between the in vivo actions in rodents and the effects in humans will not occur. However, human ES cells are associated with bioethical issues; therefore, if possible, we would like to avoid their use. Human induced pluripotent stem cells (hiPSCs), which have the same differentiation properties as human ES cells, have recently become popular in the toxicology field, and without the problem of bioethics (6, 7). Therefore, we tried to study the teratogenic potential of thalidomide using hiPSCs instead of mouse ES cells. We also assessed in the present study the effects of sodium valproate and ascorbic acid, which have previously been confirmed to have teratogenic effects and no effects in rodent studies (8–10), as a positive and negative control, respectively.

Materials and Methods

The present study was conducted following the company policy on the research ethics review committee of our institution.

Test and differentiation-inducing drugs

Thalidomide, sodium valproate and recombinant human activin-A (activin-A) were purchased from Wako Pure Chemicals (Osaka); ascorbic acid, from Nacalai Tesque (Kyoto); recombinant human Wnt-3a (Wnt-3a) and recombinant human noggin (noggin), from R&D Systems (Minneapolis, MN, USA); and recombinant human bone morphogenetic protein-4 (BMP-4) from HumanZyme (Chicago, IL, USA). Thalidomide was dissolved in dimethylsulfoxide (Dojindo, Kumamoto) and diluted in appropriate medium, as described below. Sodium valproate and ascorbic acid were dissolved in medium and used after filter sterilization (0.20 μm).

Cell culture medium

Modified Tenneille serum replacer 1 (mTeSRTM1) and AggreWellTM media were obtained from STEMCELL Technologies (Vancouver, Canada) and dermal fibroblast growth medium was purchased from DS Pharma Biomedical (Osaka). B-27[®] serum-free supplement (50 $\times$) (B27), 200 mM non-essential amino acids (NEAA),

and dulbecco's modified eagle medium F-12 Ham's with GlutaMaxTM-1 (DMEM/F12) were purchased from Life Technologies (Carlsbad, CA, USA). Monothioglycerol 50 mM StemSure[®] solution (MTG) and fetal bovine serum (FBS) were from Wako Pure Chemicals. The following DME/F12 basal media were prepared for the cardiac differentiation test: B27-DMEM/F12 medium supplemented with 2 vol% B27, 2 mM NEAA and 0.5 mM MTG, and FBS-DMEM/F12 medium supplemented with 5 vol% FBS and 2 mM NEAA.

Cells

The hiPSCs were derived from human bone marrow mononuclear cells which were purchased from AllCells (Emeryville, CA, USA) (11). Human dermal fibroblasts isolated from adult normal skin which were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA).

Test procedure

Differentiation test: Cardiac differentiation was assessed using the modified mEST method. The hiPSCs maintained in feeder-free condition was harvested by 1 mg/mL dispase (STEMCELL Technologies). The cells were suspended in AggreWellTM medium supplemented with Y-27632 (10 μM), a Rho-associated protein kinase (ROCK) inhibitor, seeded on AggreWellTM 800 plates (STEMCELL Technologies), and incubated at 37°C, 5% CO₂ with saturated humidity. One to two days later, embryonic bodies (hiPSCs aggregates) were formed, and the medium was replaced with B27-DMEM/F12. Embryonic bodies were transferred to Nunc[®] ultra-low-cell-binding 24-well culture plates (Thermo Fisher Scientific, Rochester, NY, USA) and the test drug or vehicle was added to each well except for the control well. Subsequently, activin-A (100 ng/mL), Wnt-3a (100 ng/mL), and BMP-4 (100 ng/mL) were added to all wells. Twenty-four hours later, the medium was replaced with B27-DMEM/F12 supplemented with noggin (300 ng/mL) or noggin and the test drug or vehicle, and thereafter, the medium was replaced daily with fresh medium containing the same components for 3 days. Embryonic bodies were transferred to 96-well culture plates (one embryonic body/well, 24-well/group) coated with 0.1 w/v% StemSureTM gelatin (Wako Pure Chemicals), and the media were replaced with FBS-DMEM/F12. The beating cardiomyocytes were observed morphologically under a microscope every day.

Cytotoxicity tests: Cytotoxicities of the drugs to the hiPSCs and human dermal fibroblasts were assessed using the mEST method (13) with some modifications. The cytotoxicity was determined using the Cell meterTM colorimetric cell cytotoxicity assay kit (AAT Bioquest,

Sunnyvale, CA, USA), which is based on resazurin as an indicator of mitochondrial function, according to the manufacturer's manual. The hiPSCs were harvested by 1 mg/mL dispase, and the human dermal fibroblasts were detached by 0.05% trypsin-EDTA (1%) (Life Technologies). Each cell was suspended in medium and seeded into Nunc® 96-well optical-bottom plates (Thermo Fisher Scientific), coated with matrigel (BD Biosciences, Bedford, MA, USA) for hiPSCs and poly-L-lysine (Trevigen, Gaithersburg, MD, USA) for human dermal fibroblasts, at 1000 cells/0.1 mL per well, and then incubated at 37°C, 5% CO₂ with saturated humidity. Two hours later, medium with the test drug (two-fold of the final concentration) was added at 0.1 mL/well, with 3 or 4 wells being treated at each concentration. The medium with drug was refreshed daily for 4 days. The medium with drug was then discarded, and 0.1 mL of medium was added to all wells. One hour later, 0.02 mL of Cell meter™ assay solution was added to all wells. The absorbance change at 570 and 605 nm was monitored using a microplate reader (SpectraMax® M3, Molecular Devices, Sunnyvale, CA, USA), and the cell viability in each well was determined as the absorbance ratio of 570 nm to 605 nm. The results were calculated as a relative percentage to the control cells.

The hiPSCs were cultured in mTeSR™1, and the human dermal fibroblasts were cultured in dermal fibroblast growth medium. The sensitivities to the drugs were not significantly different based on the data when the cells were treated for 4 or 10 days. Therefore, we selected the treatment period of 4 days in order to simplify the experimental procedures in this assay.

Data analyses

Calculation of IC₅₀ values: The IC₅₀ value of the agent with regard to inhibiting the differentiation of embryonic

body cells into beating cardiomyocytes, as well as the IC₅₀ value (IC₅₀PS) for hiPSCs and the IC₅₀ value (IC₅₀F) for human dermal fibroblasts, were calculated by the logit method using the SAS software program ver. 9.2 (SAS Institute Japan, Tokyo).

Classification of test drugs

mEST criteria: Each function value (I, II, and III) was calculated by individual linear discriminant functions based on the IC₅₀, IC₅₀F, and IC₅₀PS values, as shown in Table 1. To classify the embryotoxic potential of the test drugs into one of three different classes, the values of each function were compared and classified, as shown in Table 1.

Our new criteria: In order to classify the embryotoxic potential of the test drugs into one of three different categories, the IC₅₀, IC₅₀F, and IC₅₀PS values were compared and classified, as shown in Table 2. We established our own criteria, because the mEST criteria are thought to be affected by the intensity of the test drug activity, including cytotoxicity. In order to eliminate the affect of the intensity of the test drug activity, our criteria was made to compare relative toxicity to embryonic cells and other cells (maternal). We believe that using our own criteria to judge the three IC₅₀ values may thus enable us to more appropriately evaluate the embryotoxic potential.

Results

All of the embryonic bodies (hiPSCs aggregates) differentiated into beating cardiomyocytes in the control group of all tests.

Sodium valproate

Sodium valproate inhibited the differentiation of

Table 1. Classification of the embryotoxic potential in the mouse embryonic stem cell test

Linear discriminant functions I, II, and III		
Function I	$5.916 \text{ Ig(IC}_{50}\text{F)} + 3.500 \text{ Ig(IC}_{50}\text{PS or IC}_{50}\text{ES)} - 5.307 [(\text{IC}_{50}\text{F} - \text{IC}_{50}) / \text{IC}_{50}\text{F}] - 15.27$	
Function II	$3.651 \text{ Ig(IC}_{50}\text{F)} + 2.394 \text{ Ig(IC}_{50}\text{PS or IC}_{50}\text{ES)} - 2.033 [(\text{IC}_{50}\text{F} - \text{IC}_{50}) / \text{IC}_{50}\text{F}] - 6.85$	
Function III	$-0.125 \text{ Ig(IC}_{50}\text{F)} - 1.917 \text{ Ig(IC}_{50}\text{PS or IC}_{50}\text{ES)} + 1.500 [(\text{IC}_{50}\text{F} - \text{IC}_{50}) / \text{IC}_{50}\text{F}] - 2.67$	
Classification criteria		
Class 1	Non-embryotoxic	I > II and I > III
Class 2	Weakly embryotoxic	II > I and II > III
Class 3	Strongly embryotoxic	III > I and III > II

The above prediction model is quoted from the European Union Reference Laboratories (EURL)-the European Center for the Validation of Alternative Methods (ECVAM) database service on alternative methods to animal experimentation, protocol No. 113: embryonic stem cell test. IC₅₀: 50% inhibition of the differentiation of embryonic bodies into beating cardiomyocytes, IC₅₀F: 50% inhibition of the viability of human or mouse fibroblasts, IC₅₀PS: 50% inhibition of the viability of human induced pluripotent stem cells, IC₅₀ES: 50% inhibition of the viability of mouse embryonic stem cells. Functions are calculated using the IC₅₀ values obtained from cells of the same species.

Table 2. Categorization of embryonic toxicity based on our original criteria

Category 1	Non-embryotoxic	$IC_{50}F < IC_{50}$ and $IC_{50}F < IC_{50}PS$ (or $IC_{50}ES$)
Category 2	Suspected embryotoxic	$IC_{50}F < IC_{50}$ and $IC_{50}F > IC_{50}PS$ (or $IC_{50}ES$), or $IC_{50}F > IC_{50}$ and $IC_{50}F < IC_{50}PS$ (or $IC_{50}ES$)
Category 3	Embryotoxic	$IC_{50}F \geq IC_{50}$ and $IC_{50}F \geq IC_{50}PS$ (or $IC_{50}ES$)

IC_{50} : 50% inhibition of the differentiation of embryonic bodies into beating cardiomyocytes, $IC_{50}F$: 50% inhibition of the viability of human or mouse fibroblasts, $IC_{50}PS$: 50% inhibition of the viability of human induced pluripotent stem cells, $IC_{50}ES$: 50% inhibition of the viability of mouse embryonic stem cells. The IC_{50} values obtained from cells of the same species are compared.

Fig. 1. The effects of the test drugs on cardiac differentiation and cell viability. A) The effects of sodium valproate, B) the effects of ascorbic acid, and C) the effects of thalidomide. Each point represents the mean for cardiac differentiation and the mean $\pm$ S.E.M. for the cell viability. Squares: human dermal fibroblasts, circles: human induced pluripotent stem cells, triangles: cardiac differentiation.

embryonic bodies into beating cardiomyocytes with an IC_{50} value of 125 μ g/mL (Fig. 1A, Table 4). This drug decreased the viability of human dermal fibroblasts and hiPSCs at $IC_{50}F$ value of 300 μ g/mL and at $IC_{50}PS$ value of 121 μ g/mL, respectively (Fig. 1A, Table 4).

In the mEST criteria shown in Table 1, the values of functions I, II, and III as calculated from the IC_{50} , $IC_{50}F$, and $IC_{50}SP$ values were 3.6, 6.0, and -2.1, respectively (Table 5). From the comparison of the data of these functions, $II > I$ and $II > III$, sodium valproate was classified as a weak embryotoxin of class 2 (Table 6). Based on our original criteria shown in Table 2, sodium valproate was classified as a category 3, embryotoxin, according to the comparison of IC_{50} values: $IC_{50}F > IC_{50}$ and $IC_{50}F > IC_{50}PS$ (Table 6).

Sodium valproate was validated to have developmental toxicity according to this test system based on the hiPSCs.

Ascorbic acid

Ascorbic acid inhibited the differentiation of embryonic bodies into beating cardiomyocytes with an IC_{50} value of 622 μ g/mL (Fig. 1B, Table 4). This drug decreased the viability of human dermal fibroblasts and hiPSCs at $IC_{50}F$ value of 567 μ g/mL and at $IC_{50}PS$ value of 658 μ g/mL, respectively (Fig. 1B, Table 4).

Based on the mEST criteria shown in Table 1, the values of functions I, II, and III calculated from IC_{50} , $IC_{50}F$, and $IC_{50}SP$ values were 11.4, 10.2, and -3.2, respectively (Table 5). From the comparison of the data of these functions, $I > II$ and $I > III$, ascorbic acid was classified as non-embryotoxic and was considered to be a class 1 agent (Table 6). Based on our original criteria shown in Table 2, ascorbic acid was also classified as non-embryotoxic, category 1, according to the comparison of IC_{50} values: $IC_{50}F < IC_{50}$ and $IC_{50}F < IC_{50}PS$ (Table 6).

Ascorbic acid was validated not to have any developmental toxicity according to this test system based on the hiPSCs.

Thalidomide

Thalidomide inhibited the differentiation of embryonic bodies into beating cardiomyocytes with an IC_{50} value

Table 3. Classification by our original criteria based on data quoted from mEST validated by ECVAM

Drug	Embryotoxicity ^a	mEST criteria	Our criteria	
		Class ^b	Results quoted from mEST ^b ($\mu\text{g/mL}$)	Category ^c
Ascorbic acid	Negative in animals	1	$\text{IC}_{50}\text{F} < \text{IC}_{50}\text{ES} < \text{IC}_{50}$ (25.5) (138) (408)	1
Isoniazid	Positive in animals Avoid in pregnant females for the transition to placenta Positive in adding amino salicylic acid for epidemiological survey	1	$\text{IC}_{50} < \text{IC}_{50}\text{F} < \text{IC}_{50}\text{ES}$ (360) (375) (750)	2
Penicillin G	No evidence in animals and pregnant females Avoid in pregnant females because safety is not proved	1	$\text{IC}_{50}\text{F} < \text{IC}_{50}\text{ES} < \text{IC}_{50}$ (1586) (2950) (3450)	1
Aspirin	Positive in rats, but not in humans Not recommended in pregnant females	2	$\text{IC}_{50}\text{ES} < \text{IC}_{50}\text{F} < \text{IC}_{50}$ (220) (230) (248)	2
Caffeine	No evidence in animals and pregnant females Avoid in pregnant females for the transition to placenta	2	$\text{IC}_{50}\text{F} < \text{IC}_{50}\text{ES} < \text{IC}_{50}$ (155) (165) (185)	1
Dexamethasone	Positive in animals	2	$\text{IC}_{50} < \text{IC}_{50}\text{ES} < \text{IC}_{50}\text{F}$ (23) (18.3) (26)	3
Diphenhydramine	Negative in animals Avoid in pregnant females for the epidemiological survey of H1 blockers	2	$\text{IC}_{50} < \text{IC}_{50}\text{ES} < \text{IC}_{50}\text{F}$ (6.7) (29.5) (30)	3
Phenytoin	Positive in humans	2	$\text{IC}_{50} < \text{IC}_{50}\text{ES} < \text{IC}_{50}\text{F}$ (20) (27.3) (35)	3
Valproate	Positive in humans and animals	2 ^d	$\text{IC}_{50} < \text{IC}_{50}\text{ES} < \text{IC}_{50}\text{F}^{\text{d}}$ (23.73) (364.04) (638.69)	3
Busulfan	Positive in mice, rats, and rabbits Suspected positive in humans	3	$\text{IC}_{50}\text{ES} < \text{IC}_{50} < \text{IC}_{50}\text{F}$ (2.1) (4.6) (4.8)	3
Cytarabine	Positive in animals Contraindicated in pregnant females	3	$\text{IC}_{50}\text{ES} < \text{IC}_{50} < \text{IC}_{50}\text{F}$ (0.024) (0.029) (0.033)	3
5-Fluoro uracil	Positive in mice and rats Contraindicated in pregnant females	3	$\text{IC}_{50} < \text{IC}_{50}\text{ES} < \text{IC}_{50}\text{F}$ (0.0289) (0.103) (0.17)	3
Hydroxyurea	Positive in rats Contraindicated in pregnant females	3	$\text{IC}_{50} < \text{IC}_{50}\text{ES} < \text{IC}_{50}\text{F}$ (1.7) (2.0) (7.2)	3
Retinoic acid	Positive in animals and pregnant females Contraindicated in pregnant females	3	$\text{IC}_{50} < \text{IC}_{50}\text{ES} < \text{IC}_{50}\text{F}$ (0.000105) (0.005) (1.0)	3
Thalidomide	Negative in rodents, but positive in rabbits Positive in humans	No data	No data	No judgement

^aDrug interview form, detailed drug information, prepared by the pharmaceutical company. ^bThe European Union Reference Laboratories (EURL)-ECVAM database on alternative methods to animal experimentation (DB-ALM), protocols No. 113: embryonic stem cell test (EST), Class 1: non-embryotoxic, Class 2: weakly embryotoxic, Class 3: strongly embryotoxic. ^cCategory; 1: non-embryotoxic, 2: suspected embryotoxic, 3: embryotoxic. ^dData were quoted from Toxicol Sci. 2011;124:460–471. mEST: mouse embryonic stem cell test validated by ECVAM. IC_{50}F : IC_{50} value for the viability of mouse skin fibroblasts, IC_{50}ES : IC_{50} value for the viability of mouse embryonic stem cells, IC_{50} : IC_{50} value for the differentiation of mouse embryonic stem cells into cardiomyocytes. ECVAM: the European Center for the Validation of Alternative Methods.

of 117 $\mu\text{g/mL}$ (Fig. 1C, Table 4). This drug decreased the viability of human dermal fibroblasts and hiPSCs at IC_{50}F value of 578 $\mu\text{g/mL}$ and at IC_{50}PS value of 207 $\mu\text{g/mL}$, respectively (Fig. 1C, Table 4).

In the mEST criteria shown in Table 1, the values of functions I, II, and III calculated from IC_{50} , IC_{50}F , and IC_{50}SP values were 4.9, 7.1, and -1.8 , respectively (Table 5). From the comparison of the data of these

functions: $\text{II} > \text{I}$ and $\text{II} > \text{III}$, so thalidomide was classified as a weak embryotoxin of class 2 (Table 6). Based on our own original criteria shown in Table 2, thalidomide was classified as embryotoxic of category 3, according to the comparison of IC_{50} values: $\text{IC}_{50}\text{F} > \text{IC}_{50}$ and $\text{IC}_{50}\text{F} > \text{IC}_{50}\text{PS}$ (Table 6).

Thalidomide embryotoxicity was detected in this test system.

Table 4. The IC₅₀ values for cardiac differentiation and cell viability

Drug	IC ₅₀ F (for Fibroblasts, $\mu\text{g/mL}$)	IC ₅₀ PS (for hiPSCs, $\mu\text{g/mL}$)	IC ₅₀ (for Cardiac differentiation, $\mu\text{g/mL}$)
Sodium valproate	300 [254–368]	121 [113–128]	125 [122–128]
Ascorbic acid	567 [547–584]	658 [635–682]	622 [621–624]
Thalidomide	578 [564–589]	207 [182–232]	117 [114–120]

The brackets contain the 95% confidence interval ($\mu\text{g/mL}$). hiPSCs: human induced pluripotent stem cells, IC₅₀: 50% inhibition of the differentiation of embryonic bodies into beating cardiomyocytes, IC₅₀F: 50% inhibition of the viability of human or mouse fibroblasts, IC₅₀PS: 50% inhibition of the viability of human induced pluripotent stem cells, IC₅₀ES: 50% inhibition of the viability of mouse embryonic stem cells.

Table 5. The values calculated from the linear discriminant functions of the mEST classification

Drug	Function		
	I	II	III
Sodium valproate	3.6	6.0	–2.1
Ascorbic acid	11.4	10.2	–3.2
Thalidomide	4.9	7.1	–1.8

mEST: mouse embryonic stem cell test.

Table 6. Classification of drugs based on the mouse embryonic stem cell test and our original criteria

Drug	Classification	
	Our criteria	mEST criteria
Sodium valproate	Category 3 (IC ₅₀ F > IC ₅₀ and IC ₅₀ F > IC ₅₀ PS)	Class 2 (II > I and II > III)
Ascorbic acid	Category 1 (IC ₅₀ F < IC ₅₀ and IC ₅₀ F > IC ₅₀ PS)	Class 1 (I > II and I > III)
Thalidomide	Category 3 (IC ₅₀ F > IC ₅₀ and IC ₅₀ F > IC ₅₀ PS)	Class 2 (II > I and II > III)

Categorization by our criteria: 1, non-embryotoxic; 2, suspected embryotoxic; 3, embryotoxic. Classification by the mouse embryonic stem cell test (mEST) criteria: 1, not embryotoxic; 2, weakly embryotoxic; 3, strongly embryotoxic. I: function I, II: function II, III: function III. IC₅₀F: IC₅₀ value for the viability of human dermal fibroblasts, IC₅₀ES: IC₅₀ value for the viability of human induced pluripotent stem cells, IC₅₀: IC₅₀ value for the differentiation of human induced pluripotent stem cells into cardiomyocytes.

Discussion

The developmental toxicity of thalidomide, in addition to sodium valproate, was detected in the present study, while ascorbic acid showed negative results. The positive results of thalidomide have not been reported in the mEST using cardiac differentiation as a marker of toxicity. The mouse embryonic stem cells differentiate

spontaneously into cardiomyocytes in the presence of fetal bovine serum, and the removal of murine leukemia inhibitory factor maintains them in an undifferentiated state (13). In the same culture conditions, hiPSCs hardly differentiate into cardiomyocytes (data was not shown). Therefore, it is suggested that the cardiac differentiation pathway is different between mice and humans. The adverse effects of thalidomide have been shown to be caused by the inhibition of cereblon, a component of the ubiquitin ligase that is important for the expression of an essential regulator of limb development. However, zebra fish and chickens were used in this study because the adverse effects of thalidomide are difficult to detect in rodents (4, 5, 14). For these reasons, the in vitro developmental toxicity test based on hiPSCs would be a useful tool to assess the developmental toxicity of candidate drugs in humans. We considered that the in vitro developmental toxicity test based on the hiPSCs may have much greater predictive ability than that based on mouse ES cells because of the potential differences in some developmental pathways in humans compared to mice.

In the present study, similar to the positive control drug (sodium valproate), thalidomide was classified as a Class 2 agent (weak embryotoxic potential), not as a Class 3 (strong embryotoxic potential) agent, by the mEST criteria, despite the fact that both are categorized as Category X agents, indicating that the use of the agent should be avoided in pregnant females and those who may become pregnant, as established by the United States Food and Drug Administration (FDA) and the Australian Drug Evaluation Committee (ADEC). One reason for the different classification of the mEST criteria may be caused by the intensity of the drug activity, including cytotoxicity, because all IC₅₀ values of Class 3 agents tend to be lower than those of Class 1 agents. It has been suggested that the results of the mEST classification would be obtained due to the weak

inhibitory activities of test drugs on cardiac development and cell viabilities. On the other hand, our proposed criteria is independent of the intensity of inhibitory activity on cardiac development and cell viabilities, from the published mEST data, and the detailed drug data prepared by the pharmaceutical company.

We considered that the effects of the drug on human dermal fibroblasts would more closely mimic maternal conditions and that the hiPSCs would reflect the embryonic state. When either the cardiac differentiation endpoint or the hiPSCs endpoint is lower than the human dermal fibroblast endpoint, the test drug is judged to have the suspected embryotoxic potential (e.g., to cause irreversible structural malformations, the death of embryos, and others). When both endpoints are lower than the human dermal fibroblast endpoint, the test drug is judged to have embryotoxic potential. Then, based on the benefits/risks for patients, the continuation of drug development is assessed. Based on our original criteria, both thalidomide and valproate were classified as Category 3 agents, with embryotoxicity, including a teratogenic potential. The ability to predict the embryotoxic potential using our criteria was almost identical to that noted in the clinical data. This suggests that using our criteria in addition to the mEST criteria increases the predictive performance of the mEST classification.

In the present study, the true extent of developmental toxicity of thalidomide in humans was detected by using our in vitro test system based on the hiPSCs for the first time. Further studies using more reference drugs and the hiPSCs of other cell lines are needed in order to demonstrate the usefulness of the in vitro developmental toxicity test based on the hiPSCs. This humanized test system may be able to more accurately predict the developmental toxicity of drug candidates at an early stage in drug development by combining the mEST criteria with the criteria proposed in this study.

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