Alternative Methods for Developmental Toxicity Testing Using Mouse ESCs

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Abstract. Development of an organism is accompanied by rapid and complex changes within a relatively short period, and embryotoxic chemicals administered to a mother during pregnancy can result in persistent lesions, general growth retardation, or delayed organ growth. *In vitro* toxicity tests are useful for evaluating the safety or hazards of small quantities of chemicals. Since cell death and inhibition of differentiation in mouse embryonic stem cells (ESCs) can occur within different concentration ranges of compounds, depending on the toxic potency of the compound, these cell properties can be used as guides for classifying the embryotoxicity of a compound. The use of multiple endpoints, such as assessing the inhibition of viability in ESCs (IC_{50}ESC) and 3T3 cells (IC_{50}3T3), and reduction in embryoid body (EB) area (ID_{50}EB), has the advantage of providing a detailed baseline for the classification of a compound's toxicity level and for establishing a prediction model that utilizes those endpoints. The EB area-based toxicity test (EBT) is an animal-free, novel drug screening system that can be useful in evaluating of various embryotoxic chemicals within a short time.

Keywords: Embryonic stem cell test · Embryoid bodies
Developmental toxicity · Prediction model

Introduction

Toxicity tests are necessary for assessing the safety or hazard levels of substances in various fields. Alternative tests based on the 3R principles (reduction, refinement, and replacement of animal use) have been proposed to overcome some of the drawbacks of animal experiments and to avoid unethical procedures [1]. Developmental toxicology is an important field in which undesirable effects on the development of an organism, including malformation, growth retardation, embryo lethality, and malfunction are assessed [2]. *In vitro* systems for testing developmental toxicity of compounds are capable of providing rapid, precise, and relevant information compared to that provided by some animal-based studies, and they are an economical approach as they are characterized by a low compound requirements and a short testing duration [3]. Embryonic stem cells (ESCs) have the capacity to self-renew and the ability to generate differentiated cells. Embryoid bodies (EBs) act at the onset of differentiation and are useful for the evaluation of developmental toxicity. The mouse embryonic stem cell test

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(EST) can be used to evaluate the 50% inhibitory concentration of chemicals at three endpoints: viability of undifferentiated mouse ESCs (IC_{50}ESC), viability of mouse fibroblasts (3T3 cells; IC_{50}3T3), and differentiation of ESCs into cardiomyocytes (CMs; ID_{50}CM) (Fig. 1A green line). In our study, we used EB area to replace the assessment of cardiomyogenesis of ESCs in order to reduce the need for time-consuming and laborious processes. The replacement of CM assessment with that of EB area shortened the assessment period from 10 days to 3 days (Fig. 1A orange line). The EB area test is also referred to as the embryoid bodies test (EBT).

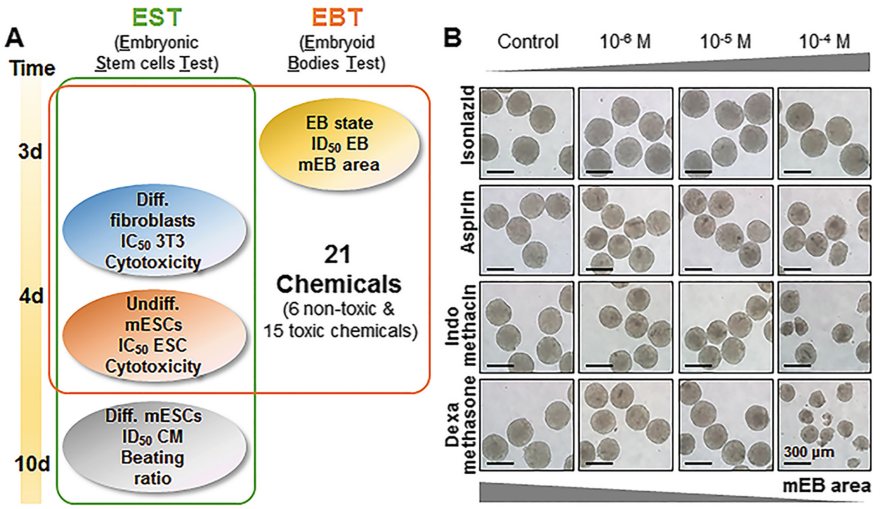

Fig. 1. Endpoints and reduction in EB area, dependent on the toxic potency of chemicals. (A) The EST evaluates the developmental toxicity of chemicals at three endpoints: Cytotoxicity in (i) ESCs. (ii) 3T3 cells, and (iii) inhibition in cardiac differentiation of ESCs at 10 days of treatment (green line). The EBT replaces cardiac differentiation endpoint with a new endpoint based on EB cross-sectional area (orange line). (B) EBs were formed over three days via the hanging drop method with 10^{-6}, 10^{-5}, or 10^{-4} M of non-toxicant (isoniazid) or toxicants (aspirin, indomethacin, or dexamethasone). Scale bars indicate 300 μm. The isoniazid-treated group maintained a EB size in 10^{-4} M treatment similar to that in the control group. Toxicant-treated groups markedly reduced the EB area at 10^{-4} M.

Materials and Methods

Twenty-one compounds (Table 1), including non-embryotoxic and embryotoxic or teratogenic chemicals, were evaluated. Cell viabilities of ESCs and 3T3 cells were measured using a cell counting kit (CCK) assay. Sectional image of EBs were photographed by a phase-contrast microscope and the EB area was analyzed by using image analysis software. The 50% inhibitory concentrations were derived from logarithmic graphs.

Table 1. 21 chemicals tested in a developmental toxicity test using mouse ESCs (N, non-toxicant; W, weak toxicant; M, moderate toxicant; S, strong toxicant)

No	Chemicals	CAS No.	Mw	Class	Function
1	Sodium bicarbonate	144-55-8	84.01	N	Food additive (EU, E500)
2	Sodium gluconate	527-07-01	218.14	N	Chelating agent
3	Saccharin	82385-42-0	205.17	N	artificial sweetener
4	Penicillin G	113-98-4	372.48	N	Antibiotic
5	Isoniazid	54-85-3	137.14	N	Antibiotic, Initial therapy of active tuberculosis
6	Ascorbic acid	134-03-2	198.11	N	Antioxidant
7	Doxylamine succinate	562-10-7	388.46	W	Antihistamine, Antiallergy
8	Pravastatin	81131-70-6	446.51	W	Hypocholesterolemic drug
9	Caffeine	58-08-2	194.19	W	Psychoactive drug, natural pesticide
10	Aspirin	50-78-2	180.16	W	Non-steroidal, Anti-inflammatory
11	Diphenhydramine	147-24-0	291.82	M	Antihistamine, Antiallergy, Antiemetic
12	Diphenylhydantoin	57-41-0	252.27	M	Anti-seizure
13	Indomethacin	53-86-1	357.79	M	Non-steroidal, Anti-inflammatory
14	Dexamethasone	50-02-2	392.46	M	Steroidal, Anti-inflammatory, Anti-Rheumatic
15	Papaverine	61-25-6	375.85	M	Opium alkaloid antispasmodic drug
16	Lovastatin	75330-75-5	404.54	M	Hypocholesterolemic drug
17	Verapamil-HCl	152-11-4	491.06	M	Calcium channel blocker, Antiarrhythmic agent
18	Methotrexate	133073-73-1	454.44	S	Abortifacient, Anti-Rheumatic, Antitumor
19	D-Penicillamine	52-67-5	149.21	S	Chelating agent
20	Ochratoxin A	303-47-9	403.81	S	Toxin produced by Aspergillus ochraceus
21	Retinoic Acid	302-79-4	300.44	S	Metabolite of vitamin A (retinol)

Results

Exposure to toxicants resulted in increased cell death and reduced EB area in mouse ESCs. Tested chemicals were roughly categorized based on their typical concentration-response curves obtained for EB area and the viability of ESCs and 3T3 cells over the tested concentration range of each test chemicals. Strongly embryotoxic chemicals including methotrexate, ochratoxin-A, and retinoic acid inhibited growth and differentiation of EBs and showed a high cytotoxicity to 3T3 cells and ESCs at very low concentrations. As the toxic potency of the chemical increased, the concentration at

which cellular viability, growth, and differentiation rapidly decreased. The EB area was reduced in a dose-dependent manner by the tested chemicals (Fig. 1B), and a decrease in EB area resulted in a decline in beating ratio during cardiac differentiation. The 50% inhibitory concentration of EB area ($ID_{50}EB$) was highly correlated with the $ID_{50}CM$ (correlation coefficient, 0.8842). Thus, the EBT can reflect not only the cytotoxicity of a chemical but also the differentiation toxicity. The developmentally toxic levels of various chemicals were evaluated and classified by using a prediction model (PM) based on $IC_{50}ESC$, $IC_{50}3T3$, and $ID_{50}EB$ (Fig. 2A–C). To classify the chemical results into four classes, the EBT-PM included four linear functions that could best divide groups with different characteristics along a plane. Among the results of functions I, II, III, and IV of the EBT-PM, if the value of linear function I is the largest, the group is classified as non-embryotoxic. If the value of function II is largest, the toxicity is weak. If function III is largest, toxicity is moderate, and if function IV is largest, toxicity is strong (Fig. 2D).

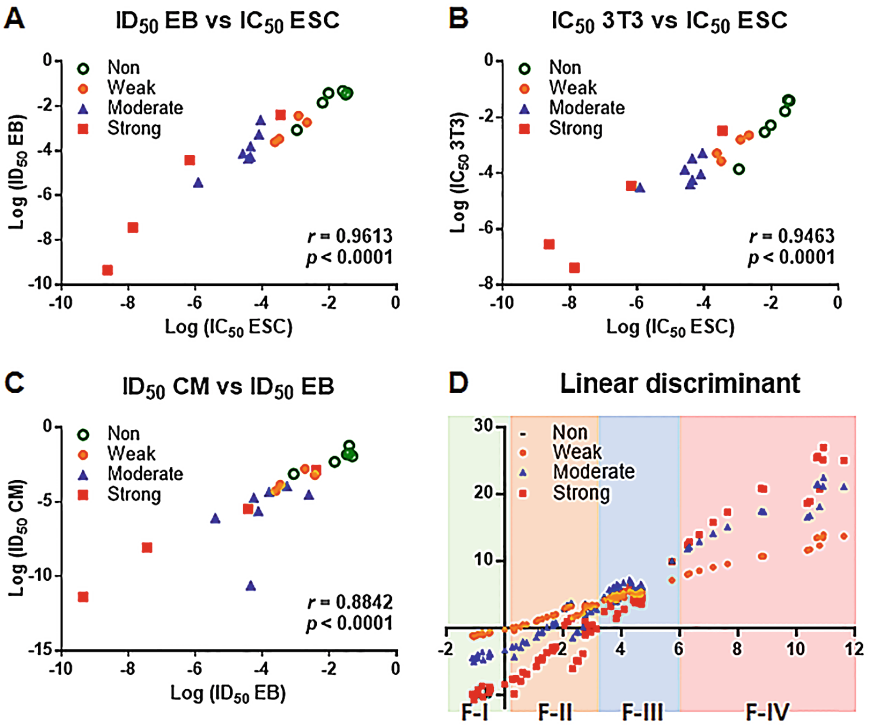

Fig. 2. Relationship between endpoints and classification for 21 chemicals. On the log scale, the correlation coefficients between (A) $ID_{50}EB$ and $IC_{50}ESC$, (B) $IC_{50}3T3$ and $IC_{50}ESC$, and (C) $ID_{50}CM$ and $ID_{50}EB$ were 0.9613, 0.9463, and 0.8842, respectively. (D) Non-, weak, moderate, and strong toxicants were classified according to the highest value among functions I, II, III, and IV.

Discussion

The EBT assesses the developmental toxicity of chemicals at three endpoints: viability of ESCs, viability of 3T3 cells, and reduction in EB area. The three EBT endpoints were verified by performing experiments independently and repeatedly (three or more times in triplicate; 3 × 3). In comparison with the toxicity classification according to *in vivo* data, the EBT-PM showed a prediction accuracy of 90.5%. Developmental toxicants at toxicity-inducing concentrations resulted in a reduction in EB area and a deterioration of EB quality, which indicate the potential for growth retardation and abnormal differentiation in embryos. Chemical treatments resulted in dose-dependent decreases in the EB area via epigenetic inhibition of differentiation and arrest of the cell cycle. To determine the reliability and relevance of the EBT, a validation process is required. In this study, the initial evaluation determined that EB area assessment could be used instead of CM assessment to indicate the differentiation-related toxicity of chemicals. The EBT is a novel toxicological screening system that can facilitate rapid evaluation of embryotoxicants.

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