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Assessment of Developmental Toxicants using Human Embryonic Stem Cells

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Embryonic stem (ES) cells have potential for use in evaluation of developmental toxicity because they are generated in large numbers and differentiate into three germ layers following formation of embryoid bodies (EBs). In earlier study, embryonic stem cell test (EST) was established for assessment of the embryotoxic potential of compounds. Using EBs indicating the onset of differentiation of mouse ES cells, many toxicologists have refined the developmental toxicity of a variety of compounds. However, due to some limitation of the EST method resulting from species-specific differences between humans and mouse, it is an incomplete approach. In this regard, we examined the effects of several developmental toxic chemicals on formation of EBs using human ES cells. Although human ES cells are fastidious in culture and differentiation, we concluded that the relevancy of our experimental method is more accurate than that of EST using mouse ES cells. These types of studies could extend our understanding of how human ES cells could be used for monitoring developmental toxicity and its relevance in relation to its differentiation progress. In addition, this concept will be used as a model system for screening for developmental toxicity of various chemicals. This article might update new information about the usage of embryonic stem cells in the context of their possible ability in the toxicological fields.

Key words: Embryonic stem cells, Embryoid body, Developmental toxicity, Differentiation

INTRODUCTION

Developmental toxicants are defined as substances that exert adverse effects on reproductive tissues or embryo; these effects are associated with birth defects, low birth weight, and biological dysfunction. About 10% of birth defects are related with environmental factors including therapeutic agents and developmental toxicants. Experimental animal or mammalian cells have been used in an attempt to assess *in vivo* or *in vitro* toxicity, respectively. Although animal study provides more precise physiological conditions for evaluation of toxic chemicals, it also has some restrictions, such as economic and ethical issues (1). In an effort to avoid these restrictions, many research

groups have focused on *in vitro* toxicity testing using cell, tissues, organ and whole embryo culture. However, none of these *in vitro* tests has been successfully validated. For these reasons, some toxicologists were interested in embryonic stem (ES) cells, which have potential for differentiation into three germ layers, endoderm, mesoderm, and ectoderm (2). Using mouse ES cells derived from the inner cell mass of pre-implantation mouse embryos (3), embryonic stem cell test (EST) was performed for assessment of the embryotoxic potential of compounds (4); this method was established by the European Centre for the Validation of Alternative Methods (ECVAM) (5-7). In more detail, the EST is assessed by formation of embryoid bodies (EBs) indicating the onset of differentiation of ES cells during early embryogenesis (Fig. 1). Using the EST method, many toxicologists have refined the developmental toxicity of a variety of compounds (8-11). Using the EST method, developmental toxicants were also examined by the 50% inhibition of cytotoxicity (IC_{50}) and 50% inhibition of differentiation (ID_{50}) values (12), and were then divided into three major classes, non-embryotoxic, weakly embryotoxic, and strongly embryotoxic (13,14). Based on the cytotoxic and differentiated endpoints of the ES-EBs system (Fig. 1), recent stud-

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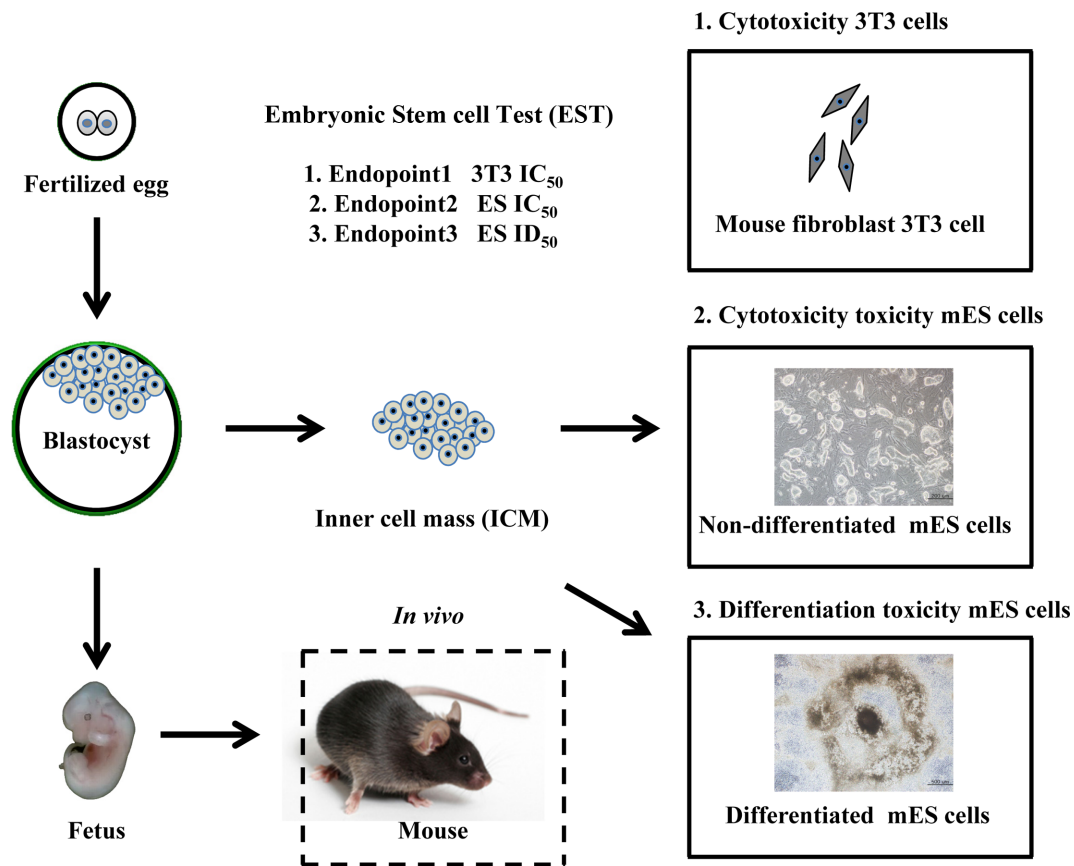E-mail: ebjeung@chungbuk.ac.kr

Fig. 1. Schematic overview of the EST method. The figure illustrates the principle and the endpoints of the EST method to assess the developmental toxic chemicals using permanent cell lines: 3T3 fibroblast and mouse ES cells.

ies have used modified EST methods, including reporter gene assay (15,16) and analysis of differentiation markers (17,18).

In this review, we introduce the EST method for developmental toxicants using an *in vitro* system featuring pluripotent ES cells, and share our knowledge of the toxicological aspect in developmental progress of toxicant-exposed embryonic stem cells.

EMBRYONIC STEM CELL TEST (EST)

Embryonic stem cell test consisted of two kinds of experiments conducted using a cytotoxicity test and a differentiation test. For the cytotoxicity test, both mouse blastocyst-derived embryonic stem cell line D3 and mouse fibroblast 3T3 cells were introduced in various ranges of concentration of test chemical, and the viabilities of these cells were determined by MTT test and schematized by dose response curve. To determine whether presence of the test chemical system in the mouse ES-EB system can influence differentiation progress, D3 mouse ES cells were cultured in the absence of murine leukemia inhibitory factor using the hanging-drop and suspension culture method until day 5

(19). After growth of EBs, they were incubated with a culture flask on day 10 and evaluated by morphological change of cardiomyocyte differentiation (4,20). In evaluation of toxic chemicals using the EST method, 78% of 20 reference compounds showed toxicity matching, as reported in several previous studies (14). Pharmaceutical and chemical companies evaluated their compounds that have proven to be useful for applications of non-clinical testing (11).

While these latter observations are interesting, the validated EST failed to address their embryotoxic potential in the 13 substances because these compounds might cause relevant defect to other tissues apart from inhibition of cardiac cell differentiation (21). More recently, many toxicologists have focused their attention on refining the EST method, including extra assays (22) or replacing some steps of the original EST Standard Operating Procedure (23,24). Because embryonic cells have characteristics of pluripotency, such as an endodermal, mesodermal, and ectodermal originated germ layers (25,26), recent issues include the additional ES cell differentiation endpoint and molecular approaches to detection of toxic effects on embryonic development. In recent studies, a luciferase reporter gene assay with constructs containing the heart and neural crest

derivatives expressed transcript 1 (Hand1) and cardiomyopathy associated 1 (Cmya1) promoters were introduced in mouse ES cells (16). In addition, several differentiation-lineage marker genes were examined in the presence of developmental toxic chemicals on mouse ES cells (27). Using a fluorescence-activated cell sorting system, the cardiac differentiation marker (sarcometric myosin heavy chains) of ES cells was used for evaluation of developmental toxic chemicals (22).

DEVELOPMENTAL TOXICANTS ON DIFFERENTIATED MOUSE ES CELLS

Using embryonic stem cells with characteristics such as cell proliferation and pluripotent differentiation, many chemicals including hydroxyurea, 5-fluorouracil, cytosine arabinoside, indomethacin and dexamethasone, have investigated to evaluate the effects of developmental toxicity. Although these chemicals are used widely as anticancer drugs and anti-inflammatory agents, they also exert developmental toxicity, causing disrupted development of the embryo due to interference in DNA synthesis or protein production (28-31). Early studies have shown that treatment of rat and rabbit embryos with cytosine arabinoside results in abnormalities of the head, mandible, and limb bud (32). In a more recent study, exposure to cytosine arabinoside during pregnancy resulted in a reduction in the number of fetuses as well as a decrease in fetal body weight in mice (33). Another chemical, 5-fluorouracil, which was highly used as an anti-cancer drug, increasingly induced malformation of rat embryos in dose and time-dependent manner

(34). In addition, 5-fluorouracil was reported to exert developmental toxic effects on pregnant rodents (29,35). Following exposure to hydroxyurea, increased levels of some apoptosis-related markers and cell cycle markers have been reported in the mouse fetal brain (36). In addition, hydroxyurea causes a significant decrease in developmental progress of rodent embryo (28). In correlation with the *in vivo* result in which hydroxyurea exhibited anti-proliferative activity on decidua of pregnant rats (37), the embryotoxicity of hydroxyurea is indicative of reduction of cell viability and inhibition of differentiation. In addition, the anti-cancer drugs used (hydroxyurea, 5-fluorouracil, and cytosine arabinoside) have been reported to reduce implantation rate and fetal/placental weight (37-39). Based on this evidence, our study provides direct evidence indicating that this also occurs in fetal development during embryogenesis. Indomethacin has been reported to have many effects on cerebral, mesenteric, and renal hemodynamics as well as renal tubular function in fetuses (40,41). Like anti-cancer drugs, anti-inflammatory agents (indomethacin and dexamethasone) inhibited implantation during early pregnancy (42,43). In parallel with above evidences, it is interesting that diameter of EBs was decreased in response to treatment with developmental toxic chemicals in our unpublished data.

Of particular interest, accumulation of dead mouse ES cells was observed in the vehicle treated control group when compared with undifferentiated mouse ES cells in medium supplemented with leukemia inhibitory factor (LIF) cytokine. Accumulation of apoptotic cells is not unprecedented because accumulation of spontaneous apoptosis occurs within differentiated mouse ES cells in medium depleted of
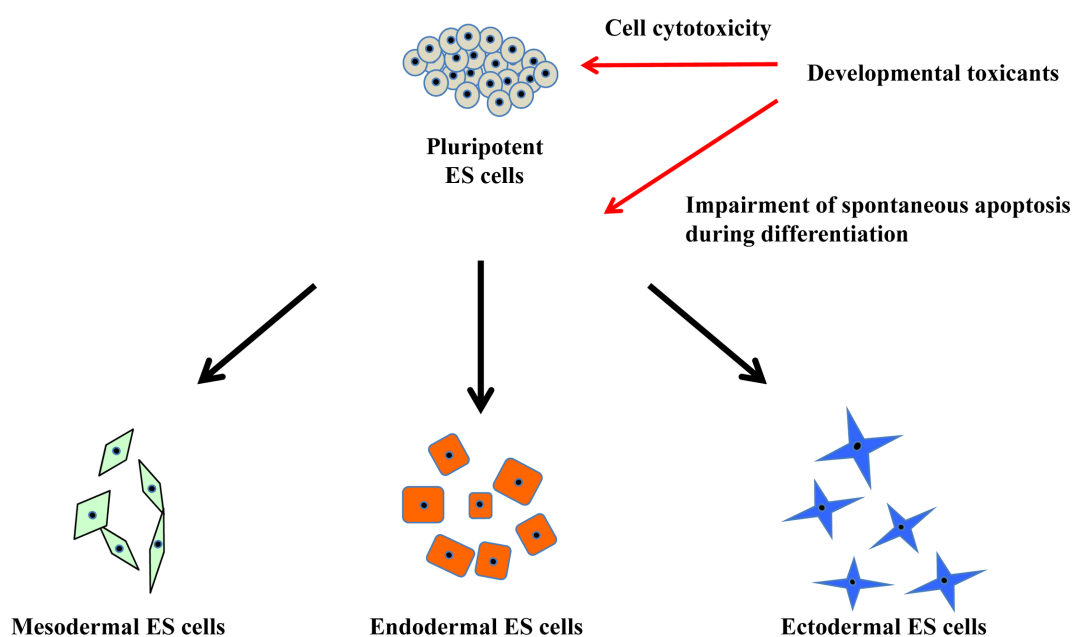

Fig. 2. Scheme for differentiation-related endpoints using developmental toxicants.

LIF cytokine (44). Although the toxicants used have been shown to induce apoptosis *via* inhibition of DNA synthesis (45-47), it was observed that an inverse relationship between cytotoxicity and apoptotic activity in differentiated mouse ES cells in response to developmental toxicants is not predominantly mediated by apoptotic activity in our recent data and that treatment with toxicants may interfere with proper early developmental mechanisms such as apoptosis. While the apoptotic markers including Caspase-3 and PARP were decreased in toxicant-exposed EBs, necrotic marker (Hmgb1) was detected in culture media of EBs. Thus, developmental toxicants may interfere with differentiation of mouse ES cells linked with spontaneous apoptotic cell death (Fig. 2). In addition, it is clear that cell deaths, which are enhanced by the presence of toxicants, are necrosis-dependent, at least in mouse ES cells.

We also demonstrated that un-differentiation marker was induced by treatment with hydroxyurea, 5-fluorouracil, and cytosine arabinoside. While one study reported that ectodermal and mesodermal markers on human ES cells were decreased after treatment with hydroxyurea (18), it was found that the relative levels of ectodermal marker and mesodermal marker were consistent with vehicle control, and endodermal marker was significantly increased in differentiated mouse EBs. As reported in a previous study in which cytosine arabinoside induced differentiation into ectoderm in parallel with inhibition into mesodermal differentiation in human ES cells, it was also related with a decrease of mesodermal markers and an increase of ectodermal markers in differentiated mouse EBs (48).

DEVELOPMENTAL TOXICANTS ON UNDIFFERENTIATED HUMAN ES CELLS

In some recent studies, it was observed that human embryo stem (ES) cells are used for detection of developmental toxicity of chemicals (49-51). Since genetic or physiological diversity between humans and mice has been reported in pre-implantation (52), the use of human ES cells might provide more accurate endpoints. While undifferentiated mouse ES cells have been used as ESTs, we evaluated embryotoxic chemicals in the vitro system using undifferentiated human ES cells because use of mouse ES cells has reached its limits due to interspecies distinction (53). When we modified the protocol for measurement of IC_{50} values after exposure of embryotoxic chemicals for up to seven days, the IC_{50} values of embryotoxic chemicals were similar to those reported by other groups using ESTs with mouse ES (14) and human ES cells (18,54). This result suggests that our system was appropriate for assessment of the IC_{50} values of embryotoxic chemicals within a relatively short period. For identification of other marker genes that are influenced by the presence of embryotoxic chemicals in human ES cells, we performed gene expression profiling

using microarray. While treatment with non-embryotoxic penicillin G induced a change in expression of a small number of genes, the tested toxicants altered the levels of a high number of genes. As expected, a number of genes involved in embryonic development, cell cycle regulation, and apoptosis were significantly affected by the toxicants, and this is consistent with previous microarray studies using mouse stem cells (55,56). For example, down-regulated expression of OCT-4 and NANOG genes is associate with impairment of embryo development because the loss of OCT-4 and NANOG expression in mouse embryos causes failed development of the inner cell mass (57,58). In this regard, we observed down-regulated expression of human ES cell markers, including OCT-4 and NANOG genes, which have an essential role in early development. In summary, our system may help to elucidate toxicant-dependent alterations of ES cell-specific gene expression in undifferentiated human ES cells. More importantly, the stimulation or repression of toxicant-responsive genes is important for assessment of new chemicals in undifferentiated human ES cells.

DEVELOPMENTAL TOXICANTS ON DIFFERENTIATED ES CELLS

In order to build a screening system for developmental toxicants that could impact the embryonic developmental stage, many toxicologists have focused on mouse ES cells. In many earlier studies, it was reported that developmental toxicants impaired the differentiation of mouse ES cells in company with cytotoxicity. In our recent study, we examined their effects on formation of EBs using human ES cells because of some limits due to species-specific differences between humans and rodents. First, we established an alternative *in vitro* system using differentiated human ES, such as neuronal differentiation or cardiomyocyte differentiation. In an investigation of differentiation markers during differentiation of human ES cells, we found that developmental toxicants within human ES cells are able to cause a marked increase or decrease in relative abundance of differentiation-related genes. These differentiation-related genes include NR4A2, an intracellular transcription factor that plays a key role in maintenance of the dopaminergic neuron (59). As a solute carrier protein, SLC1A2 is the principal transporter that clears the excitatory neurotransmitter glutamate from the extracellular space at synapses in the central nervous system (60). CNP is expressed exclusively by oligodendrocytes in the CNS, and the appearance of CNP seems to be one of the earliest events of oligodendrocyte differentiation (61). GAD1 encodes one of several forms of glutamic acid decarboxylase, which catalyzed production of gamma-aminobutyric acid (GABA) from L-glutamic acid (62). When quantitative RT-PCR measurements of specific transcripts in dexamethasone, hydroxyurea,

or cytosine arabinoside treated human ES cells were compared with those in control human ES cells, four (SLC1A2, glutamartergic neuron marker; GAD2, GABA neuron marker; CNP, oligodendrocyte marker; GAD1, GABA neuron marker) genes were highly reduced in toxicant treated human ES cells. The relative levels of NR4A2 (domainergic neuron marker), SLC1A2, and GAD2 mRNA were higher in indomethacin treated human ES cells than in control human ES cells, and the transcriptional levels of glutamartergic or GABA neuron markers were higher in 5-fluorouracil treated cells.

To determine whether or not cardiomyocyte differentiation in human ES cells is due to a developmental toxicant, a separate set of experiments was conducted in which human ES cells were incubated in medium containing toxic chemicals. As a cardiomyocyte indicating marker, the presence of cTnI is used as a marker of differentiation because it is reported in cardiac muscle cell in myocardium, and plays an important role in regulation of skeletal and cardiac muscle contraction (63). As a calcium channel, RYR2 is found primarily in cardiac muscle, and its role in cardiomyocyte contraction has been studied (64). MYL3 is a gene encoded in myosin light chain 3, which is expressed in ventricular muscle in heart (65). While the relative abundance of heart specific transcripts in human ES cells was induced by dexamethasone, the results showed that several cardiomyocyte specific transcripts in human ES cells were decreased by treatment with toxicants, including hydroxyurea, 5-fluorouracil, and cytosine arabinoside.

PERSPECTIVES

These studies have increased our understanding of how human ES cells could be used for monitoring developmental toxicity and its relevance in relation to its differentiation progress by well known-developmental toxicants. They have also provided new tools for evaluation of toxic chemicals in the context of their possible actions in the extracellular environment of embryonic stem cells. However, human ES cells also have some limitations by sensitive culture condition and low ability of growth/development compared with those of cancer cell lines. In addition, this method requires a relatively long period of time for formation of embryonic bodies. Nevertheless, the relevancy of our experimental method is more accurate than that of EST using mouse ES cells. Therefore, we suggest that human ES cells may be useful for testing the toxicity or differentiation impairment of chemicals that could have an impact on the embryonic developmental stage.

ACKNOWLEDGEMENTS

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