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Development of an In Vitro System Detecting Pro-embryotoxin

Masaaki Miyata*, Etsuko Tamura and Yasushi Yamazoe

Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-ku Sendai 980-8578, Japan

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ABSTRACT—An in vitro system for detection of embryotoxins has been developed by using primary cultures of embryo fibroblasts. Various embryotoxins, including benzo[*a*]pyrene and thalidomide, have trivial cytotoxicity in embryo fibroblast systems, which is at least in part due to a lack of capacity for metabolic activation. Introduction of steps for microsomal pre-incubation and calcium-precipitation prior to chemical contact resulted in the clear appearance of embryotoxicity toward thalidomide and benzo[*a*]pyrene. This pre-incubation method will offer advantages for the detection of embryotoxins, which require maternal metabolic activation, and for understanding the mechanisms of their metabolic activations.

Keywords: Embryo fibroblast, Embryo toxicity, Pre-incubation

Drugs and environmental chemicals exert their toxicities in the embryo or fetus through formation of their reactive intermediates. Inert compounds are converted in vivo to their highly toxic reactive intermediates by drug-metabolizing enzymes in the target organ (embryo) and/or maternal organs (1 – 3). Thus, the metabolic activation potency is a key factor to alter the embryo toxicity of chemicals, which undergo the metabolic biotransformation in the maternal body/embryo. However, many mammalian cell lines have poor metabolic activation potencies, so the cell lines generally display limited sensitivities to toxic compounds. External metabolic activating capacity has been introduced to detect the cytotoxicity and mutagenicity of chemicals in test systems with mammalian cells (4, 5). In these systems, cells were directly contacted with microsomal reaction mixtures containing an NADPH generating system and test compound.

In the present study, primary cultures of embryo fibroblasts derived from mouse embryos (gestational day 14) were used to detect the toxicity of embryo toxic compounds. Primary culture of mouse embryo fibroblasts has been shown to have metabolic activation potency (6) and to respond to treatment with xenobiotics (7 – 9). Cytotoxicity of several chemicals was, however, not detected by direct treatment (Table 1). Thus, a pre-incubation step was introduced to the embryo fibroblast assay to compensate for the metabolic activation potency of embryo fibroblasts. In this protocol, chemicals are biotransformed in microsome /cytosol reaction mixtures. Then the mixture was treated with calcium chloride to precipitate microsomal protein. The resultant supernatant is in contact with embryo cells within a short period.

Phenytoin, 7,12-dimethylbenz[*a*]anthracene (DMBA), benzo[*a*]pyrene carbamazepine, naphthalene and neutral red were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Thalidomide (racemate) was purchased from Tocris Cookson Inc. (Ballwin, MO, USA). Dulbecco's modified Eagle's medium and penicillin-streptomycin were obtained from GIBCO BRL (Santa Clara, CA, USA). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was kindly provided by Dr. Keiji Wakabayashi (National Cancer Research Institute, Tokyo). Pregnant New Zealand white rabbits and C57BL/6 mice were purchased from Charles River Japan, Inc. (Yokohama). Anti-rat microsomal epoxide hydrolase (mEH) antibody was provided by Dr. James P. Hardwick (Northeastern Ohio University College of Medicine) and anti-rat CYP1B1 antibody was provided by Daiichi Pure Chemicals (Tokyo).

All experiments were performed in accordance with Guidelines for Animal Experiments of Tohoku University. Primary cultures of embryo fibroblasts were prepared as described previously (7). Mice at GD14 were euthanized, the embryos placed in phosphate-buffered saline (pH 7.4), and the internal organs and head were removed. The remaining torsos were minced and suspended in 0.25% trypsin for 40 min at 37°C. The reaction was stopped by the addition of an incubation medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml

*Corresponding author. FAX: +81-22-217-6826
E-mail: miyata@mail.pharm.tohoku.ac.jp

Table 1. Cytotoxicity of various chemicals in mouse embryo fibroblasts

Chemical	Viability (% of control)				
	1	3	10 (μM)	30	100
Thalidomide	103 ± 7	102 ± 8	101 ± 11	98 ± 8	102 ± 9
Phenytoin	105 ± 8	118 ± 18	118 ± 10	129 ± 11	114 ± 14
Carbamazepine	113 ± 20	109 ± 2	114 ± 12	105 ± 11	96 ± 5
Naphthalene	113 ± 4	ND	109 ± 8	ND	106 ± 9
Benzo[*a*]pyrene	104 ± 7	98 ± 8	100 ± 2	97 ± 7	99 ± 17
IQ	ND	ND	ND	96 ± 14	67 ± 11
PhlP	105 ± 4	1058 ± 8	100 ± 5	81 ± 6	55 ± 6
Trp-P-2	ND	ND	ND	39 ± 10	7 ± 4
DMBA	42 ± 8	32 ± 5	20 ± 5	8 ± 3	ND

Data represent the mean ± S.D. (n = 12). IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PhlP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; DMBA, 7,12-dimethylbenz[*a*]anthracene; ND, not determined.

amphotericin B and 2 mM L-glutamine). The embryo fibroblasts were cultured at 37°C in an atmosphere of 5% carbon dioxide for 48 h. The cells were trypsinized and seeded into 96-well plates at a density of 8×10^3 cells/well in 100 μl of medium. Following incubation for 24 h, the cells were replaced to the medium (100 μl/well) containing chemicals or microsomal metabolites of chemicals and incubated for a successive 48 h. The cells were treated with neutral red solution (50 μg/ml) for 3 h and then were fixed with 1% formalin solution containing 1% $CaCl_2$. After the neutral red was extracted with 50% ethanol containing 1% acetic acid, the viability of embryo fibroblasts was measured by absorbance at 540 nm.

Hepatic microsomes were prepared from mice at gestational day (GD) 14 or rabbit at GD 19. Incubation mixtures for embryo fibroblasts in twelve wells contained 1.2 mg of microsomal protein, a substrate (benzo[*a*]pyrene or thalidomide) in DMSO (1.2 μl), and Dulbecco's modified Eagle's medium (pH 7.0) in a final volume of 1.2 ml. To initiate the reaction, an NADPH generating system (2.5 mM glucose-6-phospate, 0.8 mM $NADP^+$, 1.4 U/ml glucose-6-phosphate dehydrogenase and 8 mM $MgCl_2$, as final concentrations) was added to the reaction mixture. Incubations were carried out at 37°C under subdued lighting for 30 min, and then 2.4 μl of 4 M $CaCl_2$ solution was added into the reaction mixture to terminate the reaction (10). The reaction mixtures were immediately centrifuged for 5 min at $14,000 \times g$ to remove microsomes. Embryo fibroblasts in twelve wells were treated with the supernatant of the reaction mixture in which fetal bovine serum (final concentration of 10%) and antibiotics were included.

Results are expressed as the mean ± S.D. for each experiment and analyzed by the unpaired Student's *t*-test. A *P* value of less than 0.05 was considered significant.

To evaluate the susceptibility of embryo fibroblasts, mouse embryo fibroblasts were treated with various chemicals including DMBA, PhIP, benzo[*a*]pyrene, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), thalidomide, phenytoin, carbamazepine and naphthalene for 48 h (Table 1). Some chemicals such as DMBA, Trp-P-2, IQ and PhIP significantly reduced embryo fibroblast viability at a concentration less than 100 μM. Interestingly, polyaromatic hydrocarbon, DMBA, but not benzo[*a*]pyrene produced a strong cytotoxicity in embryo fibroblasts. DMBA is shown to undergo activation by cytochrome P450 (CYP) 1B1 and mEH (7, 8, 11). To understand the metabolic potency of embryo fibroblasts, microsomal levels of CYPs and mEH were quantified or determined by immunoblotting (Fig. 1). Immunoblots indicated the presence of CYP1B1 and mEH in embryo fibroblasts, while CYP3A and CYP1A forms

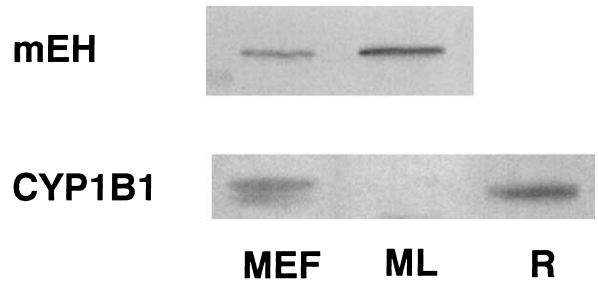

Fig. 1. Immunoblot analyses of mEH and CYP1B1. Microsomes were prepared from mouse embryo fibroblasts cultured for 3 days and pregnant (GD14) mouse livers. Microsomes loaded were 10 μg (mEH) and 25 μg (CYP1B1). Mouse mEH protein was detected with anti-rat mEH antibody, and mouse and rat CYP1B1 proteins were detected with anti-rat CYP1B1 antibody. MEF, mouse embryo fibroblast; ML, mouse liver; R, rat CYP1B1.

were not detected (data not shown). These results indicate the limited metabolic potency of embryo fibroblasts, which is not sufficient to activate a broad class of teratogens and carcinogens. Thus, we have tried to establish a cytotoxicity detecting system combined with a pre-incubation step to detect a broad class of embryo toxic chemicals. It is well known that metabolically activated forms of benzo[*a*]pyrene induce teratogenesis (12). The cytotoxicity of benzo[*a*]pyrene was, however, not observed at a concentration less than 100 μM in embryo fibroblasts. Thus, benzo[*a*]pyrene was selected as a first model compound to develop the detection system combined with the pre-incubation step. Microsomes for pre-incubation were prepared from livers of pregnant animals. To remove any influence of microsomal components on cell viability of embryo fibroblasts, microsomal components in the pre-incubation mixture were precipitated with $CaCl_2$ and removed by centrifugation (10). Inclusion of the pre-incubation step resulted in reduced cell viability. To optimize the pre-incubation condition, the experiment was done with various microsomal protein amounts and pre-incubation periods. Decrease in the viability of embryo fibroblasts was dependent on the amount of microsomes in the pre-incubation mixture (data not shown). Benzo[*a*]pyrene metabolites produced during 30-min incubation with microsomal protein (100 μg/100 μl reaction mixture) and 100 μM benzo[*a*]pyrene caused more than 50% reduction of cell viability in embryo fibroblasts. Microsomal metabolites of 100 μM benzo[*a*]pyrene for a 30-min incubation produced higher cytotoxicity of embryo fibroblasts than those for a 15-min incubation (data not shown). Thus, chemicals were pre-incubated with a 100 μg/100 μl reaction mixture of hepatic microsomes for 30 min in the following experiments.

Viability of embryo fibroblasts treated with mouse hepatic microsomal metabolites of benzo[*a*]pyrene was reduced in a substrate-concentration-dependent manner (Fig. 2A). Although the viability of embryo fibroblasts was not affected by the treatment with mouse hepatic microsomal metabolites of thalidomide, the viability of embryo fibroblasts was decreased to less than 80% of the control level with rabbit microsomal metabolites of thalidomide (Fig. 2B). These results demonstrate the usefulness of a cytotoxicity assay using embryo fibroblasts in combination with the pre-incubation method to detect embryo toxic compounds, although further experiments using other toxic compounds were necessary to evaluate the assay system.

In the present study, calcium-precipitation was used to remove microsomes and NADPH generating system. Introduction of steps for microsomal pre-incubation and calcium-precipitation resulted in an approximately 10% increase in cell viability of the vehicle control. To identify whether calcium concentration in the culture medium is involved in the cell toxicity, we have examined the cell toxicity of thalidomide by the ultracentrifugation (125,000 × *g* for 5 min) method, instead of calcium-precipitation. The ultracentrifugation method also showed a decrease in the cell viability to the same extent as the calcium-precipitation method, suggesting that the cell toxicity was independent of calcium concentration in this experimental condition.

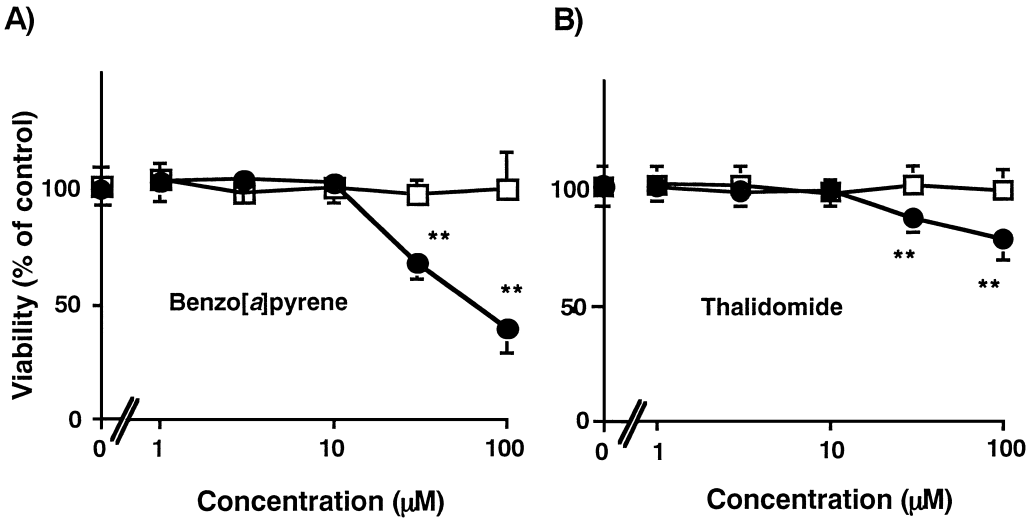

Fig. 2. Effect of pre-incubation with hepatic microsomes on cytotoxicity of benzo[*a*]pyrene and thalidomide. Benzo[*a*]pyrene (A) or thalidomide (B) was incubated with mouse liver or rabbit liver microsomes (1.2 mg), respectively, in the reaction mixture (1.2 ml). Mouse embryo fibroblasts were treated with the supernatant of the reaction mixture by calcium-precipitation for 48 h. Cell viability was measured by neutral red assay. Substrate concentration in the reaction mixture is indicated in the figure. Values represent the mean ± S.D. from 12 experiments. Significant difference from the vehicle control (**P<0.01). Closed circles, 30-min incubation; open squares, no incubation.

This assay system has the advantage that cytotoxicity derived from microsomes and NADPH generating system is omitted. Furthermore, this assay system which separates the metabolic activation of chemicals from culture of embryo fibroblasts might be useful for analyzing the metabolic activation mechanism of embryo toxic compounds, because chemical inhibitors or antibodies to drug metabolizing enzymes as well as microsomes from different species or different organs are applicable to this assay system. Our preliminary experiments show that the addition of P450 inhibitors or anti-P450 antibody suppresses the thalidomide-induced cytotoxicity in this system. We are currently analyzing the mechanisms for the bioactivation of thalidomide using this assay system.

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