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Methods for Evaluating Teratogenic Activities of Chemicals and Serum Fluids Using Micromass Culture System

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Summary

Mouse or rat embryonic midbrain (MB) and limb bud (LB) cells were prepared from day 10–13 embryos and cultured as micromass cell islands for 5–7 days. Differentiation was determined by the number of stainable foci of differentiated cells, and the concentration at which each compound inhibited the formation of differentiated foci by 50% of the control value (IC50) was estimated for evaluating the teratogenic potentials of compounds.

We apply the micromass teratogen test to the studies on the causes of TBZ-induced teratogenesis and species differences in the ETU-induced teratogenesis.

Introduction

In vitro test systems for detecting teratogens have been developing in recent years.[1] Although these tests could not completely replace whole animal testing, in vitro tests appeared to offer several advantages over the whole animal toxicologic approaches.

In vitro micromass teratogen test using mouse and rat embryonic midbrain (MB) and limb bud (LB) cells has been investigatED for several years in our laboratory.[2~4] The experimental procedures for micromass teratogen test were different among the laboratories, but the usefulness of this systems was reported by many researchers.[5~7]

In vitro micromass teratogen test involves exposing undifferentiated embryonic MB and LB cells to test compounds and observing the subsequent effect on cell differentiation. Some of the most important aspects of cell behavior involved in embryogenesis, such as cell adhesion, movement, communication, division, and differentiation are retained by the micromass cultures, though full pattern formation is not achieved. Therefore, we consider that micromass teratogen test can detect the inhibitory effects of various types of teratogens on the behavior of embryosnic cells.

We report the mechanistic studies on the teratogogenesis induced by thiabendazole (TBZ), and the interpretation of species difference in the teratogenesis induced by ethyrenethiourea (ETU).

Materials and Methods

Animals and treatment.

Pregnant or female JcL/ICR mice (10–12 weeks) were obtained from Nihon Kurea Co. Pregnant Wistar-Imamichi rats (10–12 weeks) were obtained from the Institute for Animal Reproduction (Saitama, Japan). For an *in vivo/in vitro* test system,[8] ETU (50, 100, 200 mg/kg) in distilled water or control vehicle (distilled water, 11 ml/kg) was orally administered to the pregnant females on Day 11 of gestation (day of discovery of copulatory plug=Day 0). The uteri were removed 16 hr later, and the embryos were pooled from two test or control animals. The forelimb buds and midbrain were removed according to the method described previously.[4]

Serum prepartaion.

ETU in distilled water was administered orally to female Wistar-Imamichi rats (10–12

weeks) at doses of 50, 100, or 200 mg/kg. As a control, distilled water (11 ml/kg) was administered to the animals. Animals were anesthetized with ether 2 hr after administration, and blood was gently withdrawn from the dorsal aorta with a syringe and then placed in a centrifuge tube for preparation of the serum. After centrifugation at 3000 rpm for 5 min, the supernatant of the serum sample was placed into filtration tubes (Millipore UFC3TTKOO) and further centrifuged at 6000 rpm for 30 min. These serum filtrates were used as the assay samples in the embryonic MB cell culture system.

Culture and assay methods for rat and mouse embryonic MB and LB cells.

Both MB and LB tissues wree dissociated into individual cells by successive washings in calcium- and magnesium-free balanced salt solution (CMF) and by trypsin (1% in CMF) digestion (15 min, 37°C). Cell suspensions were prepared in culture medium consisting of Ham F12 plus 10% FCS and were adjusted to give 5×10^6 MB cells/ml or 1.4×10^7 LB cells/ml. Twenty-microliter aliquots of the cell suspensions were delivered to each well of 24-well tissue culture plates. The cells were allowed to settle and to adhere to the dish for 2 hr at 37°C. The plate was then flooded with 0.5 ml of culture medium. The adhering cells formed separate micromass islands and were cultured for 5 days at 37°C in 5% CO_2/95% air. The cultures were stained with hematoxylin (MB cultures) or alcian blue (LB cultures) after fixation for the cell differentiation assay. The number of individual foci was counted by use of a dissecting microscope (SZH, Olympus Co., Tokyo).

Assay of cartilage proteoglycan in a mouse imb bud cell system.

The LB tissues were isolated from pregnant mice and dissected from the day 10 mouse embryos, then dissociated in a solution of 0.1% trypsin and 0.1% EDTA in calcium-magnesium-free saline to obtain a single cell suspension. Aliquots of 20 μl of this cell suspension were dropped in the center of the wells of 24 wells Costar dishes. The dishes were placed in an humidified CO_2 incubator at 37°C for 2 hours to allow the cells to attach and then CMRL medium containing 10% fetal bovine serum was added (0.5 ml) to each well. Medium containing various concentrations of the compounds was changed on days 1,3 and 5. The accumulation of sulfated proteoglycan, a measure of the extent of cartilage differentiation, was determined by staining with 0.5% alcian blue on day 6. The bound dye was extracted from the cultures with 0.3 ml of 4 M guanidine HCl, and the absorbance of the extracted solution was measured with a spectrophotometer.

ATP determination

Fore and hind limb buds were quickly-frozen, and extracted with ATP-free 50 mM Tris-4mM EDTA buffer, pH 7.75 for 3 min at 100°C. An aliquot of the extract was mixed with luciferin-Inciferase (lumac), and ATP was determined by using an ATP photometer (Lumac cell tester 1030).[9]

Incorporation of $^{35}SO_4^{2-}$ into APS, PAPS and endogenous acceptor.

Incorporation of $^{35}SO_4^{2-}$ into APS, PAPS and endogenous acceptor was determined as reported.[10]

Analysis of ETU concentration in the serum.

The serum samples were analyzed on a Waters high-pressure liquid chromatograph at 253 nm using a μ Bondapak C18 column (25 cm length, 4.6 mm i.d.). As a mobile phase, a mixture of methanol: H_2O: 0.1 M phosphate buffer (pH 6.4) (30: 60: 10) was used at a flow rate of 1 ml/min. The retention time of ETU was 3.26 min.

Results and Discussion

1. Application of micromass culture for mechanistic studies on teratogenesis induced by thiabendazole (TBZ).

In relation to the mouse limb malformation caused by thiabendazole (TBZ)[11], we tested the teratogenicity of TBZ and its metabolites in a mouse limb bud culture system. The main metabolic pathways of TBZ in mice were

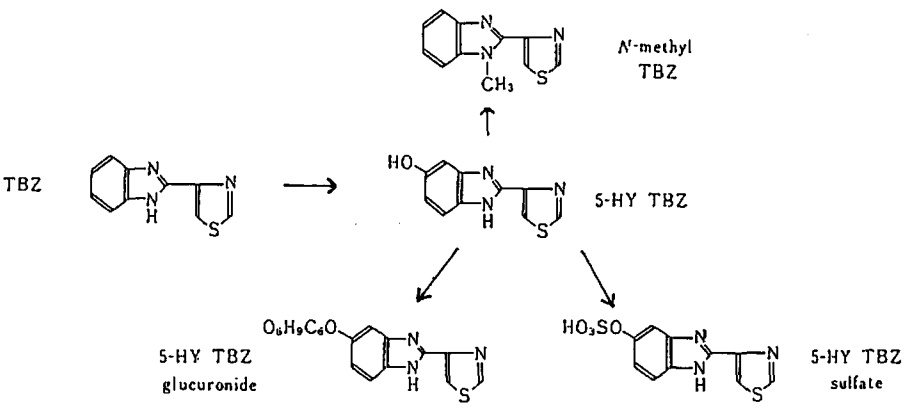

Chart 1. Metabolic pathways of thiabendazole in mice. TBZ: thiabendazole, 5-HY TBZ: 5-hydroxy thiabendazole.

Table 1. Teratogenic potentials of thiabendazole (TBZ) and its metabolites in the limb bud cell culture system.

Compound	IC50 (μg/ml)
Thiabendazole (TBZ)	18
5-hydroxy TBZ	19
5-hydroxy TBZ glucuronide	1000
5-hydroxy TBZ sulfate	1000
N-methyl TBZ	52

IC50: the concentration of compound necessary to reduce alcian blue staining by 50%.

indicated in Chart 1.[12] The teratogenic potentials of the metabolites were estimated and compared as shown in Table 1. The data showed that the IC50 of TBZ, 5-hydroxy TBZ and N-methyl TBZ are about 18, 19 and 52 ug/ml, respectively. However, the conjugated metabolites such as 5-hydroxy TBZ glucuroniode and 5-hydroxy TBZ sulfate were very weakly inhibitory on chondrogenesis. The IC50's of these conjugates were high values of 1000 μg/ml.

Studies on the biosynthesis of proteoglycan as an index of limb chondrogenesis demonstrated that TBZ and 5-hydroxy TBZ directly impaired its biosynthesis in the limb bud cell system. these results may indicate that TBZ and 5-hydroxy TBZ directly causes the abnormalities in mouse limb development.

The IC50 of benzimidazoles, benzoxazoles and benzothiazoles are shown in Table 2. It was found that benzoxazole and benzothiazole showed no inhibitory effet on chondrogenesis at concentrations <0.14 mM. While benzimidazoles exhibited inhibitory activities at concentrations <0.14 mM. Thus, imidazole NH proton in TBZ seemed to be important in the inhibitory action in the mouse cell system.

Next, the effects of the substituted group in benzene ring of 2-(2-pyridyl) benzimidazole were compared using a mouse limb bud cell

Table 2. IC50 values of benzimidazoles, benzoxazoles, and benzothiazoles tested in the mouse limb bud cell culture system.

Compound	IC50 (mM)
2-(4-Thiazolyl)-benzimidazole (TBZ)	0.088
2-(2-Pyridyl)-benzimidazole	0.082
2-(2-Pyridyl)-benzoxazole	>0.14
5-Methoxy-2-(2-pyridyl)-benzimidazole	0.076
5-Methoxy-2-(2-pyridyl)-benzothiazole	<0.14

IC50: the concentration of compound necessary to reduce alcian blue by 50%.

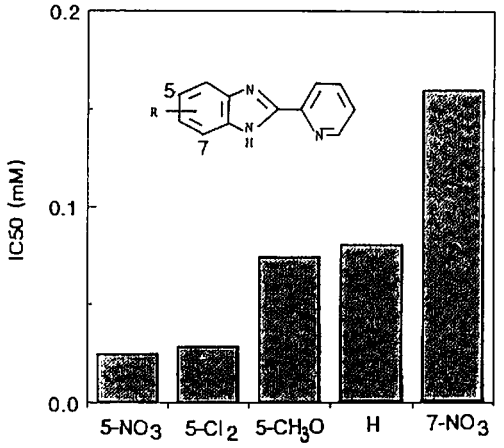

Fig. 1. Inhibitory effects of benzimidazoles on the amounts of cartilage proteoglycan in the mouse limb bud cell culture system.
IC50: the concentration of compound necessary to reduce alcian blue staining by 50%.

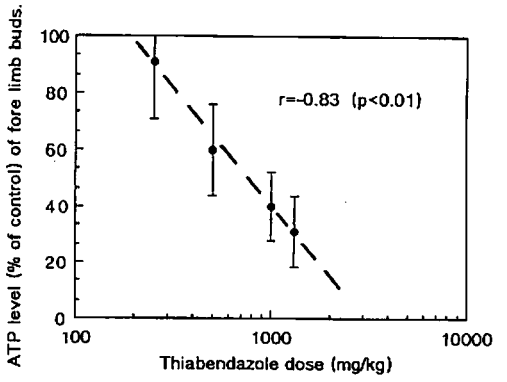

Fig. 2. Correlation between the ATP levels of mouse limb buds and the dose of thiabendazole suspended in olive oil administered to the dams on day 9 of gestation. Bars indicate SD.

culture system (Fig. 1). We found that nitro and chloro groups at 5 position were potent substituens, while 7-nitro derivative showed a weaker inhibition than the orginal compound.[13]

Figure 2 shows the relationship between ATP levels and the concentrations of TBZ administered to mice. ATP levels in the limb buds were measured at day 10 of gestatin in controls and mice treated with the deoses up to 1300 mg TBZ/kg 24 hr previously. The results showed that there was a correlation between the dosage of TBZ and the ATP levels of fore and hind limbs, respectively. This suggests that there may be a relationship between the teratogenic and ATP-depressing actio of TBZ on the limb buds of mouse embryos. A close correlation existed between the concentration of TBZ and ATP levels in fore and hind limb buds.

Table 3 shows a comparison of ATP levels and proteoglycan amounts in the rat limb bud cells after cultivation with TBZ. ATP levels decreased with increasing TBZ concentration. Further, the extents of ATP depletion in rat cells were similar to those of the decrease in cartilage proteoglycan present in the cellular matrix (Table 3).

Table 3. Comparison of cartilage proteoglycan amounts and ATP levels in the rat limb bud cells after exposure of thiabedazole.

Thiabendazole conc. (mM)	Cartilage proteoglycan (%)	ATP level (%)
0	100	100
0.155	70.7	83.1
0.311	57.5	57.9
0.622	33.3	31.7

ATP level of the control group was 1354 ng per well.

^{35}S-APS, ^{35}S-PAPS, and ^{35}S-labeled endogenous product were simultaneously determined by paper electrophoresis after incubation of fore limbs with H_2 $^{35}SO_4$. Fore limbs were pooled from the pregnant day 10 mice treated with TBZ at the dose of 1000 mg/kg 24 hr previously, and incubated with H_2 $^{35}SO_4$. After 100 min of incubation, incorporation of $^{35}SO_4^{2-}$ into endogenous acceptors with TBZ dosed limbs attained only 25% of the control level. The accumulated ^{35}S-APS and ^{35}S-PAPS in the limbs of TBZ-dosed mice were only 53% and 27% of those in control limbs, respectively. Thus, the decreased incorporation of $^{35}SO_4^{2-}$ into endogenous acceptors is presumably due to the limited supply of ^{35}S-PAPS. APS and PAPS ar known to be biosynthesized from ATP.

From these results, the possible association between TBZ-induced teratogenicity and ATP was clarified.[14]

2. In vivo/in vitro test for ETU using micro-

mass culture, and studies on the cause of the species differences in the ETU induced-teratogenesis between rats and mice.

We used an *in vivo/in vitro* approach[8] to determine the teratogenicity of ETU. MB and LB cells were prepared from Day 12 rat embryos after maternal exposure to three different doses of ETU. Figure 3 shows the changes in the differentiated foci produced in each cell island of the MB and LB cells. There was a correlation between the dose of ETU and the levels of differentiated foci in MB and LB cells of rats ($p<0.001$).

It can be seen clearly from the equations in Fig. 3 that the differentiation of MB cells was inhibited more effectively than that of LB cells. There was no difference in the number of differentiated foci between the control group (no treatment) and the group given distilled water only (11 ml/kg) (data not shown).

We are able to evaluate the teratogenicity of ETU using an *vivo/in vitro* test (Fig. 3). Teramoto *et al.*[15] showed that malformations, especially brain and digit defects, in the fetuses of Wister-Imamichi rat were caused by a single oral dose of 50, 100 or 200 mg/kg of ETU. This study[15] reported that the neural tube was more sensitive than the limb buds to the teratogenic action of ETU. Our results (Fig. 3) also showed that the differentiation of MB cells was inhibited to a greater degree than that of LB cells. Thus, the cellular sensitivities coincided with the degree of the malformations observed in the fetus.

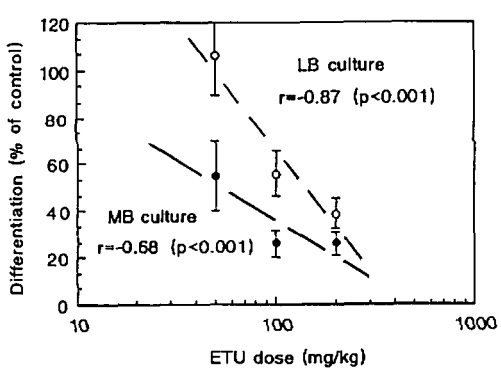

Fig. 3. *In vivo/in vitro* test after exposure of dam to various doses of ETU. Bars indicate SD.

From the equations in Fig. 3, the calculated ETU dose that would caused a 20% inhibition of differentiation was 14.5 mg/kg. In the *in vivo experiments*,[16] ETU induced early histologic changes in the fetal central nervous system. The changes in the fetal neuraxis were unequivocal at the 30 mg/kg dose. At a dose of 15 mg/kg, the changes were far less striking and obscured by spontaneously occurring cell necrosis. Further, the postnatal mortality at 15 mg/kg was not statistically different from that of control. The cellular experiments appeared to be more sensitive than the in vivo experiments reported. It was assumed that inhibitory effects below 20% of the control (Fig. 3) were restored before birth.

The chemical similarlity of ETU to thiourea and thiouracil suggests that ETU acts by blocking the iodination of thyroxine precursors, thus reducing synthesis of the thyroid hormones. Thyroid hormones are thought to play an important role in the differentiation of neuronal tissues.

A recent *in vitro* study[17] with propylthiouracil, a thyroid inhibitor with a mode of action similar to that of ETU, had confirmed that monkeys are much less sensitive than rats. Therefore, it is assumed that rat embryonic midbrain cells may be more sensitive than mouse embryonic cells to the thyroid peroxidase inhibition by ETU. As a result, ETU is teratogenic for rats but not for mice.

The teratogenic activities in the serum samples from rats and mice after ETU dosing were measured using embryonic MB cell cultures (Table 4). When the rat MB cells were used for monitoring the teratogenic activities, both rat and mouse sera exhibited inhibitory activities, however, neither rat nor mouse sera caused an inhibitory effect when the mouse MB cells were used (Table 4).

ETU has a half-life of 9–10 hours in rats and 5 hours in mice. Iverson et al.[19] suggested that the ability of the cat to metabolize extensively ETU may contribute to the absence of teratogenic effects in this species.

Table 4. Monitoring of teratogenic activity in rat and mouse serum samples obtained from animals after administration of ETU using rat and mouse midbrain cell culture systems.

Cell culture System	Group	Rat-serum (% of cont.)	Mouse-serum (% of cont.)
Rat midbrain Cells	Control	100	100
	ETU 50 mg/kg	67	—
	100	27	—
	200	5	12
Mouse midbrain Cells	Control	100	100
	ETU 200 mg/kg	94	116

Table 5. ETU concentration in the serum.

Animals	ETU dose (mg/kg)	ETU concentration (μg/ml)
Rat	50	4.2
	100	9.4
	200	21.8
Mouse	200	10.9

Therefore, it was concluded that mice perhaps metabolize ETU more rapidly than rats. Analyses of the concentration of ETU in the serum filtrates of both species showed that at a dose of 200 mg/kg, mouse serum contained about one-half that present in rat sera (Table 5).

These results indicate that the marked difference of the effects of ETU in th rat and mouse was confirmed in the cell studies. One cause of the species difference in the teratogenicity of ETU between rats and mice may be the different sensitivities of the MB cells in the two species.

The micromass teratogen test is simple, allowing numerous compounds to be rapidly tested at a very low cost. Further, it is useful for the mecahnistic studies and the monitoring of teratogenic activities in biological fluid as described in this report.

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