4. Discussion

A number of international development projects run by organizations such as OECD are being conducted to establish the validity of screening tests for the detection of endocrine disrupting chemicals. In the OECD project, the immature rat uterotrophic assay and the classical uterotrophic assay using ovariectomized female rats have been proposed as potential screening methods, and validation studies are now in progress. The US Environmental Protection Agency has also developed a chemical screening and testing program consisting of a tiered system for evaluating the endocrine disrupting effects of chemicals. The uterotrophic assay has been proposed as a Tier 1 screening test in this system [12]. The immature rat uterotrophic assay is advantageous because the test animals are not traumatized by ovariectomy. However the endocrine status of the immature animal is not stable, and important changes in several hormone levels can occur during the course of the assay [6–9]. Antide is a long-acting gonadotropin-releasing hormone antagonist that inhibits the secretion of endogenous gonadal estrogen by blocking gonadotropin-releasing hormone secretion [10,11]. In this study, uterine and ovarian weights of antide-treated animals were markedly lower than those of control animals.

In response to pituitary FSH and LH, granulosa cells of the primordial follicle undergo hyperplasia and hypertrophy and secrete estrogen. Our finding suggests that this endogenous gonadal estrogen may already be acting on the uterus in the immature animal, producing a proliferative response. Ashby et al. [13] reported that the use of gonadotropin-releasing hormone antagonist in the immature female rat uterotrophic assay enhanced the resolving power of this assay and enabled direct and centrally-mediated endocrine effects to be distinguished from one another. Assays using ovariectomized rats cannot detect centrally-mediated effects of estrogenic chemicals. Although endogenous gonadal estrogen-blockade may enhance the sensitivity of the immature rat uterotrophic assay, the possibility that this protocol may interfere with the ability of the immature rat uterotrophic assay to detect centrally mediated effects cannot be discounted.

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The efficacy of endocrine disruptor screening tests in detecting anti-estrogenic effects downstream of receptor–ligand interactions

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Abstract

Several predictive test methods for endocrine disrupters have been evaluated by international organizations. In this study, we performed a series of predictive tests for endocrine disrupters, i.e. the receptor binding assay, reporter gene assay, and immature rat uterotrophic assay, on all-*trans* retinoic acid (tRA), which may cause antiestrogenic activity via their receptors, interfere with estrogenic action at estrogen responsive element level, and we examine the efficacy of endocrine disruptor screening tests in detecting anti-estrogenic effects downstream of receptor–ligand interactions. Despite showing complete lack of binding affinity to ER in the receptor binding assay, tRA exhibited clear antagonist activity without any agonist activity in the reporter gene assay. In the in vivo test, tRA was subcutaneously administered to immature Crj:CD (SD) IGS rats at doses of 5 and 25 mg/kg per day for 3 days, beginning at 20 days of age. Additional groups of rats given tRA at the above doses were also subcutaneously injected with ethinyl estradiol (EE) at a dose of 0.6 μg per rat per day. A vehicle control group given olive oil alone and a positive control group given EE alone were also established. Although no uterotrophic activity was detected in any of the rats given only tRA, co-treatment with 5 and 25 mg/kg tRA and EE reduced the EE-induced increases in uterine weight. We confirmed that the ER antagonist activity of tRA may be mediated by transcriptional interference after ER–ligand complex binding to an estrogen responsive element of the gene by the gel mobility shift analysis. These findings suggest the reporter gene assay and uterotrophic assay can detect anti-estrogenic effects downstream of receptor–ligand interactions, but the receptor binding assay can not detect this type of interference. In any case, a screening strategy for endocrine disrupters, especially the primary screening battery for prioritizing the chemicals to be tested in the higher screening stages, should be designed to detect various kinds of chemicals possessing endocrine modulating activity including a retinoid-like endocrine modulator. Accordingly, reporter gene assay or uterotrophic assay should be conducted in the early stage of screening process for endocrine disrupting chemicals, because they can detect antagonist activity caused by both inhibition of receptor–ligand interaction and transcriptional interference. Particularly, the reporter gene assay may be a promising prescreening procedure, because it can be adopted in the

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high throughput screening process for thousands of chemicals and it requires no use of experimental animals.

Keywords: Endocrine disrupter; All-*trans*-retinoic acid; Binding assay; Reporter gene assay; Utrotrophic assay

1. Introduction

Several predictive test methods for endocrine disrupters have been evaluated by the Organization for Economic Cooperation and Development (OECD, 2001), and the United States Environmental Protection Agency of the US has developed a tiered system of chemical screening and testing to evaluate the endocrine disrupting effects of chemicals (EDSTAC, 1998). In this tiered system, the hormone-receptor-binding assay and receptor-mediated reporter gene assay are proposed for pre-screening and a Tier 1 screening battery to detect interaction with the endocrine system. The theory of these in vitro predictive tests is based on detection of hormone-receptor-mediated interference with hormonal action.

Retinoids are a family of natural and synthetic compounds structurally related to vitamin A. All-*trans*-retinoic acid (tRA) is known as a teratogen (Elmazar et al., 1996; Kochhar et al., 1995; Mulder et al., 1998; Tsuiki et al., 1996); it possesses anti-estrogenic activity and exerts an inhibitory effect on the growth of breast cancer cells (de Cupis et al., 1995; Eck-Enriquez et al., 2000; Nesaretnam et al., 2000; Wilcken et al., 1997). In addition, anti-estrogenic effect of tRA was reported to be caused by their receptors, interfere with estrogenic action at estrogen responsive element level. In this study, we performed a series of predictive tests for endocrine disrupters, the receptor binding assay, reporter gene assay and immature rat uterotrophic assay with tRA, and examined the efficacy of each predictive test against tRA.

2. Materials and methods

2.1. Chemicals

The all-*trans*-retinoic acid (tRA, Lot No. A0138879, 96.9%) was obtained from Acros Organics (Ceel, Belgium); 4-hydroxytamoxifen, (Lot No. 108H4069, 98.0%) from Sigma Chemical Company (MO, USA), 17β-estradiol (E2, Lot No. ACK5754, 97.0–103.0%) from Wako Pure Chemicals Industries Ltd. (Osaka, Japan), ethinyl estradiol (EE, Lot No. KSF1601, 98.0%) from Wako Pure Chemicals Industries Ltd., ICI 182 780 (Lot No. 2/15 495, purity not specified.) from Tocris Cookson Ltd. (Bristol, UK), olive oil (Lot No. 014OOY) from Fujimi Pharmaceutical, Co. (Osaka, Japan).

2.2. Receptor binding assay

Assay was performed according to the method previously described (Nakai et al., 1999). Briefly, a solution (10 μl) of recombinant human estrogen receptor and its ligand binding domain (expressed in *E. coli* fused with glutathione *S*-transferase) at a concentration of approximately 10 nM was dissolved in Tris–HCl (pH 7.4, 70 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 10 mg/ml γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM leupeptin. After the sample solution (10 μl) and 5 nM [2, 4, 6, 7, 16, 17-^{3}H] 17β-estradiol (10 μl) were added, the solution was incubated for 1 h at 25 °C. Free radioligand was removed by incubation with 0.2% activated charcoal and 0.02% dextran in phosphate buffered saline (PBS) (pH 7.4) for 10 min at 4 °C followed by filtration. Data was analyzed by the computer program ALLFIT (DeLean et al., 1978).

2.3. Reporter gene assay

The full open reading frame of rat ERα cDNA (Genbank Accession No. X61098) was cloned into the pCI mammalian expression vector (Promega Corp., Madison, WI, USA) to make rER/pCI. An oligonucleotide containing three copies of the *Xenopus laevis* vitellogenin A estrogen responsive element (ERE), 5′-GGTACCAAAG TCAGGT-

CACA GTGACCTGAT CAAAAGTCAG GTCACAGTGA CCTGATCAAA AGTCAGGTCA CAGTGACCTG ATCAGAGCTC-3′, was synthesized and concatenated in the upstream region of the rat α_{2u} globulin promoter containing TATA signal. This concatenated fragment was cloned into the multiple cloning site of pGL3 Basic vector (Promega Corp.) to create reporter vector ERE-TATA-Luc+.

A human cervical carcinoma cell line (HeLa229, ATCC No. CCL-2.1) grown in 90 mm dishes to 60–80% confluence was washed twice with PBS. The cells were then transiently transfected with both 2 μg of rERα/pCI designed to express rat ERα and 4 μg of ERE-TATA-Luc+ designed to detect ERE-mediated transcriptional activation with 12 μl of Lipofect AMINE (GibcoBRL) and 60 μl of PLUS reagent (GibcoBRL) according to the manufacturer's protocol. After overnight incubation, the cells were trypsinized, resuspended in Eagle's Minimal Essential Medium (EMEM) without phenol red (Nissui Pharmaceuticals Co. Ltd., Tokyo, Japan) containing 10% DCC-treated fetal calf serum (FCS) (Miller et al., 2000), and plated on a flat-bottomed microplate (Corning Coster Corp., Cambridge, MA, USA) at a density of 10^4 cells per well. To detect agonist activity, each test chemical diluted in DMSO was added to the wells to final concentrations of 10, 1 μM, 100, 10, 1 nM, 100 and 10 pM in quadruplicate. Positive control wells ($n = 6$) treated with a natural ligand (1 nM of 17β-estradiol) and negative control wells ($n = 6$) treated with DMSO alone were prepared at the same time. After adding the chemicals to induce the reporter gene product, the assay plates were incubated for 24 h. After washing three times with PBS, the cells were lysed with cell culture lysis reagent (CCLR, Promega Corp). Luciferase activity was measured with the commercial luciferase assay reagent (Promega Corp.) and a luminometer (LumiStar, BMG Instrument) as integrated value for 5 s. The luminescence signal data were processed and the average and standard deviation (S.D.) for the negative control wells were calculated. The integrated value for each test well was divided by the average integrated value of the negative control wells to obtain individual relative transcriptional activity. The average transcriptional activity was then calculated for each concentration of the test chemical. To detect antagonist activity, E2 (25 pM at the final concentration) was added to the assay medium prior to adding the test chemicals to the wells at final concentrations of 10, 1 μM, 100, 10, 1 nM, 100 and 10 pM, in quadruplicate. In the antagonist assay, luciferase activity was measured in the same manner as in the agonist assay, the transcriptional activity at each concentration of the test chemicals was calculated as a percentage of the positive control wells. Then the IC50 value was calculated by the computer program GraphPad PRIZM® (Graphpad Software Inc., San Diego, CA, USA).

2.4. Gel mobility shift analysis

The binding buffer system in the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) was used for the gel mobility shift analysis according to the manufacturer's instruction. The double-strand oligo DNA probe containing a *X. laevis* vitellogenin A ERE, 5′-CTAGAAAGTCAGGTCACAGTGACCT-GATCAAT-3′, was synthesized, and its 5′ ends were labeled with fluorescein isothiocyanate (FITC). Baculovirus-derived recombinant human ER-alpha (PANVERA, Madison, WI, USA) was used in this assay. Oligonucleotide bearing ERE and ER were incubated for 30 min in the presence of 1 nM E2 with non-labeled oligonucleotide or 1 μM of 4-hydroxytamoxifen or 1 μM of tRA. The protein-bound oligonucleotide was then separated by 5% polyacrylamide gel in low ionic strength buffer (0.5X TBE), and visualized and quantified with the EPIPRO IMAGER and image analysis software (AISIN COSMOS R&D, Ltd., Aichi, Japan) under UV light. The assay was performed in duplicate, and the relative signal intensities to the control were calculated.

2.5. Uterotrophic assay

Crj:CD (SD) rats at postnatal day (PND) 13 and dams were purchased from Charles River Japan, Inc. (Shiga, Japan). Dams and pups were kept in polycarbonate cages until weaning. All

rats were weaned at PND 19 and then housed in three rats per cage during the study. The immature rats were weighed, weight-ranked, and assigned randomly to each of the treatment and control groups. Each group consisted of six rats. Body weight and clinical signs were recorded on a daily basis throughout the study. Rats were provided with tap water and a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) ad libitum before weaning, and with water automatically and a commercial diet (MF, Oriental Yeast Co.) ad libitum after weaning. The animal room was maintained at a temperature of 23 ± 2 °C and a relative humidity of $55 \pm 5\%$, and it was artificially illuminated with fluorescent light on a 12-h light:12-h dark cycle (07:00–19:00 h). All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by the *Japanese Association for Laboratory Animal Science*.

tRA was dissolved in olive oil and subcutaneously injected at doses of 5 and 25 mg/kg for 3 days beginning at PND 20. And EE in olive oil was injected subcutaneously in the back at a dose of 0.6 μg/kg per day for 3 days. The concentration and stability of tRA and EE was confirmed prior to the injection. The doses were based on the results of a preliminary study. The volume of the olive oil solutions containing each chemical was 5 ml/kg. A vehicle control group injected with olive oil alone and a positive control group injected EE alone were also included. ICI 182 780, which is known to be a complete antagonist of estrogenic properties (de Cupis et al., 1995), was administered at dose levels of 50 and 100 μg/kg per day with EE as a comparative control group. tRA was administered first, and the EE was injected within 5 min. Approximately 24 h after the final dosing, the animals were killed by bleeding from the femoral vessels under deep ether anesthesia. At necropsy, the uterus (without oviduct) was carefully dissected and trimmed of fascia and fat to avoid loss of luminal contents, and it was weighed with and without its luminal contents.

Body weight and the uterine weight data were tested by Bartlett's test for homogeneity of variance. When homogeneity of variance ($P < 0.05$) was evident from Bartlett's test, analysis of variance (ANOVA) was performed. When significant differences ($P < 0.05$) were indicated by ANOVA, Dunnett's test was used to compare each group with the vehicle control group or positive control group. When homogeneity of variance was not evident by Bartlett's test, the Kruskal–Wallis test was performed. Whenever there was a significant difference according to this test, a nonparametric Dunnett's test was used to compare each group with the corresponding vehicle control group or positive control group.

3. Results

3.1. Receptor binding assay

The results of the receptor binding assay are shown in Fig. 1 as percentages of ER-bound radio-labeled E2. As shown in Fig. 1, E2 and

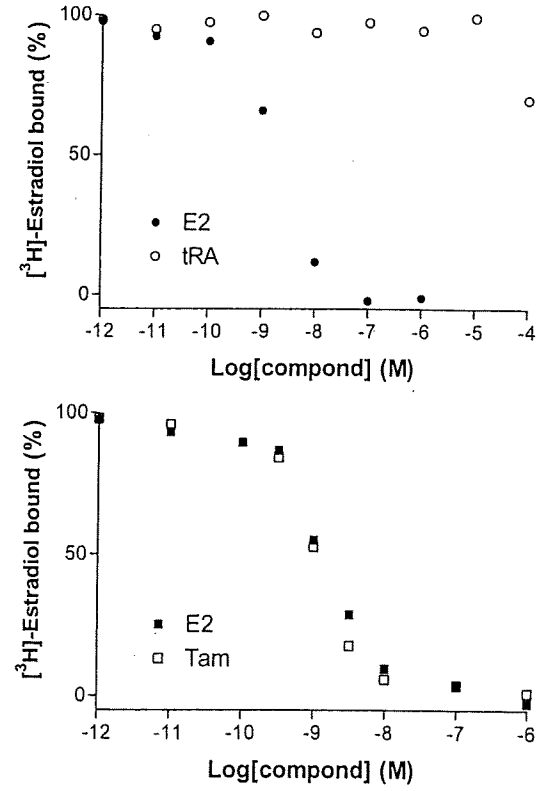

Fig. 1. Effects of all-*trans* retinoic acid (tRA) and 4-hydroxytamoxyfen (Tam) on the positive cooperativity of [^{3}H] estradiol binding to the ligand binding domain of human estrogen receptor alpha.

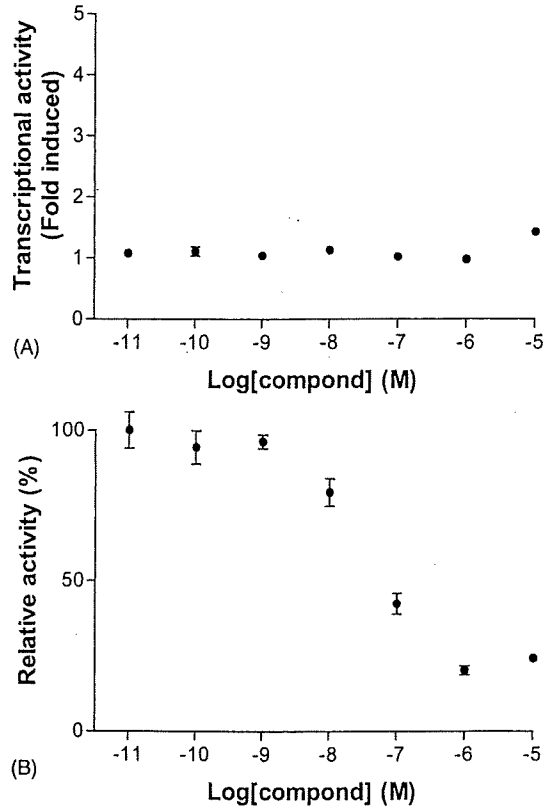

Fig. 2. Agonist (A) and antagonist (B) activities of tRA tested with rat ER alpha mediated reporter gene assay. *Data represents a mean and S.D. of quadruplicate analysis.

4-hydroxytamoxifen (Tam) inhibited the specific [^{3}H]-E2 binding to ER in dose-dependent manner, and IC50 of 4-hydroxytamoxifen was estimated to be 1.05×10^{-9} M. In the case of tRA, although a slight inhibition of specific bound E2 to ER was observed at the highest concentration (100 μM), no binding affinity to ER was noted in the concentration range from 10 pM to10 μM.

3.2. Reporter gene assay

The results of the reporter gene assay are shown in Fig. 2. In the agonist assay, a slight transcriptional activation was observed at the highest concentration (10 μM) of tRA, no transcriptional activation was noted in the concentration range from 10 pM to 1 μM. However, the chemical displayed clear antagonist activity in the concentration over 10 nM, and the IC50 value of tRA was estimated to be 1.56×10^{-8} M in antagonist assay. In this reporter gene assay system, 1 nM of E2 induced 20-fold transcriptional activation compared with the control (data not shown).

3.3. Gel mobility shift analysis

The results of the gel mobility shift analysis are shown in Fig. 3. In the presence of 1 nM E2 and either non-labeled oligonucleotide or 4-hydroxytamoxifen, the signal intensity of the shifted band dropped to approximately 70% compared with the positive control with 1 nM E2 alone. At the same time, tRA had no effect on the signal intensity of the shifted band.

3.4. Uterotrophic assay

The results of the uterotrophic assay are shown in Table 1. Although decreased body weight gains were observed 3 days after administration and on

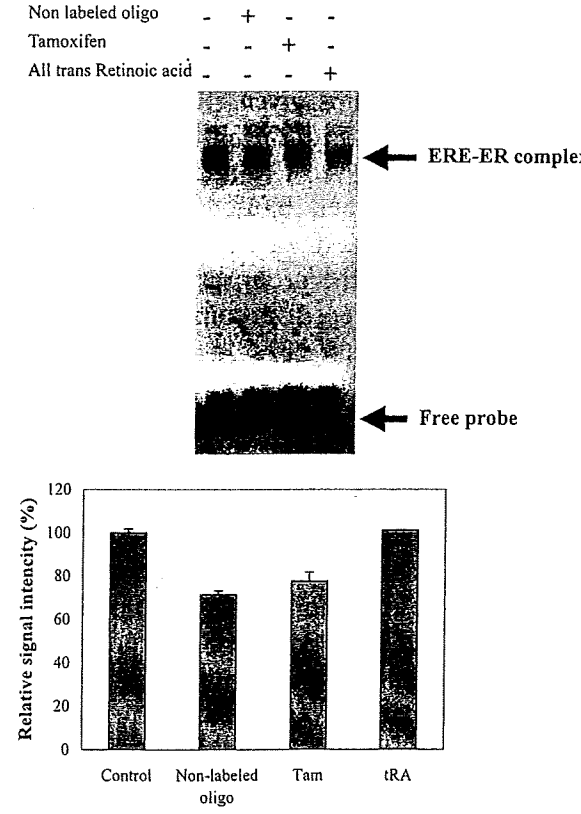

Fig. 3. Inhibitory effect of non labeled oligonucleotide probe, 4-hydroxytamoxifen (Tam) and all-*trans*-retinoic acid (tRA) on gel mobility shift assay results of ERE and ER–ligand complex. *Values were represented as a mean and S.D. of duplicate analysis.

Table 1
Relative uterine and ovarian weights of rats injected tRA or ICI subcutaneously for 3 days

Doses			Uterine weight (mg/100 g bw)		Body weight (g)	
tRA (mg/kg per day)	ICI (μg/kg per day)	EE (μg/kg per day)	Wet	Blotted	Initial	Final
–	–	–	53.6 ± 9.1	52.5 ± 9.0	42.4 ± 2.1	59.2 ± 2.9
5	–	–	42.3 ± 4.7	41.4 ± 4.6	42.5 ± 2.1	56.9 ± 4.9
25	–	–	47.9 ± 7.9	46.9 ± 7.7	42.5 ± 2.0	47.7 ± 3.7[a]
–	–	0.6	193.2 ± 19.3	176.4 ± 12.8	42.6 ± 2.5	59.7 ± 1.9
5	–	0.6	148.3 ± 14.0[b]	143.8 ± 11.7[b]	42.6 ± 2.5	58.2 ± 2.4
25	–	0.6	136.6 ± 24.2[b]	131.0 ± 20.0[b]	42.7 ± 2.4	51.0 ± 2.8[b]
–	50	0.6	91.2 ± 12.6[b]	88.6 ± 12.1[b]	42.4 ± 2.5	61.3 ± 1.9
–	100	0.6	65.5 ± 7.1[b]	63.7 ± 6.7[b]	42.7 ± 2.6	61.1 ± 1.5

Animals were treated as described in Section 2. Values represent mean ± S.D.
[a] Significantly different from the vehicle control (tRA-, ICI-, EE-) at $P<0.01$ (Dunnett's test).
[b] Significantly different from the EE control (tRA-, ICI-, EE 0.6) at $P<0.01$ (Dunnett's test).

the day of necropsy in the group given 25 mg/kg tRA and the group given 25 mg/kg tRA plus EE, respectively, there were no clinical or gross abnormalities in any of groups. The organ weights showed no significant differences in uterine wet or blotted weights between the vehicle control group and any of the groups given tRA. By contrast, co-treatment with 5 and 25 mg/kg tRA plus EE significantly reduced the EE-induced increases in uterine wet weight and blotted weight. Uterine wet weight and blotted weight were also significantly decreased in the group given ICI 182 780, compared with the positive control group given EE.

4. Discussion

Several environmental contaminants may interfere with the actions of endogenous steroid hormones. Steroid hormones control the development and maintenance of reproductive tissues, and thus interference with these hormones by environmental estrogens may cause serious dysfunction of reproductive organs. A number of chemicals have been newly synthesized and tested for their safety in mammals. However, there is still no validated test method for detecting the hormonal modulating potential of chemicals.

A great effort to establish new test methods, such as the uterotrophic assay for detecting estrogenic activity of chemicals are made under the international organization (OECD, 2001). And the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) proposed a conceptual framework for detecting the endocrine disrupting properties of chemicals (EDSTAC, 1998). In the EDSTAC's framework, the hormone receptor binding assay and hormone receptor mediated reporter gene assay have been proposed for the pre-screening and the Tier 1 screening batteries, and rodent uterotrophic assay is proposed for Tier 1 screening battery. We conducted series of these screening tests, to detect the ER agonist and antagonist activity of tRA. The tRA is one of retinoids that are a family of natural and synthetic compounds structurally related to vitamin A, and it is known to have anti-estrogenic activity and exerts an inhibitory effect on the growth of breast cancer cells (de Cupis et al., 1995; Eck-Enriquez et al., 2000; Nesaretnam et al., 2000; Wilcken et al., 1997). In this study, the anti-estrogenic activity of tRA was confirmed by the reporter gene assay and uterotrophic assay, but the receptor binding assay indicated that tRA lacks ER binding affinity, and no agonist activity of tRA was detected in reporter gene assay or uterotrophic assay. We then performed a gel mobility shift analysis with oligonucleotide probe containing a single *X. laevis* estrogen responsive element and ER with tRA or 4-hydroxytamoxyfen in the presence of E2 to elucidate the ER inhibitory

mechanism of tRA. The results suggest that tRA exerts its anti-estrogenic effect without interfering with receptor–ligand interaction. Although we have no evidence of expression of RARs in *HeLa* cells, Pratt et al. (1996) have suggested that the AF-2 region of retinoic acid receptor (RAR) alpha mediates RA inhibition of ER-induced transcription in breast cancer cells. Another groups also reported that the antiestrogenic effect of retinoic acid was due to an inhibition of estrogen receptor activity, for example by altering the amount of estrogen receptor protein bound to the ERE or affecting the transcriptional efficiency of this complex (Demirpence et al., 1992, 1994; Prakash et al., 2001). The mechanism of the anti-estrogenic effect of retinoids is still not clearly understood at present; nevertheless our results support these findings.

In any case, a screening strategy for endocrine disrupters, especially the primary screening battery for prioritizing the chemicals to be tested in the higher screening stages, should be designed to detect various kinds of chemicals possessing endocrine modulating activity including a retinoid-like endocrine modulator. Our results showed the reporter gene assay and uterotrophic assay could detect a retinoid-like endocrine modulating effect of chemicals, but the receptor binding assay could not detect this type effect. Accordingly reporter gene assay or uterotrophic assay should be conducted in the early stage of screening process for endocrine disrupting chemicals, because they can detect antagonist activity caused by both inhibition of receptor–ligand interaction and transcriptional interference. Particularly, the reporter gene assay may be a promising prescreening procedure because it can be adopted in the high throughput screening process for thousands of chemicals and it requires no use of experimental animals.

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Toxicology Letters

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Development of non-radio isotopic endpoint of murine local lymph node assay based on 5-bromo-2′-deoxyuridine (BrdU) incorporation

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Abstract

Allergic contact dermatitis is a serious health problem. Over the last decade, the murine local lymph node assay (LLNA) has been developed to detect chemical allergens, and international validation studies have been conducted. We have tried to establish an alternative non-radioisotopic endpoint for the LLNA by using 5-bromo-2′-deoxyuridine (BrdU) incorporation in place of radioisotopes, such as [^{3}H]thymidine, employed in the standard method. BrdU was given as a single administration at 5 mg/animal 2 days following three consecutive daily applications of a test chemical. BrdU incorporation into draining lymph node cells was measured using an enzyme immunosorbent assay technique. In this study, *p*-benzoquinone(PBQ), trimellitic anhydride (TMA), citral(CT) and dextran (DEX) were used as pilot chemicals. PBQ, TMA and CT, which are classified as moderate to strong sensitizers in the guinea pig maximization test and were positive in the original LLNA, were also found to elicit positive responses in the alternative LLNA using BrdU incorporation. In contrast, DEX tested negative in the modified assay consistent with previous guinea pig and LLNA data. Consequently, the modified LLNA endpoint using BrdU incorporation may represent a useful alternative to the standard assay in situations, where there is a need to avoid the use of radioisotopes.

Keywords: Local lymph node assay; Chemical-hypersensitivity; 5-bromo-2′-deoxyuridine (BrdU); non-RI

1. Introduction

Allergic contact dermatitis is a serious health problem.

For a long time, skin sensitization tests using guinea pigs, such as the guinea pig maximization test (Magnusson and Kligman, 1969) and the

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Buehler occluded patch test (Buehler, 1995), have been used for predicting the allergic potential of chemicals. Over the last decade, the murine local lymph node assay (LLNA) has been developed for identifying chemicals with sensitization potential, and international validation studies have been conducted (Scholès et al., 1992; Kimber et al., 1994; Loveless et al., 1996; Gerberick et al., 2000). The endpoint of the guinea pig skin sensitization tests is based on measurement of challenge-induced cutaneous reactions. In contrast, the activity in the LLNA is measured as a function of the proliferative activity of draining lymph node cells after topical application of a test chemical. The LLNA appears to offer several advantages compared with guinea pigs predictive tests: it is quicker and cheaper, provides a quantitative endpoint and reduces the potential trauma to animals. However, in the standard LLNA, cell proliferation is measured using the incorporation of radiolabeled thymidine or uridine into draining lymph node cells, and this requires specific facilities and handling conditions. In present study, we have assessed the potential utility of a non-radioisotopic endpoint for the LLNA based on 5-bromo-2′-deoxyuridine (BrdU) incorporation into lymph node cells.

2. Materials and methods

2.1. Chemicals

Oxazolone (OX, Lot No. 106H3650, SIGMA) was used in Section 2.3 to determine the optimum BrdU dosing point. *p*-bebzoquinone BZQ, Lot No. 012D2294, Kanto Chemical Co.), citral (CT, Lot No. M7K6160, Nakalai Tesque), trimellitic anhydride (TMA, Lot No. 101D2118, Kanto Chemical Co.), and dextran (DEX, Lot No. A011667001, Kanto Chemical Co.) were used as pilot chemicals. OX, BZQ, CT, and TMA were dissolved in acetone/olive oil (AOO; 4:1), and DEX was dissolved in dimethyl sulfoxide (DMSO). 5-Bromo-2′-deoxyuridine (BrdU, Nakalai Tesque) was dissolved in physiological saline at a concentration of 10 mg/ml.

2.2. Animals

Male BALB/cAnN mice were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Mice were housed in animal rooms maintained at a temperature of 23 ± 2°C and relative humidity of 55 ± 15%. The rooms were ventilated at a frequency of 10–15 cycles/h, and lighted artificially for 12 h daily.

2.3. Experiment 1

To determine the optimal BrdU administration schedule, mice were randomly allocated to six groups (four mice/group). A 25 μl volume of 0.5% OX in AOO was applied to the dorsum of both ears of the mice daily for 3 consecutive days. A single or two injections (5 mg/mouse/injection) of BrdU were administered intraperitoneally accord-

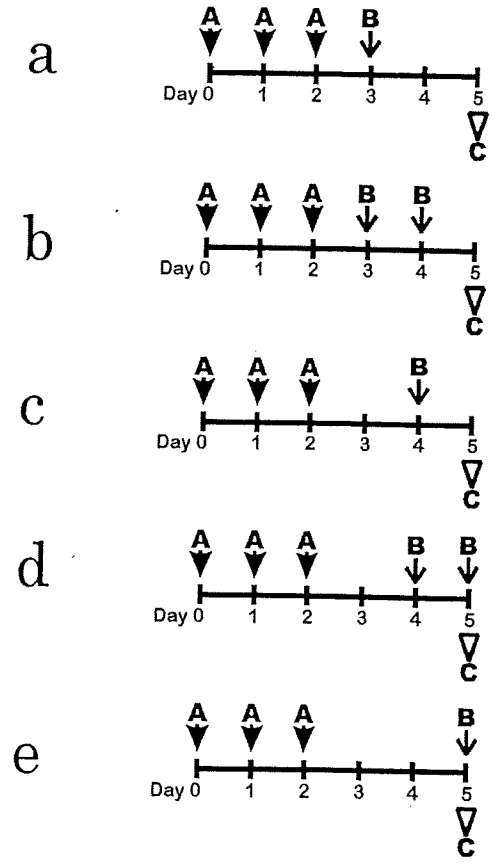

Fig. 1. Experimental designs for determining the BrdU injection time points. (A) Application of chemical, (B) BrdU injection, (C) Collection of lymph nodes.

Table 1
Results of Experiment 1 †

Group	Lymph node weight (g)		BrdU labeling index ($A_{540-650}$)		No. of BrdU injections
	Mean	SE	Mean	SE	
Control	0.004[a]	0.001	0.224[a]	0.034	2
a	0.034[b]	0.002	0.714[b]	0.019	1
b	0.032[b]	0.002	1.406[c]	0.039	2
c	0.027[b]	0.003	1.188[c]	0.105	1
d	0.031[b]	0.001	1.359[c]	0.097	2
e	0.031[b]	0.002	0.943[d]	0.052	1

† Results represent mean values and standard errors in four mice. Mean values with no common letters are significantly different ($P < 0.05$, Student's t-test).

ing to the protocols shown in Fig. 1. Auricular lymph nodes were removed, weighed, and stored at −20°C until analysis by an enzyme-linked immunosorbent assay (ELISA) to measure BrdU incorporation.

2.4. Experiment 2

Four pilot chemicals, *p*-benzoqionone (PBQ), trimellitic anhydride (TMA), citral (CT), and dextran (DEX), were used to determine the sensitivity and specificity of the assay system. Mice were randomly allocated to 14 groups (four animals/group), as shown in Table 1. A 25 μl volume of each test chemical solution was applied to the dorsum of both ears of the mice daily for 3 consecutive days. BrdU solution was injected intraperitoneally on day 4 according to the results of Section 2.3. The lymph nodes were collected, weighed, and stored at −20°C for ELISA for BrdU incorporation.

2.5. ELISA for BrdU incorporation

BrdU incorporation into the lymph node cells was determined using a commercial cell proliferation assay kit (Boehringer Mannheim Corp., Indianapolis, IN, USA, Cat. No. 1647229). Briefly, lymph nodes were crushed, and after passage through a #70 nylon mesh, the cells were suspended in 15 ml of physiological saline. The cell suspension (100 μl) was added to the wells of a flat-bottom microplate (Coster 3595, Corning Inc., NY, USA) in quadruplicate. After centrifugation (3000 × *g*, 10 min), the supernatants were removed, 200 μl of Fix-Denat solution was added to each well, and then the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted *anti*-BrdU antibody solution (100 μl, Boehringer Mannheim Corp.) was added to each well, and after rinsing three times with washing buffer (phosphate-buffered saline), 100 μl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. The absorbance was defined as the BrdU labeling index.

2.6. Statistical analysis

Means and standard errors were calculated for lymph node weights and the optical density values obtained by ELISA for each treatment group. In the experiment 1, the one-way analysis of variance (ANOVA) was performed at first, then the Student's t-test was employed to detect significant differences between means. In the experiment 1, the ANOVA was performed at first, and the stimulation index (SI) was calculated by dividing the mean value obtained in each treatment group by that of the control group. Positive responses were defined as those where a SI of three or more

was recorded according to the criteria described in Kimber et al., 1995.

3. Results

3.1. Experiment 1

Lymph node weights after administration of OX, and BrdU incorporation values, are shown in Table 1. The mean lymph node weights of animals given 1% OX were markedly increased, but no significant differences were noted between the treatment groups. In contrast, the BrdU labeling indices of animals given 1% OX ranged from 0.714 to 1.406. Among the groups given OX, groups b, c and d showed significantly higher indices than the other groups, although the differences between these three groups were not significant.

3.2. Experiment 2

The lymph node weights of animals given BZQ, CT or TMA increased in a dose-dependent manner, but DEX did not caused any increases in lymph node weight at any concentration (Table 2). PBQ showed positive reactions in the 0.5 and 1% groups in terms of both increased in lymph node weight and BrdU labeling. CT displayed a positive reaction in terms of the BrdU labeling index only in the 25% group. TMA gave positive reactions in terms of BrdU labeling at all concentrations tested, although the 1.25% group did not show a positive reaction in terms of lymph node weight.

4. Discussion

The LLNA has been developed for detecting chemicals that have the potential to induce skin sensitization, and its validity has been confirmed by studies conducted internationally (Scholes et al., 1992; Kimber et al., 1994; Loveless et al., 1996; Gerberick et al., 1999). In the present study, we investigated the utility of BrdU incorporation as an alternative endpoint for the LLNA. BrdU

Table 2
Results of the non-RI murine local lymph node assay with four pilot chemicals[a]

Group	Concentration (%)	Lymph node weight (g)			BrdU labeling index		
		Mean	SE	SI[b]	Mean	SE	SI[b]
Control (AOO)	–	0.0035	0.0002		0.148	0.012	
p-Benzoquinone	0.25	0.0070	0.0008	2.00	0.411	0.094	2.78
	0.5	0.0107	0.0010	**3.06**	0.494	0.048	**3.35**
	1	0.0127	0.0014	**3.63**	0.642	0.041	**4.35**
Citral	6.25	0.0036	0.0002	1.03	0.174	0.021	1.18
	12.5	0.0047	0.0003	1.34	0.215	0.024	1.46
	25	0.0098	0.0002	2.80	0.502	0.067	**3.40**
Trimellitic anhydride	1.25	0.0085	0.0011	2.43	0.464	0.033	**3.14**
	2.5	0.0137	0.0004	**3.91**	0.554	0.026	**3.75**
	5	0.0198	0.0014	**5.66**	0.715	0.041	**4.85**
Control (DMSO)	–	0.0057	0.0001		0.246	0.031	
Dextran	2.5	0.0053	0.0003	0.93	0.232	0.025	0.94
	5	0.0066	0.0003	1.16	0.217	0.034	0.88
	10	0.0060	0.0005	1.05	0.201	0.003	0.82

[a] Results represent mean values and standard errors in four mice.
[b] The stimulation index (SI) was calculated by dividing the mean value obtained in each treatment group by that of the control group. The cases showing three or greater SI values were defined as positive (Bold-faced).

is an analogue of thymidine, and when incorporated into DNA during cell proliferation is easily detectable by immunochemical methods such as ELISA (Huong et al., 1991). As the principle of the [^{3}H]thymidine-based method for detecting cell proliferation is the same as that for BrdU incorporation, equivalent results might be expected from the two methods.

In experiment 1, a single injection of BrdU at day 4 (protocol c) was found to be as effective as the two-injection methods (protocols b and d). This showed that allergen-induced LNC proliferative responses were induced between day 4 and 5, and that injected BrdU is incorporated efficiently during this period. Accordingly, we decided to employ the single injection protocol (c) to minimize basal incorporation of BrdU in the control group.

The BrdU-based LLNA using the optimized protocol was conducted with four pilot chemicals, PBQ, TMA, CT, and DEX, to determine the sensitivity and specificity of the modified assay. With this protocol, PBQ and TMA caused positive reactions in terms of both weight- and BrdU-based endpoints, and CT caused positive reactions in terms of only BrdU-based endpoint. The BrdU-based endpoint was able to detect positive reactions at dose levels lower than those required for a measurable increase in lymph node weight, and this resulted in a positive result for CT. The SI values of 1% BPA, 25% CT, and 5% TMA in the standard assay were reported as 42.3, 5.0 and 45.3, respectively (Basketter and Scholes, 1992). The SI values in the alternative assays were markedly lower than those of the standard assay. These low SI values observed in the alternative method were considered to be caused by narrow dynamic range of BrdU–ELISA.

PBQ is reported to be a strong sensitizer, and TMA and CT are reported to be moderate sensitizers in guinea pig maximization tests. In contrast, DEX is reported to be a non-sensitizer (Basketter and Scholes, 1992).

There is no doubt that the standard LLNA provides a robust and reliable method for the identification of skin sensitzing chemicals. In the accepted protocol activity is determined as a function of the incorporation of radiolabelled thymidine by draining LNC. Necessarily the use of radioisotopes requires appropriate containment and as a consequence there has been some interest in the development of alternative endpoints for the LLNA, including the histochemical detection of BrdU, the measurement of interleukin 2 production by draining lymph node cells, and flow cytometric analysis of lymph node cell populations (Boussiquet-Leroux et al., 1995; Hatao et al., 1995; Gerberick et al., 1999; Hariya et al., 1999). We reported here another approach, in which the standard LLNA protocol is followed more closely and the incorporation of BrdU in draining lymph nodes is measured by ELISA. Although the SI values obtained by the alternative BrdU methods are smaller than those in the standard method, the preliminary results reported here are largely in line with the standard method, insofar as chemicals that are known to be contact allergens and which elicit positive responses in the conventional LLNA were also positive in this modified protocol. Likewise, a chemical which is known not to cause allergic contact dermatitis and which fails to induce responses in a standard LLNA also tested negative in the modified method. Clearly there is a need for more rigorous evaluation of this modified method with a wider range of test chemicals before it is possible to determine to what extent BrdU offers the same degrees of specificity and sensitivity displayed by the conventional LLNA. Nevertheless, these preliminary data are very encouraging and suggest that this approach might form the basis of a modified test method, which would avoid the use of radioisotopes.

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Intrauterine position and postnatal growth in Sprague–Dawley rats and ICR mice

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Abstract

In rodents, steroid hormones are thought to be transported between adjacent fetuses, and male or female fetuses that develop in utero between female fetuses may have higher serum levels of estradiol, and lower serum levels of testosterone, relative to siblings of the same sex that develop between two male fetuses. The consequence in the variation of postnatal growth, development, and function in the intrauterine position, using various parameters such as anogenital distance, preputial separation and vaginal opening, estrous cycle, locomotor activity, and growth of reproductive organs, were examined in Sprague–Dawley rats. ICR mice were treated with 17β-estradiol before copulation and during pregnancy to address the interaction with endogenous estradiol during pregnancy. In rats, no evidence of effects of prior intrauterine position was observed for any of the parameters examined. Mouse fetal exposure via the mother to low-dose 17β-estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. The results of this study suggested that the intrauterine position of the embryos/fetuses did not affect the postnatal growth of the reproductive organs, sexual maturation, or behavior in rats and mice.

Keywords: Intrauterine position; Postnatal growth; Sexual maturation; Behavior; Anogenital distance; Rats; Mice

1. Introduction

The development of sexually differentiated phenotypes depends upon the hormonal environment during a critical period of growth [1]. Testosterone secretion by the fetal testis causes a longer anogenital distance (AGD), seen in neonatal males, relative to females. The AGD of newborn rats, mice, and gerbils is longer in males than in females and varies as a function of the intrauterine position of the animals [1–4]. A longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus. Females with a male fetus on only one side are immediate [4].

In all litter-bearing species that have been examined to date, the intrauterine position that a fetus occupies relative to fetuses of the same or opposite sex has profound effects on its reproductive, behavioral, and morphological traits measured during adult life [4–7]. Gerbil males and females that developed in utero between two female fetuses or two male fetuses, respectively, did not differ in relative hippocampal size [8].

The effects of intrauterine position are apparently not the result of the position itself, but rather of the movement of steroid hormones between the fetuses, and variations in the hormonal environment relative to the proximity of an individual fetus to other fetuses of the same or opposite sex [9]. Male rats located between two females had elevated serum estradiol and larger prostates than males located between two males, which had elevated serum testosterone and larger seminal vesicles [10]. The effect of intrauterine position in mice has been correlated with concentrations of steroid hormones in amniotic fluid and subsequent sexual activity [11,12].

Recently, intrauterine position has been the focus of discussions in the toxicology community because of its potential to alter the susceptibility of fetuses to endogenous hormones and endocrine disrupting chemicals [13,14]. In this regard, failure to account for intrauterine position in endocrine disrupting chemical toxicology studies could lead to false negative results, especially when adverse alterations

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are produced by low doses in fetuses from only one intrauterine position [14,15]. This possibility has been raised because of investigations into estrogenic compounds in mice. In rats, consistent effects due to intrauterine position on testosterone concentrations, and therefore potential interactions with endocrine disrupting chemicals, have not been found. Howdeshell and vom Saal [16] demonstrated that the greatest response to the estrogenic chemical, bisphenol A, occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A within the range of human exposure, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development. It has been demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin interacted with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses [17].

The objectives of this study were to determine the effects of intrauterine position, under normal physiological conditions, on the development of rat offspring, as well as sexual maturation, estrous cycle, behavior, and reproductive organ development. Another objective of this study was to determine whether the intrauterine position of mouse fetuses, which is related to background levels of estradiol and testosterone, would influence the response of the postnatal growth of gonads, including sexual maturation, to low dose 17β-estradiol.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (Crj:CD, IGS), and ICR mice (Crj:CD-1) were purchased from Charles River, Laboratories, Inc. (Atsugi, Japan). Twenty-seven male rats (9 weeks of age), 84 female rats (8 weeks of age), 130 male mice (9 weeks of age), and 130 female mice (8 weeks of age), were used. The rats and mice arrived with mean weights of 301.1 ± 7.9 g for males and 216.2 ± 8.1 g for females, and 37.2 ± 1.2 g for males and 29.1 ± 0.9 g for females (mean ± S.D.), respectively. The animals were acclimated to the laboratory for 7–14 days prior to the start of the experiments to evaluate weight gain and any gross signs of disease or injury. The animals were housed individually in stainless steel, wire-mesh cages in a room with controlled temperature (22–25 °C) and humidity (50–65%), with lights on from 07:00 to 19:00 h daily. The animals were given access to food (NIH-07-PLD: phytoestrogen low diet, Oriental Yeast Co., Japan) and tap water through metal pipes (distilled water, Wako Pure Chem., Japan) ad libitum. In a few instances, the temperature and humidity were outside the standard ranges, but the magnitude and duration of these incidents were minimal and judged to be of no consequence. The contents of genistein and daidzein in the diet and wood bedding (ALPHA-dri, Shepherd Specialty Paper, USA) used in the present study were determined. Neither genistein nor daidzein were not detected in the diet or wood bedding (detection limit: 0.5 mg/100 g in each individual phytoestrogen, by HPLC).

Animal care and use conformed to published guidelines [18].

2.2. Experiment I (examination of intrauterine position effect on postnatal growth in rats)

2.2.1. Cesarean delivery and fostering

Estrous female rats at 10–11 weeks of age were cohabited overnight with a single male to obtain 66 pregnant females within 4 days. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation. Thirty-three pregnant females were killed by CO_2 asphyxiation and cervical dislocation, and subjected to cesarean sectioning on day 21 of gestation. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed. Anogenital distance (AGD) was measured with a digital micrometer (reproductive precision of 0.01 mm, Digimatic caliper CD-15C, Mitutoyo Co., Kanagawa, Japan) under an Olympus dissecting microscope for each fetus, and the average was taken. The subject was held steady and in the same position during measurement. Measurements were made without knowledge of intrauterine position by one person. The AGD was measured from the center of the phallus to the center of the anus. The fetuses obtained by cesarean delivery were fostered to 33 dams that had just given birth naturally (one litter to each female). The original littermates remained together when cross-fostered. The litter sizes were similar for each cross-fostered dam. The day of cesarean section was considered as postnatal day (PND) 0. Pup body weights were recorded on PND 21 (day of weaning). Following weaning, and until 10 weeks of age, offspring were weighed once a week.

Neonates from 33 pregnant females were categorized as occupying six different intrauterine positions: 2M (male fetus located between two male fetuses; number of pups and litters on PND 0 = 36 and 19); 1M (male fetus that located between a male fetus and a female fetus; $n = 73$ and 27); 0M (male fetus located between two female fetuses; $n = 45$ and 24); 2F (female fetus located between two female fetuses; $n = 38$ and 18); 1F (female fetus located between a female fetus and a male fetus; $n = 83$ and 29); 0F (female fetus located between two male fetuses; $n = 41$ and 27). Fetuses adjacent to dead embryos (resorptions or macerated fetuses), and fetuses that were closest to each ovary or the cervix, were discarded from further analyses.

2.2.2. Observations of postnatal growth

2.2.2.1. Measurement of AGD and reproductive organ weights, and evaluation of sexual maturation. On PND

4, the AGD was measured for pups in each group using calipers with a reproductive precision of 0.01 mm. On PND 21, all pups were weaned and half of the pups in each group (2M = 13, 1M = 37, 0M = 11, 2F = 14, 1F = 43, 0F = 12) were subjected to necropsy, and the testes, epididymides, and prostates with seminal vesicles (fluid was not removed and all lobes were included) in males, and uteri and ovaries in females, were weighed. For the remaining male and female pups in each litter (2M = 21, 1M = 32, 0M = 30, 2F = 23, 1F = 36, 0F = 26), as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 28), and preputial separation for males (beginning on PND 35), were assessed, and each rat was weighed when these criteria were achieved.

2.2.2.2. Postweaning tests of behavior, evaluation of estrous cycle, and histological observation of reproductive organs. One male and one female were randomly selected from each litter in each group (number of rats examined: 2M = 18; 1M = 27; 0M = 25; 2F = 17; 1F = 27; 0F = 25), and were subjected to an open field test and wheel cage activity test to assess the emotionality and regulatory running activity, respectively. At 4 weeks of age, the rats were placed into a circular area (140 cm in diameter) surrounded by a wall (40 cm in height). The light and noise levels averaged 500 lx and 50 dB, respectively, at the center of the circular area. Rearing, grooming, defecation, and urination were counted, and ambulation was recorded automatically on a computer (Unicom, Inc., Japan), during a 3-min trial between 13:00 and 16:00 h on one day. At 7 weeks of age, the rats were placed into a wheel cage (Nippon Cage, Inc., Japan), 32 cm in diameter and 10 cm in width, as a measure of spontaneous activity. Each rat was kept within the wheel for 24 h with free access to food (NIH-07-PLD) and distilled water in the same animal room. The number of revolutions was automatically recorded with a 20-channel digital counter (Seiko Denki, Inc., Japan).

Each morning (9:00–10:00 h), from 6 to 10 weeks of age, all females in each group were subjected to vaginal lavage. The lavage fluid was applied to a glass slide, air-dried, and stained with Wright–Giemsa stain. Cytology was evaluated and the stage of the estrous cycle was determined using the method of Everett [19].

At 10 weeks of age, 3–5 males in each group were weighed, and anesthetized. Transcardial perfusions were carried out with a mixture of 0.1 M phosphate-buffered 1.25% glutaraldehyde and 2% paraformaldehyde. Following fixation, the prostate gland was sampled, rinsed three times in phosphate buffer, postfixed for 2 h at 4 °C in 2% osmium tetroxide, and dehydrated in alcohol; the prostate gland was embedded in epoxy resin. Ultrathin sections of the prostates were stained with uranyl acetate and lead citrate, and observed with an electron microscope (H-7100, Hitachi, Japan). The remaining males in each group (2M = 18, 1M = 27, 0M = 25) were weighed and subjected to necropsy, and the testes, epididymides, ventral prostate, and dorsal prostates with seminal vesicles, were weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. All females (2F = 17, 1F = 27, 0F = 25) were weighed and subjected to necropsy when the stage of the estrous cycle was diestrus. The ovaries and uteri were then weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. These reproductive organs were embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.3. Experiment II (examination of low-dose in utero effects of 17β-estradiol in mice)

The objective of this experiment was to determine whether the intrauterine position of male fetuses, which is related to background levels of estradiol (elevated in males located between two female fetuses) and testosterone (elevated in males located between two male fetuses), would influence the response of the developing prostate to low dose 17β-estradiol. In addition, we examined whether the intrauterine position of male and female fetuses would affect the postnatal growth of other reproductive organs and sexual maturation.

2.3.1. Administration, cesarean delivery and fostering

Thirty female mice at 9 weeks of age were administered 17β-estradiol (Sigma Chem. Co., MO, USA) subcutaneously at a dose of 0.05 μg/kg per day for 7 days before mating, during a mating period of 7 days at the longest, and on day 0 through 17 of gestation. In a preliminary study, the offspring of the ICR pregnant females exposed to 17β-estradiol at 0.05 μg/kg per day on day 0 through 17 of gestation showed no changes in weight and histological morphology of reproductive organs in adulthood. However, the offspring of dams exposed to 17β-estradiol at 0.1 μg/kg per day on these gestational days showed changes in the parameters in adulthood (data not shown). In the present study, 30 control females were administered corn oil (Nacalai Tesque, Co., Tokyo). After the administration for 7 days before mating, female mice were caged with untreated males overnight and examined for a vaginal plug the next morning. The day on which a plug was found was termed day 0 of gestation. In this study, 30 female mice in the 17β-estradiol exposed group and the control group copulated and became pregnant. On day 18 of gestation, pregnant females were killed by CO_2 asphyxiation, and subjected to cesarean sectioning. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed, and then the AGD was measured. The fetuses obtained by cesarean delivery were fostered to 60 dams that had just given birth naturally (one litter to each female). The day of cesarean section was considered as PND 0. Pup body weights were recorded on PND 21 (day of weaning), and at 5, 7, and 10 weeks of age.

Neonates from 30 pregnant females exposed to corn oil and 30 pregnant females exposed to 17β-estradiol were categorized as occupying four different intrauterine positions:

2M (the number of neonates in the 17β-estradiol exposed group and the control group: 38 and 41) and 0M (33 and 32), and 2F (41 and 32) and 0F (28 and 37). Fetuses adjacent to dead embryos, and fetuses that were closest to each ovary or the cervix, were discarded. In this experiment, fetuses of two intrauterine positions, 1M and 1F, were also discarded.

2.3.2. Observations of postnatal growth

2.3.2.1. Evaluation of sexual maturation. On PND 21, all male and female pups (2M, 0M, 2F, 0F) in each litter were weaned. For all male and female mice in each litter, as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 25), and preputial separation for males (beginning on PND 30), were assessed, and each pup was weighed when these criteria were achieved.

At 10 weeks of age, five males in each group were weighed and processed to the transcardial perfusion to observe the histological alteration of the prostate by electron microscope. The remaining males in each group were weighed and subjected to necropsy, and the testes, epididymides and seminal vesicles, were weighed. All females were weighed and subjected to necropsy. The ovaries were then weighed. These reproductive organs including prostates and uteri were fixed in 0.1 M phosphate-buffered 10% formalin solution and embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.4. Data analyses

Statistical analysis of the data for the offspring (AGD, body weight and organ weight, organ/body weight ratios, timing of vaginal opening and preputial separation) was performed using the litter as the unit [20,21]. The AGD, body weight and organ weight, organ/body weight ratios (relative organ weight), timing of vaginal opening and preputial separation, were analyzed using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect the significances among the groups. If a significant difference was detected among the groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous, or there was a group whose variance was zero, Kruskal–Wallis analysis of ranks was applied. If a significant effect was detected among the groups, Dunnett's test was applied for multiple comparisons. Comparisons between groups were made using $P \leq 0.05$ as the level of significance.

3. Results

3.1. Experiment I

3.1.1. AGD and body weights of fetuses at cesarean section and pups at PND 4

Table 1 shows the AGD, body weight, AGD/body weight (AGDI: anogenital distance index), and AGD/$\sqrt[3]{\text{body weight}}$ of fetuses at various intrauterine positions and pups at PND 4. It is reasonable to anticipate that the AGD might vary with body weight of fetus or pup. It has been proposed that the relationship between AGD and body weight should be more properly evaluated using the cube root of the body weight [22–25]. If it is desirable to normalize AGD to body weight, the AGD/$\sqrt[3]{\text{body weight}}$ seems to provide a more appropriate adjustment.

There were no statistically significant differences in any parameter evaluated at cesarean section (PND 0) or PND 4

Table 1
Effects of prior intrauterine position on anogenital distance in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
AGD of fetuses at cesarean section						
No. of litters	19	27	24	18	29	27
No. of pups	36	73	43	38	83	41
Body weight (g)	5.6 ± 0.4[a]	5.6 ± 0.3	5.7 ± 0.4	5.2 ± 0.3	5.4 ± 0.3	5.3 ± 0.4
AGD	2.43 ± 0.22	2.42 ± 0.22	2.42 ± 0.28	1.21 ± 0.20	1.23 ± 0.19	1.22 ± 0.24
AGD/body weight	0.43 ± 0.04	0.42 ± 0.04	0.42 ± 0.05	0.23 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
AGD/$\sqrt[3]{\text{body weight}}$	1.36 ± 0.12	1.36 ± 0.14	1.35 ± 0.18	0.69 ± 0.08	0.70 ± 0.07	0.70 ± 0.08
AGD of pups on PND 4						
No. of litters	19	27	24	18	29	27
No. of pups	34	69	41	37	79	38
Body weight (g)	10.9 ± 1.5	11.2 ± 1.5	10.8 ± 1.1	10.4 ± 1.4	10.3 ± 1.1	10.4 ± 1.3
AGD	4.57 ± 0.54	4.41 ± 0.48	4.43 ± 0.51	2.00 ± 0.22	1.99 ± 0.19	2.00 ± 0.21
AGD/body weight	0.42 ± 0.06	0.40 ± 0.05	0.41 ± 0.03	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.04
AGD/$\sqrt[3]{\text{body weight}}$	2.06 ± 0.22	1.99 ± 0.19	1.99 ± 0.19	0.92 ± 0.12	0.91 ± 0.11	0.92 ± 0.13

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

Table 2
Effects of prior intrauterine position on reproductive organs before maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight on PND 21						
No. of litters	18	27	24	17	27	25
No. of offspring	13	37	11	14	43	12
Body weight (g)	40.9 ± 6.3[a]	40.5 ± 6.5	40.2 ± 9.6	38.8 ± 6.3	38.2 ± 7.0	40.7 ± 7.0
Testes (mg)[b]	169.3 ± 27.5	172.2 ± 22.2	164.9 ± 26.1			
Testes[c]	416.3 ± 48.4	429.2 ± 40.3	418.2 ± 46.7			
Epididymides (mg)[b]	23.3 ± 3.1	23.5 ± 4.8	21.9 ± 4.4			
Epididymides[c]	60.5 ± 10.8	58.2 ± 7.8	55.2 ± 7.0			
Prostate + SV (mg)[b,d]	47.2 ± 9.9	46.7 ± 10.3	45.9 ± 7.9			
Prostate + SV[c,d]	115.6 ± 18.0	115.7 ± 19.0	117.2 ± 19.5			
Ovaries (mg)[b]				24.3 ± 4.0	22.9 ± 3.9	24.8 ± 3.6
Ovaries[c]				63.5 ± 10.1	60.8 ± 9.5	61.5 ± 7.3
Uterus (mg)[b]				10.2 ± 2.0	11.2 ± 3.7	11.8 ± 2.9
Uterus[c]				26.4 ± 4.5	28.8 ± 6.8	29.1 ± 6.0

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (g or mg per 100 g body weight).
[d] Seminal vesicle.

between groups 2M, 1M and 0M in males, or groups 2F, 1F and 0F in females.

No significant differences in viability of fetuses at cesarean section (PND 0), or that from PND 0 to PND 4 (the number of pups died; 2M = 2, 1M = 4, 0M = 2, 2F = 1, 1F = 4, 0F = 3), were detected between the groups. In addition, there were no statistically significant differences in body weight at PND 0 and 4.

3.1.2. *Body weight and reproductive organ weight of offspring at PND 21*

The absolute and relative weights of testes, epididymides, and prostates with seminal vesicles in males, and ovaries and uteri in females, as well as body weight of offspring at PND 21 are shown in Table 2. Irrespective of the intrauterine position, no significant differences were detected between the groups in absolute or relative reproductive organ weights, or body weights of male and female weanlings, suggesting that the intrauterine position did not affect postnatal growth before weaning in rats.

3.1.3. *Sexual maturation and estrous cycle of offspring*

Table 3 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups. The estrous cycle of female offspring from 6 to 10 weeks of age is shown in Table 4. No significant differences were detected between the groups in mean estrous cycle length, or the frequency of females showing each stage of estrous cycle.

Table 3
Effects of prior intrauterine position on sexual maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
No. of litters	18	27	24	17	27	25
No. of offspring	21	32	30	23	36	26
Day of preputial separation	43.3 ± 1.3[a]	43.4 ± 1.2	44.0 ± 1.8			
Body weight (g)[b]	211.8 ± 5.5	212.1 ± 4.3	212.9 ± 5.2			
Day of vaginal opening				33.8 ± 2.2	33.8 ± 1.8	34.1 ± 1.7
Body weight (g)[b]				125.6 ± 4.1	124.6 ± 4.4	126.1 ± 3.9

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Body weight when the criterion was achieved.