sists of two each of the core histones H2A, H2B, H3, and H4, all of which have a basic, unstructured amino terminal tail. These basic proteins are susceptible to a variety of posttranslational modifications, e.g., acetylation (Howe et al., 1999; Wu et al., 1986). One of the well-characterized modifications is acetylation of specific lysine residues, which is reversibly catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC).

The GCN5-related *N*-acetyltransferase family includes GCN5 and PCAF, which share a remarkable degree of homology throughout their sequences and are present in a multisubunit complex consisting of more than 20 distinct polypeptides (Ogryzko et al., 1998). GCN5 and PCAF are transcriptional coactivators with intrinsic HAT activity; they contribute to transcriptional activation by acetylating chromatin (Sterner and Berger, 2000). Disruption of *Gcn5* and *Pcaf* genes revealed that they play distinct but functionally overlapping roles during embryogenesis (Yamauchi et al., 2000). Other well-characterized coactivators possessing HAT activity are CBP and P300, which are ubiquitously expressed global transcriptional coactivators that have critical roles in a wide variety of cellular processes, including development (Giles et al., 1998; Giordano and Avantaggiati, 1999; Yao et al., 1998).

Recent studies have demonstrated that some environmental pollutants affect the hormonal system and produce adverse effects on animals and probably also humans (Colborn et al., 1996; Van der Kraak et al., 1992). These pollutants are referred to as endocrine-disrupting chemicals (EDCs). The major targets of EDCs are nuclear hormone receptors, which bind steroid hormones and regulate transcription of their target genes (Nishihara et al., 2000; Nishikawa et al., 1999). For ligand-dependent gene activation, nuclear hormone receptors require coactivators that link the basal transcriptional machinery with the hormone receptors (Chen, 2000). Recent studies have shown that the nuclear hormone receptor coactivators possess HAT activity and recruit two other types of HATs, CBP and PCAF (Chen et al., 1997; Spencer et al., 1997). Hormone-dependent gene activation mediated by nuclear receptors involves the mutual recruitment of at least three classes of HATs.

These observations raise the possibility that HATs may be the targets of EDCs, and we tested the effects of suspected EDCs on HAT activity. Interestingly tributyltin (TBT) and triphenyltin (TPT) enhanced HAT activity, but other EDCs did not. These organotin chemicals have been used in such applications as wood preservation and as antifouling agents in marine paints, and are ubiquitous in the environment. TBT and TPT have been found to induce imposex (the superimposition of male sex organs in female gastropods) in the rock shell *Thais clavigera* and are known EDCs in marine species (Horiguchi et al., 1997). These compounds are also reported to affect not only the hormone system but also embryogenesis in mammals (Harazono et al., 1998; Nakanishi et al., 2002). Organotins caused behavioral and neurological symptoms and pancreatic and hepatic toxicities in rodents (Brown et al., 1979; Merkord et al., 2001). In the immune system, at low doses TBT inhibits immature thymocyte proliferation, whereas at higher doses in particular TBT induces apoptotic cell death (Gennari et al., 2002). However, the biological mechanism of the effects of organotin compounds on marine species and mammals awaits further characterization. The present study showed that some organotin compounds enhanced HAT activity when both core and nucleosomal histones were used as substrates. These data suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.

2. Materials and methods

2.1. Chemicals

All chemicals were dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemicals, Osaka, Japan). Organotin and related chemicals tested are listed in Table 1.

2.2. Preparation of rat liver nuclear extracts and HAT fraction

All animal care and handling procedures were approved by the animal care and use committee of Osaka University. Rat liver nuclear extracts (RLNE) were prepared as described previously (Osada et al., 1995). For binding RLNE to Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany), nuclei were suspended in a nuclear lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 10% glycerol, 3 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and

Table 1
Effect of organotin and related chemicals on HAT activity

Chemical	Abbreviation	Relative HAT activity[a]	CAS no.	Purity (%)	Source
Triphenyltin chloride	TPT	$2.03 \pm 0.13^{**}$	639-58-7	>95	Aldrich Chemicals
Diphenyltin dichloride	DPT	$1.63 \pm 0.061^{**}$	1135-99-5	>96	Aldrich Chemicals
Monohenyltin trichloride	MPT	0.97 ± 0.026	1124-19-2	>98	Aldrich Chemicals
Tributyltin chloride	TBT	$2.22 \pm 0.13^{**}$	1461-22-9	>95	Tokyo Kasei Kogyo
Dibutyltin dichloride	DBT	$1.81 \pm 0.045^{**}$	683-18-1	>97	Tokyo Kasei Kogyo
Monobutyltin trichloride	MBT	1.01 ± 0.037	1118-46-3	>95	Aldrich Chemicals
Tin chloride	$SnCl_4$	0.91 ± 0.025	10025-69-1	>97	Nacalai tesque
Tetrabutyltin	TetBT	1.05 ± 0.042	1461-25-2	>93	Aldrich Chemicals
Trimethyltin chloride	TMT	0.95 ± 0.011	1066-45-1	>98	Aldrich Chemicals
Triethyltin bromide	TET	$1.27 \pm 0.034^{*}$	2767-54-6	>97	Aldrich Chemicals
Tripropyltin chloride	TPrT	$3.09 \pm 0.080^{**}$	2279-76-7	>98	Merck Schuchardt
Triphenylsilanol	TPSiOH	1.14 ± 0.23	791-31-1	>95	Merck Schuchardt
Triphenylmethane	TPM	0.95 ± 0.12	519-73-3	>98	Kanto Chemical
Triphenylethylene	TPE	0.91 ± 0.12	58-72-0	>98	Kanto Chemical

[a] Core histones and 10 μM of chemicals were used for the HAT assay. Relative HAT activity shows mean HAT activities (±S.D.) relative to a control treated without chemical (three independent experiments). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)), and 3 M KCl was added to a final concentration of 0.55 M. Nuclear lysate was gently mixed on ice for 30 min and centrifuged at 40,000 rpm in a Beckman 50.2 Ti rotor (Beckman, Fullerton, CA, USA) for 40 min at 4 °C. The RLNE was diluted by adding the same volume of nuclear lysis buffer without KCl and $MgCl_2$ and then incubating with preequilibrated Ni^{2+}-NTA agarose overnight at 4 °C on a rotating wheel. This suspension was poured into a column and the flowthrough was collected. The column was then washed with five column volumes of the nuclear lysis buffer without KCl and $MgCl_2$, and the retained proteins were eluted with a solution consisting of 20 mM imidazole (pH 7.5), 100 mM KCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 1% protease inhibitor cocktail.

2.3. Western blotting and antibodies

Ten microliters of each fraction of column eluate was electrophoresed on an SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with an ECL Western blotting analysis detection system (Amersham Biosciences, Piscataway, NJ, USA). Antibodies against P300, CBP, and GCN5 were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-PCAF antibody was kindly provided by Dr. Yoshihiro Nakatani (Harvard Medical School, Boston, MA, USA).

2.4. HAT assay

HAT assays were performed as follows: 1.5 μg core histones or nucleosome histones was incubated together with 5 μl of eluate from the Ni^{2+}-NTA agarose column, ^{3}H-labeled acetyl-CoA (0.25 μCi), and 1.2 μl of the test compound in 30 μl of HAT buffer (50 mM Tris–HCl, pH 8.0, 50 mM KCl, 5% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate) at 30 °C for 30 min. Histones were prepared as described previously (Owen-Hughes et al., 1999). After incubation, 15 μl of reaction mixture was transferred to a P81 phosphocellulose filter (Whatman, Brentford, UK) and allowed to air-dry. Filters were washed three times in wash buffer (50 mM $NaHCO_3$-$NaCO_3$, pH 9.2) and air-dried. The samples were counted in a scintillation counter (Beckman) for 10 min. The remaining 15 μl was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). All gels were stained with Coomassie Brilliant blue to ensure loading of equivalent amounts of histone in each lane, then destained and flourographed.

2.5. Statistics

All results are expressed as means ± standard deviations (S.D.). Statistical analysis was performed by Dunnett's method.

3. Results

3.1. Partial purification of HAT complex from RLNE

To observe the effects of suspected EDCs on HAT activity, we used partly purified HAT complex from RLNE. Many HATs function as catalytic subunits in HAT complexes, and the specificity and the activity of HAT complexes are different from those of recombinant HATs. For example, recombinant GCN5 can acetylate core histones well, but it exhibits poor nucleosomal HAT activity (Balasubramanian et al., 2002; Grant et al., 1997). Recombinant SAS2 does not show HAT activity, but a complex including SAS2 can acetylate histones (Sutton et al., 2003). Grant and coworkers reported that some native HAT complexes in yeast bind to Ni^{2+}-NTA agarose (Grant et al., 1997). We wondered whether native mammalian HAT complexes bind to Ni^{2+}-NTA agarose. RLNE was incubated with Ni^{2+}-NTA agarose, and bound proteins were eluted with a buffer containing imidazole. Bound fraction included at least four HATs: GCN5, PCAF, P300, and CBP (Fig. 1).

3.2. Effect of TBT and TPT on core HAT activity

Nickel and curcumin (diferuloylmethane) were found to inhibit the acetylation of histones in vitro using recombinant histone acetyltransferase (Balasubramanyam et al., 2004; Broday et al., 2000), indicating that heavy metals and low molecular compounds may affect HAT activity. Suspected EDCs were screened for inhibition and activation of the HAT activity of partly purified HATs by the standard HAT assay procedure using core histone as substrate (Eberharter et al., 1998) and 20 chemicals in the list of chemicals suspected of having endocrine disrupting effects, as published by the Strategic Programs on Environmental Endocrine Disrupters '98, the Japan Environmental Agency (www.env.go.jp/en/pol/speed98/sp98.pdf). Tested 20 chemicals (benzophenone, octachlorostyrene, diethyl phthalate, butyl benzyl phthalate, diethylhexyl adipate, TPT, diethylhexyl phthalate, dicyclohexyl phthalate, di-*n*-butyl phthalate, TBT, 4-nonylphenol, *p*-octylphenol, bisphenol A, 2,4-dichlorophenol, 4-nitrotoluene, di-*n*-pentyl phthalate, dipropyl phthalate, pentachlorophenol, amitrole, and dihexyl phthalate) did not inhibit HAT activity. Interestingly TBT and TPT enhanced HAT activity but other chemicals did not (Fig. 2A and data not shown). HAT activity in the presence of various concentrations of TBT and TPT was assayed, and both chemicals demonstrated dose-dependent enhancement of HAT activity (Fig. 2A). TBT and TPT at both 1 and 10 μM promoted HAT activity but at 0.1 μM had little, if any, effect. Fluorography indicated that partly purified HATs from RLNE acetylated histones H2A, H3 and H4 and that these histones were more effectively acetylated in the presence of TBT or TPT (Fig. 2A, middle panel). A partly purified HAT fraction includes several kinds of HATs and HDACs, but TBT and TPT did not show HDAC inhibitory activity (data not shown). HDACs are classified into three groups, class I, II, and III. Assay reaction mixtures include butyrate, an inhibitor for class I and II HDACs (Ajamian et al., 2004). Further, TBT and TPT enhanced HAT activity in the presence of nicotinamide, an inhibitor for class III HDACs (Bitterman et al., 2002) (data not shown). These results also indicate that TBT and TPT do not inhibit HDAC activity.

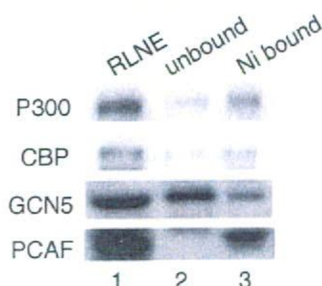

Fig. 1. Partial purification of HATs from RLNE. Western blots were performed with RLNE (lane 1), flow-through from a Ni^{2+}-NTA agarose column (unbound fraction, lane 2), and eluate from a Ni^{2+}-NTA agarose column (Ni-bound, lane 3) using antibodies against P300, CBP, GCN5, and PCAF.

We investigated the effects of organotin and related chemicals on HAT activity. TBT is metabolized to dibutyltin (DBT), monobutyltin (MBT), and inorganic tin; and TPT is metabolized to diphenyltin (DPT), monophenyltin (MPT), and inorganic tin (Horiguchi et al., 1997). The effects of these metabolites of the organotin compounds on HAT activity were also analyzed (Table 1). DBT and DPT enhanced HAT activity, but MBT, MPT, and $SnCl_4$ had no effect. DBT and DPT showed less enhancement of HAT activity than TPT and TBT, but tetrabutyltin did not affect HAT activity (Table 1). These results indicate that trialkyltin com-

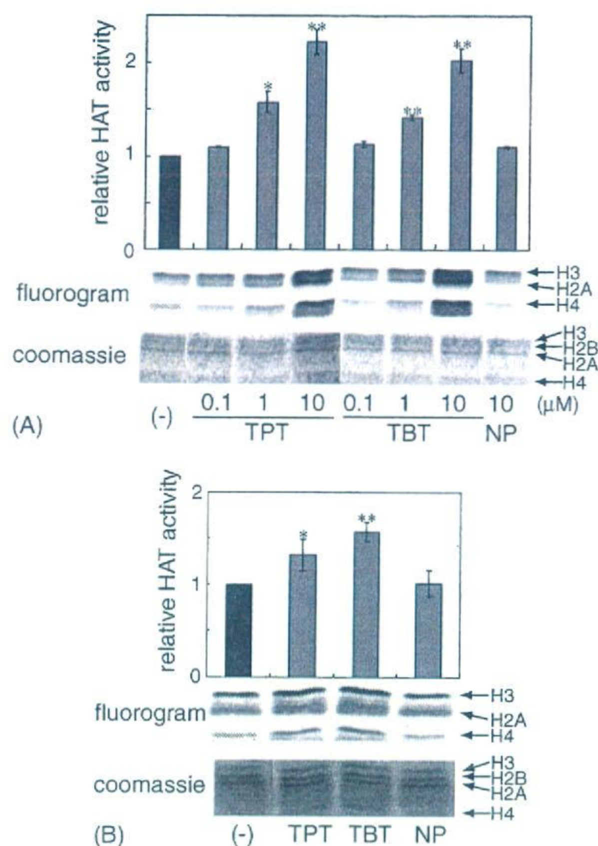

Fig. 2. Effect of TPT and TBT on HAT activity. (A) HAT assays with core histones as substrates and partly purified HAT fraction from RLNE as enzyme. The bar graph shows mean HAT activities (±S.D.) relative to a control treated without chemical (three independent experiments). 4-nonylphenol (NP) was used as a negative control. $^{*}P<0.05$ and $^{**}P<0.01$ compared to control. Products of HAT assays were separated by SDS-PAGE on 18% polyacrylamide gels to resolve the histones. The gels were stained with Coomassie Brilliant blue to visualize proteins (lower panel) and were then dried and visualized by fluorography (middle panel). (B) HAT assays in the absence or presence of chemicals (10 μM), TPT, TBT, and NP, using nucleosome histones as substrates were performed in triplicate as described in the legend to (A). A typical fluorogram from three independent experiments that showed similar results is shown.

pounds are more effective enhancers than other alkyltin compounds.

Next, we focused on the number of carbon alkyl chain in the trialkyltin compounds. Trimethyltin (TMT) did not affect HAT activity, and triethyltin (TET) was a poor promoter of HAT activity. Interestingly, tripropyltin was a more effective enhancer than the compounds containing one or more butyl or phenyl groups. The compounds containing carbon or silicon in place of tin were used to determine whether tin is essential for the promotion of HAT activity by organotin compounds. However, triphenylsilanol, triphenylmethane, and triphenylethylene did not affect HAT activity (Table 1).

3.3. *Effect of TBT and TPT on nucleosomal HAT activity*

Several transcription co-activators possess HAT activity, and the acetylation of nucleosomes associated with the promoter is correlated with transcriptional activation (Ikeda et al., 1999; Sterner and Berger, 2000; Utley et al., 1998). To investigate the effect of TPT and TBT on the nucleosomal HAT activity, we used nucleosomal histones as substrates instead of core histones (Fig. 2B). The partly purified HAT fraction from RLNE includes HAT activity for nucleosome histones, which was promoted by adding TPT and TBT to the reaction. This means that these compounds enhance the core HAT activity as well as the nucleosomal HAT activity.

4. Discussion

We demonstrated that HAT activity is enhanced by certain organotin compounds, including TPT and TBT. Here we found that: (1) trialkyltin compounds are more effective enhancers of HAT activity than mono- and dialkyltin compounds; (2) tin compounds with short alkyl chains showed no effect; and (3) the tin atom is important for the enhancement of HAT activity. On the basis of these results, it is likely that the acetyl CoA binding pocket (active site) or the substrate-binding site of HATs can tolerate a small compound, such as TMT or TET. These results suggest that both a carbon chain of a suitable length and a charge of tin are required for the regulating HAT activity. The crystal structure of HATs with acetyl CoA and/or histone has been determined (Dutnall et al., 1998; Rojas et al., 1999; Yan et al., 2000). Therefore, this information might be useful for analyzing the molecular mechanism of the enhancement of HAT activity by organotin compounds. However, we cannot rule out an alternative possibility that the organotin compounds affects histones. For example, organotin compounds may release histones from a inhibitor of acetyltransferase complex, which binds to histones and masks them from being HAT substrates

(Seo et al., 2001), or change the structure of the histone tails and making them better substrates. Zoroddu et al. propose that the binding of Ni (II) can produce a secondary structure with organized side-chain orientation in the amino terminal tail of histone H4 (Zoroddu et al., 2002). Some compounds including heavy metal(s) may affect histones. We used a partly purified HAT fraction so that the HAT complexes would be in their native form. To clarify the mechanism of HAT activity enhancement by organotin compounds, we are proceeding with identification of the specific HAT whose activity is activated by organotin compounds.

A cDNA microarray analysis revealed that expression of about 130 genes was induced by treatment of the ascidian *Ciona intestinalis* with TBT (Azumi et al., 2004). We previously reported that the level of the mRNA for aromatase/CYP19, which is essential for converting androgenic to estrogenic steroids, was increased by treatment of human choriocarcinoma JAR cells with TBT (Nakanishi et al., 2002). The mechanism of the induction of these mRNAs by TBT has not been elucidated yet. Aberrant HAT activity induced by TBT treatment might cause unusual expression of these genes.

HAT activity is required for the regulation of gene expression and histone acetylation has a fundamental biological role. Organotin compounds have various influences on physical function including the hormone and immune systems, embryogenesis, development, etc. In previous studies, reasonable butyltin concentrations were detected in human liver and blood (Kannan et al., 1999; Lo et al., 2003). Aberrant HAT activity in vivo induced by organotin compounds may cause abnormal development in human and wildlife. Our data indicate that the organotin compounds have unique effects on HATs independent of their EDC activities and suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.

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Involvement of the Retinoid X Receptor in the Development of Imposex Caused by Organotins in Gastropods

JUN-ICHI NISHIKAWA,[†]
SATORU MAMIYA,[†]
TOMOHIKO KANAYAMA,[†]
TOMOHIRO NISHIKAWA,[‡]
FUJIO SHIRAISHI,[‡] AND
TOSHIHIRO HORIGUCHI*,[‡]

Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan, and National Institute for Environmental Studies, Endocrine Disrupters & Dioxin Research Project, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

Organotin compounds released from antifouling paints, such as tributyltin (TBT) and triphenyltin (TPT), are potent inducers of imposex (a superimposition of male genital tracts, such as penis and vas deferens, on females) in marine gastropods. Little is known about the induction mechanism of gastropod imposex. Here, we show that organotins bind the human retinoid X receptors (hRXRs) with high affinity and that injection of 9-cis retinoic acid (RA), the natural ligand of hRXRs, into females of the rock shell (*Thais clavigera*) induces the development of imposex. Cloning of the RXR homologue from *T. clavigera* revealed that the ligand-binding domain of rock shell RXR was very similar to vertebrate RXR and bound to both 9-cis RA and to organotins. These suggest that RXR plays an important role in inducing the development of imposex, namely, the differentiation and growth of male genital tracts in female gastropods.

Introduction

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s and released into the marine environment resulting in a worldwide pollution (*1*). TBT and TPT are very toxic to organisms, including marine species (*2–5*). One of the most interesting toxic effects of TBT and TPT to marine organisms is the induction of the development of imposex in gastropods (*6*). Imposex (as an abbreviation of imposed sexual organs) is defined to be an irreversible syndrome imposing male genital tracts, such as penis and vas deferens, upon female gastropods (*7*). Gastropod imposex is known to be typically induced by very low concentrations of TBT and/or TPT (*7–18*). Reproductive failure is involved at severely affected stages of imposex, due to either oviduct blockage by vas deferens formation or ovarian spermatogenesis, resulting in population declines and/or mass extinction (*6, 19, 20*). Approximately 150 species of gastropods including the rock shell (*Thais clavigera*) have been observed to be affected by imposex in the world (*6*). Gastropod imposex is thought to be one manifestation of endocrine disruption in wildlife (*6*). Despite several hypotheses about imposex induction mechanisms, such as those involving aromatase inhibition, testosterone excretion–inhibition, functional disorder of female cerebropleural ganglia, and involvement of a neuropeptide–APGWamide (*21–25*), the exact physiological/biochemical pathway is still unclear.

The occurrence of reproductive abnormalities in wildlife may be associated with exposure to environmental pollutants capable of mimicking the action of natural hormones (*26*). The nuclear receptors of intrinsic hormone systems are likely to be targets of industrial chemicals because they are originally mediators for fat-soluble, low molecular weight agents such as steroid hormones, thyroid hormones, fat-soluble vitamins, and fatty acids. Forty-eight members of the nuclear receptor family have been shown to exist in the human genome (*27*). Information on the ability of chemicals to bind nuclear receptor family members is therefore important for environmental risk assessment.

To determine if environmental pollutants can bind to members of the nuclear receptor family, we constructed assay systems for human nuclear receptors including ERα, ERβ, AR, PR, GR, MR, RARα, RARβ, RARγ, TRα, TRβ, VDR, RXRα, RXRβ, RXRγ, CAR, and SXR based on a yeast two-hybrid system (*28*). In the course of the study on suspected endocrine disrupters, we found that TBT and TPT strongly enhanced the protein–protein interaction between human RXRs (hRXRs) and coactivator TIF2 to a somewhat greater extent than 9-cis retinoic acid (RA), the natural ligand of RXR.

Here, we will show the results of interaction between organotin compounds, such as TBT and TPT, and hRXR. We will also report the results of cloning of the RXR homologue from the rock shell (*T. clavigera*), its binding characteristics to both 9-cis RA and organotins, and results of the in vivo injection experiment of 9-cis RA using the rock shell. On the basis of these results, we will discuss involvement of the RXR in the development of imposex caused by organotins in gastropods.

Experimental Methods

Yeast Two-Hybrid Assay. We cloned the ligand-binding domain of nuclear receptors including ERα, ERβ, AR, PR, GR, MR, RARα, RARβ, RARγ, TRα, TRβ, VDR, RXRα, RXRβ, RXRγ, CAR, and SXR by RT-PCR from human mRNA (Origin Technologies, Inc.). All sequences were confirmed to be identical to the database by sequencing. These genes were subcloned into pGBT9 (Clontech, Palo Alto, CA) so that they were in the same translational reading frame as the vector's GAL4 DNA binding domain. pGBT9-NRs and pGAD424-TIF2 were introduced into *Saccharomyces cervisiae* Y190. Transformed yeasts were incubated with test chemicals for 4 h at 30 °C, and then β-galacosidase activity was measured as described in Nishikawa et al. (*28*).

Ligand Binding Assay. The LBD of hRXRα (codons 201–693), hRXRβ (codons 275–534), hRXRγ (codons 172–455), and the rock shell RXR (sRXR) (codons 177–431) were subcloned into pGEX-4T (Pharmacia, Uppsala, Sweden). GST-RXRs fusions were expressed in *Escherichia coli* BL21 and purified according to the standard procedure (Pharmacia, Uppsala, Sweden). The purified proteins (30 μg/mL) were incubated with increasing concentrations of 9-cis-[20-methyl-^{3}H]retinoic acid (69.4 Ci/mmol, NEN Life Science Products, Inc.) with or without a 400-fold molar excess of

* Corresponding author telephone: +81-29-850-2522; fax: +81-29-850-2870; e-mail: thorigu@nies.go.jp.
[†] Osaka University.
[‡] National Institute for Environmental Studies.

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TABLE 1. Body Size of Female Rock Shells (*Thais clavigera*) Used in the Injection Experiment (February 14–March 14, 2003)[a]

	control	RA	TPT
shell height (mm)	21.6 ± 1.8	21.5 ± 1.5	20.3 ± 1.4
shell width (mm)	14.5 ± 1.2	14.4 ± 1.2	14.2 ± 0.5
shell weight (g)	2.0 ± 0.5	1.9 ± 0.5	1.7 ± 0.3

[a] Mean ± standard deviation.

TABLE 2. Quality of Artificial Seawater during the Experimental Period (February 14–March 14, 2003)[a]

	control	RA	TPT
water temp (°C)	18.1 ± 0.1	18.2 ± 0.1	18.2 ± 0.1
pH	8.28 ± 0.02	8.31 ± 0.02	8.31 ± 0.04
salinity (‰)	33.5 ± 1.0	33.5 ± 0.9	33.4 ± 1.0

[a] Mean ± standard deviation.

unlabeled 9-cis RA. After incubation at 4 °C for 1 h, specific binding was determined by hydroxyapatite binding assay (*29*). Similarly, organotin compounds were used to compete for 9-cis RA in this assay to determine the binding preference for RXRs.

DNA Cloning. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using total RNA derived from male *T. clavigera*. Degenerate primers used for amplification of RXR were synthesized as follows: F-primer, 5′-TGYGARGGNTGYAARGGNTTYTTYAARMG-3′; R-primer, 5′-RAAGTGNGGVABNMKYTTVGCCCAYTC-3′. A single 390-bp fragment was obtained and sequenced. The fragment was used as a probe for screening in a cDNA library made with λ-ZAP II phagemid vector (Stratagen, Kirkland, WA). The 5′ end of the cDNA was cloned using 5′-Full RACE Core Set (Takara Bio, Shiga, Japan). The amplified products were analyzed by agarose gel electrophoresis, isolated from the gel, cloned into a pBluescript. Five independent clones were sequenced.

In Vivo Injection Experiment. The rock shell specimens were collected at Hiraiso in Ibaraki Prefecture, Japan, in December 2002 for experiments to investigate the effect of 9-cis RA. The rock shells were reared in a laboratory aquarium for approximately 2 months in artificial seawater (Senju Pharmaceutical Co. Ltd., Japan) with live mussels (*Septifer virgatus*) collected at Hiraiso as feed. Before the experiments, the rock shells were narcotized by exposure to a 72 g/L solution of magnesium chloride hexahydrate to allow the selection of females. As a male rock shell has a large penis behind the right tentacle, female shells were easily recognized by its absence (*16, 18*). The female rock shells were divided

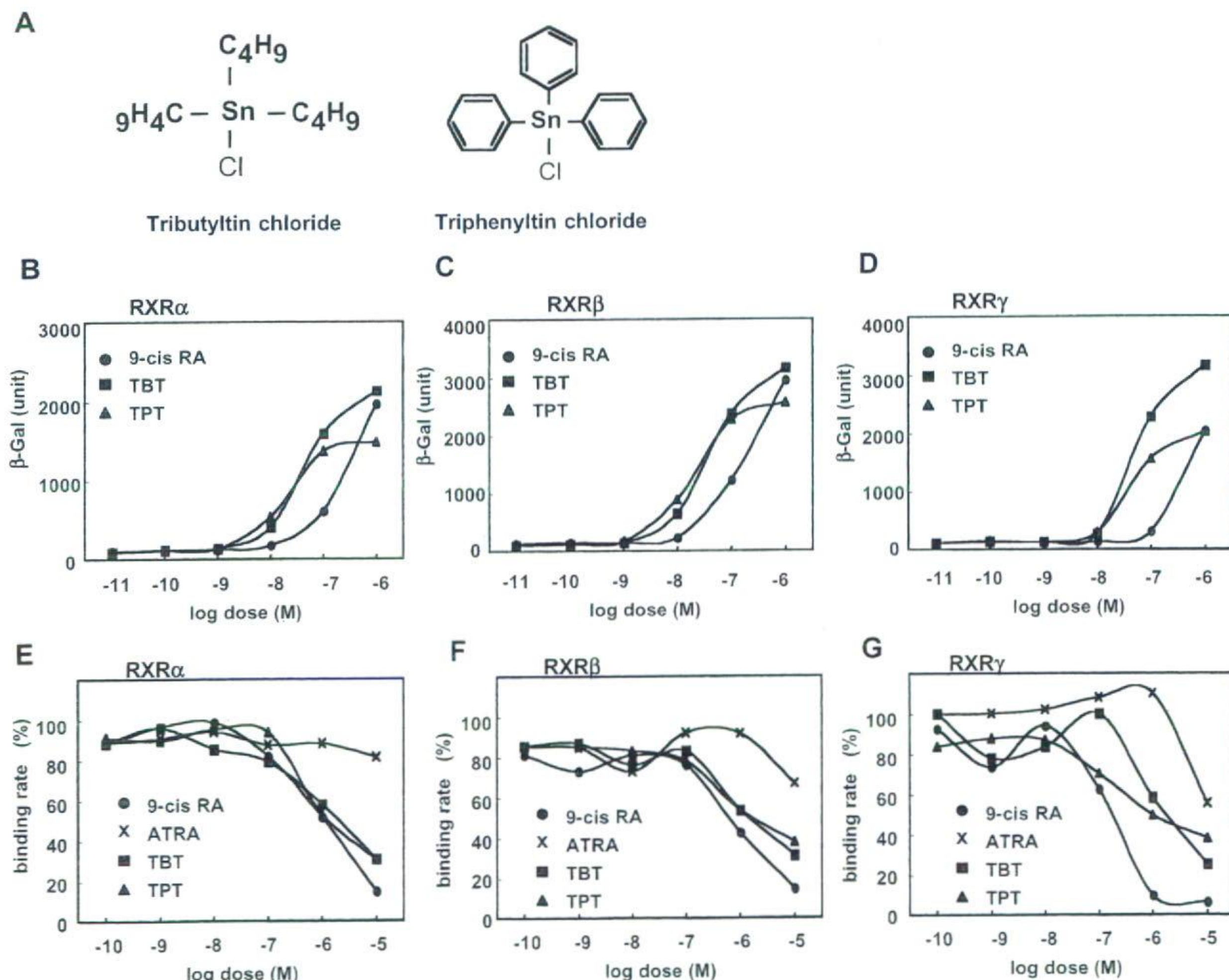

FIGURE 1. RXR activation by TBT and TPT. Structures of organotin compounds are shown in panel A. Yeast strain Y190 was transformed with GAL4AD fused to TIF2 and GAL4DBD fused to LBD of human RXRα (B), RXRβ (C), or RXRγ (D). Chemicals were added to yeast cultures in doses ranging from 10^{-11} to 10^{-6} M. Following 4 h incubation, yeasts were disrupted and assayed for β-galactosidase activity. Data points are means of three independent experiments. For in vitro binding assay, LBDs of RXRα (E), RXRβ (F), or RXRγ (G) were expressed in *E. coli* as fusion proteins with GST. Increasing amounts of chemicals were added to RXRs with 9-*cis*-[20-methyl-^{3}H]retinoic acid for competitive binding assays.

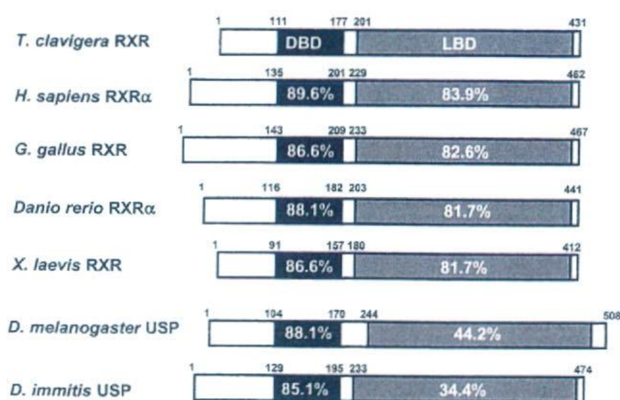

FIGURE 2. Comparison of the deduced amino acid sequences of rock shell RXR with related nuclear receptors. The similarity in the DBD and LBD between rock shell RXR and related nuclear receptors is indicated as percentage amino acid identity. The database accession numbers for the sequences are as follows: *T. clavigera*, AY704160; *H. sapiens*, NM 002957; *G. gallus*, X58997; *Danio rerio*, U29940; *X. laevis*, X87366; *D. melanogaster*, NM 057433; *D. immitis*, AF438230.

into three experimental groups of 20 animals each: for 9-cis RA injection, for triphenyltin (TPT) injection, and for control. 9-cis RA (Wako Pure Chemicals Industries, Ltd., Japan) was prepared in a fetal bovine serum (FBS; Flow Laboratories Inc.) and was injected into the foot at an application rate of approximately 1 μg/g wet wt of soft tissue of the rock shell. FBS was injected to the control animals. Triphenyltin chloride (TPTCl; Tokyo Kasei Kogyo Co., Japan, 98% pure) was used as a positive control agent and was also injected at a rate of approximately 1 μg/g wet wt of soft tissue of the rock shell. The body size of the female rock shells used in the injection experiment is shown in Table 1. After the injection of each test solution, the rock shells were kept in 2 L glass beakers in separate groups, in flow-through systems of artificial seawater saturated with oxygen (10 L/d), with live mussels as feed, for 1 month. Temperature of experimental seawater was maintained to be 18 $\pm$ 1 °C. The quality of artificial seawater during the experimental period (February 14–March 14, 2003) is summarized in Table 2. After this time, animals were removed for imposex examination (*16*). Parameters concerning gastropod imposex [the incidence of imposex (percentage occurrence of imposex individuals among females used in the experimental group), mean values of penis length (measured by automatic/digital caliper), and the vas deferens sequence (VDS) index (an index for the degree of development of vas deferens in the imposex-exhibiting female; the VDS index for the rock shell is similar to that for the dog-whelk reported by Gibbs et al.; *12*)] were calculated for each experimental group (*12, 16*), and the statistical significance of any difference to the control group was tested. The statistical significance of the incidence of imposex was determined using Fisher's *t*-test, and an analysis of variance (ANOVA) was carried out for penis length and VDS index (*24*).

Results and Discussion

Interaction between Organotin Compounds (TBT and TPT) and hRXR. We found that TBT and TPT (Figure 1A) strongly enhanced the protein–protein interaction between hRXRs and coactivator TIF2 (Figure 1B–D) to a somewhat greater extent than 9-cis RA, the natural ligand of RXR (*30, 31*). Because the interaction of nuclear receptor with coactivator correlated with transcriptional activity (*32*), organotin compounds, such as TBT and TPT, were thought to be agonists for hRXRs. However, TBT and TPT showed no activity to other nuclear receptors including retinoic acid receptors

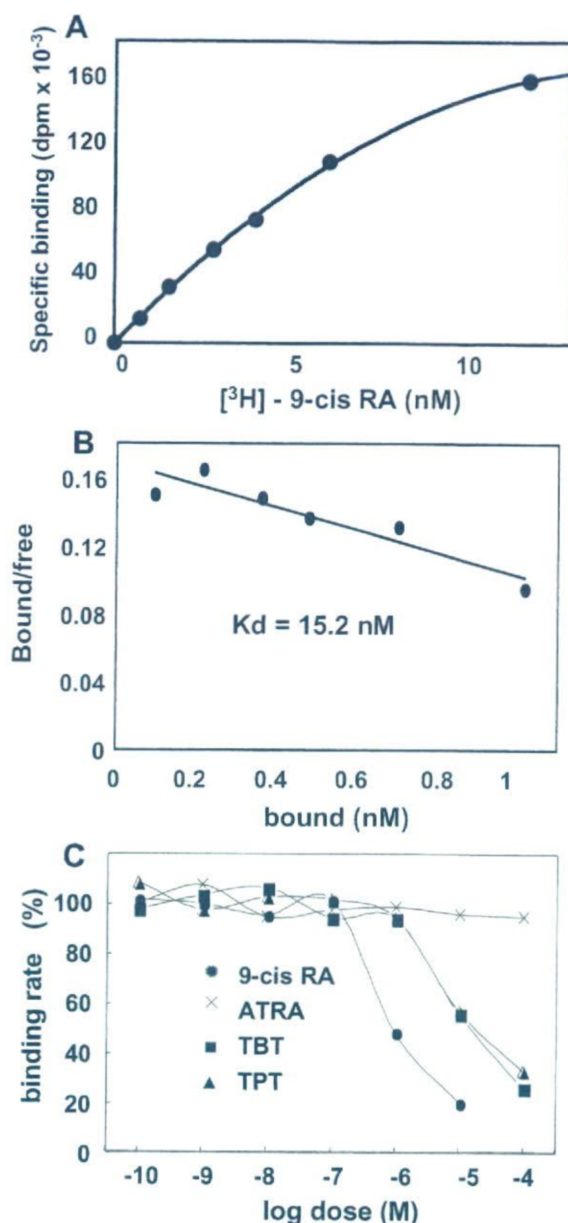

FIGURE 3. 9-cis RA and organotin compounds bind rock shell RXR in vitro. (A) The LBD of rock shell RXR expressed in *E. coli* was incubated with increasing concentrations of ^{3}H-labeled 9-cis RA in the absence (total binding) or presence of 400-fold nonlabeled 9-cis RA (nonspecific binding). Nonspecific binding was subtracted from total binding and plotted as specific binding. (B) Scatchard analysis. Specific 9-cis RA binding to rock shell RXR was transformed by Scatchard analysis and plotted. Linear regression yielded K_d = 15.2 nM. (C) Competition assay. The LBD of rock shell RXR was incubated with increasing concentrations of nonlabeled 9-cis RA, ATRA, TBT, or TPT in the presence of ^{3}H-labeled 9-cis RA.

(RARs) (*33*). While 9-cis RA is known to be a ligand for RARs as well as RXRs (*30*), organotin compounds are specific for RXRs. To confirm the binding of organotin compounds to hRXRs, we carried out an in vitro competition assay against ^{3}H-labeled 9-cis RA and found that TBT and TPT bound to RXRs as well as 9-cis RA (Figure 1E–G). The observation that TBT and TPT could act as agonists for hRXRs led us to investigate the involvement of RXR in the development of imposex in gastropods.

Cloned Rock Shell RXR (sRXR). We tried to clone the RXR cDNA from *T. clavigera*. Comparison of the RXR protein sequences in various species revealed significant similarities in the P-box in the DNA binding domain (DBD) and

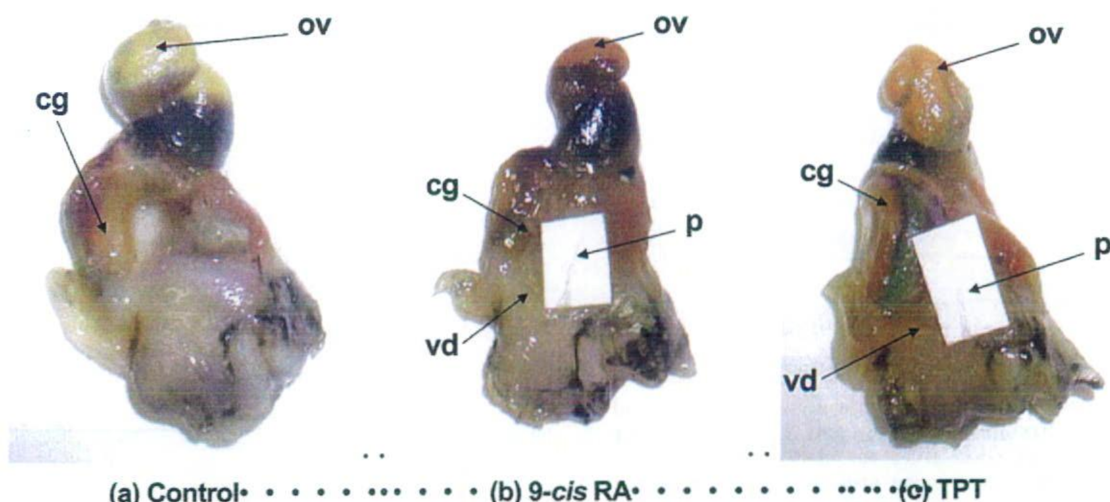

FIGURE 4. Substantial penis growth observed in the female rock shells after 1 month of 9-cis RA injections: cg, capsule gland; ov, ovary; p, penis; vd, vas deferens. (A) Neither penis nor vas deferens was observed in the control female (after shell removal). (B) Substantial penis growth as well as vas deferens development was observed in the female which received 9-cis RA injection (after shell removal; penis length: 6.06 mm). (C) Substantial penis growth as well as vas deferens development was also observed in the positive control female that received TPT injection (after shell removal; penis length: 6.50 mm). Imposex symptoms based on penis length and vas deferens sequence (VDS) index of the females that received 9-cis RA injections were clearly promoted, similar to those of females receiving TPT injections.

helix 4 in the ligand-binding domain (LBD) (*34, 35*). By using degenerate primers deduced from these peptide sequences, we obtained a segment of *T. clavigera* RXR. Next, the *T. clavigera* cDNA library was screened to high precision using the RT-PCR product as a probe. Given that the cDNA isolated by screening was truncated, the 5′ end was amplified by RACE. Comparison of the rock shell RXR (sRXR) protein sequence with the Genebank database revealed that sRXR is closely related to vertebrate RXRs and invertebrate homologues (Figure 2). The highest homology with other species is in the DBD where 85–90% of the amino acids residues are identical (Figure 2). The LBD of sRXR also shows considerable homology with vertebrate RXRs but much less homology to ultraspiracle (USP), the RXR homologue found in *Drosophila*.

Ligand Binding Assay. Vertebrate RXRs bind to 9-cis RA, but insect USP does not (*30, 31, 36*). The LBD of sRXR protein, expressed after fusion with GST in bacteria, bound to 9-cis RA with $K_d = 15.2$ nM (Figure 3A,B), similar to values reported for vertebrate RXRs (*30*). These data implied that *T. clavigera* RXR could bind to 9-cis RA, even though *T. clavigera* is a gastropod mollusk. The sRXR fusion protein also bound to organotin compounds, such as TBT or TPT (Figure 3C). On the other hand, sRXR did not bind to all-trans RA (ATRA) in contrast to human RXRs that bind to ATRA even with low affinity (*30*) (Figure 3C; Figure 1E–G). The jellyfish RXR has also been reported to bind 9-cis RA with high affinity but not to ATRA (*37*).

In Vivo Injection Experiment To Examine the Involvement of RXR in the Development of Imposex in *Thais clavigera*: Effect of 9-cis RA Inducing and/or Promoting the Development of Imposex. To further verify the involvement of RXR in the development of imposex in gastropods, live female rock shells (*T. clavigera*) collected at Hiraiso in Ibaraki Prefecture, Japan (an area of low organotin contamination: see Horiguchi et al.; *18*) were injected with 9-cis RA. Results of these experiments are shown in Table 3 as well as Figure 4. Imposex was significantly induced in female *T. clavigera*, which received the injection of 9-cis RA ($p < 0.01$; Table 3), and substantial penis growth was observed in them after 1 month of 9-cis RA injections (Table 3; Figure 4). Their increased penis length and VDS index were significant when compared with controls ($p < 0.01$ and $p < 0.001$, respectively; Table 3).

TABLE 3. Incidence of Imposex (IOI), Penis Length (PL), and Vas Deferens Sequence Index (VDS) in Female Rock Shells (*Thais clavigera*) after 1 Month of Injections[a]

	control	RA	TPT
IOI (%)	10	50**	80**
PL (mm)	0.04 ± 0.13	2.87 ± 2.39**	3.77 ± 2.16***
VDS	0.20 ± 0.63	3.80 ± 0.42***	3.63 ± 0.74***

[a] Mean ± standard deviation. **, $p < 0.01$. ***, $p < 0.001$.

These results suggest that much 9-cis RA could bring about induction and/or promotion of the development of imposex in *T. clavigera* through its binding to RXR. Relatively large variance for the penis length in females that received injections of 9-cis RA may have resulted from differences in the rate of metabolism of 9-cis RA among female rock shells used in the experiment, although it is not known if *T. clavigera* inherently has a biosynthetic system for RA.

9-cis RA is the first substance, except for certain organotin compounds, that has been confirmed to induce and/or promote the development of imposex in gastopods, especially in terms of penis growth in females. As both TBT and TPT were observed to have agonistic activity to the RXR, it is strongly suggested that gastoropod imposex could be mediated by RXR.

Mode of Action of Organotins on the Development of Imposex in Gastropods. Several hypotheses have been proposed concerning the imposex induction mechanism, and they can be summarized as (i) increased androgen levels, such as testosterone, due to aromatase inhibition by TBT (*21*); (ii) inhibition by TBT of the excretion of sulfate conjugates of androgens (*22*); (iii) disturbance by TBT of penis morphogenic/retrogressive factor released from pedal/cerebropleural ganglia (*23*); and (iv) increase in a neuropeptide, APGWamide, level caused by TBT (*24, 25*). Experimental evidence, however, is weak for these four hypotheses. There is a lack of correlation between the time course of the increase in testosterone titers and penis growth in females in the aromatase inhibition hypothesis (*21*), and there is a possibility that the results given in support of the testosterone excretion–inhibition hypothesis (*22*) may reflect a phenomenon that is at least partly short-term and/or associated with acutely

toxic TBT concentrations (*20*). The effect of APGWamide to induce and/or promote the development of imposex also appears weak based on experimental results of incidence of imposex and penis growth (*24, 25*).

In addition, it should be noted that substantial penis length has been observed in natural populations of imposex-exhibiting females distributed in coastal areas severely contaminated with TBT and/or TPT, as well as in females that received injections of or were exposed to TBT or TPT in the laboratory (*8–12, 16, 18*), and that little is known about basic endocrinology in invertebrates including mollusks (*38*). The penis length in female gastropods observed in the experiments given in support of the aromatase inhibition hypothesis, and the APGWamide involvement hypothesis was small (*21, 24, 25*). This contradiction concerning imposex development, especially penis length in imposex-exhibiting females, strongly suggested that gastropod imposex could be primarily induced and promoted by a factor other than increased androgen levels caused by aromatase inhibition or the neuropeptide, APGWamide. Moreover, there has not been any experimental evidence on purified aromatase protein itself (or aromatase at the protein level) in invertebrates, but only reports on aromatase-like activity in invertebrates including mollusks (*39–41*). The role of steroid sex hormones, similar to those of vertebrates, are still uncertain in invertebrates, because certain peptides have been reported to act as sex hormones in invertebrates such as *Aplysia californica* (Mollusca: Opisthobranchia), *Lymnaea stagnalis* (Mollusca: Plumonata), and *Armadillidium vulgare* (Arthropoda: Malacostraca) (*42–44*). In contrast, RXR is rather well-conserved from invertebrates to vertebrates (Figure 2).

In this paper, we have shown that TBT and TPT are high affinity ligands for RXR and that the natural ligand of RXR significantly caused the development of imposex in female rock shells. These results imply that RXR plays an important role in the induction/differentiation and growth of male genital tracts in female gastropods. Further studies on a heterodimer partner, coupling factors, and target genes of sRXR with molecular biological and immunohistochemical techniques are necessary to clarify the entire mode of action of TBT and/or TPT on the development of imposex in gastropods.

RXRs are key factors involved in the mediation of several hormone response systems via their association with other nuclear receptors as heterodimer partner (*45*). The knockouts of RXRs in the mouse have provided important information in the physiological functions of these receptors. RXRα null mice died in utero and exhibited a hypoplasic ventricular myocardium and ocular abnormalities (*46, 47*). Approximately 50% of RXRβ null mice died before or at birth, and males of the remaining null mutants were sterile, owing to the aberrant lipid metabolism in Sertoli cells (*48*). On the other hand, 9-cis RA is difficult to detect in vivo, and its action is remained to be obscure (*49*). Our result that injection of 9-cis RA into female gastropods induced and/or promoted the development of imposex may provide some insight into the physiological function of 9-cis RA.

Acknowledgments

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新しい視点からみたトキシコロジー
−発生・成長・老化−

井上 達
国立医薬品食品衛生研究所

はじめに

医薬品・食品関連の安全性諸法規が本邦に制定されたのは明治初頭のことである。文明開化の波に乗って，新しい国家として整えるべき多くのシステムが導入された。筆者らの所属する現・国立医薬品食品衛生研究所（当初の名称は東京司薬所，のちの国立衛生試験所）の設立にも遠因をなしたと伝えられるオランダ人アントン・ヘールツ[*1]の指導的影響の下で，最初の局方が作られたのは120年前（1886），東京司薬所の設立後まだ10年余りの時期であった[*2]。引き続いて食品添加物や食品に関する法規がつぎつぎと定められていった（表1）。ついでながら米国が，腐敗したミルクを飲まされたたくさんの乳幼児が死亡したのをきっかけにFood & Drug Actを制定してそれまでの無法状態に終止符をうったのは，そののち，1906年のことである。

近代トキシコロジーの研究がはじまったのは，さらにあとのことである。ここでは，近代トキシコロジーがどのようにして成立し，その後どのような歩みのもとに今日に至り，いまどんな課題をもって将来を展望しているのか，このやや大きな目標にたって，これまでのトキシコロジーを通覧してみたい。

表１　明治初期の食品と医薬品の安全性関連法規

1888	最初の日本薬局方［The Japanese Pharmacopoeia］
1898	最初の添加物規制法［Anilines for food additive dyes］
1900	最初の食品関連法規［Regulation of milk and milk products］
1947	最初の食品関連食品衛生包括法規［Law for Food Hygiene］

参考：U.S.A.

1906　US Food and Drug Act
before regulation:
Food unrestricted sale of unsafe foods
no safety and efficacy testing
Drug unregulated sale and use of worthless or risky drugs

(After Leonard M Schechtman, ATLA 32, Suppl 663-668, 2004)

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*1 Anton Johannes Cornelis Geerts（1843-1883）。オランダ局方第1版をもとに本邦局方原案を作成。
（江本龍雄「ゲールツの日本薬局方蘭文草案（1877）について」医薬品研究, 14: 457, 1983.）

*2 1886年（明治19年），ヘールツの没後，オランダ局方第2版を底本として初版を出版。（国立衛生試験所百年史,（昭和50年）1975.）

1．トキシコロジー研究組織の誕生（表2）

トキシコロジーの領域を毒物学的に考えると，この語源は毒矢の鏃に塗った毒（toxicum）に由来しているとのことだから，大いに歴史の古い分野だということになる。他方，これを生体の異物応答作用に力点をおいた毒性学としての見地[1]から考えると，その歴史は割合短く，半世紀に充たないと云うことになる。

表2　トキシコロジー研究組織の誕生と関連事項

トキシコロジー研究組織の誕生

出来事		トキシコロジー研究組織	
1956	関連する最初のPhocomelia (Widukind Lenz)	・	
1959	FDA医薬品等のガイドラインを公布	1959	Toxicol And Applied Pharmacol創刊
1961	GrünentalがThalidomideの製造を停止	1960	先天異常学会発足（日本）
1962	KelseyにDFCSA（連邦公務員最高栄誉章）授与	1961	SOTの設立準備会　CARTの発足
1963	胎児に及ぼす影響に関するガイドライン薬務局長通知	1962	ESSDT（=EUROTOX）発足
		1964	国立衛試に毒性部新設
1968	配合剤一般毒性試験法	・	
1973	化審法公布	1975	EST/SOT Joint Meet.
1975	PMA自主規制GLP（案）	1977	ICT (Toronto)
1978	FDA GLP公布（翌79発効）	1978	国衛試安全センター発足
・		1980	IUTOX発足 (Sakai form Japan)
・			ICT II (Brussels)
1981	OECD化学物質毒性試験指針	1981	日本毒科学会発足
1984	厚生省医薬品毒性試験法ガイドライン通知	1983	ICT III (San Diego)

トキシコロジーの研究組織が，1960年代にアメリカ，カナダ，ヨーロッパで急速に整備されるに至った当初のきっかけは，いわゆるサリドマイド（thalidomide）による奇形，アザラシ症（phocomelia）の発症にあった。この奇形は稀に片側性の例が観察される。両側性のそれの異常な頻度の発症に気づいたWidukind Lenzが遡行的に第1例と考えたのは1956年の症例であった[2]。米国医薬品局（FDA）は，59年には医薬品のガイドラインの整備に着手しており，同じ年，トキシコロジーの創生的学術誌となるTAAP（Toxicology and applied Pharmacology）が米国薬理学会から創刊されている。

「胎児に及ぼす影響に関する厚生省ガイドライン」が本邦で最初の「医薬品安全性ガイドライン」として薬務局長通知の形で通達されたのはこの時期であり，日本先天異常学会の発足も期を一にしている。これに前後して米国でトキシコロジー学会（SOT）が誕生した。

【SOTの発足】

SOTは，1961年4月，米国学士院（National Academy of Sciences）の担当部長Harry Haysの指導によって催されたワシントンD.C.での準備会議をその起源としている。そこで議論された内容が次のような記録として残されている（図1）。そこでは，

➢新しいトキシコロジー学会が，様々な分野の人々によって構成される学際的[*3]な研究組織となるべきこと[*4]，

➢併せて様々な科学の諸分野にToxicologyの亜領域組織[*5]が生まれるという状態をさけ，SOTがToxicologyの代表的な学会組織，"The Society of Toxicology"になるべきこと，

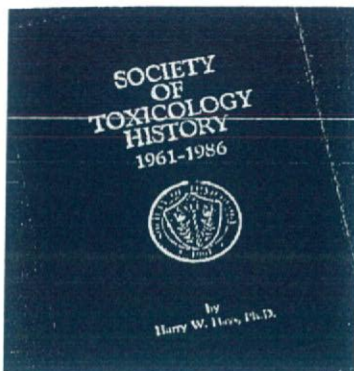

図1トキシコロジー研究組織の誕生

と云った内容になっている。そこには，トキシコロジー学会の発足が既存の薬理学に対する分派行動としての意味を持ちかねない状況への危惧なども記されており，学会発足のニーズとあるべき形との間の桎梏が反映されている。そうした危惧を乗り越えて文字通り学際的で且つ単なる学会にとどまらず，この領域の中心組織としての役割をよく果たして発展してきたSOTの今日そのものは，この時点での申し合わせ意義と米国科学アカデミーのその後の健全な指導力をよく物語っている。

2．試験法の整備

2.1トキシコロジーに用いられる毒性試験法の特性

前述のとおり，SOTの成立に前後して諸々の試験法が整備されてゆく。新しく生まれたトキシコロジーに求められた最も基本的な性格は，グローバリズム（網羅性）と云う考え方であった。すなわち，網羅的試験法の整備（標的臓器，標的表現型，時間軸を含む網羅性）にあったと考えられ，これは，それまでになかった視点であったといってよい。標的の前提を全身に置き，予想外の傷害性（adverse effect）を網羅的にスクリーニングするということの重要性は，予想外の不幸を引き起こしたサリドマイド以後の基本的な視点となった。それは，野生型動物に被験物質を投与し，標的臓器（群）と標的表現形をスクリーニングするという，いわば安全性に関する極めて初歩的認識の再確認であり，毒性メカニズムの探索以前の基本であった。余談ながら，かくして，時には*in vitro*の試験さえも拒否をしてきたトキシコロジーが，今日，後述するようにゲノムの遺伝子発現をトキシコロジーに採用しようとする将来展望にたどりつく原点は，奇しくもこの遺伝子発現の網羅的把握が可能になった現状が基礎になっている。科学における認識の発展の妙味に感じ入るものがある。

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*3 Interdisciplinary

*4 多くの薬理学系のメンバーの重複参加の見通しに鑑みて，薬理学会の分派組織化したりして問題を起こすことのないよう独立した学際性を発揮することを特段に留意している。

*5 Subdisciplines

この時期，がん原性試験も整備された。このものの立脚根拠は，今日的に見ると，これまたたいへん大胆な論理展開になっている。そこでは当然の事ながら発がんのメカニズムは不明であることを大前提としているのであるが，・・・その論理は，メカニズムが不明であっても動物における催腫瘍性を検索し，その結果をヒトへ外挿することによって，ヒトでの発がん性のリスクを予知することができるはずだ，というものである[※6]。これは学術の側から考えるならば，“画期的な認識の飛躍”と云ってよいであろう。爾来，がんのメカニズムが不明のまま発がん性試験を今日まで推進したトキシコロジーには，よい意味での近代プラグマティズムの神髄を見て取ることができる。そしてこれは，トキシコロジーにとって大切な視点と考えられる。

表3　医薬品関連非臨床試験ガイドライン制定の経緯

◆ 1963（昭和38年）4月	「胎児に及ぼす影響に関するガイドライン」厚生省薬務局長通知 （本邦最初の医薬品のための毒性試験法ガイドライン）
◆ 1967（昭和42年）9月	「医薬品製造承認等の基本方針」厚生省薬務局長通知（医薬品承認申請のための添付資料範囲の明確化）（急性毒性・亜急性毒性・慢性毒性・胎児およびその他の特殊毒性）
◆ 1968（昭和43年）7月	「配合剤に関する急性毒性試験法等一般毒性試験法ガイドライン」厚生省薬務局長通知 （1975（昭和50年）3月依存性試験を追加，胎児影響試験，生殖影響試験の追加・訂正）
◆ 1980（昭和55年）	「医薬品承認申請用の添付資料の範囲」が規定される（新医薬品として9種類の試験データを指定）， 同，12月 ECによる医薬品の毒性試験法ガイドライン勧告案 （ついでOECDも「化学物質毒性試験指針」を採択）
◆ 1984（昭和59年）2月	「一般毒性試験法（急性，亜急性，および慢性毒性試験法）およびがん原性試験法，生殖試験法，および変異原性試験法」が毒性試験法ガイドラインとして通知
◆1994（平成6年）以降	国際化の枠組みに応えてICHガイドラインが順次受け入れられてゆく。

数次に亘ってガイドラインは整備されてゆく。表3は医薬品の特に重要な節目の改訂に限って列挙したものである[3]。

以下は，主だった毒性試験法の概要を列挙するが，これらとても時宜に応じて種々変遷してきている。

2.2 急性毒性試験法

急性毒性試験は，短期間曝露による有害作用と致死量（LD_{50}）の検索を目的として行われてきた。これはLD_{50}があたかも化学物質の化学定数の如くに理解され取り扱われてきたことにもとづいている。実際には実験条件の差によって大きく値が変動する生物指標であり，極限高用量の適用が通常適用量での毒性のつよさを必ずしも反映するものとは限らない事などの認識により，無為な実験動物の殺傷を減らすことをも意図して，そうしたデータを相互に受け入れないことが，まずICH[※7]にて，続いてOECD（2002年12月）にて申し合わせられるに至った。これにより，必要適切な条件下で行われる反復投与試験での予備試験結果を代替利用すること，評価目標本位に動物数

※6 日本トキシコロジー学会名誉会員・元国立衛試安全センター長の林 裕造は，がん予防のために確立のなされた「基本原則の1」にこの点をあげてその歴史的経緯を回顧している。（林 裕造 毒性学ノート-化学物質との共存. 2001, pp. 94-98.）

※7 三極調整会議（International Conference of Harmonization of Technical Requirements for Regulation of Pharmaceuticals for Human Use）：日，米，欧の行政および製薬工業界の担当者および専門家による医薬品許認可に関する協調討議のための枠組み。1990年より協議を開始。品質性（Quality），有効性（Efficacy），安全性（Safety）などの論点に整理してトピックを討議している。

を設定すること，臨床使用経路本位に投与経路を設定することなど，裁量の範囲が対象となる医薬品の目的本位で柔軟に判断されるようになってきた。急性毒性試験に関する限り，近年，EU内では完全廃止の方向性を模索する動きも見られるほどである。

2.3 反復投与毒性試験法

反復投与毒性試験は，被検物質を哺乳綱動物に繰り返し投与したときに生ずる毒性変化を用量及び投与時間との関係で把握することとされている。試験用量には，毒性量と無毒性量を含むことが必要なことはもちろんであるが，投与期間については，これまで臨床投与量に応じて行われてきた1ヶ月，6～12～24ヶ月などの4段階に分けた試験に対して，医薬品では，ICHにおける申し合わせに沿って，齧歯類では原則として6ヶ月，非齧歯類では9ヶ月の毒性試験をもって充分との考え方も承認されている。動物試験で得られる結果の範囲で試験を行うこととして，無為な実験の義務づけを排除する動きは，ICHにおける取り組みと共に近年急速に活発になってきた。

2.4 その他の毒性試験法

その他に個別の生体機能への傷害発生予測として，表4に掲げるような基本的な毒性試験が必要要件として求められている。更に，特定の生体機能への傷害発生予測として行われる特殊毒性試験としては，トキシコキネティクス，局所刺激性試験，安全性薬理試験，皮膚感作性試験，光感作性試験，抗原性試験，免疫毒性試験，皮膚・眼毒性（局所刺激性）試験，および毒性機作・標的臓器毒性試験などがあげられる。

表4　その他の毒性試験法

生殖発生毒性試験

新生児への毒性予測のために，受胎能および着床までの初期発生に関する試験。
出生前および出生後の発生ならびに母胎の機能に関する試験，胚・胎児発生に関する試験の3試験（三節）が選択される。

遺伝毒性試験

被験物質の細胞遺伝物質（DNA）に障害性を示す性質を評価することを目的とする。
細菌を用いる復帰突然変異試験，ほ乳類の培養細胞を用いる染色体異常試験，マウスリンホーマー・チミジンキナーゼ試験，および齧歯類を用いる小核試験が選択される。

がん原性試験

被験物質の動物での催腫瘍性の検索により，ヒトでの発がん性リスクを予知する。
ラット，マウスなどを用いて，24ヶ月（18ヶ月で50%の生存率を標準とする）投与され，発がん性について検討する。
GM動物を用いた易発がんモデルや中期2段階発がんモデルなども実施されることがある。

神経発生毒性試験

被験物質を妊娠・授乳期間中に投与し，出生児について身体発達，反射発達，活動性，運動・感覚機能，学習と記憶を検索し，生後発達期と成熟期に脳重量測定と神経病理学的検索を行う。

催奇形性試験は，胎児の発生過程での一刻一刻が特異的な形態形成に掛かっているので，全体的なスクリーニングとしての把握の難しい試験と云うことができよう。遺伝子型によってもそれらの環境因子との相互作用も，すべて特異性があり，傷害の発生感受性には，これまた発育段階特異性が見られること[4]は，携わったことのあるものに周知の通りである（表5）。

表5　Principle of Teratologyに沿った発生毒性試験法の開発

下記，WilsonのPrincipleを満足するよう試験が実施されている：

・奇形発生感受性の，遺伝子型特異性と，そのものと有害環境因子の相互作用
・奇形発生感受性の発生・発育段階特異性
・催奇形性物質の細胞と組織に対する作用の，発生病理機序の特異性
・標的組織とそれらに至る経路の物質特性依存性
・発生異常の４大症状：死亡，奇形，発育遅延，および，機能障害
・逸脱性発生・発育過程の頻度と重症度の用量相関性

ひとつの試験で明らかに出来る事柄は限られている。そこで，短期試験から長期試験に至るまでの種々の試験の組合せバッテリーで当該物質の生体との相互作用を明らかにしてゆく工夫も進展した。表6には，現在適用されている食品添加物に関する必要な試験項目を例示した。

表6　安全性試験必要項目の設定（例：食品添加物）

一般毒性試験	28日間反復投与毒性試験	実験動物に28日間繰り返し与えて生じる毒性を調べる
	90日間反復投与毒性試験	実験動物に90日間以上繰り返し与えて生じる毒性を調べる
	1年間反復投与毒性試験	実験動物に1年以上の長期間にわたって与えて生じる毒性を調べる
特殊毒性試験	繁殖試験	実験動物に二世代にわたって与え，生殖機能や新生児の生育に及ぼす影響を調べる
	催奇形性試験	実験動物の妊娠中の母体に与え，胎児の発生，生育に及ぼす影響を調べる
	発がん性試験	実験動物にほぼ一生涯にわたって与え，発がん性の有無を調べる
	抗原性試験	実験動物でアレルギーの有無を調べる
	変異原性試験	細胞の遺伝子や染色体への影響を調べる
	（発がん性試験の予備試験）	細胞の増殖性や遺伝子の修復への影響を調べる

以上の毒性試験法の結果を総合し，生体有害性として整理すべき事と生体有害性から区別すべきことなどを分けて，無影響量ないし無毒性量に相当するものを判断し，耐容一日摂取量（Tolerable daily intake, TDI）を定めてゆくことは，既存のトキシコロジーの教科書にあるとおりである。蛇足ながら，安全性試験というものはリトマス試験紙のような白黒を見る仕組みになってはいない。

存在するのは種々の毒性試験法であり，それら毒性試験法で明瞭な毒性が認められないことを以て，安全である可能性を推論する仕組みになっているにすぎない。

3. トキシコロジーの概念と問題点

このようにして整備されてきたトキシコロジー関連の毒性試験で，これまでの約半世紀の経過の中で，あらたに整理すべきことがらが次第に明らかになりつつある。それらの中にはまず，1）動物実験関連の諸課題があり，これには種特異性を巡る問題，実験動物代替法を巡る技術革新のための課題，動物愛護のための諸問題が含まれる。つづいて，2）用量反応相関性と低用量特異性反応に関する課題，3）発がん性と閾値に関する課題，そして，4）リスクアセスメントに関する新しい課題などが問題点として浮上している。

主だったものを，順を追って簡単に通覧してみよう。

3.1 実験動物関連の諸課題

実験動物関連の諸課題では，信頼性保証（Good Laboratory Practice: GLP）に関わる適正な飼育や記録の保管，動物愛護の推進のための技術革新，近頃では，環境中に人工的な変種が拡散しないようにとの配慮にもとづいたカルタヘナ条約[※8]の批准など，あらたな配慮すべき技術的あるいは事務的な事柄が増えている。もとより，神経毒性に比較的鋭敏であることの知られる鳥類，アナフィラキシーを引き起こしやすく免疫毒性試験に多く用いられるモルモットといった動物毎の毒性に関する特徴が知られるなど，動物試験にあっては科学面で留意すべき事柄が多岐におよぶほか，さらに近年では種々の遺伝子改変動物（GM動物）[※9]の普及も日進月歩である。たくさんの課題の山積する中で，抗腫瘍作用などで見直されつつあるサリドマイドのバイオアベイラビリティにおけるAUC（area under the curve）なども，マウス，ウサギ，ヒトのあいだで図2にみられるような大きな差があることが報告されたのは最近のことにすぎない[5)]。

種特異性(動物毎に特性があるが・・・)

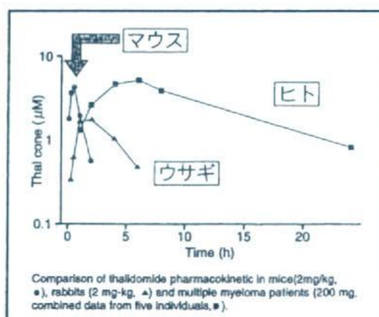
Thalidomide Pharmacokinetics and Metabolite Formation in Mice, Rabbits, and Multiple Myeloma Patients

Francisco Chung, Jun Lu, Brian D. Palmer, Philip Kestell, Peter Browett, Bruce C. Baguley, Malcolm Tingle, and Lai-Ming Ching

ABSTRACT

Purpose: Thalidomide has a variety of biological effects that very considerably according to the species tested. We sought to cstablish wether differences in pharmaconkinetics could form a basis for the species-spectific effects of thalid-omide.

Results: Plasma concentration-time profiles for the individual patients were very similar to cach other, but widely different pharmacokintic propertics were lound between patience compared with those in mice or rabbits. Area under the concentration curve values for mice, rabbits and multiple myeloma patients were 4, 8, and 81 μmo/L · hour, respectively, and corresponding elimination half-lives were 0.5. 2.2. and 7.3 hours. respectively. Large differences weree



Comparison of thalidomide pharmacokinetic in mice(2mg/kg, ●), rabbits (2 mg-kg, ▲) and multiple myeloma patients (200 mg. combined data from five individuals,■).

図2　実験動物関連諸課題[5)]

[※8] 2000年1月の生物多様性条約特別締約国会議において採択。バイオテクノロジーにより改変された生物であって，生物の多様性の保全及び持続可能な利用に悪影響を及ぼす可能性のあるものについて，その安全な移送，取扱い及び利用を取り決めている。研究などによる移送にあたっても文書で記録を残すことを厳しく義務づけ，違反の際の罰則も規定している。

[※9] GM動物（genetically modified animals）

こうした中で，種間外挿の課題とも相俟って「ヒト型動物」の作製のニーズが取り上げられることになる[6]（図3）。連絡するシグナル経路のすべてをヒト型遺伝子に植え替えるワケには行かないので，得られる結果に限界があることはやむを得ない。ヒト型反応をする薬物代謝酵素改変動物を作製したりして，種間差を乗り越える努力が重ねられる一方，さらに図4に示すような，ヒトの胚性幹細胞や，造血あるいは神経幹細胞，そして，通常の株細胞を用いた代替法の展望も活発に探索されるようになっている。

ヒトに特異的な免疫反応様式や薬物代謝様式を持つ実験動物を，「ヒト型モデル」とよぶ。医薬品などに起因する種々の薬物傷害のなかには，実験動物とヒトとの生物学的差異そのものに基づくものが少なくなかった・・・。

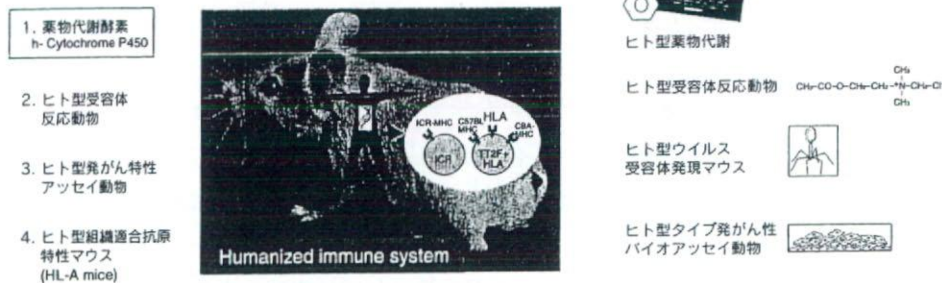
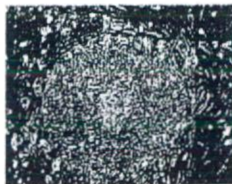


図3　ヒト型モデル動物作製プログラム

Stem cells:



ヒト胚性幹細胞

Embryonic stem cells
neuron, hemopoietic system, vascular system, cartilage-osteocyte system

Hemopoietic stem cells
variety of hemopoietic lineages

Neuronal stem cells, etc.
neuron, retinal stem cells, glia, and other tissue lineages

Tissue cells: Primary hepatic cells
Primary renal cells
Variety of cell lines

metabolism
cell cycle modulators
proliferators and variety of inhibitors
terminal differentiation

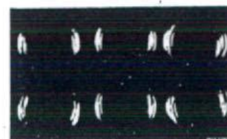


図4　実験動物関連諸課題
（“*in vitro*”“*in vivo*”の相互外挿性 ⟶ 代替法への展望）