Constitutively Active Aryl Hydrocarbon Receptor Expressed Specifically in T-Lineage Cells Causes Thymus Involution and Suppresses the Immunization-Induced Increase in Splenocytes[1]

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The aryl hydrocarbon receptor (AhR) is a transcription factor belonging to the basic helix-loop-helix-PER-ARNT-SIM superfamily. Xenobiotics, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, bind the receptor and trigger diverse biological reactions. Thymocyte development and T cell-dependent immune reactions are sensitive targets of AhR-dependent 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity. However, the exact role of the AhR in T cells in animals exposed to exogenous ligands has not been clarified because indirect effects of activated AhR in other cell types cannot be excluded. In this study, we generated transgenic (Tg) mice expressing a constitutively active mutant of AhR under the regulation of a T cell-specific CD2 promoter to examine AhR function in T cells. The mRNAs of the constitutively active mutant of AhR and an AhR-induced gene, CYP1A1, were expressed in the thymus and spleen of the Tg mice. The transgene expression was clearly detected in the thymocytes, CD4, and CD8 T cells, but not in the B cells or thymus stromal cells. These Tg mice had a decreased number of thymocytes and an increased percentage of CD8 single-positive thymocytes, but their splenocytes were much less affected. By contrast, the increase in number of T cells and B cells taking place in the spleen after immunization was significantly suppressed in the Tg mice. These results clearly show that AhR activation in the T-lineage cells is directly involved in thymocyte loss and skewed differentiation. They also indicate that AhR activation in T cells and not in B cells suppresses the immunization-induced increase in both T cells and B cells.* ***The Journal of Immunology*, 2005, 174: 2770–2777.**

Xenobiotics, such as polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons, bind and activate the aryl hydrocarbon receptor (AhR),[3] a transcription factor belonging to the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) superfamily (1, 2), and elicit diverse biological and physiological responses (3–6). These findings suggest that the AhR functions physiologically as a ligand-dependent transcription factor, whereas the endogenous ligands and intrinsic role of the AhR have yet to be identified. The decreased fertility and abnormalities found in various organs, including the liver, spleen, vascular structures, ovary, mammary gland, and bone marrow lymphocytes, in AhR-deficient mice (7–12) also imply intrinsic roles of the AhR in normal developmental processes. In the absence of ligands, the AhR exists in the cytoplasm in an inactivated form complexed with a dimer of heat shock protein 90 and the immunophilin homologue hepatitis B virus X-associated protein 2 (13). Upon binding with ligands, such as its most potent ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the AhR becomes activated, dissociates from the protein complex, and translocates into the nucleus, where the receptor dimerizes with another basic helix-loop-helix-PAS transcription factor, aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT heterodimer specifically binds DNA sequences, called xenobiotic responsive elements (XREs), distributed in the enhancer regions of various genes, including one of the most sensitive targets, CYP1A1, and modulates their expression (14). The receptor complex also interacts with various nuclear proteins, such as retinoblastoma, NF-κB, and estrogen receptors (15–17). However, determination of the functions of the AhR requires identification of the genes and proteins that it modulates and the cell types in which the individual biological or physiological reactions occur.

The immune system is one of the sensitive targets of TCDD (6). Although a major portion of TCDD toxicities, such as thymus involution, suppressed CTL activity, and reduced Ab production, have been demonstrated to be mediated through the AhR by studies in AhR-deficient mice (18–20), the precise mechanisms of AhR function, including the primary cellular targets and biological reactions involved in these toxic effects, remain to be clarified. The thymus involution induced by administration of TCDD or other AhR ligands to mice is characterized by decreases in tissue weight and cell number that are mainly attributable to a decrease in $CD4^+CD8^+$ double-positive (DP) cells, the predominant population of thymocytes. Skewing of thymocyte differentiation toward CD8 single-positive (SP) T cells is another peculiar feature of the

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[3] Abbreviations used in this paper: AhR, aryl hydrocarbon receptor; PAS, PER-ARNT-SIM; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic responsive element; DP, double-positive; SP, single-positive; FTOC, fetal thymus organ culture; Tg, transgenic; CA-AhR, constitutively active mutant of AhR; h, human; DIG, digoxigenin; 7-AAMD, 7-aminoactinomycin D; DN, double negative.

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response to TCDD exposure (18, 21, 22). All of these features are reproduced in vitro by direct addition of TCDD to fetal thymus organ culture (FTOC) (19, 23, 24), indicating that the target cells responsible for the alterations are present in the thymus. The results of previous studies that have included histological examination have led to the hypothesis that thymic stromal cells, and not thymocytes themselves, are the direct targets of TCDD that induce thymus involution (25). This hypothesis was supported by a study showing that the stroma of a fetal thymus reaggregation culture treated with an AhR-binding halogenated aromatic hydrocarbon, not the thymocytes, induced thymus involution (24). By contrast, a recent study in which chimeric mice having AhR-deficient hemopoietic cells and wild-type stromal cells or vice versa were exposed to TCDD demonstrated that the AhR in the hemopoietic compartment, that is in the thymocytes or their precursor cells, is responsible for the TCDD-induced thymus involution (18).

The results of other studies have also shown or suggested that the AhR in T cells plays an essential role in TCDD-induced immunotoxicity. A recent study by Kerkvliet et al. (26) in a mouse graft-vs-host model injected with $AhR^{+/+}$ or $AhR^{-/-}$ T cells showed that AhR activation in T cells is critical to the suppression of CTL activity by TCDD. In our own study examining the effect of TCDD on OVA-specific Ab production in mice (27), TCDD exposure suppressed the increase in T cell number in the spleen and production of IL-2 and Th2-type cytokines before the inhibition of Ag-induced Ab production, suggesting that the AhR activation in T cells causes suppression of T cell activation and subsequent immune reactions leading to Ab production. However, it is difficult to determine the specific role of AhR activation in T cells alone in TCDD-exposed mice, because the AhR in all cell types, including B cells and APCs, is simultaneously activated, and indirect effects cannot be excluded. Chimeric mouse models and a T cell transfer system produced by using AhR-deficient mice or their cells are very useful tools for studying the primary cell target of TCDD, but they are inconvenient, because generation of chimeric mice and reconstitution by T cell transfer require highly specialized techniques. In addition, when chimeric mice are used, it must be borne in mind that their hemopoietic cells contain precursor cells not only for T cells but for B cells and APCs as well (28).

To investigate the role of AhR activation in TCDD-induced immunotoxicity, in the present study we generated transgenic (Tg) mice that specifically express a constitutively active mutant of AhR (CA-AhR) in T-lineage cells by expressing a CA-AhR with a minimal deletion in the PAS-B domain (29) under the regulation of a CD2 promoter. The AhR mutant constitutively localizes to the nucleus, heterodimerizes with ARNT, and activates transcription by binding XRE sequences in a ligand-independent manner (29, 30). The results of the present study demonstrate that AhR activation in T-lineage cells alone directly induces the thymocyte changes. They also show that the increase in number of splenocytes after immunization is suppressed in the Tg mice, whereas resting splenocytes in nonimmunized mice are much less affected, suggesting that the AhR plays a role in the growth of activated and proliferating T cells.

Materials and Methods

Generation of Tg mice

The CA-AhR expression construct (VA hCD2-CA-AhR) was generated by subcloning PAS B-domain-deleted mouse AhR cDNA with poly(A) signal (29) into the *EcoRI/BamHI* site of the VA human CD2 (hCD2) minigene, an improved version of a human CD2 minigene-based vector (31). Tg founder mice were obtained by microinjecting the transgene expression construct into C57BL/6J × DBA/2 eggs as described previously (32). In some lines, VA hCD2-GFP was coinjected with VA hCD2-CA-AhR. One line carrying both CA-AhR and GFP constructs (line A) and two lines with the CA-AhR construct alone (lines K and N) were chosen for further studies and subsequently were backcrossed into C57BL/6J mice. Founders and subsequent littermates were genotyped by PCR of tail DNA using primers for VA hCD2-CA-AhR (5′-GAACAGAGAGTTTGTCCAGC-3′, located in hCD2 promoter, and 5′-CTTCCAAAGGTAAGCATAAGAGTC-3′, located in N terminus of CA-AhR). Integrated CA-AhR copy number was determined by Southern blot analysis. Genomic DNA from a tail sample was digested with *EcoRI* and *PstI*, separated by agarose gel electrophoresis, blotted onto a Hybond filter (Amersham), and hybridized with a digoxigenin (DIG)-labeled probe. The DIG-labeled probe was synthesized from the *HincII* digestion fragment of pEB6CAG-CA-AhR-GFP (29) with a DIG-high prime DNA labeling and detection starter kit I (Roche Diagnostics) and was detected with CSPD as a substrate according to the manufacturer's instructions. Heterozygous ($CA\text{-}AhR^{+/-}$) mice were used for experiments after crossing into C57BL/6 mice for two to six generations. Their nontransgenic ($CA\text{-}AhR^{-/-}$) littermates (designated as wild type) were used as controls.

Cell preparation

Single cell suspensions of thymus and spleen were prepared by forcing cells in RPMI 1640 medium supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (complete medium) through a stainless-steel mesh. Spleen cells and bone marrow cells prepared from thigh bones were treated with ammonium chroride/EDTA solution (0.83% NH_4Cl, 0.1% $KHCO_3$, 0.37% EDTA (pH 7.4)) for 2 min at room temperature to eliminate RBCs and then were washed with PBS (33). Cells were counted with a hemocytometer after staining with trypan blue.

RT-PCR

Total RNA was isolated from cells or tissues with an RNeasy Mini kit (Qiagen). After checking the quality of the RNA by electrophoresis, RT-PCR was performed with an RNA LA PCR kit (AMV) ver1.1 (TaKaRa Biomedicals) according to the manufacturer's instructions. The amplification was conducted by heating at 94°C for 2 min, cycling at 94°C for 30 s, 60 or 66°C for 30 s, and 72°C for 30 s, and then extension at 72°C for 10 min after the final cycle. The primer sequences and annealing temperatures for each gene are shown in Table I. The primers for detecting mRNA expression of CA-AhR were designed to span the region coding for PAS B domain to distinguish PCR products between CA-AhR and wild-type AhR. The PCR products were separated with a 1.2% Synergel (Diversified Biotech) containing 0.5 μg/ml ethidium bromide, and the gel images were captured and visualized using an Electrophoresis Documentation and Analysis System 290 (Eastman Kodak).

Flow cytometry

Cells were stained with mAbs against lymphocyte surface markers or streptavidin-allophycocyanin (BD Pharmingen) for 20 min on ice. After staining, the cells were washed, treated with 7-aminoactinomycin D (7-AAMD; Sigma-Aldrich) to label dead cells, and measured with a FACSCalibur (BD Biosciences). Live cells were gated and analyzed (22). The following mAbs, all purchased from BD Pharmingen, were used: PE-conjugated anti-CD4 (anti-CD4-PE, clone GK-1.5), FITC-conjugated anti-CD8

Table I. *List of primers used for RT-PCR*

Description	Primer Sequence (5′-3′)	Annealing Temperature (°C)	Product Size (bp)
CA-AhR	TTACCTGGGCTTTCAGCAGT AACTGGGGTGGAAAGAATCC	66	506
CYP1A1	CCATGACCGGGAACTGTGG TCTGGTGAGCATCCTGGACA	60	344
Adseverin	GTGCTTCTAAGCATTTCCCC GAGTGAATGGCATCCAAGTG	60	121
CD4	AAGGGCTCTCCCTGAGAGTC AAAGAGGAAAAAAGGGGAAGG	60	104
Spatial	GAAGGTGACAGCGAAAATCA AAGGCATTAGACAGGTTGGG	60	112
β-Actin	GAGGCCCAGAGCAAGAGAG GGCTGGGGTGTTGAAGGT	60	225
HPRT	GCTGGTGAAAAGGACCTCT CACAGGACTAGAACACCTGC	60	249

(anti-CD8-FITC, clone 53-6.7), biotinylated anti-CD8 (anti-CD8-biotin, clone 53-6.7), anti-CD3-PE (clone 145-2C11), anti-CD19-biotin (clone ID3), anti-B220-FITC (clone RA3-6B2), anti-CD127(IL-7Rα)-biotin (clone B12-1), and anti-CD61-PE (clone 2C9.G3). Biotinylated rat IgG2a was used as an isotype-matched control.

Immunization

OVA (albumin, chicken egg, grade VII) was purchased from Sigma-Aldrich. Alum-precipitated OVA (OVA/alum) was prepared as follows (27, 34). OVA (1 mg/ml) in PBS was mixed with an equal volume of 9% (w/v) $AlK(SO_4)_2$, and pH of the mixture was adjusted to 6.5 with KOH. The precipitate was washed three times with PBS and then resuspended in PBS at 0.5 mg/ml. Mice were i.p. immunized with the OVA/alum (100 μg OVA/mouse).

TCDD treatment

TCDD (50 μg/ml in nonane) purchased from Cambridge Isotope Laboratories was diluted with corn oil to adjust it to a dose volume of 10 μl/g body weight. TCDD was administered to the mice orally.

Fetal thymus organ culture

Line A heterozygous Tg mice backcrossed into C57BL/6J mice for five generations were mated, and homozygous CA-$AhR^{+/+}$ Tg mice were obtained. Male CA-$AhR^{+/+}$ mice were mated with female C57BL/6J mice, and thymuses were collected from fetuses on gestation day 16.5. One or two lobes of the thymuses were placed on a nitrocellulose filter (45-μm pore size) set in a 24-well culture plate with 1 ml of complete medium and were cultured for 4 days (35). To deplete them of thymocytes, the lobes were cultured in the presence of 1.35 mM 2-deoxyguanosine (Sigma-Aldrich) for 4 days (36, 37).

Results

Generation of T cell-specific CA-AhR Tg mice

We used the VA hCD2 vector to generate Tg mice expressing a CA-AhR mutant (Fig. 1*A*) specifically in T-lineage cells. We chose one line carrying both CA-AhR and GFP constructs (line A) and two lines with only the CA-AhR construct (lines K and N) functioning under the control of the VA hCD2 vector for the subsequent experiments. The transgene-positive mice were mated with C57BL/6 mice and maintained as heterozygotes. Heterozygous mice were used in all experiments unless otherwise specified, and their nontransgenic (CA-$AhR^{-/-}$) littermates (designated as wild type) were used as controls. Integrated CA-AhR copy numbers were determined by Southern blotting to be 2 for line A, 6–7 for line K, and 9–11 for line N. All of the lines were fertile, exhibited a normal sex ratio at birth, showed no increase in mortality after birth, and appeared healthy.

Fig. 1*B* shows CA-AhR mRNA expression in various organs in line A Tg mice. CA-AhR mRNA was detected in the thymus and spleen as expected, and was also found in the lung and, to a very minor extent, in the kidney. Expression of the AhR-responsive gene CYP1A1 was also detected in the thymus and spleen in the Tg mice in contrast with their wild-type littermate mice (Fig. 1*C*). In the lung, CYP1A1 mRNA was detected in the wild-type mice. The lung is reported to express the highest level of AhR mRNA among the tissues examined, including the thymus and spleen, in the mice (38). Recently, endogenous ligand was isolated from porcine lung (39). Thus, the lung may contain abundant AhR and endogenous ligand may activate the receptor and induce CYP1A1. The expression of CYP1A1 in the lung was further increased in the Tg mice (Fig. 1*C*). Expression of CA-AhR and CYP1A1 mRNA was also confirmed in the thymus and spleen of lines K and N (see Fig. 4).

Expression of the transgene in immune cells was measured by flow cytometry analysis of the GFP expression in line A Tg mice (Fig. 2). Thymocytes showed a broader peak of the GFP-positive population (Fig. 2*A*). CD4 and CD8 T cells in the spleen were confirmed to be GFP-positive, and B cells did not express GFP (Fig. 2*B*).

We then investigated whether the bone marrow cells of the CA-AhR Tg mice expressed the transgene and, as shown in Fig. 3, the $CD3^-CD127^+$ (IL-7Rα-expressing) lymphocyte progenitor fraction (40) was found to be faintly GFP-positive (Fig. 3*C*, R3). Although a previous study reported that the VA hCD2 vector functions in megakaryocytes as well as T-lineage cells (32), $CD61^+$ megakaryocytes (41) in the bone marrow did not express the transgene (Fig. 3*C*, R4 and R5).

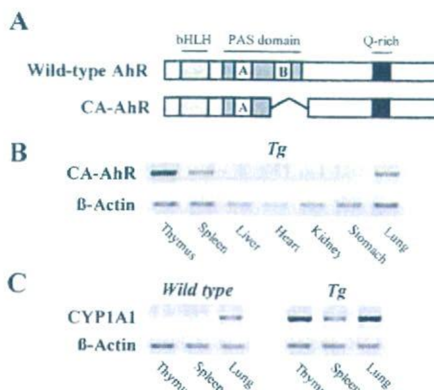
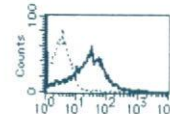
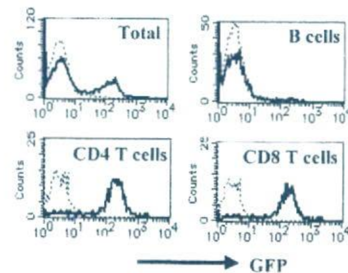


FIGURE 1. Generation of T cell-specific CA-AhR Tg mice. *A*, Schematic representation of the wild-type mouse AhR and the CA-AhR mutant lacking the minimal PAS B motif. *B*, Different tissues from line A heterozygous Tg mice were examined for CA-AhR mRNA expression by RT-PCR. C, Functional activation of the CA-AhR was confirmed by detection of CYP1A1 expression by RT-PCR.

A Thymocytes



B Splenocytes



GFP

FIGURE 2. Transgene expression in thymocytes and splenocytes. *A*, Thymocytes prepared from line A heterozygous mice were stained with 7-AAMD and analyzed with a FACSCalibur flow cytometer. 7-AAMD-negative live cells were gated and expression of coinjected GFP was analyzed. *B*, Splenocytes from line A heterozygous mice were stained with a combination of anti-CD19-biotin/streptavidin-allophycocyanin and anti-CD3-PE or a combination of anti-CD8-biotin/streptavidin-allophycocyanin and anti-CD4-PE and then were stained with 7-AAMD and analyzed with a FACSCalibur. CD4 T cells, CD8 T cells, and $CD19^+$ B cells in the 7AAMD-negative live cells were gated, and GFP expression was analyzed. The staining obtained in Tg mice is represented by the bold line, and the staining in wild-type mice is represented by the dotted line.

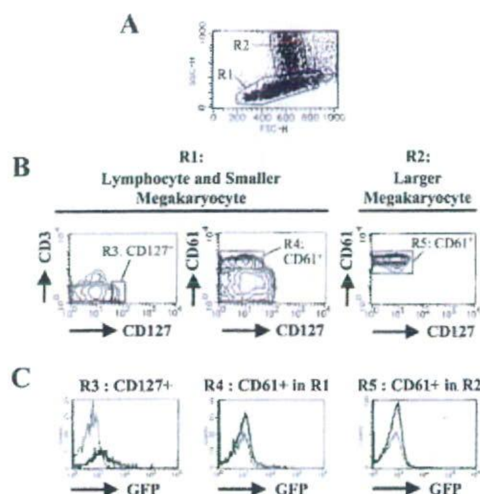


FIGURE 3. The transgene is faintly expressed in CD127$^+$ cells in the bone marrow. Bone marrow cells from line A heterozygous mice were stained with anti-CD127-biotin/streptavidin-allophycocyanin and either anti-CD3-PE or anti-CD61-PE and then were stained with 7-AAMD and analyzed with a FACSCalibur. *A*, Side scatter vs forward scatter of the cells analyzed. Region 1 (R1), including lymphocytes and smaller size megakaryocyte, and R2, including larger size megakaryocytes (41), in the 7-AAMD-negative live cells are indicated in the dot plot. *B*, R3, including CD127$^+$ cells, and R4, including CD61$^+$ cells in R1, and R5, including CD61$^+$ cells in R2, are indicated in the contour plot. *C*, Expression of GFP as analyzed by gating R3–R5 is shown in the histogram plot. The staining obtained in Tg mice is represented by the bold line, and the staining in wild-type mice is represented by the thin line.

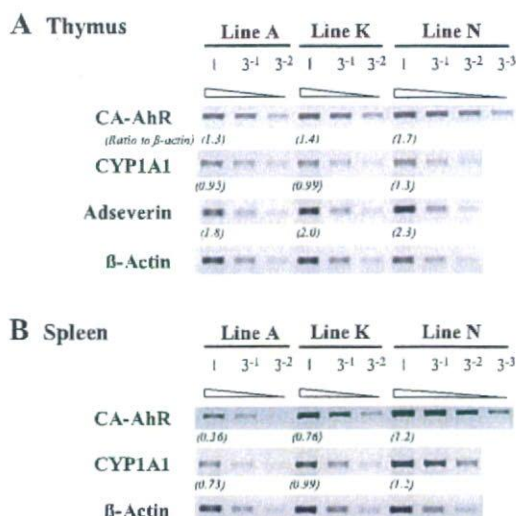


FIGURE 4. Comparison of CA-AhR and target gene expression in the thymus and spleen from line A, line K, and line N heterozygous mice. Total RNA was prepared from the thymus and spleen of the three lines with the RNeasy mini kit. cDNAs prepared from 20 ng of total RNA and serial dilutions (3^{-1}–3^{-3}) were amplified by PCR using primers for CA-AhR, CYP1A1, adseverin, or β-actin as a housekeeping gene. The expression of genes was quantified by densitometrically scanning gel images, and the values normalized to β-actin mRNA are indicated in parentheses. The numbers of PCR cycles for CA-AhR in thymus and spleen were 32 and 34, respectively, 26 and 32 for CYP1A1, and 20 for β-actin in both tissues. Mice were used after crossing into C57BL/6 mice for six generations in line A and for three generations in lines K and N.

CA-AhR expression induces target gene mRNA in both thymus and spleen, but reduces thymocyte number alone

Expression of the CA-AhR transgene and its target genes and phenotypic changes in the thymus and spleen were examined in the three lines. RT-PCR analyses showed that CA-AhR and CYP1A1 expression in the thymuses and spleens of lines A, K, and N were increased according to the integrated CA-AhR gene numbers (Fig. 4). Expression of adseverin, which was reported to be induced by TCDD in mice thymuses in an AhR-dependent manner (37), was also increased according to the transgene numbers.

The thymus weight was reduced in all three lines, by 36% in line A, 70% in line K, and 63% in line N (Fig. 5*A*). The thymocyte number was reduced by 49% in line A, 96% in line K, and 92% in line N (Fig. 5*A*). The thymocyte population defined by CD4 and CD8 expression was also affected in the Tg mice, with reduced percentages of CD4$^+$CD8$^+$ DP cells and increased percentages of CD8 SP and double negative (DN) cells (Table II). The ratios of CD4 SP/CD8 SP were significantly reduced in the Tg mice (Table II). The remarkable increases in the percentage of DN cells in line K and N were parallel to the large decreases in the total cell numbers.

By contrast, spleen weight was unaffected by the expression of CA-AhR (Fig. 5*B*). Splenocyte number was significantly reduced only in line N (by 40%), in which CA-AhR is most highly expressed (Fig. 5*B*), and the percentages of CD4 T cells, CD8 T cells, and B cells were unchanged, even in line N (data not shown). All of these findings are consistent with those observed in the thymuses exposed to TCDD (18, 21, 22).

We further confirmed that the CA-AhR is not expressed in the stromal cells and that CA-AhR expression in T-lineage cells alone is capable of inducing the thymus alteration in FTOC. mRNA expression was examined in whole thymus and stroma obtained by culturing thymus tissues in the presence of 2-deoxyguanosine to deplete it of thymocytes. As expected, CD4 mRNA was detected only in whole thymus, and Spatial mRNA, which is specifically expressed in thymic stromal cells (42), was detected in both the

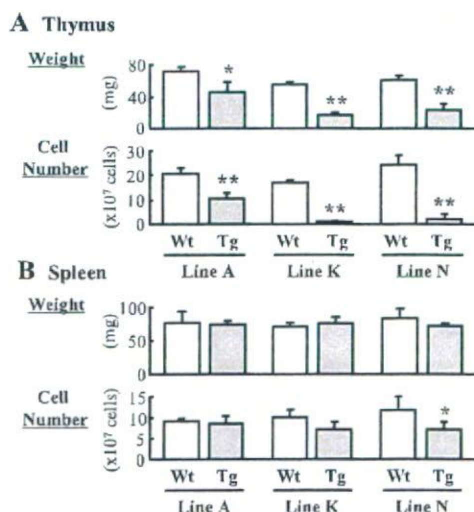


FIGURE 5. CA-AhR expression in T-lineage cells reduces thymus weight and cell number, but affects the spleen less. Thymus and spleen from female heterozygous Tg mice and nontransgenic littermate wild-type mice of line A (8 wk old, n = 4/each group), line K (10 wk old, n = 5), and line N (8–9 wk old, n = 5) were examined. Mice were used after crossing into C57BL/6 mice for two generations in line A and for three generations in lines K and N. The differences between Tg mice and wild-type mice were analyzed by Student's t test. The data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$.

Table II. *CA-AhR expression affects thymocyte population*[a]

	Line A (n = 4)		Line K (n = 5)		Line N (n = 5)	
	Wt	Tg	Wt	Tg	Wt	Tg
	(%)		(%)		(%)	
DN	4.7 ± 0.5[b]	6.4 ± 1.2	4.2 ± 1.1	52.6 ± 7.8	6.8 ± 2.8	27.9 ± 14.1
DP	82.2 ± 1.7	79.3 ± 1.4	87.2 ± 1.8	33.5 ± 7.6	83.2 ± 2.4	53.1 ± 15.9
CD4 SP	10.0 ± 1.5	9.5 ± 2.0	6.9 ± 0.7	8.7 ± 1.2	7.0 ± 0.8	10.2 ± 0.9
CD8 SP	3.1 ± 0.2	4.9 ± 0.4	1.8 ± 0.4	5.2 ± 0.5	3.1 ± 0.3	8.8 ± 1.2
CD4/CD8[c]	2.6 ± 1.1	2.0 ± 0.5	4.0 ± 0.7	1.8 ± 0.6**	2.3 ± 0.3	1.2 ± 0.1**

[a] Thymocytes from female heterozygous (CA-AhR$^{+/-}$) Tg mice and nontransgenic (CA-AhR$^{-/-}$) littermate mice (Wt) (8–10 wk old) were examined by flow cytometry.
[b] The data was expressed as means ± S.D. The differences between CD4/CD8 ratio in Tg mice and Wt mice were evaluated with Wilcoxon rank sum test. **, $p < 0.01$.
[c] Ratio of CD4 SP cells/CD8 SP cells.

whole thymus and the stroma (Fig. 6). CA-AhR was detected only in whole thymus, not in the stroma, in this system (Fig. 6). Thus, AhR activation in thymocytes alone was demonstrated to cause the cellular loss and population changes in the thymus.

CA-AhR suppresses the increase in spleen weight and splenocyte number caused by immunization

We previously reported finding that TCDD administration to mice immunized with OVA suppressed the immunization-induced increase in spleen weight and splenocyte number (27, 34). Consistent with these findings, the increase in spleen weight and splenocyte number observed in wild-type mice after immunization with OVA was suppressed in line A Tg mice (Fig. 7), although their spleen was unaffected when not immunized, as stated above (Fig. 5). Interestingly, increases of both CD4 T cells and B cells were significantly suppressed despite the specific expression of CA-AhR in T cells (Fig. 7). The number of CD8 T cells was also fewer in the Tg mice than in the wild-type mice, although the difference was not significant. Simultaneous suppression of the T and B cell increase was also observed in OVA-immunized and TCDD-exposed wild-type mice (27).

To estimate how much TCDD induces the corresponding level of AhR activation, CYP1A1 expression in the thymus and spleen of line A Tg mice and TCDD-exposed wild-type mice was compared. As shown in Fig. 8, the level of expression of CYP1A1 mRNA in the thymus of the Tg mice was slightly higher than its level of expression in wild-type mice exposed to a single dose of 20 μg/kg TCDD. CYP1A1 expression in the spleen of the Tg mice was less than in wild-type mice exposed to 20 μg/kg TCDD, which seems plausible because only T cells express CYP1A1 mRNA in the spleen of Tg mice, whereas both T and B cells express CYP1A1 mRNA in TCDD-exposed wild-type mice (43). These results show that the function of the activated AhR in line A heterozygous mice and AhR activated by 20 μg/kg TCDD are roughly comparable. In our previous study, 20 μg/kg TCDD suppressed the splenocyte number to 60% of the number in unexposed control mice 1 wk after immunization (27). The same dose of TCDD reduced the thymus weights to 40–60% (44). The fact that a similar extent of suppression was observed in the thymus and spleen of the Tg mice indicates that the major portion of the effect of TCDD in these organs is attributable to AhR activation in the T cells.

Discussion

To prove the role of activated AhR in T cells in TCDD-induced immune suppression, we generated Tg mice in which expression of CA-AhR cDNA is controlled by an improved version of hCD2 promoter and their T cells specifically express activated form of AhR. We obtained three lines bearing different copy numbers of the transgene, ranging from 2 to 10. All three lines showed expression of CA-AhR and an AhR-induced target gene in the thymus and spleen. In line A mice, which coexpress GFP in addition to CA-AhR under the control of the same CD2 promoter, the transgene was confirmed to be strongly expressed in the thymocytes, CD4, and CD8 T cells, but not in the thymus stromal cells or in the B cells. CD2 is principally expressed on T cells and NK cells in humans (45), but low expression has also been reported on subsets of other cell types, including B cell progenitors (46, 47). Consistent with the observation, faint expression of the transgene was detected on the CD3$^-$CD127$^+$ lymphocyte progenitor cells in our

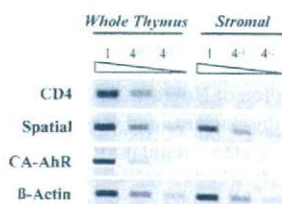


FIGURE 6. CA-AhR is expressed in the thymocytes, but not in the stromal cells, in the thymus. line A male CA-AhR$^{+/+}$ mice were mated with female C57BL/6J mice, and thymuses were collected from fetuses on gestational day 16.5. The thymuses were cultured for 4 days on a nitrocellulose filter floated on complete medium. To deplete them of thymocytes and obtain stromal cells, the lobes were cultured in the presence of 1.35 mM 2-deoxyguanosine. Fifteen or 16 lobes were pooled from each treatment group and were used to prepare RNA. cDNAs prepared from 20 ng of total RNA and serial dilutions (4^{-1}, 4^{-2}) were amplified by PCR

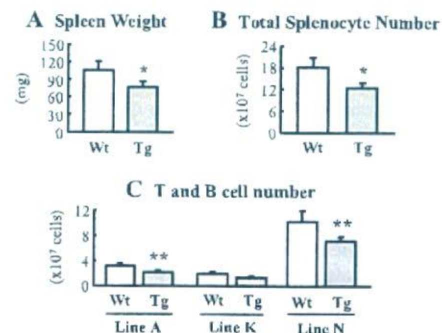


FIGURE 7. CA-AhR expression suppresses the increase in spleen weight and both CD4 T cell and B cell numbers after immunization. Line A female heterozygous Tg mice and littermate wild-type mice (8 wk old, n = 4) were immunized with OVA/alum, and their spleens were examined 7 days later. Mice were used after crossing into C57BL/6 mice for five generations. The differences between the Tg mice and wild-type mice were analyzed by Student's t test. The data are expressed as mean ± SD. *, $p <$ 0.05; **, $p < 0.01$.

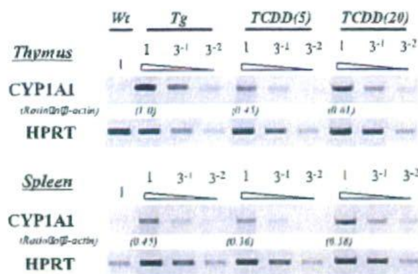


FIGURE 8. Comparison of CYP1A1 induction by CA-AhR in Tg mice and by TCDD exposure. Female C57BL/6 mice were given 5 or 20 μg/kg TCDD, and 3 days later total RNAs were prepared from thymocytes and splenocytes. CYP1A1 induction was compared with its induction in female line A heterozygous mice. cDNAs prepared from 20 ng of total RNA and serial dilutions (3^{-1}, 3^{-2}) were amplified by PCR using primers for CA-AhR or hypoxanthine phosphoribosyltransferase as a housekeeping gene. The expression of genes was quantified by densitometrically scanning gel images, and the values normalized to β-actin mRNA are indicated in parentheses. In both tissues, 30 PCR cycles were used for CYP1A1 and 20 for hypoxanthine phosphoribosyltransferase.

Tg mice. The transgene was also found to be expressed in the lung and, to a very minor extent, in the kidney in the Tg mice. Although the mechanism for the expression of CA-AhR in those tissues is unknown, presence of cells expressing CA-AhR in those tissues, such as T cells in the lung, may partly contribute to the transgene expression. Albeit the expression of the vector is found in other cell types, these CD2-based vectors, including the VA hCD2 vector, have been proved to be very useful to study specific functions of molecules in T cells. Likewise, the Tg mice we developed in the present study enable a new approach to explore the effect of AhR activation in T cells in the immune suppression. Lymphocyte progenitor cells have been reported to be affected by TCDD, and its effect was suggested to contribute to a loss of thymocytes (48, 49). Although the expression of CA-AhR in the lymphocyte progenitor cells was much fainter than that in the thymocytes and T cells (Figs. 2 and 3), the effects of low expression of CA-AhR may need to be considered.

All three lines of our Tg mice were characterized by thymus involution, including reduced thymocyte number and increased percentage of CD8 SP cells, the same as observed in TCDD-exposed mice. The fact that direct exposure of FTOC to TCDD reproduces the thymus involution induced by TCDD exposure in vivo (19, 24) shows that TCDD directly affects the thymus, in which the target cells are present. However, the results of previous studies have suggested that two types of cells in the thymus, thymocytes (18, 28) and stromal cells (24, 25), are the primary targets. The results of the present study in the Tg mice demonstrate that AhR activation in T-lineage cells alone can cause the thymus alterations, including loss of thymocytes and increase in percentage of CD8 SP thymocytes, without AhR activation in the stromal cells. Tomita et al. (50) recently produced T cell-specific ARNT-deficient mice in which the ARNT gene is disrupted under the control of T cell-specific $p56^{lck}$ proximal promoter, and showed that the thymus of the Tg mice is resistant to TCDD. Their results are consistent with our own showing that the AhR/ARNT heterodimer in the thymocytes, but not stromal cells, is essential for the occurrence of thymus involution.

Whereas thymus undergoes involution upon TCDD exposure, the splenocytes and splenic T cells of nonimmunized animals are unaffected by TCDD (18, 51). The same finding was observed in the spleen of our T cell-specific CA-AhR Tg mice, even though the CA-AhR was fully expressed in both the spleen T cells and the thymocytes. Although we examined the expression of CYP1A1 and adseverin as sensitive AhR-dependent target genes to estimate the extent of AhR activation, the genes responsible for the thymus involution remain to be identified. Previous studies have suggested suppression of thymocyte proliferation (24, 28) and induction of apoptosis (52) as the biological process involved in the thymus atrophy caused by TCDD. Our own recent study demonstrated that CA-AhR expression in Jurkat T cells inhibits cell growth by inducing both apoptosis and cell cycle arrest (29). Several genes in these CA-AhR-expressing Jurkat T cells that are related to apoptosis or cell cycle arrest, such as Fas, cyclin G_2, and growth arrest and DNA damage-inducible protein 34, were shown to be up-regulated in an XRE-mediated transcription-dependent manner (29), and these genes may be responsible for the loss of thymocytes.

In contrast with the nonimmunized mice in which splenocytes were less affected by AhR activation, as described above, the increase in splenocyte number after immunization was suppressed in the CA-AhR Tg mice, suggesting that the AhR/ARNT heterodimer inhibits cell growth in activated and proliferating T cells, but not in resting T cells. From this point of view, the effect of AhR activation in thymocytes may be also attributable to the effect on activated or proliferating cells: in thymus atrophy, the suppression of DN cell proliferation (28) and the loss of DP cells (52) are suggested to be responsible for cellular loss, DN thymocytes are vigorously proliferating cells, and DP cells receive an activation signal via their T cell receptors. Thus, activation state of the cell seems to affect the sensitivity of T-lineage cells to AhR activation.

The results of the present study also demonstrated that AhR activation in T cells alone suppresses the increase in both T and B cells in the spleen after OVA immunization. In terms of primary target cells of TCDD toxicity in immune reaction, Kerkvliet et al. (26) recently showed that AhR in both CD4 and CD8 T cells is necessary for full suppression of CTL response by TCDD in a mouse acute graft-vs-host model in which T cells, or CD4 or CD8 subsets, from $AhR^{+/+}$ and $AhR^{-/-}$ C57BL/6 mice were injected into C57BL/6 × DBA/2 F_1 host mice. Consistently, our results indicated that AhR activation in T cells is involved in changes in immune reaction. We previously reported that TCDD administration to OVA-immunized mice suppresses the growth of T and B cells and the production of Th2-type cytokines before suppression of Ab production (27, 34), which suggested that TCDD inhibits Ab production by suppressing T cell activation and the subsequent Th2-cell differentiation. The results of the present study strongly support the hypothesis that activation of the AhR directly inhibits cellular activation of the T cells and their subsequent proliferation and differentiation, leading to the suppression of T cell help on B cell proliferation. Alternatively, indirect effect of $CD4^+CD25^+$ regulatory T cells may be involved in the immune suppression. The regulatory T cells are positively selected in the thymus when their TCR receives a signal with intermediate strength (53). TCDD exposure to thymus is shown to affect thymocyte selection, possibly through up-regulation of Notch 1 (54) or activation of the ERK pathway (35) in the thymocytes, and these mechanism may alter the selection of $CD4^+CD25^+$ regulatory T cells. Further study of our Tg mice will clarify whether the suppression of T cell growth by AhR activation inhibits Th2-type cytokines and following Ab production by B cells, and it will also provide a clue for solving the mechanism of inhibition.

Andersson et al. (55) recently produced Tg mice expressing a CA-AhR mutant (30), which has a structure very similar to the one we used, under the control of an Ig H chain enhancer (Eμ), which promotes transgene expression in both B- and T-lineage cells (56). In addition to exhibiting thymus atrophy, their mice have a reduced life span and develop tumors in the glandular part of the stomach

(55). The Tg mice generated in our study, in contrast, are fertile and do not exhibit any overt phenotype differences except thymus atrophy, showing that AhR activation in T cells is not responsible for the stomach tumors.

Although T cells contain functional AhR and directly respond to TCDD (57), phenotypic changes caused by TCDD in T cells, such as changes in proliferation and differentiation, are difficult to detect in vitro, possibly because of optimized culture conditions that compensate for the effects of TCDD (6). The Tg mice expressing CA-AhR in T cells will be a useful model for investigating the role of activated AhR in the T cells. In particular, immunization of the Tg mice is expected to show suppression of various immune reactions, including Ab production and CTL activity, the same as observed in TCDD-exposed mice. Dioxins are persistent environmental contaminants and as such animals are continually exposed to them. TCDD maternally exposed is transferred to fetus and pups through the placenta or milk and activates their AhR (58, 59). Our Tg mice express CA-AhR mRNA in the fetal thymuses (data not shown) and the expression continues in the T-lineage cells after birth. Thus, these Tg mice will also be a useful model for clarifying the effect of persistent activation of AhR in T cells. Studies using our CA-AhR Tg mice should shed light on the role of the AhR in T cells in immune suppression by TCDD and also in physiological reactions.

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Disclosures

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A dioxin sensitive gene, mammalian *WAPL*, is implicated in spermatogenesis

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Abstract 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an endocrine disruptor that produces a variety of toxic effects. We have isolated a mouse homolog of the *hWAPL* gene, termed *mouse WAPL* (*mWAPL*), as a target of TCDD by cDNA representational difference analysis from mouse embryonic stem cells. A statistically significant increase in *mWAPL* expression was observed at 0.1 μM TCDD in $AhR^{-/-}$ mouse embryonic fibroblast cells. Interestingly, at 1 μM TCDD, *mWAPL* mRNA levels decreased in $AhR^{+/+}$ cells, but further increased in $AhR^{-/-}$ cells. *hWAPL* and mWAPL were highly expressed only in testes among normal tissue samples, and we observed mWAPL localization in the synaptonemal complex of testicular chromosomes. In addition, mouse testes decreased the expression of *mWAPL* mRNA after a single intraperitoneal injection of TCDD. Thus, mammalian *WAPL* such as *hWAPL* and *mWAPL* may be involved in spermatogenesis and be target genes mediating the reproductive toxicity induced by TCDD.
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Keywords: WAPL; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Spermatogenesis; Synaptonemal complex

1. Introduction

In *Drosophila melanogaster*, the protein encoded by the *wings apart-like* (*wapl*) gene regulates heterochromatin structure [1]. The *wapl* product is required to hold the sister chromatids of meiotic heterochromatin together. In addition, wapl is implicated in both heterochromatin pairing during female meiosis and the modulation of position-effect variegation (PEV). Moreover, a *P*-element screen of *Drosophila* identified wapl as a modifier of chromosome inheritance [2]. Recently, we have identified a novel human gene *hWAPL* that is a homolog of *wapl* [3]. *hWAPL* is overexpressed in invasive human cervical cancers and is often associated with cervical carcinogenesis. However, the essential function of hWAPL in normal cells is still unknown.

Dioxins, classified as endocrine disruptors, are ubiquitous in the environment. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic dioxin and causes a variety of effects, including immunotoxicity, hepatotoxicity, teratogenicity, and tumor promotion [4,5]. Changes in gene expression induced by TCDD and related chemicals are initiated by the binding of the compounds to the aryl hydrocarbon receptor (AhR). AhR then dimerizes with the aryl hydrocarbon receptor nuclear translocator to form a complex that interacts with gene regulatory elements containing a xenobiotic response element (XRE) motif [6]. AhR mediates many of the TCDD-induced changes in gene expression. Many of the target genes responsible for the symptoms of toxicity, however, remain unidentified.

Previously, we performed a cDNA representational difference analysis (RDA) of the cDNA derived from mouse ES cells treated or not with TCDD in order to isolate genes induced by TCDD. This procedure identified three genes, temporarily termed *Dioxin Inducible Factor 1, 2,* and *3* (*DIF-1*, *DIF-2*, and *DIF-3*), that were induced in TCDD-treated ES cells. *DIF-1* is identical to the gene encoding histamine releasing factor (HRF) [7] and *DIF-3* is the gene encoding a novel protein with a C2H2 zinc-finger domain [8]. However, *DIF-2* has not yet been characterized.

In this study, we have identified the gene corresponding to *DIF-2* as a mouse homolog of *wapl* and *hWAPL*, termed *mouse WAPL* (*mWAPL*). We have also confirmed the effects of TCDD on *mWAPL* expression and investigated the localization of mWAPL protein in normal adult mice to reveal the participation of mWAPL in TCDD-induced toxicity.

2. Materials and methods

2.1. cDNA cloning and sequencing

To isolate the complete mWAPL cDNA, we used total RNA from mouse testes. The full-length mWAPL cDNA was amplified by reverse transcription-PCR with primers designed from a computer search. We determined the nucleotide sequence of the cDNA with an Applied Biosystems 310 automated DNA sequencer.

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; MEF, mouse embryonic fibroblast

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2.2. Cell cultures and chemicals

$AhR^{-/-}$ embryos were generated from intercrossed $AhR^{+/-}$ mice [9]. Mouse embryonic fibroblasts (MEFs) of wild-type or mutant genotypes were harvested from day 14.5 mouse embryos. MEFs were grown as previously described [10]. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (Cambridge Isotope Laboratories, Inc., Andover, MA) was prepared in dimethylsulfoxide (DMSO).

2.3. RNA isolation, quantitative real time PCR, and Northern blot analysis

RNA isolation was performed as described [10]. First strand cDNA synthesis was performed as described [11]. Real time PCR analysis was performed using the Smart Cycler System (Cepheid, Sunnyvale, CA) with SYBR Green I (Cambrex, Washington, DC). Real time PCR utilized *mWAPL* specific primers, 5′-ACCTGGTGGAGTATAGTGCCC-3′ and 5′-TGGCAGAGACACCCAAGAAGC-3′; mouse *β-actin* specific primers, 5′-AGCCTTCCTTCTTGGGTATGG-3′ and 5′-CACTTGCGGTGCACGATGGAG-3′; or *CYP1A1* specific primers, 5′-TTTGGTTTGGGCAAGCGA-3′ and 5′-GTCTAAGCCTGAAGATGC-3′. Reaction mixtures were denatured at 95 °C for 30 s, then subjected to 40 PCR cycles at either 95 °C for 3 s, 68 °C for 30 s, and 86 °C for 6 s for *mWAPL*, or 95 °C for 3 s, 68 °C for 30 s, and 85 °C for 6 s for mouse *β-actin* and *CYP1A1*. mRNA levels of *mWAPL* and *CYP1A1* were determined by normalization of their signals to *β-actin* signals. We performed the experiments to evaluate mRNA levels in triplicate. The data were analyzed using Student's t test, and $Ps < 0.01$ were considered to indicate significant differences.

For Northern blot analysis, the 567-bp *Dpn*II fragment of *mWAPL* cDNA and a PCR-amplified mouse *β-actin* cDNA fragment using the primers described above were used as probes and labeled with ^{32}P using the Rediprime II random prime labeling system (Amersham Biosciences, Piscataway, NJ). To examine *hWAPL* expression in various human tissues, we used Human MTN Blot I, II and III (Clontech, #7760-1, #7759-1 and #7767-1, respectively).

2.4. Immunoblot analysis and Immunohistochemistry

To generate polyclonal antibodies against hWAPL, we immunized rabbit against a 6× histidine-tagged hWAPL COOH terminus (amino acids 814–1037) fusion protein and obtained an anti-hWAPL polyclonal antibody (480-02). The anti-hWAPL polyclonal antibody was used at the dilution of 1:2000 for Immunoblot analysis and Immunohistochemistry. Immunoblot analyses were performed as previously described [7]. Protein samples from adult mouse tissues were prepared in RIPA buffer [7] and quantified using the BioRad protein assay (Nippon Bio-Rad Laboratories, Tokyo, Japan). Immunohistochemical analysis was performed on formalin-fixed paraffin-embedded sections using Ventana HX System Benchmark (Ventana Medical Systems INC., Tucson, AZ). As a control of specificity, the anti-hWAPL polyclonal antibody (480-02) was pre-incubated for 18 h at 4 °C with a recombinant hWAPL protein and then applied to immunohistochemistry.

2.5. Immunocytology

The slides with surface-spread spermatocyte nuclei were prepared for immunocytological analysis as previously described [12]. Then, the anti-hWAPL polyclonal antibody (480-02) was diluted 1:400 and used as the primary antibody. After incubation with the primary antibody, the slide was reacted with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. DNA was visualized by counter-staining with 4′, 6-diamidine-2-phenylindole (DAPI).

2.6. Animals and treatment

Guidelines for the care and use of animals were approved by the animal research center at Tokyo Medical University. C57/BL6 male mice (13 weeks old) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Each mouse received one intraperitoneal injection of 200 μl saline and 12.5 μl DMSO containing TCDD at a dose of 600 μg/kg of body mass. Control mice were injected with the same solution without TCDD. Testis samples were harvested 24 h after injection and subjected to real time PCR analysis.

3. Results and discussion

3.1. Molecular cloning of mouse WAPL

Using BLAST, we found that the nucleotide sequence of the DNA fragment corresponding to *Dioxin Inducible Factor-2* (*DIF-2*) is included in mKIAA0261, which is a mouse KIAA-homolog [13]. This suggested that *DIF-2* might be a homolog of the *hWAPL* gene because *hWAPL* corresponds to the KIAA0261 cDNA fragment [3]. Thus, we renamed the *DIF-2* gene *mouse WAPL* (*mWAPL*). Based on the mKIAA0261 sequence, we cloned and confirmed the nucleotide sequence of the full-length coding region of the cDNA for *mWAPL* (Database Accession No. AB167349). Multiple sequence alignment of the proteins encoded by *mWAPL*, *hWAPL*, and *wapl* demonstrated that the three proteins are similar not only in the WAPL conserved region [3], but throughout the entire protein (Fig. 1) (mWAPL is 92% identical and 96% similar to hWAPL, and 24% identical and 45% similar to wapl). Thus, the protein encoded by *mWAPL* may be involved in heterochromatin organization, PEV modification and chromosome inheritance like the wapl protein in *Drosophila*.

3.2. Effects of TCDD on MEFs

To confirm the effects of TCDD on *mWAPL* expression, we examined *mWAPL* mRNA levels in $AhR^{+/+}$ and $AhR^{-/-}$ MEFs treated with 0, 0.01, 0.1, and 1 μM TCDD for 2 h by Northern blot analysis. Although *mWAPL* signals were on the whole extremely weak and barely visualized by strong enhancement, we found that *mWAPL* mRNA levels in $AhR^{-/-}$ MEFs showed the highest at 1 μM TCDD; in $AhR^{+/+}$ MEFs, on the other hand, *mWAPL* mRNA level at 0.1 μM TCDD was maximum (Fig. 2A). The two hybridization signals observed for *mWAPL* are similar to those observed in Northern blots for *hWAPL* and, as previously discussed [3], may reflect the difference of the length of the untranslated regions of the *WAPL* mRNAs.

To evaluate the *mWAPL* mRNA levels more accurately, we performed quantitative real time PCR analysis and confirmed the expression pattern of *mWAPL* mRNA in $AhR^{+/+}$ and $AhR^{-/-}$ MEFs (Fig. 2B). We did not find significant differences of cell cycle profile of these MEF cells by flow cytometric analysis (data not shown). Thus, although further investigation is required, the *mWAPL* gene may be inducible by TCDD independent of the AhR-mediated pathway but downregulated by a direct target molecule of TCDD in the AhR-dependent pathway.

3.3. Expression of mammalian WAPL in testes

Our previous study showed that *hWAPL* is expressed only in uterine cervical cancer among human tumor and normal control tissue samples examined [3]. Here, we examined mWAPL expression in normal mouse tissues by Western blot analysis and detected strong expression of mWAPL protein in the testes (Fig. 3A). Therefore, we also investigated *hWAPL* expression in various normal human tissues by Northern blot analysis, and confirmed that *hWAPL* mRNA was expressed abundantly in the testes, with weak expression in all other normal human tissues (Fig. 3B). Two hybridization signals for *hWAPL* mRNAs were visible in testes similar to MEFs (Fig. 2A) and previously reported results in cervical cancer tissues [3].

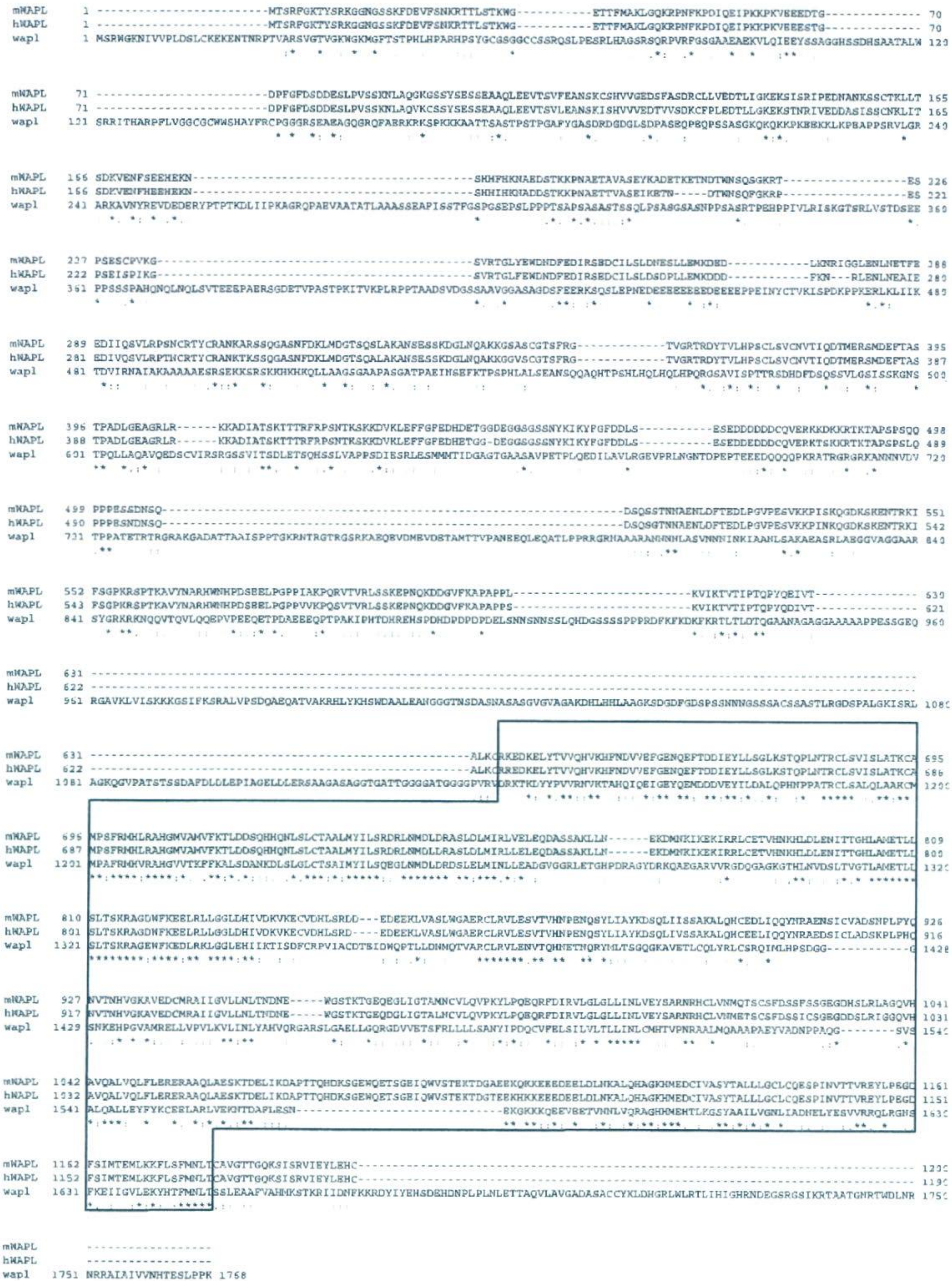

Fig. 1. Sequence alignment of WAPL homologs. The deduced amino acid sequences of mWAPL, hWAPL (Database Accession No. AB065003) and *Drosophila* wapl (Database Accession No. U40214) were aligned using CLUSTAL W multiple sequence alignment program. (*) indicates positions which have a single, fully conserved residue; (:) indicates that one of the following 'strong' groups is fully conserved: (STA), (NEQK), (NHQK), (NDEQ), (QHRK), (MILV), (MILF), (HY) and (FYW); and (·) indicates that one of the following 'weaker' groups is fully conserved: (CSA), (ATV), (SAG), (STNK), (STPA), (SGND), (SNDEQK), (NDEQHK), (NEQHRK), (FVLIM), and (HFY). The boxed region indicates the WAPL-conserved region.

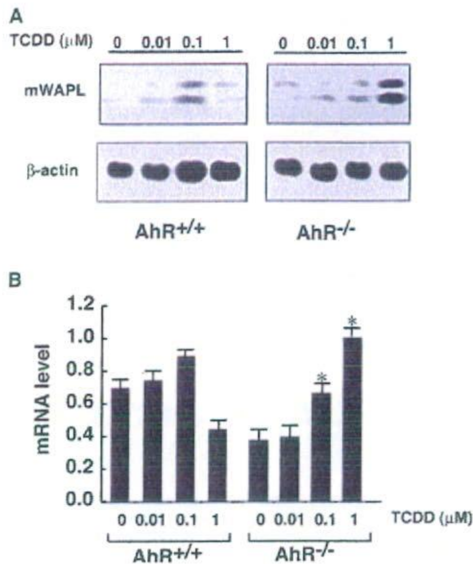

Fig. 2. Effects of TCDD on $AhR^{+/+}$ and $AhR^{-/-}$ MEFs. (A) Northern blot analysis of *mWAPL* in $AhR^{+/+}$ and $AhR^{-/-}$ MEFs treated with 0.1% DMSO and 0, 0.01, 0.1, or 1 μM TCDD for 2 h. The blots were hybridized with a probe for *mWAPL* (upper) and then reprobed with a *β-actin* probe as loading control (lower). (B) *mWAPL* mRNA levels in $AhR^{+/+}$ and $AhR^{-/-}$ MEFs treated with 0.1% DMSO and 0, 0.01, 0.1, or 1 μM TCDD for 2 h were determined with quantitative real time PCR analysis. The *mWAPL* mRNA levels were determined by normalization of *mWAPL* signals to *β-actin* signals, and the maximum mRNA expression level was arbitrarily set to 1 in the graphical presentation (*Y*-axis). The data were obtained from three independent experiments. Columns: means; bars: S.D. *, $P < 0.001$ versus the $AhR^{-/-}$ MEFs at 0 μM of TCDD.

We next examined the expression pattern of mWAPL in testes by immunohistochemical analysis using formalin-fixed mouse samples (Fig. 3C). The results showed that mWAPL was expressed abundantly in large pachytene spermatocytes, which are conspicuous for their size and loose organization of their chromatin, whereas it was undetectable in condensing forms of sperm (Fig. 3C, panel 2). We did not find any positive staining with the antibody after pre-absorption with a recombinant hWAPL protein (Fig. 3C, panel 3). These results suggested that mWAPL is expressed predominantly in early stages of spermatogenesis. similar results were observed in frozen sections (data not shown).

We have also characterized the subcellular localization of mWAPL in early spermatocytes by immunocytological staining. mWAPL immunoreactivity was detected at both zygotene (Fig. 3D) and pachytene stage (Fig. 3E). The antibody stained both unsynapsed and synapsed axial element components in early zygonema, and localized to the fully synapsed bivalents (synaptonemal complex) and the partially synapsed X and Y chromosomes (Fig. 3E, panel 3). From these results, hWAPL and mWAPL may be implicated in spermatogenesis. Although additional evidences are required, we suspect that mammalian WAPL may play a significant role in meiosis as does *Drosophila wapl* [1].

Furthermore, because of the localization of mWAPL in pachynema, we expect that mammalian WAPL functions in homologus recombination and DNA recombination. DNA double-strand breaks (DSB) constitute the most dangerous type of DNA damage induced by ionizing radiation.The gene

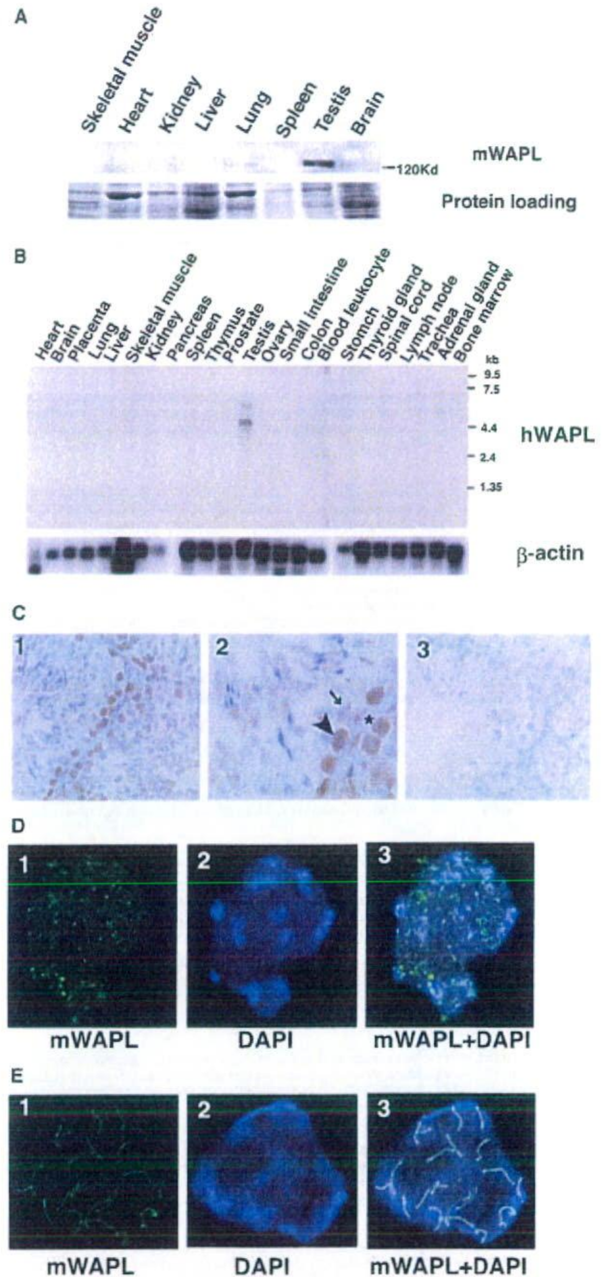

Fig. 3. Expression of mammalian WAPL in testes. (A) Western blot analysis of mWAPL in various normal mouse tissues. A Ponceau-stained nitrocellulose membrane is also shown as a control for protein loading (lower panel). (B) Northern blot analysis of *hWAPL* in various normal human tissues. *β-actin* signals are also shown as loading control. (C) mWAPL immunostaining in adult mouse testes. Formalin-fixed and paraffin-embedded 5 μm sections of mouse testes were treated with an anti-hWAPL antibody (panels 1 and 2), or the antibody after pre-absorption with a recombinant hWAPL protein as a negative control (panel 3), a horseradish peroxidase-conjugated secondary antibody, developed with diaminobenzidine, and counterstained with hematoxylin. Specific cell types in the seminiferous epithelium are identified in panel 2: arrowhead, pachytene spermatocyte; arrow, round spermatid; asterisk, Sertoli cell. (D) Chromosomal spread of spermatocyte at early zygotene fixed and stained with anti-hWAPL antibody and DAPI (panel 1, mWAPL; panel 2, DAPI; panel 3, mWAPL + DAPI). (E) Chromosomal spread of spermatocyte at mid pachytene fixed and stained for mWAPL and DAPI (panel 1, mWAPL; panel 2, DAPI; panel 3, mWAPL + DAPI).

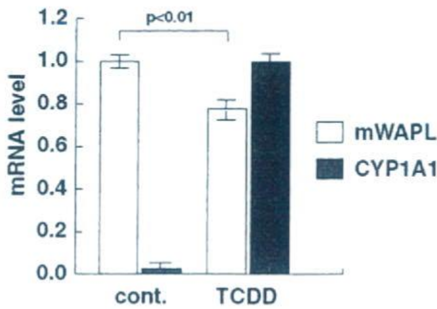

Fig. 4. Mouse testes exhibited a decreased level of mWAPL after TCDD treatment. *mWAPL* mRNA levels in testes of mice treated with or without TCDD (at a dose of 600 μg/kg of body mass) for 24 h were determined by quantitative real time PCR analysis. The data represent the means of eight samples. *CYP1A1* mRNA levels were also determined as a control for the effects of TCDD on testes. The *mWAPL* and *CYP1A1* mRNA levels were determined by normalization of their signals to *β-actin* signals, respectively, and the maximum mRNA expression level was arbitrarily set to 1 in the graphical presentation (*Y*-axis). Bars: S.E.

that is implicated in homologous recombination might work at DSB repair [14,15] and the alterations in recombination promote carcinogenesis by causing genomic instability. Therefore, unscheduled expression of mammalian WAPL by human papilloma virus (HPV) [16], TCDD or other agents may cause an inaccurately repaired or unrepaired DSB and result in mutations or genomic rearrangements in surviving cells, which in turn leads to genomic instability and subsequently results in malignant cell transformation or defects in embryogenesis.

Recently, we have characterized another dioxin inducible gene named *Dioxin Inducible Factor 3* (*DIF-3*) that is highly expressed in testes [8]. Interestingly, DIF-3 is expressed most strongly in the large pachytene spermatocytes [8] similar to mWAPL. However, we have not found any evidence of functional association between mWAPL and DIF-3 until now.

3.4. Downregulation of mWAPL expression by TCDD in testes

These results prompted us to examine whether the expression of mWAPL was influenced by TCDD in testes. Twenty hours after the injection of TCDD into the abdominal cavities of C57/BL6 mice, we harvested the testes and analyzed *mWAPL* expression by quantitative real time PCR analysis. Because the *CYP1A1* gene is a well-known target of TCDD, we also calculated *CYP1A1* mRNA levels to confirm the effects of TCDD on testes. The mice exhibited a decrease in *mWAPL* expression and a marked increase in *CYP1A1* expression compared to control mice (Fig. 4). This result suggests that TCDD exposure affects mWAPL expression levels in testes.

In recent years, several reports have focused on certain man-made toxins known as endocrine disrupting chemicals (EDCs) that persist in the environment. These chemicals are capable of altering the endocrine homeostasis of an animal, thereby causing serious reproductive and developmental defects as well as testicular oncogenesis [17–19]. Several studies have also provided evidence of a decline in semen quality and/or sperm counts over the same period [19]. Our previous study demonstrated that hWAPL overexpression induces carcinogenesis and tumor progression, and that hWAPL reduction by small interfering RNA (siRNA) induces cell death [3]. These observations suggest that unscheduled changes in hWAPL expression can cause severe damage to cells. Thus, although more experiments are needed to provide direct evidence linking mammalian *WAPL* with TCDD-induced reproductive toxicity, the present study suggests that mammalian *WAPL* may be a target gene mediating the reproductive toxicity of TCDD.

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特集　環境化学物質の作用メカニズムを解き明かす

重金属汚染による生物撹乱作用の分子基盤

Molecular Targets of Organotin Compounds in Endocrine Disruption

中西 剛　西川淳一

Tsuyoshi Nakanishi, Jun-ichi Nishikawa

重金属は生物に対し強い毒性を示すものが多いが，一般的にこのような低分子化合物の毒性は作用点が多岐に渡っており，分子レベルでの毒性発現機構の解明は困難であることが多い．一方で，近年の内分泌撹乱物質問題で話題となった有機スズ化合物は，貝類などの特定の生物種に特徴的な生殖毒性を誘引するが，最近，有機スズ化合物が核内受容体であるRXRやPPARγの強力なアゴニストとして作用することで，その毒性を発揮することが明らかとなってきた．本稿では，有機スズ化合物の核内受容体を介した生物撹乱作用について概説する．

key words

有機スズ，アロマターゼ，インポセックス，RXR，PPARγ，内分泌撹乱

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はじめに

重金属は生物に対し強い毒性を示すものが多く，鉱山や工場，産業廃棄物などから排出される重金属が，しばしば水源や土壌などの環境中に放出されてヒトの健康に影響を与えるなど，公害の原因となったりする．我々日本人にとって重金属毒性と言えば，おそらく多くの人がイタイイタイ病や水俣病といった公害を思い浮べるだろう．現在では，これら公害は過去に起こった歴史上の出来事であり，その作用機構の解明を含め，すでに解決した問題であるかのように認識されているかもしれない．しかしながら，低分子化合物の毒性発現には多くの分子や作用が関わっていると考えられ，その分子レベルでの作用機構解明は一般的に非常に困難である．イタイイタイ病や水俣病においても，これらの原因物質がカドミウムやメチル水銀であることは明らかとなっているが，その分子レベルでの毒性発現機構について解明されたとは言いがたいのが現状である．

一方で，近年話題となった内分泌撹乱物質（endocrine disrupting chemical；EDC）問題においても，船底塗料や漁網防汚剤などに使用されてきたトリブチルスズ（tributyltin；TBT）やトリフェニルスズ（triphenyltin；TPT）に代表される有機スズ化合物（図1）が，貝類に対してではあるものの，極低濃度で雌を雄性化し，繁殖不能にする状態（インポセックス）を誘導する[1)~3)]ことから，ヒトを含めた生物へのEDC作用が懸念されてきた．このような有機スズ化合物の貝類への影響は，EDC問題が提唱される以前から問題視されていたが，EDC問題が社会問題化してからは，有機スズ化合物の性ステロイドホルモンの受容体や合成経路に対する影響を中心に研究が行われてきた．これまでにも有機スズ化合物の毒性発現機構については様々な仮説が提唱されてきたが，有機スズ化合物においても他の重金属化合物と同様に，インポセックスの分子メカニズムやそれ以外の生物への毒性発現作用については不明な点が多く残されていた．しかしながら最近，これらの有機スズ化合物については，その毒性発現に関わる分子メカニズムが明確化しつつある．本稿では，有機スズ化合物の生物撹乱作用とその分子メカニズムについて，筆者らが最近得た知見を含めて紹介したい．

Ⅰ．有機スズ化合物のアロマターゼ阻害説

有機スズ化合物は，スズ原子にアルキル基やフェニル基が共有結合する構造を有し，官能基が1個結合するモノ体から4個結合するテトラ体まで多くの化合物から構成される人工化合物群である（図1）．これらの有機スズ化合物は，プラスチックの可塑剤や化学反応の触媒として各種化学工業で使用されるとともに，その殺生物能を利用して農薬や木材防腐剤，船底塗料，漁網防汚剤として使用されてきた．しかしながら，世界各地でこれらの化合物の水域汚染（主に海洋汚染）が顕在化し，また有機スズ化合物が貝類の雌に対してペニス様の突起を発生させるインポセックスを誘導することが明らかとなってからは，有機スズ化合物の水棲生態系へのみならず，汚染した海産物を食したヒトへの影響も問題視されるようになってきた．さらに有機スズ化合物は，元来毒性が強いことから，比較的古くから哺乳動物に対し

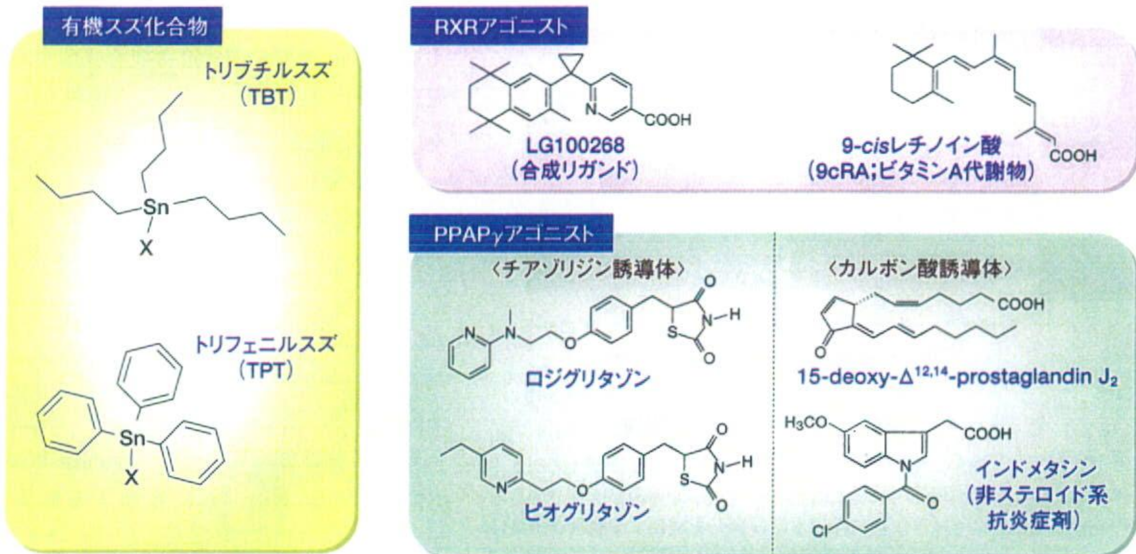

図1. RXR, PPAR γ アゴニストと有機スズ化合物の化学構造

TBTやTPTなどの有機スズ化合物は，スズ原子（Sn）にアルキル基やフェニル基が共有結合する構造を有している．官能基が1個結合するモノ体から4個結合するテトラ体まで存在している．RXRアゴニストは，一般的に極性官能基と疎水性炭化水素基をリンカーで連結したような構造を有しているが，有機スズ化合物はそのような構造を有していない．またPPAR γ アゴニストであるインスリン抵抗性糖尿病治療薬のようなチアゾリジンジオン構造も有していない．

て神経毒性や免疫毒性などを示すことが知られていた[4]が，その毒性発現機構についてはこれまでに統一した見解は得られていない．

一方で，有機スズ化合物の毒性発現には，いくつかのユニークな点が認められる．それは，①特定の生物種（貝類）に対して明確な生殖異常（インポセックス）を誘導する，②EDC問題においては，大部分の化合物が雌性化またはエストロゲン様作用が疑われているのに対し，雄性化作用が疑われている，③雄性化作用が疑われているが，エストロゲンレセプター（estrogen receptor：ER）やアンドロゲンレセプター（androgen receptor：AR）にはまったく親和性を示さない，④非特異的な細胞毒性を示す濃度よりも，かなり低濃度でインポセックスを誘導する，という点である．有機スズ化合物のインポセックスにおけるメカニズムについては様々な仮説が提唱されているが，②や③の観点から，有機スズ化合物はホルモンレセプターに直接作用するのではなく，ステロイドホルモン代謝に影響を与えることで誘導されるという説が有力視されていた[1), 2)]．

エストロゲンおよびアンドロゲンは，コレステロールを出発物質とし，モノオキシゲナーゼであるシトクロムP450と水酸基またはケト基の酸化または還元を触媒する脱水素酵素により生成されるが，有機スズ化合物はアンドロゲンからエストロゲンへの変換酵素であるアロマターゼの活性を阻害するのではないかと考えられた（図2）．すなわち，アロマターゼの酵素活性を阻害することで，体内のエストロゲン濃度の上昇を抑制し，アンドロゲンの濃度を上昇させる結果，雄性化を引き起こすのではないか？という"アロマターゼ阻害説"である[2)]．では，有機スズ化合物は本当にアロマターゼの活性を阻害するのであろうか？

CookeやHeidrichらのグループは，ヒトアロマターゼタンパク質を用いて，TBTがアロマターゼ活性を基質競合的に阻害することを報告している[5), 6)]．筆者らもヒト絨毛細胞株のミクロソーム分画を用いて，同様の検討を行ったが，確かにTBTおよびTPTともにアロマターゼの活性を阻害する[7)]．しかし，いずれの実験においても，その作用濃度は数μM〜数十μMとかなり高濃度であり，通常の動物細胞は完全に死滅する濃度である[7)]．またこの他にも，高濃度のTBTおよびTPTが，アロマターゼ以外のステロイドホルモン合成関連酵素の活性を阻害するという報告が多数存在する[8), 9)]ことを考慮すると，有機スズ化合物のアロマターゼに対する酵素特異性についてはかなり疑問が残るところである．

インポセックスのアロマターゼ阻害説は，同じ水棲動物である魚類が，性ステロイドホルモンにより雌雄の表現型が決定されることに加え，貝類にTBTを投与すると体内のテストステロン濃度が上昇したり，またテストステロンやアロマターゼ阻害剤などで処理をするとインポセックスが誘導されるという結果に基づいている[1), 2)]．しかしながら，同じ水棲動物でもステロイドホルモンの生理的意義は，脊椎動物と無脊椎動物では大きく異なっていると考えられており，少なくとも無脊椎動物においては，古典的ステロイド

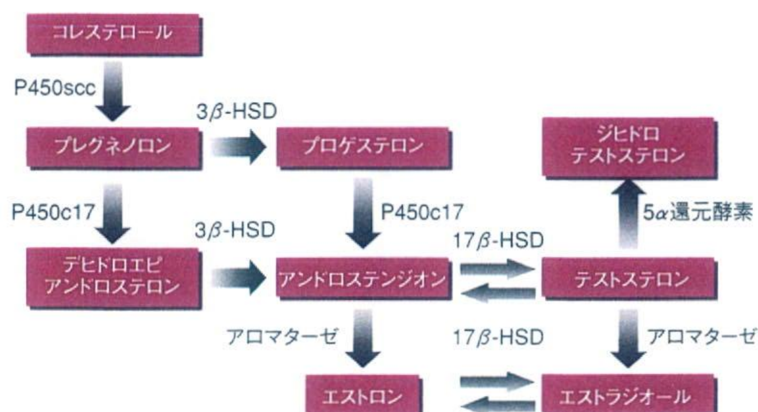

図2. コレステロールから性ステロイドホルモンへの合成経路

性ステロイドホルモンは,コレステロールを出発点としてモノオキシゲナーゼであるシトクロムP450と,水酸基またはケト基の酸化または還元を触媒する脱水素酵素により生成される.アロマターゼはアンドロゲンをエストロゲンに変換する律速酵素であることから,性ステロイドホルモンのバランスは主にアロマターゼにより保たれていると考えられる. HSD;hydroxysteroid dehydrogenase.

ホルモン(エストロゲン, アンドロゲン, プロゲステロン, 糖質コルチコイド,鉱質コルチコイド)の受容体は存在しないと報告されている[10].また貝類において,アロマターゼは同定されておらず, ERやARの存在が不確かな貝類に,果たして脊椎動物と同様の古典的ステロイドホルモンの合成および代謝経路が本当に存在するのか? といった疑問もある.貝類のインポセックスにおいては,これまでに提唱された説の中で,“アロマターゼ阻害説”が圧倒的な支持を得てきた感がある.しかしながら,本説はこのような不確定な根拠の上に存在していることから,その信憑性についてはもう少し慎重に判断されるべきではないだろうか?

Ⅱ.ヒト胎盤のアロマターゼ発現に対する有機スズ化合物の影響

アロマターゼ活性が阻害されることで,体内の性ステロイドホルモンのバランスが崩れ,雌が雄性化するというインポセックスにおける“アロマターゼ阻害説”で想定されたような現象は,じつはヒトの発生段階においても認められる. ヒトの場合,妊娠期のエストロゲン産生(アンドロゲン代謝)の場は,卵巣から胎盤へと移行し,胎児-胎盤ユニットを形成することで,胎児および母体中の性ステロイドホルモンのバランスを維持している.そのホルモンバランスの維持において,特に重要な分子が胎盤のアロマターゼである. 現に,胎盤のアロマターゼが欠損した女児においては,体内のアンドロゲン濃度が上昇し,ペニス様の突起が発生する仮性半陰陽(外性器は男性型であるが,内生殖器は女性型)となる[11]. このことは,性ステロイドホルモンのバランスや胎盤のアロマターゼが,ヒト胎児の生殖器官形成に非常に重要であることを示しており,化学物質が胎児に直接的な影響を与えなくとも,胎盤の内分泌機能を修飾することで胎児に少なからず影響を与える可能性を示唆している.

そこで筆者らは,有機スズ化合物をはじめとする様々な化学物質のヒト胎盤内分泌機能への影響を,非特異的な細胞毒性を示さない濃度域で検討を行った. その結果, TBTとTPTなどの有機スズ化合物が,濃度依存的にヒト絨毛細胞株のアロマターゼ活性とそのmRNAの発現を上昇させることが確認された[7,12]. さらにこれらの有機スズ化合物は,ヒト胎盤においてエストロンを活性型エストロゲン(エストラジオール)に変換する酵素(17 β-hydroxysteroid dehydrogenase type Ⅰ;17 β-HSD Ⅰ)をも活性化し,エストラジオール産生を促進することも確認された[7,13]. これらの結果は,これまでに貝類で予測されていたものとはまったく正反対であり,第Ⅰ章で述べた“アロマターゼ阻害説”に対する疑問を支持する結果であると言える.さらにEDC問題において有機スズ化合物は,雄性化を引き起こす典型的な化学物質であると認識されていたが,少なくともヒト胎盤に対してはエストロゲン産生を亢進することから,すべての生物種に対し雄性化作用を示すわけではないことが明らかとなった.このことは,有機スズ化合物がアロマターゼ以外の分子を標的として毒性を誘発する可能性を示唆している.

Ⅲ.核内受容体アゴニストとしての有機スズ化合物とアロマターゼ発現

ステロイドホルモン受容体をはじめとする核内受容体は,リガンド誘導性転写調節因子であり,ステロイドホルモンなどの脂溶性リガンドの情報を受け,特定の標的遺伝子群の発現を転写レベルで制御する. またこれらの内因性脂溶性リガンドは,それぞれの産生および代謝を互いに制御し合うことによって,生殖系や内分泌系などのホメオスタシスを維持していると考えられる. したがって,化学物質がERやAR以外の核内受容体に作用した場合においても,そのホメオスタシスが破綻する可能性は十分に考えられる.このような観点から,筆者らは,転写共役活性化因子と大腸菌アルカリホスファターゼとの融合タンパク質,および各種核内受容体のリガンド結合領域(ligand binding domain;LBD)を含むタンパク質とを利用した無細胞系リガンドスクリーニング法(CoA-BAP法;図3)[14]を用いて, EDC作用が疑われている40種類の化学物質についてリガンドスクリーニングを行った[15]. その結果, TBTおよびTPTが,ビタミンAの代謝物(9-*cis*レチノイン酸;9cRA)をアゴニス

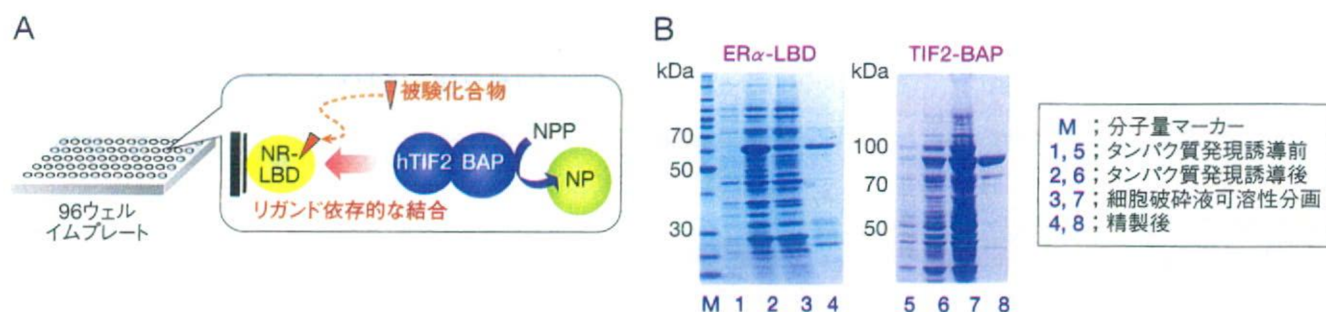

図3. CoA-BAP法[14)]

A：CoA-BAP（coactivator-bacterial alkaline phosphatase）法の概念図．核内受容体のリガンド結合領域（LBD）にアゴニストが結合したときに，コアクチベーターが結合できるようになる性質を利用している．精製したGST（glutathione-*S*-transferase）と核内受容体（NR）のLBDの融合タンパク質（NR-LBD）をGSH（glutathione）で固相化したマイクロプレートのウェルに，コアクチベーターであるTIF2とBAP（bacterial alkaline phosphatase）の融合タンパク質（TIF2-BAP）および被験化合物を加え，一定時間反応させる．被験化合物が核内受容体アゴニストとして機能する場合には，TIF2がNR-LBDに結合し，TIF2とBAPの融合タンパク質がウェル内に留まる．反応後，未反応の融合タンパク質を除き，BAPの基質であるNPP（*p*-nitrophenyl phosphate）を加える．NPPは無色であるが，BAPによって黄色の*p*-nitrophenolへと変換される．被験化合物の核内受容体に対する親和性が強いほどウェルに留まるTIF2とBAPの融合タンパク質は増加するため，*p*-nitrophenolによる発色の程度によって，被験化合物の核内受容体に対するアゴニスト活性を評価することができる．
B：ERαに対する評価系を作成した際に用いた精製タンパク質の電気泳動像．
C：構築したERαに対する評価系におけるエストラジオールの反応性．

トとするRXR（retinoid X receptor）と，インスリン抵抗性糖尿病治療薬であるチアゾリジン誘導体をアゴニストとするPPARγ（peroxisome proliferator-activated receptor γ）のアゴニストとしての作用を有する可能性を見いだした[15)]．これらの知見をもとに，ヒトやマウスの細胞を用いた検討を行ったところ，TBTとTPTは各受容体を介する転写を活性化し，そのEC_{50}は10～20nMであった[12), 15)]．また，精製タンパク質を用いた各受容体のLBDに対する解離定数の検討においては，RXRα，β，γに対しては既知のアゴニストである9cRAの各々約5，15，11倍[12)]，PPARγに対してはチアゾリジン誘導体であるロジグリタゾンとほぼ同等であった（中西ら；未発表データ）．既知のPPARγやRXRアゴニストとは構造がまったく異なるTBTやTPTが，これほどまでに強力なアゴニスト活性を示すのは驚きである（図1）．さらにTBTやTPT以外の有機スズ化合物についても検討を行ったところ，スズ原子に結合している官能基の構造や官能基の数によって，各受容体に対する転写活性化能や親和性が変動し，そのアゴニスト活性には明確な構造相関が認められた[12)]．

有機スズ化合物のこのようなアゴニスト活性が与える細胞機能への影響についても検討を行った．PPARγとRXRは互いにヘテロ二量体を形成し，各々のアゴニスト依存的に転写を活性化することで，支配遺伝子の発現や脂肪細胞分化を誘導するが，TBTとTPTは前述の結果を反映して，マウスの脂肪細胞分化をPPARγ/RXRの支配遺伝子の発現上昇を伴って誘導することも確認された[15)]．では，ヒト胎盤に対する影響はどうであろうか？ ヒト絨毛細胞株に，PPARγアゴニストを添加しても，アロマターゼのmRNA発現や胎盤型のアロマターゼプロモーターの転写活性には影響は認められない[12)]．このことは，ヒト胎盤のアロマターゼの発現にはPPARγ/RXRは関わっていないことを示唆している．しかしながら，RXR特異的アゴニストを添加した場合には，ヒト絨毛細胞株のアロマターゼ発現が上昇するうえ，胎盤型のアロマターゼプロモーターの活性も上昇する[12)]．RXRは，PPARγ以外にもPPARα，PPARβ，FXR（farnesoid X receptor），LXR（liver X recepter）ともヘテロ二量体を形成したり，またRXR自身でホモ二量体も形成する．これらの二量体も，PPARγ/RXRと同様にRXRアゴニストで転写が活性化されるが，胎盤のアロマターゼ発現には前述のヘテロ二量体は関与していない[12)]．したがって，RXRアゴニストによるヒト胎盤でのアロマターゼ発現誘導は，RXRホモ二量体を介した作用であると考えられる．このことは，有機スズ化合物によるアロマターゼの発現誘導においても同様であり，有機スズ化合物はRXRホモ二量体を介して，ヒト胎盤のアロマターゼ発現を誘導するものと考えられる（図4）[12)]．

Ⅳ．核内受容体を介した有機スズ化合物の毒性

冒頭にも述べたとおり，有機スズ化合物は前述のEDC作用以外にも様々な毒性を誘導することが報告されているが，

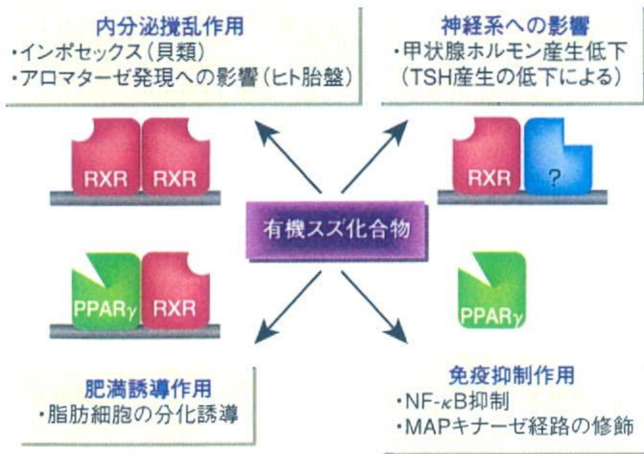

図4. 核内受容体を介した有機スズ化合物の様々な生物撹乱作用
有機スズ化合物はPPAR γやRXRを介することで，これらの受容体を有する動物に様々な影響を与える可能性がある．

これらの毒性発現においてもPPAR γやRXRが関わっている可能性がある（図4）．TBTは，ラットにおいて甲状腺刺激ホルモン（thyroid stimulating hormone；TSH）の産生低下を伴う甲状腺機能低下症を引き起こすことが報告されている[4]が，その一方で，RXR特異的アゴニストがマウスやラットにおいて同様の作用を引き起こす[16]ことから，TBTによるTSHや甲状腺ホルモンの産生低下はRXRを介して誘導されている可能性が考えられる．また有機スズ化合物は，免疫抑制作用やアレルギー反応の誘導などの免疫毒性も有する[4]が，PPAR γが転写制御因子であるNF-κB（nuclear factor-κB）の転写活性を抑制したり，MAPキナーゼ（maitogen-activated protein kinase）経路によるシグナル伝達を修飾したりすることで，免疫担当細胞のサイトカイン産生などに影響を与えることが報告されている[17]ことから，有機スズ化合物による免疫毒性においてはPPAR γが関わっている可能性が考えられる．さらに有機スズ化合物は，前述のとおり，PPAR γ/RXRを介してマウスの脂肪細胞分化を誘導する[15]が，妊娠マウスにTBTを投与すると胎仔の肝臓などで脂肪組織の過形成が認められることも報告されていることから，最近では肥満誘導因子として作用する可能性も指摘されている[18]．これらの知見は，有機スズ化合物がPPAR γやRXRを介することで，これらの受容体を有する動物に様々な影響を与える可能性があることを示唆している．

V. インポセックスとRXR

RXRは，核内受容体ファミリーの中でも例外的に種を超えて保存されている受容体であり，昆虫類などの下等無脊椎動物においてもUSP（ultraspiracle）と呼ばれるオルソログが存在する．しかしながら，これら受容体のLBDは動物種によって大きく異なり，哺乳動物の内因性アゴニストとされている9cRAなどに対してUSPは応答しない．その一方で，最近イボニシなどの貝類においては，脊椎動物のRXR-LBDと相同性の高いRXRが存在することが報告された（図5）[19, 20]．これらの貝類のRXRは，ヒトのRXRと同じく9cRAや有機スズ化合物と高い結合能を有する．また興味深いことに，これらの貝類に9cRAを投与すると，有機スズ化合物と同様にインポセックスが誘導されることから（図5），貝類のインポセックスはRXRを介して誘導されている可能性が示唆された（図4）[19, 20]．有機スズ化合物を妊娠マウスなどに投与しても，インポセックスのような表現型は認め

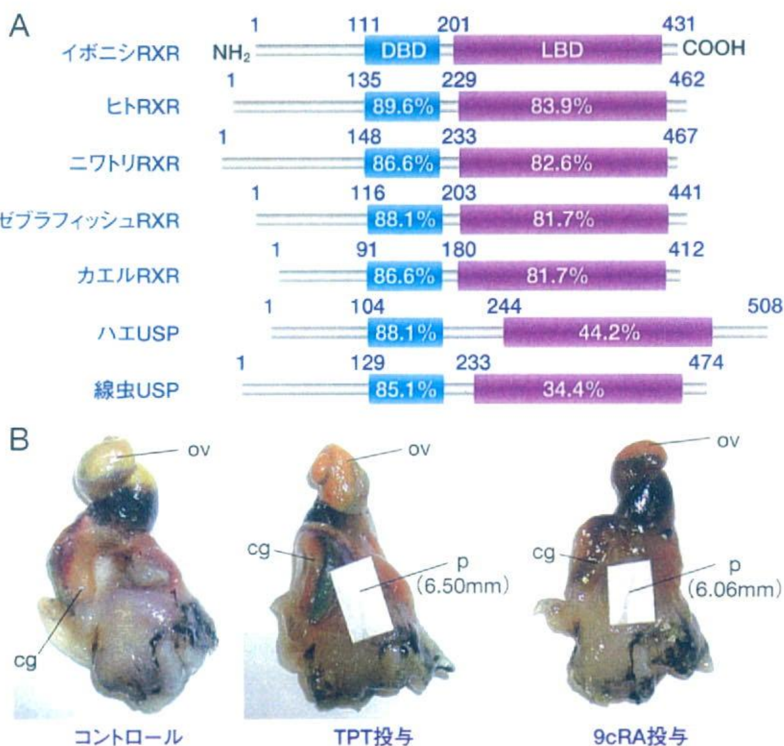

図5. 貝類のRXRと9cRAによるインポセックスの誘導
A：様々な生物種におけるRXR（USP）の構造の比較．すべての生物種においてDNA結合領域（DBD）の相同性は高いが，LBDの相同性はRXRとUSPでは大きく異なる．
B：9cRAとTPTによるイボニシのインポセックスの写真．各被験物質（1 μg/g wet wt.）をそれぞれ足部に注射して，人工海水中で1カ月間飼育後，取り上げて解剖した．ペニス部分には，ペニスを際立たせるために白い紙をのせている．cg；capsule gland（卵嚢腺），ov；ovary（卵巣），p；penis（ペニス：数値はペニス長）．Nishikawa J, et al: Environ Sci Technol (2004) 38: 6271-6276を一部改変．
写真提供：国立環境研究所 堀口敏宏先生

られないが，おそらくそれは，生殖器官形成などにおけるRXRの生理的意義が，生物種によって大きく異なるからであると考えられる．

おわりに

本稿では，有機スズ化合物の貝類とヒトアロマターゼへの影響を中心に，その分子メカニズムについて概説した．有機スズ化合物は，EDC作用が疑われている化学物質の中でも，きわめて低濃度で明確なEDC作用を誘導するが，それは核内受容体アゴニストとして作用することに起因することが明らかとなった．本来アゴニストとなることを意図されていない合成化学物質が，このように生理的アゴニストや合成アゴニストに匹敵するような影響を示す例はきわめてまれである．有機スズ化合物は，生物種によって誘発する表現型は異なるものの，様々な生物種に対してRXRまたはPPARγを介した毒性を引き起こす可能性が考えられる．今後は，PPARγやRXRなどの核内受容体に対する化学物質の作用や，様々な生物種におけるこれらの核内受容体の生理的意義を解明することによって，有機スズ化合物の生物攪乱作用がより明確になることを期待したい．

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for beginners

本稿では誌面の都合上，核内受容体の転写調節機能に関する基本的な事項の説明については割愛したが，以下の総説を参考にされたい．

・「核内受容体と創薬」山岡一良ら：実験医学（増刊）24: 152-160 (2006)