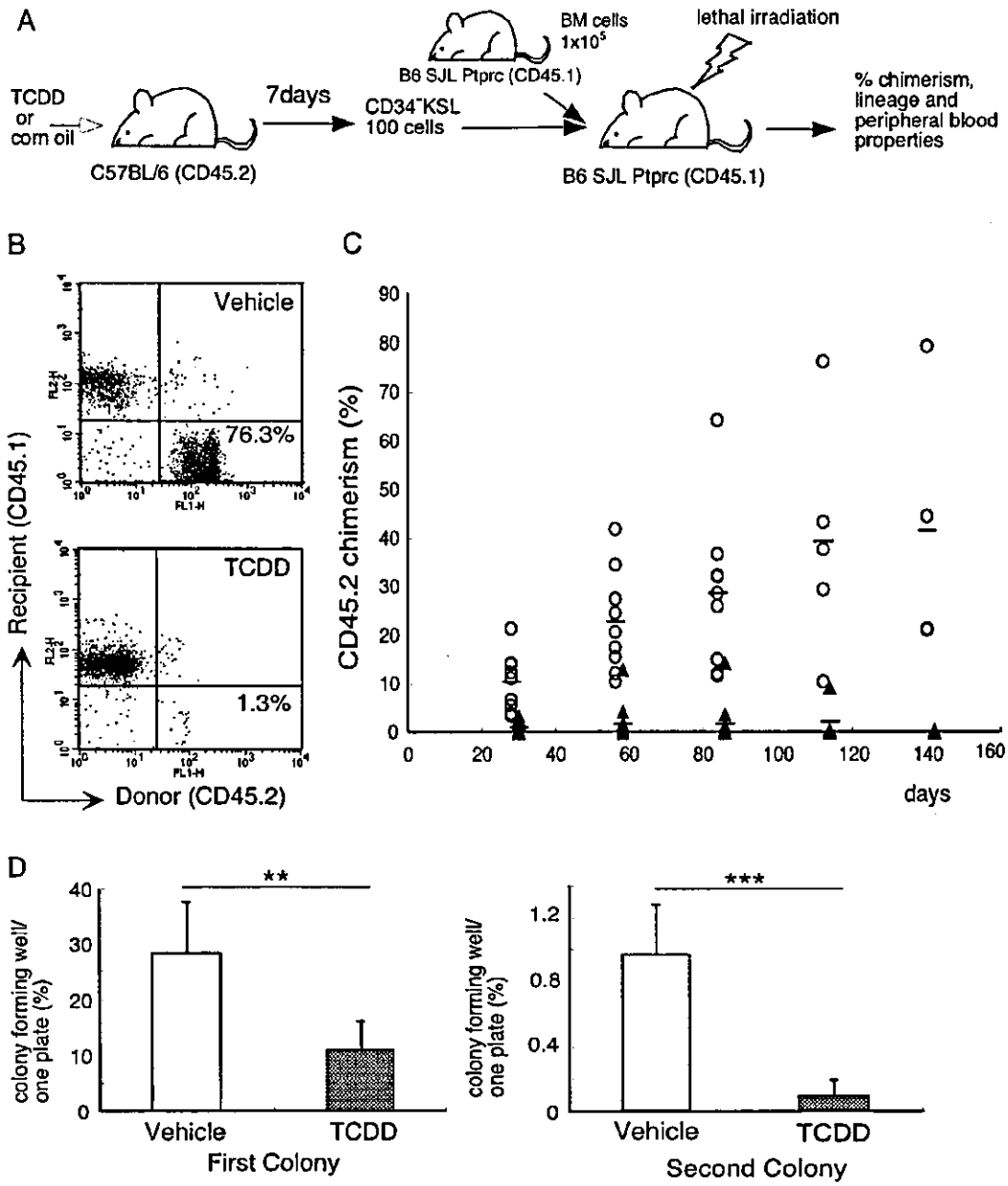

FIG. 3. Function of CD34⁻KSL cells isolated from TCDD-treated mice. (A) BM transplantation experimental design. For details, please see Materials and Methods. (B) Representative flow cytometric dot plots of CD45.2 (donor cells)/CD45.1 (recipient and competitor cells) staining of PB at 16 weeks after BM transplantation. (C) Time course profile of chimerism. While mice transplanted with CD34⁻KSL cells isolated from vehicle-treated mice exhibited more than 5% chimerism, those transplanted with cells isolated from TCDD-treated mice exhibited less than 1% chimerism. Only 2 of 12 mice in the TCDD group had more than 1% chimerism, but they died at 20 weeks. (D) *In vitro* single cell methylcellulose colony assay. Both the first colony and the second colony were counted in the number of total colonies, regardless of the cell types. Total rates of first colony formation in one plate from single CD34⁻KSL cells isolated from vehicle- and TCDD-treated mice were 30 and 10%, respectively. Second colony formation rates of cells isolated from first colonies generated from vehicle- and TCDD-treated mice were 1 and 0.1%, respectively. These experiments were performed more than 3 times with at least 5 mice per group.

assess the long-term reconstitution capability of CD34⁻KSL cells from $AhR^{-/-}$ mice treated with vehicle or TCDD. We used the same assay system described in Figure 3. We prepared three donor groups: CD34⁻KSL BM cells from the $AhR^{-/-}$ vehicle group, $AhR^{-/-}$ TCDD group, and WT($AhR^{+/+}$) TCDD group. Interestingly, we detected high percentages of chimer-

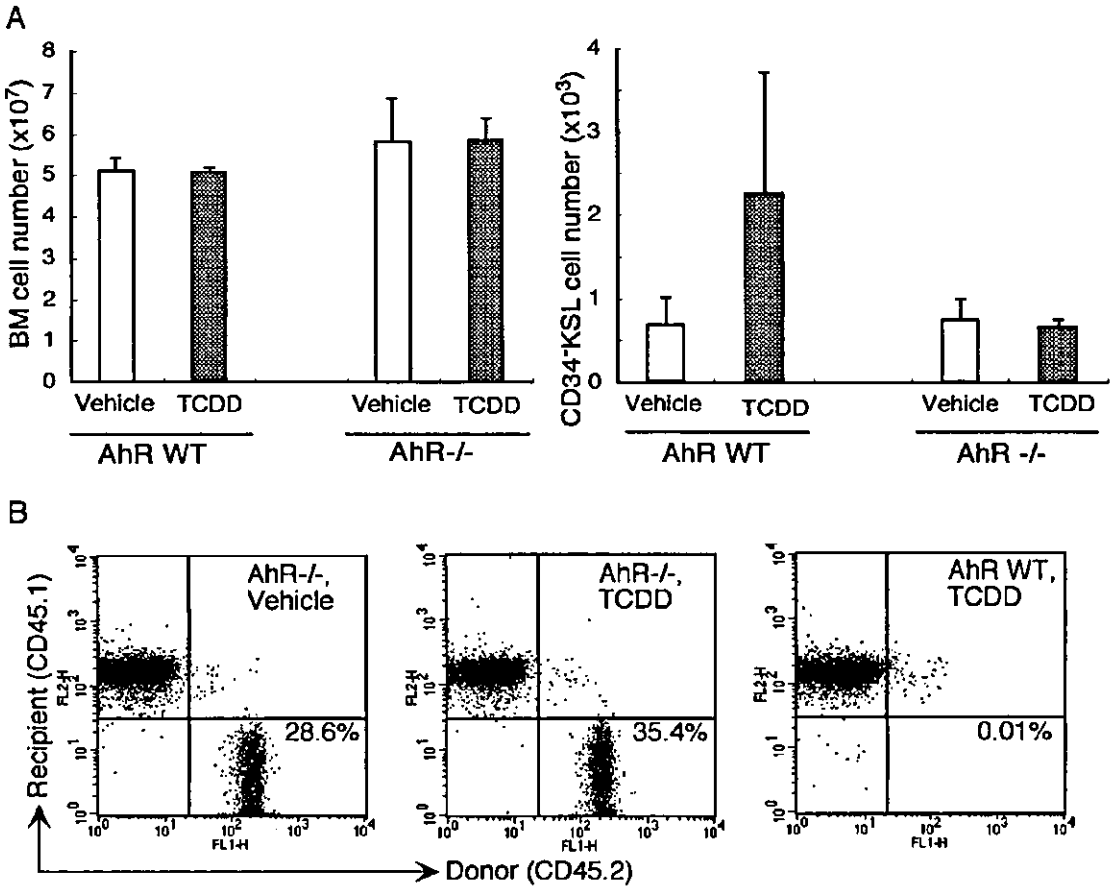

FIG. 4. Participation of AhR in TCDD function. (A) Number of BM cells and CD34$^-$KSL cells from *AhR*$^{-/-}$ mice and *AhR* WT mice (littermate). There were no differences between vehicle- and TCDD-treated conditions in the number of BM cells in either *AhR*$^{-/-}$ or WT mice. In WT mice, TCDD treatment increased the number of CD34$^-$KSL cells compared to vehicle-treated control. However, no difference was seen in the number of CD34$^-$KSL cells isolated from vehicle- or TCDD-treated *AhR*$^{-/-}$ mice. (B) Representative flow cytometric dot plots of CD45.2 (donor cells)/CD45.1 (recipient and competitor cells) staining of PB cells at 12 weeks after BM transplantation. Experimental procedure of BM transplantation was the same as described previously. These experiments were performed more than 3 times with at least 5 mice per group.

ism (more than 10%) in both the *AhR*$^{-/-}$ vehicle group and the *AhR*$^{-/-}$ TCDD group (Fig. 4B), when assayed at 24 weeks. However, we could not detect significant chimerism (at least 1%) in the WT TCDD group.

These observations strongly suggest that both TCDD-induced increase in CD34$^-$KSL cell number and suppression of CD34$^-$KSL long-term reconstitution activity are AhR-dependent. Therefore, it is likely that TCDD affects long-term reconstitution activity through the AhR/ARNT pathway.

DISCUSSION

In spite of high levels of concern about both HSC and biological effects of TCDD, there were only a few reports that investigated the influence of TCDD on HSCs (Murante and Gasiewicz, 2000). Their data revealed increases in the number of bone marrow KSL cells, relative to control, over 24 h through 31 days following treatment of TCDD. They suggested that proliferation and/or differentiation processes of HSCs were affected by TCDD and that these effects contribute to a reduced capacity of bone marrow to generate pro-T lymphocytes.

The capacity for extensive self-renewal is commonly regarded as a property that is reserved for stem cells. Recently, a clonal BM-HSC transplantation system was used to study the function of HSCs. Here, we also used the BM-HSC transplantation system (competitive repopulation assay) to examine the effects of TCDD on long-term reconstitution activity. Our data demonstrate an unexpected elimination of long-term reconstitution activity in the population of CD34$^-$KSL cells in BM. The abrogation seems to encompass all HSC behaviors, including self-renewal, cell death, homing, and lineage contribution

(development). It strongly suggests that TCDD-induced alteration of all hematopoetic processes appeared from the beginning of hematopoiesis.

Therefore it is important to hypothesize that some regulation systems that are affected by TCDD might modulate hematopoiesis from the beginning. Recent studies have demonstrated the ability of intrinsic factors such as HOXB4, cyclin-dependent kinase (CDK) inhibitors, and Wnt signals to control HSC self-renewal and/or reconstitution activities. We hypothesized that misexpression of these intrinsic factors (genes) might abrogate HSC reconstitution activity after TCDD administration.

In the case of HOXB4, the quality of the HSCs induced by *HOXB4* overexpression is not impaired, as demonstrated by their ability to fully repopulate all lineages (Antonchuk *et al.*, 2002). Enhanced HSC regenerative ability in *HOXB4*-transduced bone marrow cells has also been demonstrated (Antonchuk *et al.*, 2001; Sauvageau *et al.*, 1995; Thorsteinsdottir *et al.*, 1999). Studies in RAT-1 cells showed that *HOXB4* overexpression activates the expression of AP-1 complex genes *Fra-1* and *Jun-B*, with subsequent upregulation of *cyclin D1* (Krosl and Sauvageau, 2000). It was also demonstrated that *HOXB4* overexpression enhances *ex vivo* growth of total bone marrow cultures and that this effect is due to increased proliferation rather than reduced apoptosis (Antonchuk *et al.*, 2001). Furthermore, the *HOXB4*-mediated growth advantage was found to be largely restricted to the most primitive fraction of hematopoietic cells (Antonchuk *et al.*, 2002). The primitive cell-specific growth advantage suggests that *HOXB4* overexpression either enhances HSC proliferation or self-renewal, or some combination of both. Indeed, we noticed that the upstream region of the *HOXB4* gene possesses two xenobiotic response elements (TNGCGTG), which might interact with AhR/ARNT upon TCDD administration (M. Kanno, unpublished observation). Therefore, we speculate that AhR/ARNT might negatively control *HOXB4* expression in HSCs.

Recently, the function of CDK inhibitor in HSCs has been studied quite extensively. In the absence of p21 ($p21^{cip1/waf1}$), the G1 checkpoint-regulating CDK inhibitor, HSC proliferation and number are increased under normal homeostatic conditions. Furthermore, self-renewal of primitive cells is impaired in serially transplanted bone marrow from $p21^{-/-}$ mice, leading to hematopoietic failure (Cheng *et al.*, 2000a,b; Enan *et al.*, 1998; Kolluri *et al.*, 1999).

In the case of the Wnt pathway, a central role of *Wnt* in the establishment and maintenance of cell fates has been demonstrated in vertebrates and invertebrates. To examine whether *Wnt* are involved in the regulation of hematopoietic stem/progenitor cell populations (HSCPs), several groups have investigated *Wnt* and *frizzled* gene expression in hematopoietic tissues and the response of HSCPs to WNTs (Austin *et al.*, 1997; Van Den Berg *et al.*, 1998).

There have been several reports describing two states of HSCs (resting and active). Seventy-five percent of HSCs are usually in a resting state, but external stimuli can induce cell cycle progression (Cheshier *et al.*, 1999; Wright *et al.*, 2001). HOXB4 and Wnt might be the candidates for this stimulus. It is conceivable that the signal pathways used by these stimuli are antagonized by the modulation AhR/ARNT system activity. This process of HSC activation has yet to be explored.

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Aryl hydrocarbon receptor-mediated induction of microsomal drug-metabolizing enzyme activity by indirubin and indigo☆

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Abstract

Indirubin and indigo, which are thought to be natural ligands for aryl hydrocarbon receptor (AhR), showed marked AhR ligand activities in a reporter gene assay using recombinant yeast. Their activities were comparable with or more potent than that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. When indirubin and indigo were administered to mice, ethoxyresorufin-*O*-dealkylase and methoxyresorufin-*O*-dealkylase activities in the liver were increased, but subsequently decreased within 2 days. Indirubin was more potent than indigo. Levels of cytochrome P450 1A1/2 proteins and mRNAs in the liver of mice dosed with indirubin were also enhanced. These enhancing effects of indirubin and indigo were not observed in *AhR* knock-out mice. Ethoxyresorufin-*O*-dealkylase and methoxyresorufin-*O*-dealkylase activities in rat hepatocytes and HepG2 cells were enhanced by the addition of indirubin or indigo, but less potently than by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Indigocarmine, a sulfate derivative of indigo, which is used as food additive, did not show these inducing effects on drug-metabolizing enzymes. Our results suggest that indirubin and indigo act as inducers for cytochrome P450 1A1/2 mediated by AhR in mammals in vivo.

Keywords: Cytochrome P450; Indirubin; Indigo; Induction; Aryl hydrocarbon receptor; Ethoxyresorufin-*O*-dealkylase; Methoxyresorufin-*O*-dealkylase

Indigo, which is produced by fermentation of plant material from *Isatis tinctoria*, *Indigofera tinctoria*, and *Polygonum tinctorium*, has been used as dye for cloth used in denims, blue jeans, and other fabrics [1]. The chromophore consists of a single C=C linkage substituted by two donor groups (NH) and two acceptor groups (CO), which are linked by hydrogen-bonding. Indigo-producing plants have also been used in traditional Chinese medicine [2]. Indirubin is a pink-colored by-product of indigo synthesis, and contaminates indigo dye. It was also identified as an anti-leukemia active ingredient [3,4] and shown to be an inhibitor of cyclin-dependent kinases [5]. Gillam et al. [6,7] proposed that indirubin and indigo are formed endogenously in the human body. They demonstrated that human cytochrome P450s (P450s) catalyze the formation from indole of indoxyl and isatin, which could then undergo dimerization to form indigo and indirubin (Fig. 1). Recently, Adachi et al. [8] proposed that indirubin and indigo are endogenous ligands of aryl hydrocarbon receptor (AhR), using a recombinant yeast assay. The ligands were extracted from acid-treated

☆ *Abbreviations:* AhR, aryl hydrocarbon receptor; EROD, ethoxyresorufin-*O*-dealkylase; MROD, methoxyresorufin-*O*-dealkylase; P450, cytochrome P450; PROD, pentoxyresorufin-*O*-dealkylase; 3-MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachloro-*p*-dioxin; HPLC, high-performance liquid chromatography.

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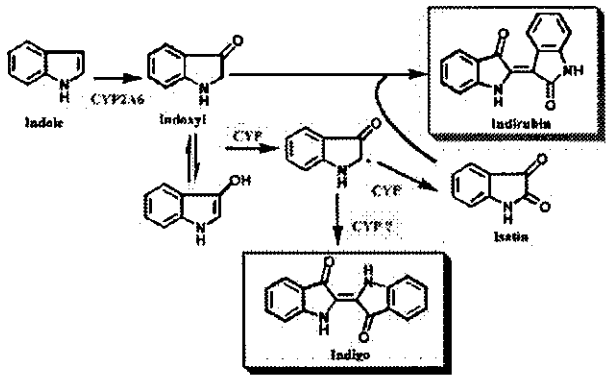

Fig. 1. Postulated scheme for the formation of indigo and indirubin in the body.

human urine and fetal bovine serum, and their levels in the body were shown to be high enough to activate AhR, i.e., 0.2 and 0.07 nM, respectively.

AhR is a ligand-binding transcription factor which was isolated as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) receptor. Binding of TCDD to cytosolic AhR is considered to be the initial event leading to the manifestation of the biological and toxicological responses elicited by TCDD [9]. AhR exists as cytoplasmic aggregates bound to heat-shock protein 90. Upon TCDD binding, AhR dissociates from heat-shock protein 90 and the ligand–receptor complex translocates to the nucleus. Then, the activated AhR dimerizes with the AhR nuclear translocator protein, and this complex recognizes and binds to the xenobiotic response element sequence upstream of the target genes to activate their transcription. Several xenobiotic-metabolizing enzymes, such as UDP-glucuronosyltransferase, specific forms of P450, NAD(P)H-quinone oxidoreductase, and aldehyde dehydrogenase-3, have the XRE sequence in the 5′-upstream region of their genes, and they exhibit AhR-mediated induction of gene expression by TCDD. Recently, it has been reported that AhR-null mice ($AhR^{-/-}$) do not exhibit TCDD-induced liver toxicity or teratogenicity, such as cleft palate and enlarged renal pelvis [10,11]. AhR is thus one of the key molecules mediating the toxicity of dioxins. AhR-mediated signaling is known to be required for xenobiotic ligands such as TCDD, 3-methylcholanthrene (3-MC), and β-naphthoflavone [12,13], but AhR remains an orphan receptor, because its physiological ligand and its function are not known. The AhR ligand activity of indigo was similar to that of TCDD, while indirubin was 50 times more potent [8]. Thus, these compounds are likely to have an important physiological role, and may have the potential to decrease the AhR-mediated toxicity of dioxins in vivo. In this study, we examined the inducing effects of indirubin and indigo on the P450 system in mice in vivo and in rat hepatocytes and HepG2 cells.

Materials and methods

Chemicals and animals. Indigo and 3-MC were purchased from Sigma Chemical (St. Louis, MO). Indigocarmine and 5,7,5′,7′-tetrabromoindigo were purchased from Tokyo Chemical Industry (Tokyo, Japan). Indirubin was synthesized as described by Hoessel et al. [5].

Male C57BL/6JJcl mice (5–6 weeks old) from CLEA Japan (Tokyo, Japan) and male Slc:SD rats (6–7 weeks old) from Japan SLC (Shizuoka, Japan) were housed in cages at 22 °C with a 12-h light/dark cycle, with free access to tap water and a standard pellet diet. The generation of *AhR*-deficient mice ($AhR^{-/-}$) and checking of genotypes of mice were done as previously reported [11]. $AhR^{-/-}$ mice were maintained in the Research Facilities for Laboratory Animal Science, Hiroshima University.

Treatment of animals. Male mice were given indirubin or indigo (1–50 mg/kg body weight) dissolved in Panacete 810 (a mixture of medium-chain triglycerides, Nippon Oils and Fats, Tokyo, Japan) at 5 ml/kg by gavage for three days. These mice were killed one day after the last dose. Vehicle control mice were given the same volume of Panacete 810. Male $Ahr^{-/-}$ mice (5–6 weeks old) were treated with indirubin (50 mg/kg body weight) in the same manner as $Ahr^{+/+}$ (wild: C57BL/6JJcl) mice.

Preparation of liver microsomes. One day after the last dose, the livers were excised from exsanguinated male mice and immediately perfused with 1.15% KCl. The livers were homogenized in four volumes of the KCl solution using a Potter–Elvehjem homogenizer. The microsomal fraction was obtained from the homogenate by successive centrifugation at 9000*g* for 20 min and at 105,000*g* for 60 min. The fraction was washed by resuspension in the KCl solution and resedimentation. The pellets of microsomes were resuspended in the solution to make 1 ml equivalent to 1 g liver. Protein contents in the liver microsomal preparations were determined by the method of Lowry et al. [14].

Enzyme assays of liver microsomes. Ethoxyresorufin-*O*-dealkylase (EROD), methoxyresorufin-*O*-dealkylase (MROD), and pentoxyresorufin-*O*-dealkylase (PROD) activities in liver microsomes were assayed by a fluorophotometric method [15]. The amount of resorufin was determined using about 0.1–0.5 mg protein of liver microsomes. Resorufin formation was linear with time for the 20-min duration of incubation.

Hepatocyte preparation and incubation. Rat hepatocytes were isolated from male rats by collagenase perfusion as described previously [16]. Hepatocytes (2×10^6 cells/ml) were suspended in Williams' E medium supplemented with 7% fetal calf serum in collagen I coated dishes. After 7 h of incubation at 37 °C under an atmosphere of 5% CO_2/95% air, the cells were harvested and the medium was changed. After an additional 17 h of incubation, the enzyme inducers were added to cell suspensions. Dimethyl sulfoxide was used as the solvent of these inducers. After treatment of hepatocytes with the enzyme inducers for 24 h, EROD activities in hepatocytes were assayed by micro-EROD analysis. The medium was removed, 8 μM ethoxyresorufin was added, and the amount of resorufin was measured after 1 h. Cell viability after isolation, estimated by means of the trypan blue exclusion test, was always greater than 90%.

Assays of EROD and MROD activities in HepG2 cells. Human liver cancer cell-line HepG2 cells were grown to confluence at 37 °C under 5% CO_2 in MEM (Sigma Chemical) containing penicillin and streptomycin with 5% fetal bovine serum (Life Technologies, Rockville, MD). The cells were seeded in 24-well plates at 2.5×10^4 cells/well and chemicals were added the next day. One hour after addition of the enzyme inducers, the EROD and MROD activities were assayed.

Immunoblot analysis of cyp 1a1/2. The levels of cyp 1a1/2 proteins were determined by immunoblot analysis of microsomal protein from mouse liver. Mouse microsomal proteins (5 μg) were separated on SDS-polyacrylamide gel electrophoresis (10% gel) and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA) by electroblotting. Membranes were then incubated with 5% skimmed

milk in 25 mM Tris-buffered saline (pH 7.6)—0.1% Tween 20 for 1 h and probed with an anti-rat CYP 1A1 (1:1000) (Daiichi Pure Chemical, Tokyo, Japan) for 3 h. After washing, antibody binding was detected with horseradish peroxidase-conjugated goat anti-rat IgG, followed by development with ECL Plus (Amersham–Pharmacia Biotech, Buckinghamshire, England).

Competitive RT-PCR of cyp 1a1 and cyp 1a2. Total RNA from mouse liver was obtained by extraction with ISOGEN (Nippon Gene, Tokyo, Japan). RNA was reverse-transcribed using a TaKaRa RNA LA PCR kit (Takara, Ohtsu, Japan) followed by competitive PCR with a rat P450 competitive RT-PCR set (Takara) according to the manufacturer's protocol.

RT-PCR analysis of CYP 1A1 mRNA in HepG2 cells. Total RNA from HepG2 cells was obtained by extraction with ISOGEN. First-strand cDNA was synthesized with 1 μg total RNA from HepG2 cells using AMV reverse transcriptase (Promega, Madison, WI). PCRs were undertaken with a HotStarTaq polymerase kit (Qiagen, Hilden, Germany). PCR primer sequences for amplification of CYP 1A1 were forward 5′-tctttttcctccgtggctatc and reverse 5′-ctgtctcttcccttcactct, and for β-actin, forward 5′-ccaaggccaaccgtgagaagatgac and reverse 5′-agggtacatggtggtgccgccagac. The PCRs were performed for 30 cycles of 15-s denaturation at 94 °C, 30 s at the optimal annealing temperature, which is 60 °C for CYP 1A1 and 62 °C for β-actin, and 30-s extension at 72 °C. Amplification products were separated on 1.5% agarose gel and visualized with ethidium bromide under UV transillumination. Density of bands detected for β-actin showed little variation.

Yeast assay for AhR ligand activity. The assay procedure was essentially as described by Miller [17,18]. The yeast strain YCM3 was grown overnight at 30 °C in synthetic 2% glucose complete medium lacking tryptophan. Test chemicals dissolved in dimethyl sulfoxide, 5 μl of the overnight culture, and 195 μl medium containing 2% galactose were mixed in a 96-well microplate with subsequent incubation for 18 h at 30 °C. The well densities were determined by reading the absorbance at 595 nm. The suspension in each well (10 μl) was added to 140 μl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol, and 0.2% sarcosyl, adjusted to pH 7), and the reaction was started by adding 50 μl *o*-nitrophenol-β-galactopyranoside (4 mg/ml solution in Z-buffer), followed by incubation for 60 min at 37 °C. The absorbance of *o*-nitrophenol was read at 405 nm. β-Galactosidase activity (referred to as lacZ units) was calculated by use of the following formula: absorbance at 405 nm × 1000/(absorbance at 595 nm × ml of well suspension added × min of reaction time).

Results

AhR ligand activity of indirubin and indigo in yeast AhR assay

AhR ligand activity of indirubin and indigo was examined in a yeast AhR signaling assay using human AhR and ARNT genes coexpressed in yeast, and compared with those of TCDD, 3-MC, and β-naphthoflavone. Very high binding ability of indirubin and indigo was observed in the concentration range of 1×10^{-9}–1×10^{-5} M. The EC50 values of indirubin, TCDD, indigo, and 3-MC were 0.12, 5.71, 4.15, and 46.3 nM, respectively. The ability of indirubin to act as a ligand in the yeast assay was about 50 times higher than that of TCDD, as noted by Adachi et al. [8]. Indigo was also a better ligand than 3-MC or β-naphthoflavone. α-Naphthoflavone, which is an inhibitor of the P450 1A subfamily, showed AhR ligand activity. However, indigocarmine, which consists of sulfate derivatives of indigo, and is used as a food additive, did not show this ability even at the concentration of 1×10^{-5} M. 5,7,5′,7′-Tetrabromoindigo and isatin also showed much smaller activity than that of indigo (Fig. 2). Indole and other indole derivatives, isatide and indomethacin, did not show ligand-binding activity with AhR (data not shown).

Effects of indirubin and indigo on liver microsomal enzyme activities in mice

We examined the microsomal alkoxyresorufin-*O*-dealkylase activities, EROD, MROD, and PROD, of male C57BL/6JJcl mice after treatment with indirubin or indigo (1, 5, 10, and 50 mg/kg body weight) by gavage for three days. The EROD and MROD activities were increased dose-dependently compared with that of the control mice. Indigo induced the EROD and MROD

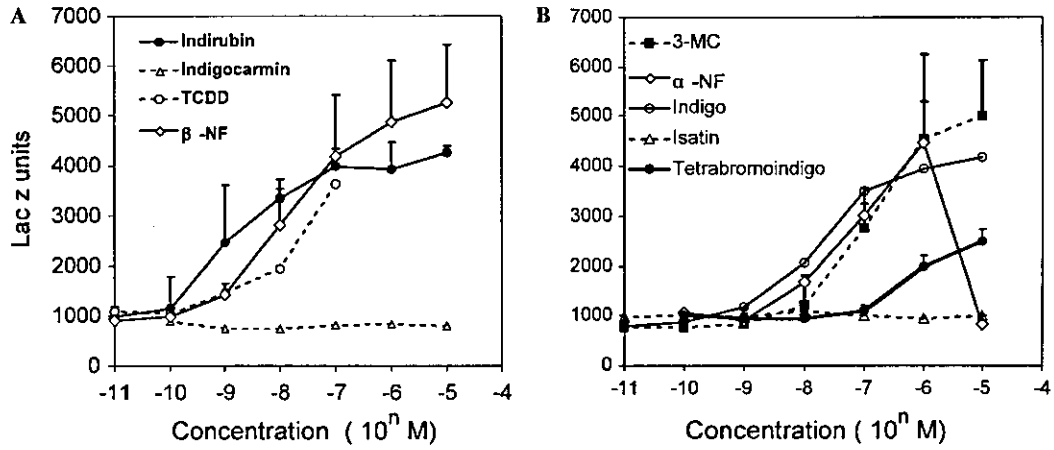

Fig. 2. AhR-binding affinity of indirubin and indigo in yeast AhR reporter assay. (A) Activities of indirubin and other ligands. (B) Activities of indigo and other ligands. Each value represents the mean ± SD of four individual experiments.

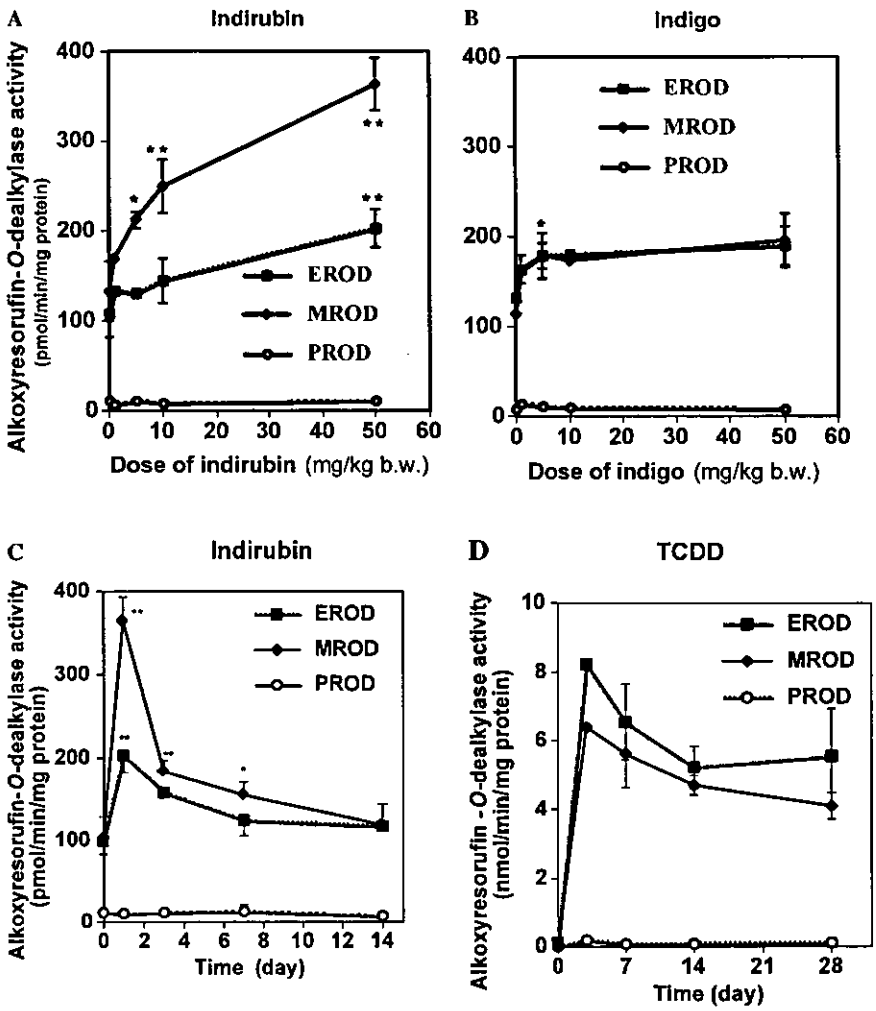

Fig. 3. Effects of indirubin and indigo on MROD, EROD, and PROD activities in mice. (A) Dose-dependent induction of EROD, MROD, and PROD activities in mice by indirubin. (B) Dose-dependent induction of EROD, MROD, and PROD activities in mice by indigo. (C) Time courses of induction of EROD, MROD, and PROD activities by indirubin. (D) Time courses of induction of EROD, MROD, and PROD activities by TCDD (40 μg/kg). Each value represents the mean ± SD of four individual mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control.

activities by 1.3- and 1.4-fold, respectively, at 50 mg/kg body weight. Indirubin induced the EROD and MROD activities by 1.9- and 2.7-fold, respectively. However, the PROD activity, which is due to phenobarbital-inducible P450, was not induced by indirubin or indigo (Figs. 3A and B). Furthermore, the activity of microsomal enzymes in mouse liver was assayed at various times after indirubin administration. The EROD and MROD activities in liver of animals dosed with indirubin increased up to 1 days after administration and subsequently decreased (Fig. 3C). In contrast, EROD and MROD activities enhanced by TCDD were maintained for at least 28 days (Fig. 3D).

When indirubin was dosed to mice at 50 mg/kg, the amount of cyp 1a2 protein in the liver was increased about 3-fold at 1 day after the dose compared with the vehicle-only control. However, the amount was lower than that of 3-MC-dosed mice (Fig. 4A). Further, the levels of cyp 1a1 and 1a2 mRNAs in nontreated, 3-MC- and indirubin-treated mouse livers were compared using competitive RT-PCR. The levels of both mRNAs after treatment with 3-MC were enhanced. In contrast, the level of cyp 1a2 mRNA after treatment with indirubin was increased about 3-fold compared with the control. However, in this case little cyp 1a1 mRNA was detected (Fig. 4B). These results suggest that enhancement of MROD and EROD activities by indirubin is mainly due to the induced cyp 1a2.

Inductive effect of indirubin and indigo on liver enzyme activities of $AhR^{-/-}$ mice

It is suggested that inductions of EROD and MROD activities by indirubin and indigo are mediated by AhR.

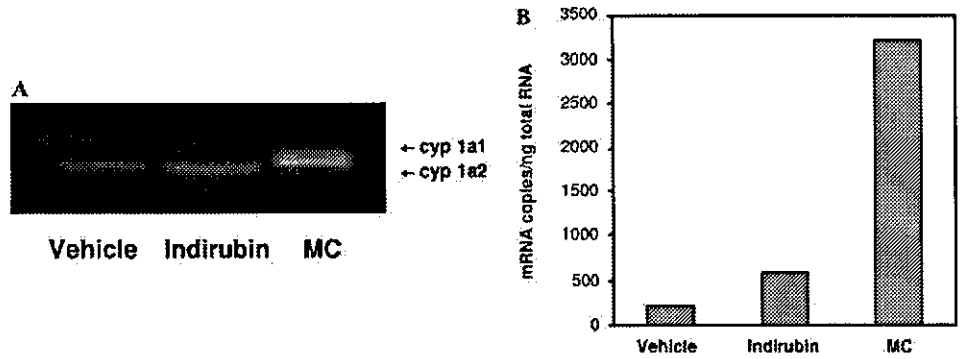

Fig. 4. Effects of indirubin on the levels of cyp 1a1/2 proteins and mRNAs in mouse liver. (A) Levels of cyp 1a1/2 proteins at 1 day after treatment with indirubin (50 mg/kg), 3-MC (25 mg/kg) or vehicle only, determined by immunoblot analysis. (B) Levels of cyp 1a2 mRNA at 1 day after treatment with 3-MC or indirubin.

When indirubin was applied to $Ahr^{-/-}$ mice, which lack AhR, no inductive effect on EROD or MROD was observed (Fig. 5). P450 content (about 0.6 nmol/mg protein) was not increased in the null mice, in contrast to the increase of P450 (1.0 nmol/mg protein) by indirubin in $AhR^{+/+}$ mice. Thus, the induction of the EROD and MROD activities by indirubin and indigo appears to be mediated by AhR.

Effects of indirubin and indigo on enzyme activities in rat hepatocytes and HepG2

The enhancing effect of indirubin and indigo on EROD and MROD activities in rat hepatocytes and HepG2 cells was examined. Both MROD and EROD activities in rat hepatocytes and HepG2 cells were enhanced by the addition of indirubin and indigo, as well as TCDD and 3-MC (Fig. 6). The enhancing ability of indirubin after 10 h was intermediate between those of TCDD and 3-MC over the concentration range of 1×10^{-9}–1×10^{-5} M in HepG2 cells. Indigo showed a similar enhancing ability to 3-MC (Figs. 6B and C). However, the enhancing effects of indirubin and indigo on EROD activity observed in hepatocytes after 24 h were smaller than that of 3-MC (Fig. 6A). In contrast, PROD activity was not enhanced by indirubin or indigo (data not shown). The time-course of enhancement of EROD activity in HepG2 cells by indirubin at 1×10^{-6} M was linear up to 16 h and the activity subsequently decreased. At 1×10^{-7} M indirubin, the EROD activity increased up to 12 h and at 1×10^{-8} M indirubin, it increased up to 10 h. In contrast, the highest level of CYP1A1 mRNA was observed at 10 h after the addition of indirubin at the concentration of 10^{-6} M (Fig. 6D). In this case, CYP 1A2 mRNA was slightly increased after the treatment. The amount of indirubin remaining in the medium and cells started to decrease immediately with HepG2 cells (Fig. 6E). This suggests that the diminished effect at 10–16 h after the addition is due to loss of added indirubin. Similar changes were induced by indigo. In contrast, indigocarmine showed no such enhancing ability in HepG2 cells at the concentration of 1×10^{-7} or 1×10^{-5} M. In this case, no enhancing effect of α-naphthoflavone on EROD and MROD activities was found, either (data not shown).

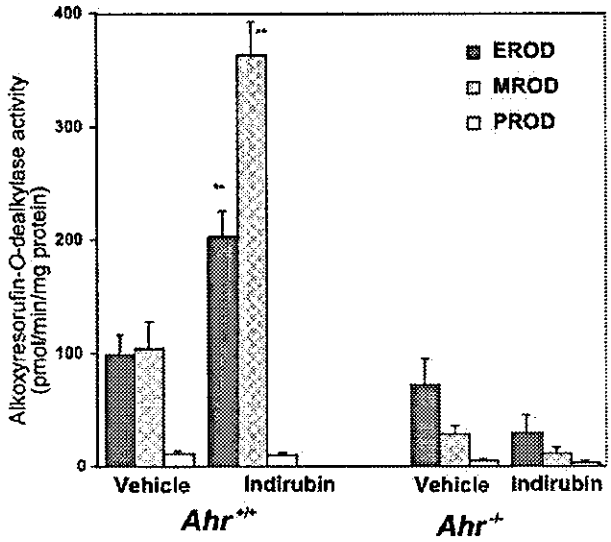

Fig. 5. Effects of indirubin and indigo on EROD, MROD, and PROD activities in liver preparations of $Ahr^{+/+}$ and $Ahr^{-/-}$ mice. Male $Ahr^{+/+}$ (wild: C57BL/6JJcl) and $Ahr^{-/-}$ mice were treated with indirubin at 50 mg/kg body weight. Each bar represents the mean ± SD of four individual mice. $^{**}p < 0.01$ compared with the control.

Discussion

High binding affinity of indirubin and indigo to AhR has been demonstrated by means of recombinant yeast assay. In this study, we have shown that indirubin and indigo induce EROD and MROD activities in mice in vivo, and the induction is mediated by AhR. In this case, MROD activity was induced more than EROD activity.

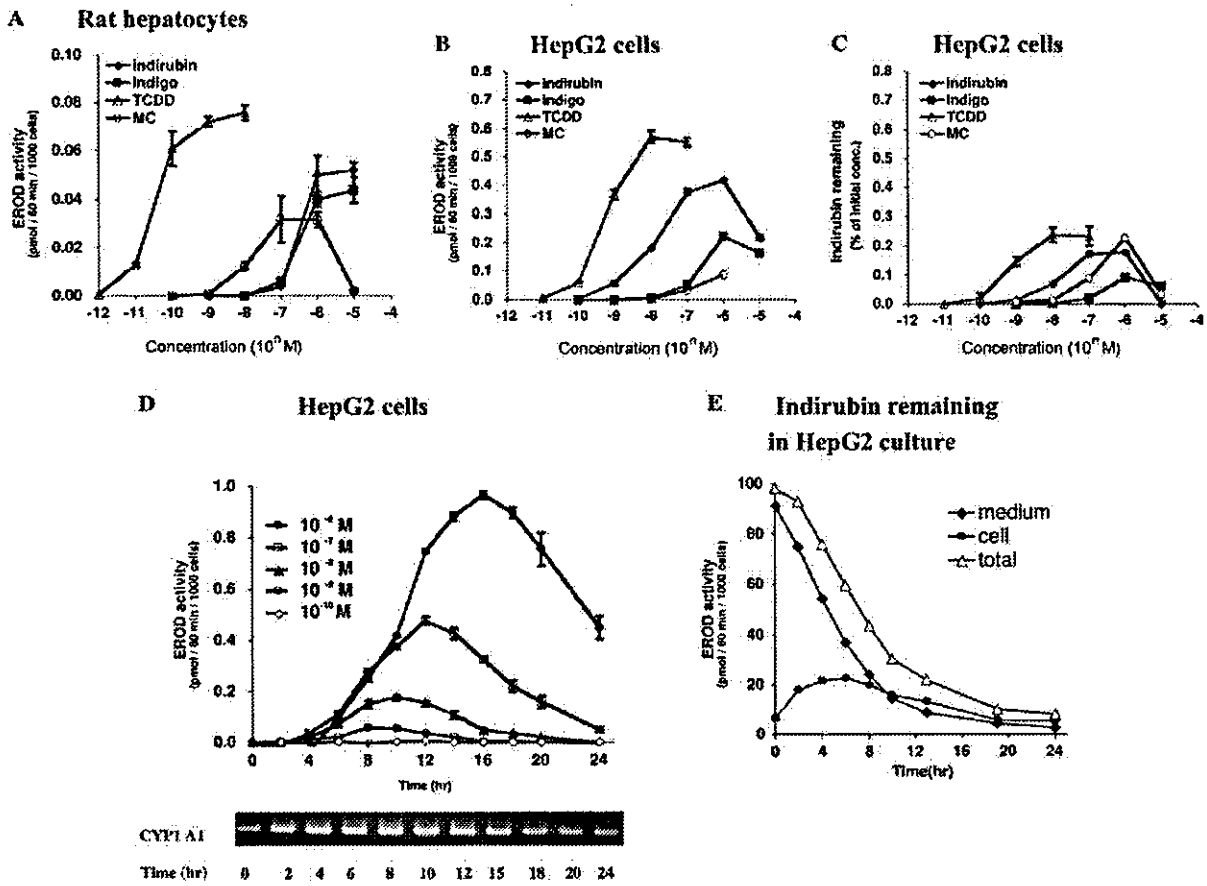

Fig. 6. Effects of indirubin and indigo on EROD and MROD activities in rat hepatocytes and HepG2 cells. (A) Effects of indirubin and indigo on EROD activity in hepatocytes after 24 h. (B) Effects of indirubin and indigo on EROD activity in HepG2 cells after 10 h. (C) Effects of indirubin and indigo on MROD activity in HepG2 cells after 10 h. (D) Time course of induction of EROD activity and CYP 1A1 mRNA by indirubin in HepG2 cells. (E) Amount of unchanged indirubin remaining in HepG2 cells and their medium after incubation. Each value represents the mean ± SD of four individual experiments. Activity was expressed as pmol/60 min/1000 cells, and the activity in the corresponding control experiment was subtracted. The amount of indirubin in (E) was measured by high-performance liquid chromatography after extraction with ethyl acetate.

In contrast, TCDD-induced EROD activity more effectively than MROD activity. It was reported that EROD activity is mainly exhibited by CYP 1A1 and MROD activity by CYP 1A2 [15]. We observed that the level of cyp 1a2 mRNA was enhanced by indirubin in mice. These facts suggest that indirubin mainly induced cyp 1a2, and this is the reason why MROD activity was enhanced more than EROD activity. TCDD and 3-MC are known to be inducers for CYP 1A1 and 1A2 [19,20]. In this study, we confirmed that 3-MC induces cyp 1a1 and 1a2, but indirubin induces only cyp 1a2 in mice. On the contrary, the level of CYP 1A1 mRNA was mainly increased by indirubin treatment in HepG2 cells, and in this case, EROD activity was induced more strongly than MROD activity. Spink et al. [21] reported that indirubin induces CYP 1A1 and 1B1 in MCF-7 cells. Thus, although indirubin induces cyp 1a2 in mouse livers, its induction of CYP isoforms might be species- and organ-dependent. Unlike caffeine, indirubin may not be a specific inducer for CYP 1A2 [22].

Although the AhR ligand activities of indirubin and indigo in the recombinant yeast assay were similar to or stronger than that of TCDD, the activities to induce drug-metabolizing enzymes in mice, in hepatocytes, and in HepG2 cells were less marked. The enhancing ability of indirubin on EROD and MROD activities was intermediate between those of TCDD and 3-MC, and indigo showed a similar ability to 3-MC in HepG2 cells. This apparent discrepancy can be explained by the finding that unchanged indirubin in HepG2 cells and their culture medium decreased time-dependently, and this would lead to a decrease of the inducing effect (Fig. 6). Liver microsomes of 3-MC-treated rats also exhibited a marked metabolizing activity toward indirubin, and in this case CYP 1A1 mainly contributed the metabolism (data not shown). Our preliminary experiments suggested that hydrophilic compounds such as isatin are formed as metabolites of indirubin by liver microsomes, and the transience of the inducing effect of indirubin and indigo is due to their rapid metabolism to

inactive products. If indigo and indirubin are endogenous AhR ligands, the actions of these compounds would be tightly controlled by their rapid metabolism. On the other hand, TCDD persists in the body and is not readily metabolized. Indeed, the inducing ability of indirubin was essentially lost by 3 days after administration. It is possible that indirubin and indigo were transformed to their leuco derivatives, as reported in the case of indigocarmine [23]. Indeed, we confirmed the conversion of indirubin, indigo, and indigocarmine to their leuco derivatives by rat liver preparations. Thus, the decrease in the inducing effect of indirubin and indigo on liver microsomal enzymes in vivo might be a consequence of metabolic conversion to product(s) that are inactive as ligands of AhR.

Indigocarmine, which is used as a food additive, did not bind with AhR in the yeast AhR assay, and showed no ability to induce microsomal enzyme activity in rat hepatocytes and HepG2 cells. Perhaps, the hydrophobicity necessary for binding with AhR was lost as a result of sulfate substitution. In other words, conjugation of indirubin and indigo with glucuronic acid or sulfuric acid may inactivate these compounds as inducers of P450. It is well known that halogenated compounds such as TCDD have a high binding affinity for AhR. Unexpectedly, the binding activity of the tetrabromo derivative of indigo was very small compared with that of indigo. This may be related to loss of the strong intramolecular hydrogen-bonding of indigo and indirubin in the tetrabromo derivative, or to steric hindrance. Indole and its derivatives, isatin and indomethacin, also did not exhibit binding affinity with AhR. This finding suggests that cleavage of the linkage of the two indole rings of indirubin and indigo, which is a possible metabolic reaction in the body, is an inactivation pathway. Thus, the interaction of indirubin and indigo with AhR may be appropriately regulated by metabolic processes.

Recently, we reported the induction of xanthine oxidase/xanthine dehydrogenase activities by TCDD in mice, and suggested that lipid accumulation in the liver causes injury to the membranes via lipid peroxidation, due to oxidative stress resulting from xanthine oxidase induction [24]. In contrast, fatty degeneration was recognized in the liver of mice dosed with TCDD [25]. Here, we attempted a histochemical analysis of livers obtained from mice after indirubin or indigo treatment (50 mg/kg body weight) and vehicle control mice. The liver sections were stained with hematoxylin–eosin or Sudan black. Indirubin and indigo caused no significant damage to the liver, and lipid droplets, which are usually observed after TCDD treatment, were not increased (data not shown). Thus, no toxicological changes were observed in liver sections from acute high-dose indirubin-treated mice. Oxidative stress following acute TCDD exposure in laboratory animals has been demonstrated to increase the production of reactive oxygen species, lipid peroxidation, and DNA damage [26–29]. The mechanism of TCDD-mediated reactive oxygen species production has been proposed to involve the P450s [30], specifically, CYP1A1 and 1A2 [24]. In contrast, the above results suggest that endogenous indirubin and indigo might be physiological ligands for AhR, and the concentrations of these ligands in tissues seem to be appropriately regulated. In this study, we showed that the induction of microsomal enzyme activity in mice by indirubin and indigo is mediated by AhR. On the other hand, AhR-mediated signaling by potent xenobiotic ligands such as TCDD and polychlorinated biphenyls produces toxic responses, such as tumor promotion, skin toxicity, reproductive impairment, endometriosis, birth defects, and immunological impairment [31–35]. The toxic potentials of xenobiotics and endogenous compounds as AhR ligands seem to be very different.

Acknowledgments

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COMMENTARY

Comments from the Behavioral Teratology Committee of the Japanese Teratology Society on OECD Guideline for the Testing of Chemicals, Proposal for a New Guideline 426, Developmental Neurotoxicity Study, Draft Document (September 2003)

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ABSTRACT **In September 2003, a new revision of the draft guideline (Organization for Economic Co-operation and Development [OECD] Guideline for the Testing of Chemicals, Proposal for a New Guideline 426, Developmental Neurotoxicity Study) was distributed. The draft guideline consists of 51 paragraphs and an appendix. The National Coordinators were requested to arrange national expert reviews of the guideline proposal in their member countries. The member of the Behavioral Teratology (BT) Committee of the Japanese Teratology Society (JTS) reviewed, discussed and commented on the draft Test Guideline proposal. The BT Committee of the JTS also commented that the International Collaborative Study to validate this protocol should be definitely performed. These comments were sent to the OECD Secretariat. The BT Committee of the JTS expects that the comments are useful for further discussion.**

Key Words: behavior, developmental neurotoxicity, OECD, test guideline

INTRODUCTION

The Organization for Economic Co-operation and Development (OECD) Working Group on Reproduction and Developmental Toxicity at Copenhagen in June 1995 (OECD 1995) recommended that a guideline for developmental neurotoxicity should be written. In June 1996 at Copenhagen, an OECD Consultation Meeting on Developmental Neurotoxicity provided the Secretariat with the draft report on the outline of a new guideline (OECD 1996). The Behavioral Teratology (BT) Committee of the Japanese Teratology Society (JTS), in association with the Meeting of Neurobehavioral Toxicology of the Japanese Society of Toxicology, commented on this draft report. After this meeting, a draft

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proposal for Test Guideline 426, Developmental Neurotoxicity Study was developed, and was submitted to the Secretariat in February 1998. The draft guideline was distributed in December 1998. The BT Committee of the JTS commented again on this draft guideline. The draft guideline proposal was extensively revised and distributed in October 1999. General issues regarding the design of developmental neurotoxicity studies were discussed in an OECD Expert Consultation Meeting and International Life Sciences Institute (ILSI) Risk Science Institute Workshop in Washington, DC, USA, in October 2000 (OECD 2003). In September 2003, a new revision of the guideline was distributed. This revised draft Test Guideline proposal is posted on the OECD public web pages of the Test Guidelines Programme at: http://www.oecd.org/document/55/0,2340,en_2649_34377_2349687_1_1_1_1,00.html. The draft guideline consists of 51 paragraphs and an appendix. National Coordinators were requested to arrange national expert reviews of the guideline proposal in their member countries. The deadline for the expert responses to this revised draft Test Guideline was January 16, 2004.

A meeting of the BT Committee (Chairman: Dr Y. Fukui, Professor, University of Tokushima School of Medicine) of the JTS was held on January 11, 2004, in Osaka, and the members of this committee reviewed, discussed and commented on the draft Test Guideline proposal. The BT Committee of the JTS also commented that the International Collaborative Study to validate this protocol as indicated in OECD ENV/EHS/HK/mc/2003.49 should be definitely performed. These comments were sent to the OECD Secretariat through the Japanese National Coordinator (Director of the Office of Chemical Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Japan) on January 16, 2004, but it is to be noted that they are not official comments from Ministry of Health, Labour and Welfare, Japan.

The BT Committee of the JTS expects that the comments are useful for further discussion.

The comments from the BT Committee of the JTS are as follows:

GENERAL COMMENTS

1 New terms such as behavioral ontogeny, instead of reflex ontogeny in the 1999 draft, are introduced in the 2003 draft, but unification of terms is insufficient in the various parts of the text.

2 The rationale for the weaning day should be stated. Day of weaning is recommended to be PND 22, but PND 21 from the previous draft still appears in some parts of the text. The description day of test performance should be unified throughout the text.

3 More flexibility of the study design must be stressed. The use of 'should' is seen too frequently.

4 Guidance for higher levels of the study, such as social behavior, pharmacologic challenge, and neurochemistry, is insufficient.

5 Examination of maternal toxicity is insufficient except for clinical signs. It is advised that dams are autopsied and examined at least macroscopically.

6 The description of use of species other than rats, such as non-human primates, is scanty.

7 Considerable recent references have been added, but there is more pertinent literature to be cited.

8 The front page (DRAFT DOCUMENT [September 2003]) should be page 1, and the present page 1 is to be changed to page 2, and so on. The final page, Appendix A, would be page 21.

SPECIFIC COMMENTS

1. Paragraph 2

The exposure period is expanded from 'lactation' to 'during early life'. This change is very welcomed, but the following explanation is limited to the exposure until weaning. Some description of administration of the test substances directly to offspring after weaning should be given, since human developmental neurotoxicity of chemicals in early childhood has become a great concern.

The phrase 'during pregnancy or' should be '*in utero* and'. Pregnancy primarily refers to dams, not to fetuses.

2. Paragraph 3

The phrase 'developmental toxicity and/or adult neurotoxicity study (e.g. Test Guidelines 415, 416, 424)' is to be changed to 'prenatal developmental toxicity, one- or two-generation study and/or adult neurotoxicity study (e.g. Test Guidelines 414, 415, 416, 424)'.

The phrase 'or as an add-on study' should be concretely explained, since the meaning is not clear.

Does 'other types of toxicity' include developmental (fetal) toxicity or is it limited to adult? It is necessary to specify this.

3. Paragraph 4

The phrase 'perinatal' in line 2 is to be 'prenatal', since the latter is the OECD term of Guideline 414.

4. Paragraph 5

The word 'and/or' in line 2 is to be 'and'.

The term 'reflex ontogeny' in line 5 is to be 'behavioral ontogeny'.

5. Paragraph 6

Since 'stand-alone' is a specific computer term, it is preferable to replace it with a more common word.

6. Paragraph 7
The usefulness of other species, especially non-human primates, for higher levels of learning and memory study, may be more circumstantially stated.

7. Paragraph 9
The third sentence should be changed to 'After evidence of copulation, individual housing of mated animals is recommended'. The sentence 'If mated animals are caged in small groups, animals should be caged separately in individual cages no later than day 15 of pregnancy' should be inserted following the third sentence.

8. Paragraph 10
It may be necessary to describe the males used for mating.

Usually, rats are obtained as a lot that may contain some brothers. Therefore, it is not practical for breeding males to be equalized across a group.

9. Paragraph 12
The numbers '8–12' in line three are to be changed to '8–10'. In cases of litter sizes of 12, many litters may be insufficient in number. When the number of pups in a litter is less than the designated number, it is not acceptable to add some pups from different dams for fostering.

Those litters with an insufficient number of pups should not be principally used for the study. These remarks are to be clearly described here.

Identification of individual pups is recommended to be performed at birth or soon after birth when the body weight is measured.

10. *'Assignment of . . .'* and paragraphs 13–15
It is recommended that this portion is placed after *Dosage* and *Administration of doses*, since dosage and administration are more directly related to dams than assignment of offspring.

11. Paragraph 14
The rationale is not clear why the same pair of male and female littermates is assigned for motor activity testing, while for all the other tests the same or separate pairs may be used.

12. Paragraph 15
'Behavioral/functional tests' in Tables 1 and 2 should be 'Functional/behavioral tests', concordant with the description in Table 3. Function is a broader category than behavior.

The contents of 'functional/behavioral test' in Tables 1 and 2 are not clear. In the text, 'functional tests' are listed in line 11. In Table 3, 'functional/behavioral endpoints' consist of three major items, motor activity, motor and sensory function, and learning and memory. Therefore, the major component of 'functional/behavioral tests' in Tables 1 and 2 would be motor and sensory function.

Note (c) to Table 1 is questionable unless the same pups are used to check the changes of findings in adolescent and young adult ages. Moreover, the number of animals tested is recommended to be 20 in Table 2. Therefore, it is generally preferable to adopt the procedures indicated in Table 2 since the offspring tested for cognitive function etc. are examined for neuropathology, and the correlation between behavioral abnormalities and neuropathological changes can be checked. Thus, Table 2 is recommended to be the first choice and treated as Table 1. The total sentences in this paragraph should be rewritten according to this consideration. Optional and Neuropathology in Tables 1 and 2 should be optional and neuropathology (small letters).

Pups no. of the female in the preweaning investigation in Table 2 is 5, not 2.

13. Paragraph 16
The phrase 'maternal or developmental toxicity or neurotoxicity' in line 10 is to be changed to 'maternal or developmental toxicity' or 'maternal or developmental toxicity including neurotoxicity', since neurotoxicity is a part of toxicity and is related to both dams and offspring.

In some cases, a high dose can not be chosen to induce maternal toxicity. Thus, it is highly recommended to add a sentence to explain the rationale in cases where no maternal toxic dose level is selected for the high dose.

A description regarding limit dose should be added.

14. Paragraph 17
The word 'should' should be changed to 'may' (lines 1 and 4).

The sentence 'However, an evaluation of direct dosing to pups has not been established yet.' should be inserted following the last sentence.

15. Paragraph 19
In case of dietary or via drinking water administration, due consideration should be taken that pups receive the test substances not only from milk but also considerably from diet or water in the later period of lactation.

The phrase 'except for the day of parturition' and the sentence 'The test substance should be administered after completion of parturition.' should be inserted following the end of the last sentence.

16. Paragraph 20
The first sentence should be deleted. In reproductive and developmental studies including teratological study and pre- and postnatal study, the dosage volume in each dam is practically calculated by two different methods: (a) based only on body weight on day 6 of gestation or (b) based on the

most recent body weight. Body weights on day 6 and day 20 of gestation are 300–320 g and 400–420 g, respectively, in SD rats. When the dosage volume is calculated based on the recent body weight, dams will be exposed to overdose (approximately 1.3 times) and excess toxicity to dams must be noted.

17. Paragraph 21
A marginal note * is to be incorporated into the text because this is an important item.

18. Paragraph 24
Delete 'secretion and' in line 3 (duplicated).

19. Paragraph 27
PND 21 is to be PND 22.

Measurement of food consumption is recommended at administration via other routes than diet since food consumption is an important indicator of maternal general toxicity.

20. Paragraph 31
The headline 'Developmental landmarks' is to be 'Physical and developmental landmarks' since body weight, described in paragraph 31, is certainly an indicator of physical development.

'Pinna reflex' is to be 'Pinna detachment'.

Add eye opening since it is an important index related to motor activity.

21. Paragraph 32
The following reference is to be cited in explanation of the usefulness of postcoital age: Tachibana T., Narita H., Ogawa T., Tanimura T. (1998) Using postnatal age to determine test dates leads to misinterpretation when treatments alter gestation length: Results from a collaborative behavioral teratology study in Japan. Neurotoxicol Teratol. 20: 449–457.

Table 3 should be carefully revised since neuropathological examination on PND 11 is no longer routinely recommended. 'Age Period' is to be 'Age period'. [Before PND 21] is to be [At and before PND 21] since PND 21 is the last day of the preweaning period. [PND 21–59(a)] is [PND 22–59(a)]. In the row of physical development, 'weekly' is to be at the level of Body weight (one line downward). In the row of Brain weight and Neuropathology, delete 'at PND 22' in the column of Preweaning since preweaning ends at PND 21. Only a remark (b) may remain in this place (for examination on PND 11). Delete 'optional' in the column of Adolescence. In Note (a), weaning (generally PND 21) is weaning (generally PND 22), and (PND 23–24) should be (PND 24–25).

22. Paragraph 33
Delete the heading 'Physical development'. The reason is given in comment 19.

It is suggested that this paragraph is moved before paragraphs 31 and 32, since the counting and sexing of live pups are the first steps for offspring observation.

23. Paragraph 34
Surface righting, cliff avoidance and swimming development should be added as examples. Also, give pertinent literature on these tests. Swimming is an especially good indicator of behavioral ontogeny.

24. Paragraph 35
The phrase 'preweaning and adult age' in line 1 should be 'preweaning, adolescence and young adult age', according to Table 3.

It is important to minimize maternal stress at the test of motor activity. Practically, the manipulation of separating the pups from the mother and returning them to the cage should be performed as gently as possible. This caution may be applied at other preweaning tests such as body weight measurement.

The description of 'Among the variables . . .' in lines 16–18 may be also applied to tests other than motor activity. Therefore, these statements should be placed in the appropriate earlier paragraphs as a general caution.

An explanation regarding the phrase '1–3 times' is needed (third line from the bottom, second column in Table 3).

25. Paragraph 36
Rotarod, open field and olfactory orientation tests are to be added as examples. As for a reference of olfactory orientation, Gregory EH, Pfaff DW. (1971) Development of olfactory guided behavior in infant rat. Physiol Behav. 6 : 573–576, is suggested.

References should be separately given for each test for the readers' convenience.

26. Paragraph 37
The headline 'Learning and memory tests' should be 'Learning and memory tests (Cognitive function tests)' or 'Cognitive function tests' (Refer to Tables 1–3).

The Biel maze (multiple T-water maze) should be added as an example. The shuttle box avoidance test (active avoidance) may be also added. Pertinent literature on these tests is also to be described.

Two or more different categories of learning and memory tests may be planned to reveal the nature of disturbances of learning and memory.

27. Paragraph 38
PND 21 is to be PND 22.

28. Paragraph 41
Some explanation of GFAP is necessary, together with references, or '(e.g. GFAP)' should be deleted.

29. Paragraph 43
The phrase '(tectum, tegmentum, and cerebral peduncles)' should be deleted.

30. Paragraph 44
The phrase 'typical of the adult brain' is not understandable. Are some words are missing?

31. Paragraph 46
The sentence 'While the use . . .' in lines 7–9 can be rewritten more simply. For instance, 'It is preferable that a pathologist who is unaware of the treatment information scores the slides to substantiate the dose–response relationship'.

32. Paragraph 48
Delete 'perinatal' in line 1. The name of this guideline is simply developmental neurotoxicity study.

The phrase 'human studies, case reports', is to be changed to 'human epidemiological studies or case reports', since case report is one of the categories of human studies.

33. Paragraph 47 after Test report
47 should be 51.

Insert water after diet in the 4th item of Test animals.

The phrase 'reflex ontogeny' in the 9th item of Results must be 'behavioral ontogeny'.

34. Literature
Try to unify the style of the reference presentation. In particular, the writing of journal titles should be uniform (e.g. compare 5 and 7 for Environ Health Perspect and italic presentations such as 28 and 32). It is recommended that the abbreviation of journal titles follows the PubMed, NLM style.

The presentation of the authors' names is also confusing (e.g. 5 vs. 9).

The placement of the published year is also variable (e.g. 3, 5 and 12).

Put a space between 18 and 19. Delete one space after 67.

Some good references as background information can be found in Massaro EJ. (2002) Handbook of neurotoxicology. Vols I and II. Humana Press, Totowa. The four papers in vol II (Henck JW, Rice SA, Cappon GD and Stump DG, and Tilson HA) are very valuable.

35. Appendix A
Totally redraw Fig. 1 according to the description in Tables 2 and 3, and also clarify in the figure legend that this scheme is based on Tables 2 and 3. A suggestion is attached.

REFERENCES

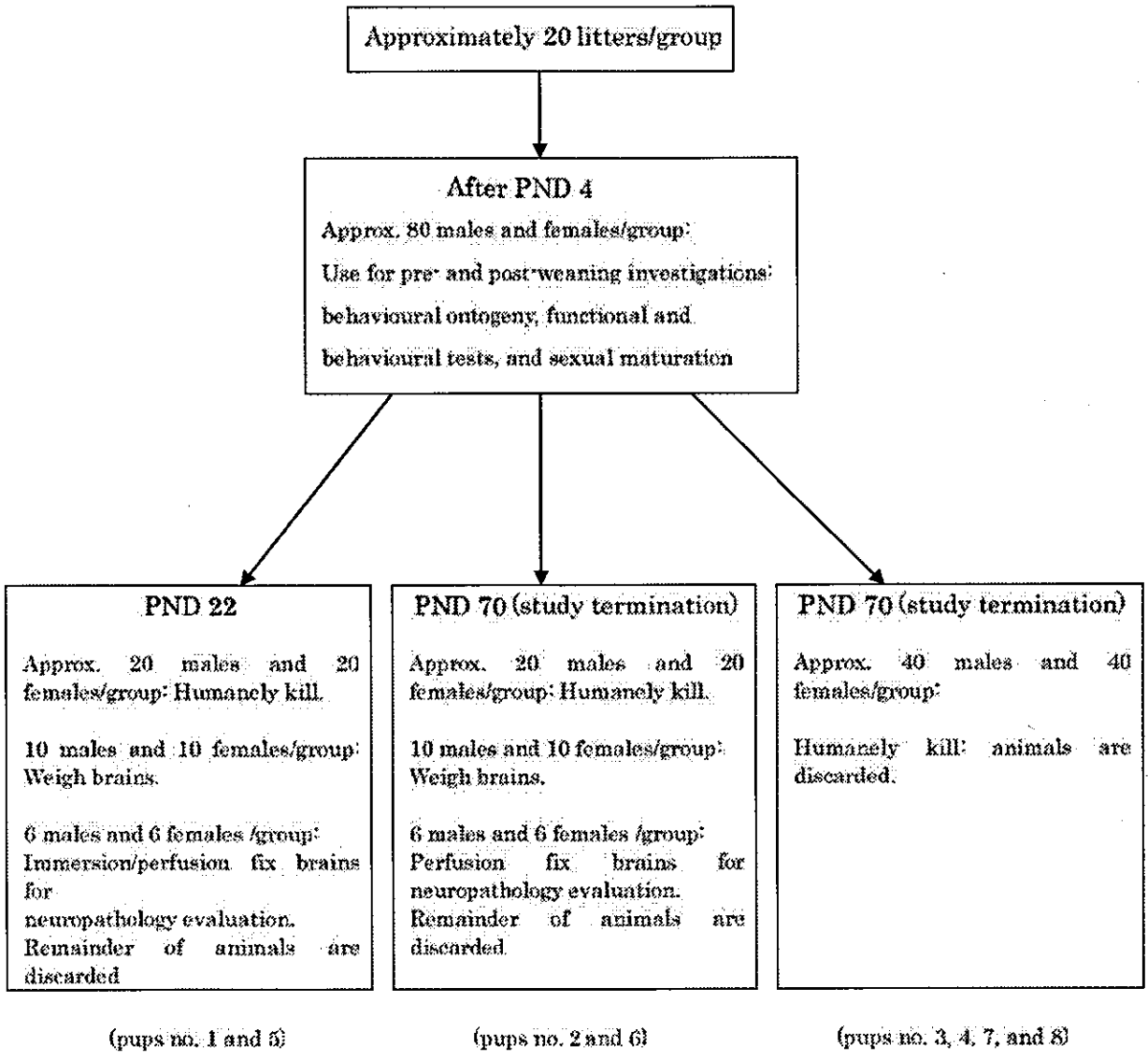
Organisation for Economic Co-operation and Development (OECD) (1995) *Draft Report of the OECD Ad Hoc Working Group on Reproduction and Developmental Toxicity.* Copenhagen, Denmark, 13–14 June 1995.

Organisation for Economic Co-operation and Development (OECD) (1996) *Final Report of the Consultation Meeting on Developmental Neurotoxicity.* Copenhagen, Denmark, 17–18 June 1996.

Organisation for Economic Co-operation and Development (OECD) (2003) *Report of the Expert Consultation Meeting in Developmental Neurotoxicity Testing.* Washington, US, 23–25 October 2000.

APPENDIX A

Fig. 1 Example of the testing scheme for assignment of animals for functional/behavioral tests, neuropathology evaluation, and brain weights, as described in paragraphs 13, 14, and 15. This diagram is based on the description in Tables 2 and 3. (PND = postnatal day).



分子呼吸器病

別　刷

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特集 COPD-病態の多様性-

気道上皮インテグリンのノックアウトマウスは肺気腫に至る

横崎恭之*

Lecture Key Notes

- ノックアウトマウスがヒトの疾患と同じ表現型を示すことがあるが，しばしばその病態解析は困難を極める．
- 今回インテグリンノックアウトマウスが肺気腫を生じることが確認，その機序が証明され，Nature 誌に掲載された．
- 気道上皮のインテグリンが肺胞マクロファージの機能を制御していること，その作用で気腫化が生じることが念入りに，手順よく 7 種類の遺伝子改変マウスを用いて示されている．
- この論文についてそのマウスが誕生した Lung Biology Center での Director Dr. Dean Sheppard を筆頭とした各人の努力の歴史を添えて紹介する．

はじめに

疾患で一体何が起っているかについて，かなり具体的に理解できるようになってきた．分子レベルでの分析の手法が格段に進歩し，いくつかの疾患で病態病理が分子機能の観点から説得力をもって説明されてきている．この推進力として，第一には *in vivo*（マウス）への遺伝子の出し入れが手技的に安定してきたこと，さらに大量の遺伝子，蛋白の網羅的な発現解析が可能になったことがあげられるであろう．2003 年，この二つのテクノロジーを組み合わせ，UCSF Lung Biology Center で肺気腫の病理が明快に説明された[1]．Lab の存続も必ずしも保証されていなかった初期に，そこに押し掛けて働いていた筆者にはとくに感慨深い論文である．このインテグリン $\beta 6$ サブユニット遺伝子のターゲティングは，致死的ではなく，1994 年マウスは文字どおり誕生した．一見表現型には異常なくインテグリン $\alpha v\beta 6$ の生命現象における役割は些細なものであるかに思われた．しかし，その後の注意深い観察によって，lab カンファレンスで体毛の脱落，気管支肺胞洗浄によ

key words
インテグリン
肺気腫
TGFβ
MMP 12
肺線維症

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