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′-tcttttcctccgtggctatc and reverse 5′-ctgtctcttccсttcactct, 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

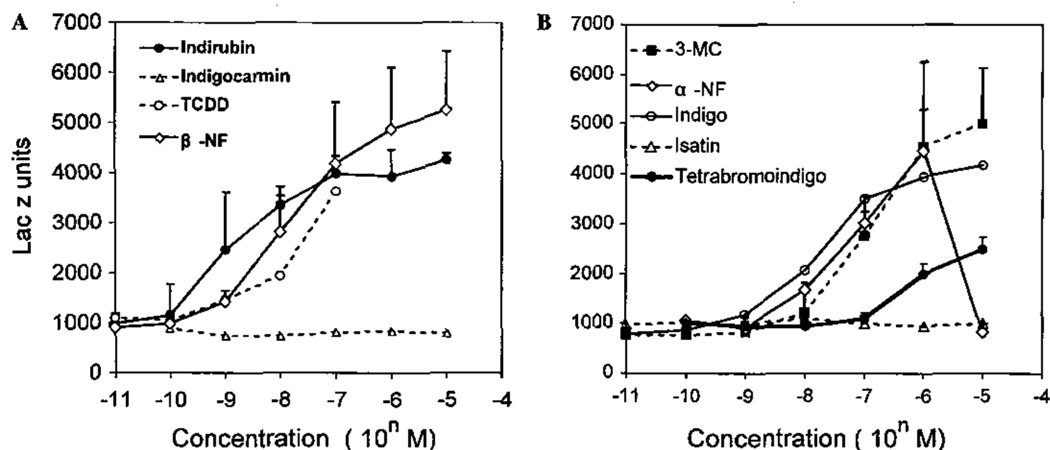

Fig. 2. AhR-binding affinity of indirubin and indigo in yeast AhR reporter assay. (A) Activities of indirubin and other ligands. (B) Activities of indigo and other ligands. Each value represents the mean ± SD of four individual experiments.

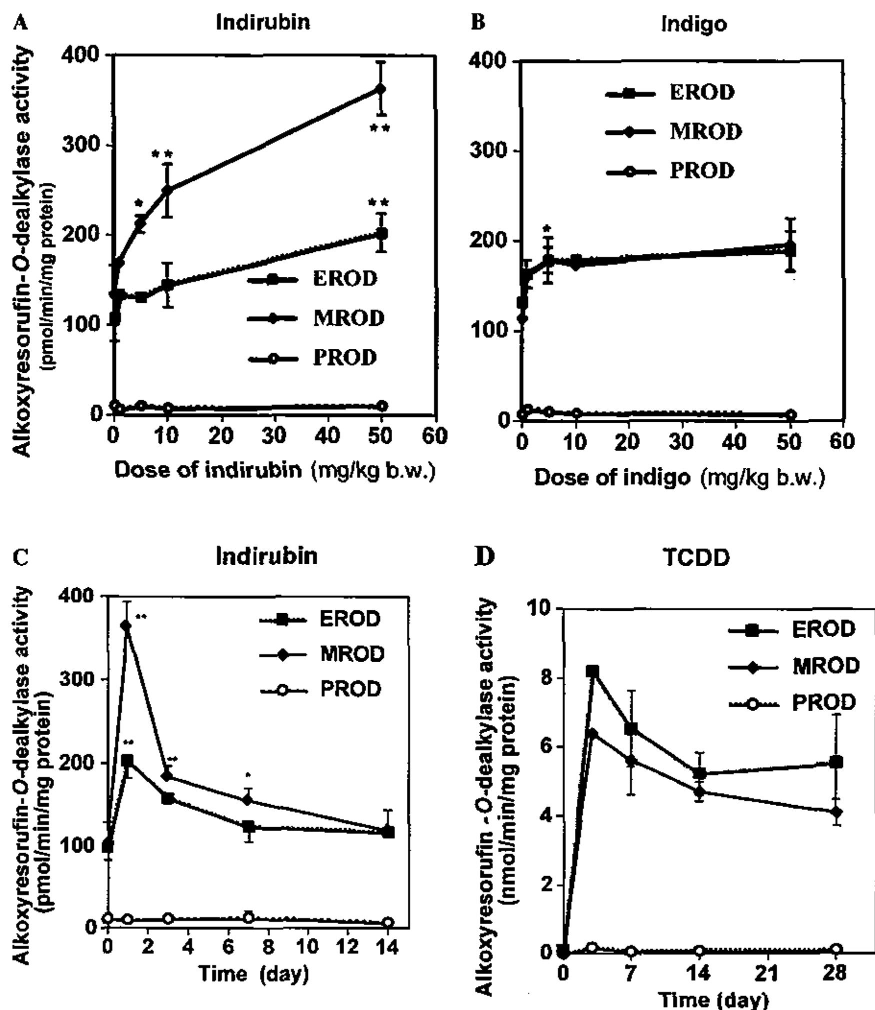

Fig. 3. Effects of indirubin and indigo on MROD, EROD, and PROD activities in mice. (A) Dose-dependent induction of EROD, MROD, and PROD activities in mice by indirubin. (B) Dose-dependent induction of EROD, MROD, and PROD activities in mice by indigo. (C) Time courses of induction of EROD, MROD, and 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.

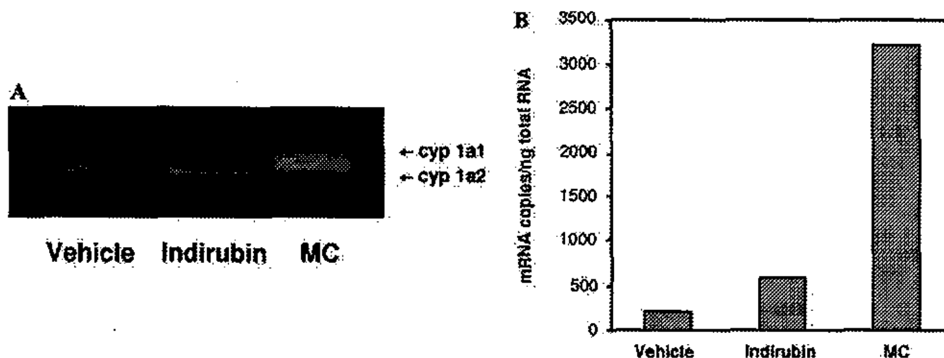

Fig. 4. Effects of indirubin on the levels of cyp 1a1/2 proteins and mRNAs in mouse liver. (A) Levels of cyp 1a1/2 proteins at 1 day after treatment with indirubin (50 mg/kg), 3-MC (25 mg/kg) or vehicle only, determined by immunoblot analysis. (B) Levels of cyp 1a2 mRNA at 1 day after treatment with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

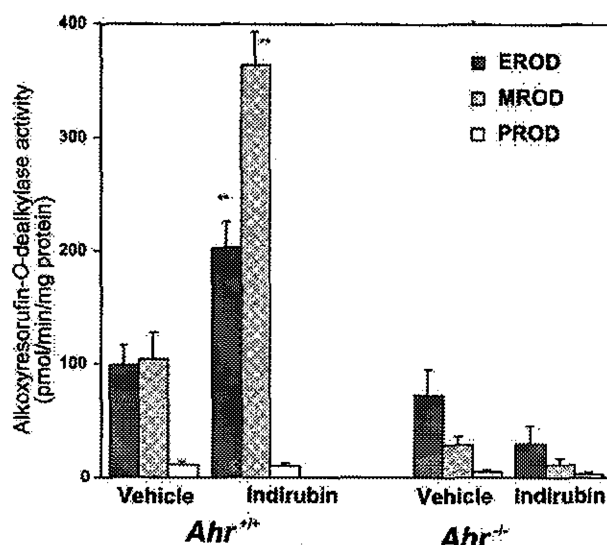

Fig. 5. Effects of indirubin and indigo on EROD, MROD, and PROD activities in liver preparations of $Ahr^{+/+}$ and $Ahr^{-/-}$ mice. Male $Ahr^{+/+}$ (wild: C57BL/6JJcl) and $Ahr^{-/-}$ mice were treated with indirubin at 50 mg/kg body weight. Each bar represents the mean ± SD of four individual mice.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

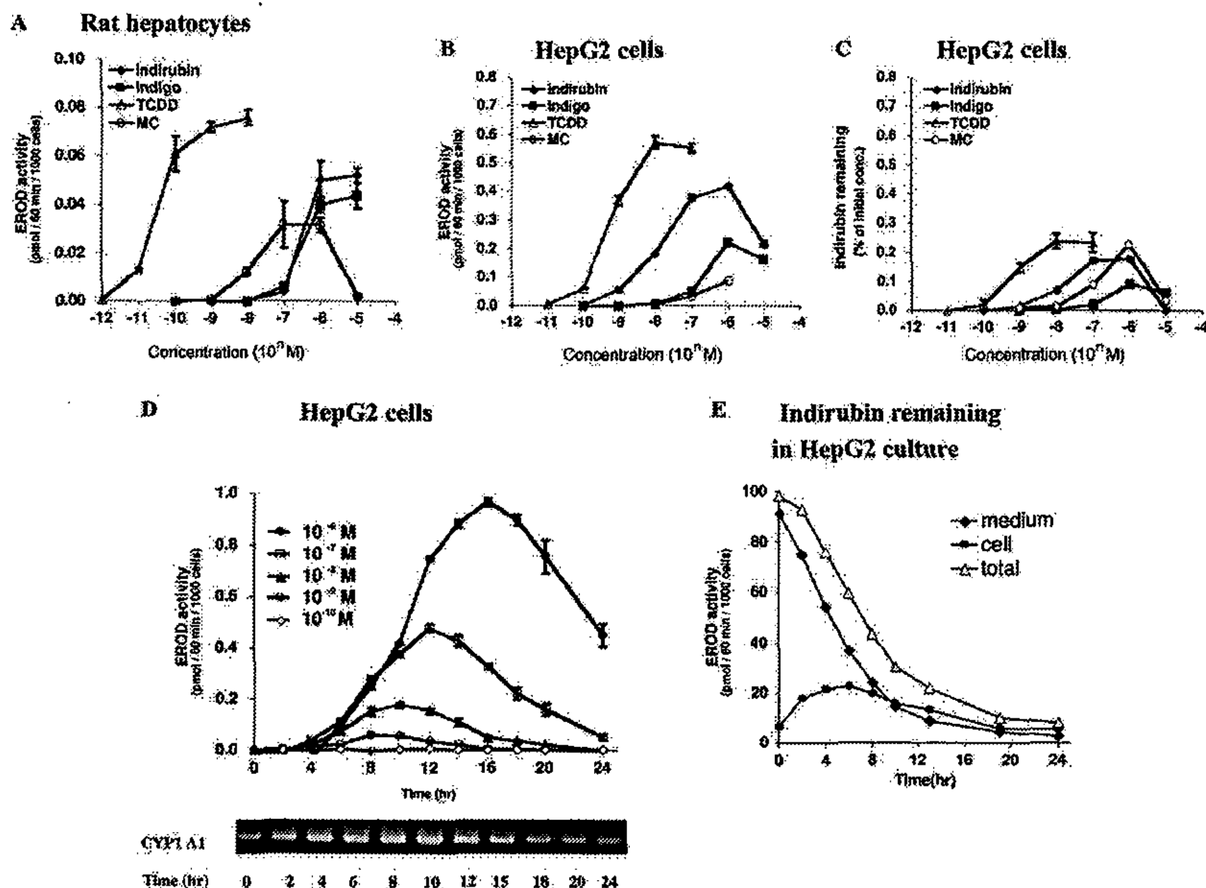

Fig. 6. Effects of indirubin and indigo on EROD and MROD activities in rat hepatocytes and HepG2 cells. (A) Effects of indirubin and indigo on EROD activity in hepatocytes after 24 h. (B) Effects of indirubin and indigo on EROD activity in HepG2 cells after 10 h. (C) Effects of indirubin and indigo on 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.**

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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

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

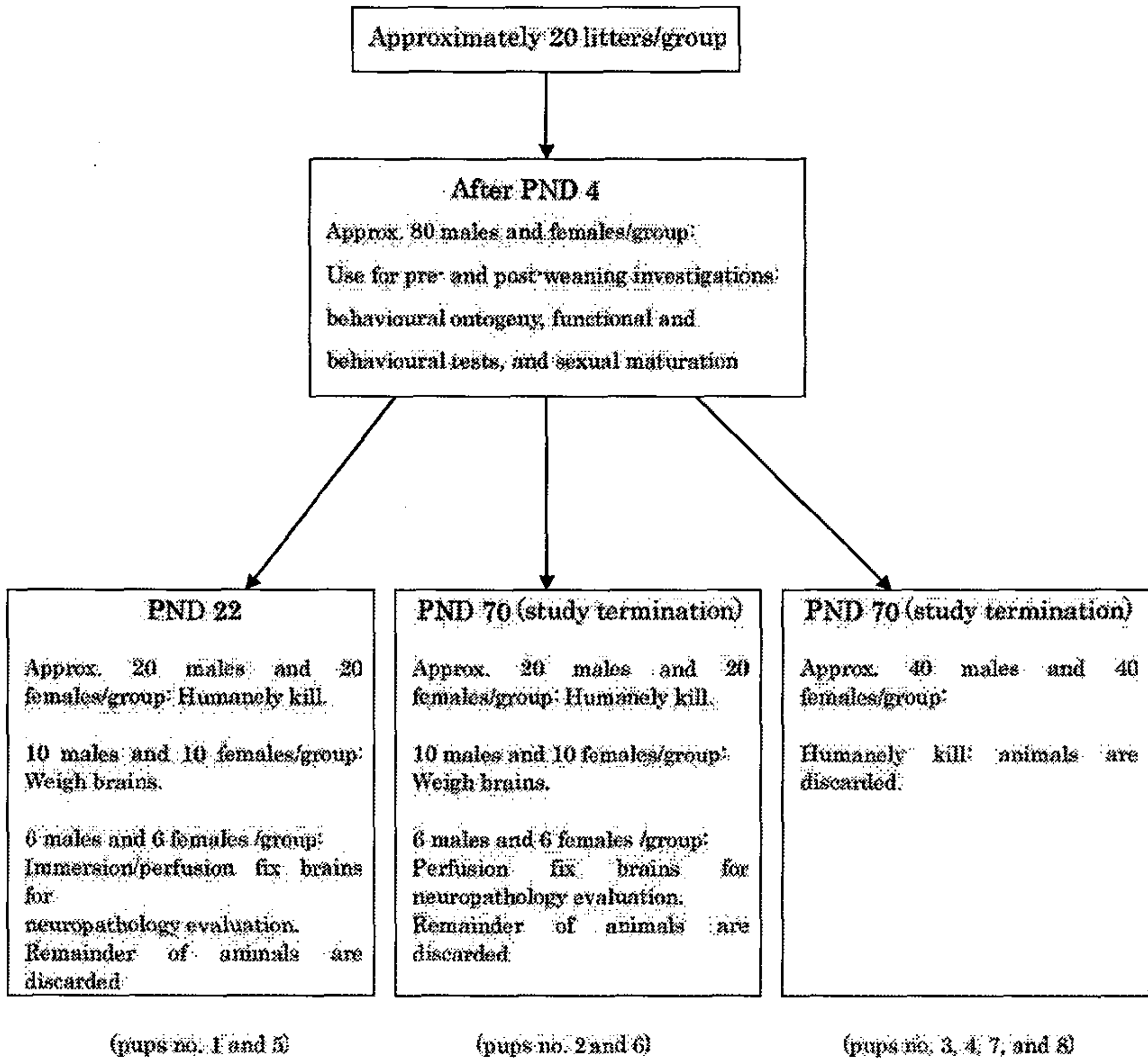
Organisation for Economic Co-operation and Development (OECD) (1995) *Draft Report of the OECD Ad Hoc Working Group on Reproduction and Developmental Toxicity.* Copenhagen, Denmark, 13–14 June 1995.

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).



Distinct structural requirements for binding of the integrins αvβ6, αvβ3, αvβ5, α5β1 and α9β1 to osteopontin

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Running title: Structural requirements of osteopontin receptors

Key words: integrin, osteopontin, thrombin, matrix metalloprotease

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Abstract

The extracellular matrix protein, osteopontin, is a ligand for several members of the integrin family, including α5β1, αvβ3, αvβ5 and α9β1. Osteopontin is a substrate for a number of extracellular proteases, including thrombin and the metalloproteases MMP-3 and MMP-7, which cleave osteopontin at sites close to or within the mapped integrin binding sites. Using affinity chromatography and cell adhesion assays, we now identify the integrin αvβ6 as an additional osteopontin receptor. Utilizing a series of recombinant forms of osteopontin we compared the structural requirements for αvβ6 binding with those for the 4 other osteopontin-binding integrins. Like α5β1, αvβ3 and αvβ5 (but not α9β1), αvβ6 binds to the RGD site in osteopontin, since RGD peptide or mutation of this site to RAA completely inhibits αvβ6-mediated cell adhesion. For both α9β1 and α5β1 the N-terminal fragment generated by thrombin cleavage is a much better ligand than full length osteopontin, whereas thrombin-cleavage does not appear to be required for optimal adhesion to αvβ3, αvβ5 or αvβ6. A recombinant fragment predicted to be generated by MMP cleavage no longer supported α5β1 or α9β1-mediated adhesion, but adhesion mediated by αvβ5 or αvβ6 was unaffected. Finally, adhesion of αvβ5 or αvβ6 was inhibited by mutation of two aspartic acid residues upstream of the RGD site, whereas adhesion mediated by αvβ3, α5β1 or α9β1 was unaffected by these mutations. These results suggest that the hierarchy of integrin interactions with osteopontin can undergo complex regulation at least in part through the action of extracellular proteases.

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INTRODUCTION

Osteopontin is an acidic phosphorylated glycoprotein with versatile functions, including roles in tissue remodeling and regulation of immunity and inflammation (Denhardt and Chambers, 1994; Denhardt et al., 2001; Weber and Cantor, 1996). Principal ways osteopontin can affect cellular behavior is through interactions with integrins or CD44 (Ashkar et al., 2000). Integrins are heterodimeric cell surface glycoproteins that mediates cell response to extracellular matrix proteins (Danen and Sonnenberg, 2003; Hynes, 2002; Sheppard, 2000). Osteopontin contains the canonical integrin recognition sequence, arginine-glycine-aspartic acid (RGD) and the integrins $\alpha v\beta 1$ (Hu et al., 1995; Liaw et al., 1995), $\alpha v\beta 3$ (Miyauchi et al, 1991), $\alpha v\beta 5$ (Hu et al., 1995; Liaw et al., 1995), $\alpha 5\beta 1$ (Barry et al., 2000; Nasu et al., 1995) and $\alpha 8\beta 1$ (Denda et al., 1998) have all been reported to bind to osteopontin through this sequence. Two other integrins, $\alpha 4\beta 1$ and $\alpha 9\beta 1$, bind to non-RGD sites of osteopontin. We have recently mapped the binding site of $\alpha 9\beta 1$ in osteopontin and shown that it recognizes the sequence 162SVVYGLR168 immediately adjacent to the RGD (Yokosaki et al., 1999). According to recent microarray studies, osteopontin is dramatically up-regulated in response to various environmental insults in many tissues (Kang et al., 2003; Boeshore et al., 2004; Ye et al., 2003). Expression of the $\alpha v\beta 6$ integrin is also upregulated in response to environmental insults. Interestingly, osteopontin itself appears to be induced at least in part by an $\alpha v\beta 6$-dependent pathway, since induction of osteopontin in response to lung injury by bleomycin is markedly attenuated in $\beta 6$-subunit knockout mice (Kaminski et al., 2000). Because $\alpha v\beta 6$ binds to RGD sites in each of its known ligands, we sought to determine whether this integrin is also a receptor for osteopontin. In the extracellular space osteopontin has been shown to be a substrate for proteolytic cleavage by thrombin (Senger, 1994) and the matrix metalloproteases MMP-3 and MMP-7 (Agnihotri et al., 2001). Thrombin cleaves osteopontin between 168R and 169S, and this cleavage event is required for $\alpha 9\beta 1$ interaction with the resultant N-terminal fragment (Smith et al., 1996). MMP-3 and MMP-7 have been reported to cleave osteopontin between 166G and 167L, within the $\alpha 9\beta 1$ binding site (Yokosaki et al., 1999) and adjacent to the RGD sequence recognized by most of the other osteopontin-binding integrins. Integrin binding to RGD sites in ligands has been shown to be modulated by the amino acid sequences adjacent to the RGD, it is thus

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conceivable that the specificity of integrin interactions with osteopontin could be regulated, at least in part by proteolytic cleavage of osteopontin. To explore this possibility, we generated recombinant forms of osteopontin to map the binding requirements for several integrins that bind to osteopontin and to determine the likely effects of proteolytic cleavage by MMPs and thrombin on the specificity of integrin binding.

RESULTS

αvβ6 integrin directly binds an N-terminal fragment of osteopontin

To determine whether the αvβ6 integrin is a receptor for osteopontin, we performed affinity chromatography by passing [^{35}S]methionine- and [^{35}S]cysteine-labeled secreted αvβ6 over Sepharose cross-linked to thrombin-cleaved N-terminal osteopontin fragment (nOPN)(or BSA as a control). Bound αvβ6 was eluted by EDTA. There were no bands detected in the eluant from the BSA column, whereas each lane of the eluted fraction from nOPN column showed bands (Fig. 1) corresponding to truncated αv (130 kDa) and β6 (85 kDa). These results indicate integrin αvβ6 binds to nOPN.

Adhesion of SW480 cells to nOPN

We tested four cell lines, mock-, β3-, β6- and α9-transfected SW480 cells for the expression of integrins, αvβ3, αvβ5, αvβ6, α5β1 and α9β1 to use for adhesion assays to various recombinant osteopontin fragments. Although levels of expression were not exactly the same among the four cell lines, all cell lines including mock-transfected cells expressed integrins αvβ5 and α5β1. β3-, β6- and α9-transfectants also expressed αvβ3, αvβ6 and α9β1 respectively (Fig. 2). These 4 cell lines were analyzed in adhesion assays to nOPN first. In mock-transfected cells, anti-α5β1 monoclonal antibody, P3D10, and anti-αvβ5 antibody, P1F6 each partially inhibited adhesion to nOPN when used separately and completely inhibited adhesion used in combination (Fig. 3A). These data confirm that α5β1 and αvβ5 are the principal osteopontin receptors on mock-transfected SW480 cells. However, these two antibodies were not sufficient to abolish adhesion of any of the other 3 transfectants. For β3-, β6-

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and α9-transfectants the further addition of anti-αvβ3, anti-αvβ6, or anti-α9β1 blocking antibodies, respectively, did result in complete inhibition of adhesion to nOPN. But each of these antibodies, when used alone, could not inhibit the adhesion completely (Fig. 3B, C, D). Therefore, in the presence of P3D10 and P1F6, these 3 transfectants adhered to nOPN utilizing a single integrin receptor.

αvβ6 binds to the RGD site in osteopontin

Since αvβ6 has not been reported as an osteopontin receptor, we further tested this interaction of β6-transfected SW480 cells with osteopontin. In the presence of blocking antibodies against α5β1 and αvβ5, β6-transfectants adhered to nOPN in a concentration-dependent fashion at concentrations within a range of 20 to 600 nM, which was completely abolished by the αvβ6-blocking antibody 10D5. Under these conditions, mock transfectants did not adhere at any concentration of nOPN (Fig. 4A). To determine if this αvβ6-mediated adhesion was RGD-dependent, β6-transfected cells were incubated with GRGDSP peptide before plating, which completely abolished adhesion to nOPN. β6-transfected SW480 cells were also plated on mutant nOPN in which the RGD sequence was mutated to RAA, and no adhesion was detected. These results demonstrate that αvβ6 binds to the RGD site in osteopontin (Fig. 4B). To confirm this interaction, we next tested adhesion of UCLA P3 lung cancer cell line that naturally express αvβ6 to nOPN. Unlike SW480 cells UCLA P3 cells do not express α5β1, but express αvβ5 (Fig. 4C). UCLA P3 cells adhered to nOPN well, which was only partially blocked by anti-αvβ5, P1F6. The residual adhesion was prominently blocked by an addition of anti-αvβ6, 10D5, although adhesion of UCLA P3 to nOPN was only partially blocked by10D5 alone (Fig. 4D). The blocking effect of 10D5 was obvious in the presence of P1F6, indicating that UCLA P3 adhered to osteopontin mediated at least in part by integrin αvβ6.

Structural requirements for binding of integrins αvβ6, αvβ3, αvβ5, α5β1 and α9β1 to N-terminal fragment of osteopontin

Several integrins overlap their binding to the region of osteopontin containing the RGD site. Sequences around the RGD site can provide specificity for binding of integrins (Ruoslahti, 1996). We therefore examined whether αvβ6 has distinct structural requirements from other osteopontin receptors that recognize RGD,

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$\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$, in comparison with $\alpha 9\beta 1$ that recognizes a non-RGD site (Yokosaki et al., 1999). The structure of the RGD-containing region in osteopontin was modified by substitution of two upstream asparatic acid residues by alanine (D154A and D157A) and by substitution of a downstream tyrosine residue that is critical for binding of $\alpha 9\beta 1$ (Y165A) (Fig. 5) (Yokosaki et al., 1999) . To examine the role of individual integrins in binding to each mutant, we performed adhesion assays in the presence of blocking antibodies to each of the other integrins present in our various cell lines. Thus, binding of integrin $\alpha v\beta 3$ was observed as adhesion of $\beta 3$-transfected SW480 cells in the presence of antibodies against $\alpha 5\beta 1$ and $\alpha v\beta 5$, $\alpha v\beta 5$ as adhesion of mock-transfectant in the presence of antibodies against $\alpha 5\beta 1$, $\alpha v\beta 6$ as adhesion of $\beta 6$-transfectant in the presence of antibodies against $\alpha 5\beta 1$ and $\alpha v\beta 5$, $\alpha 5\beta 1$ as adhesion of mock transfectant in the presence of antibodies against $\alpha v\beta 5$, and $\alpha 9\beta 1$ as $\alpha 9$-transfectants in the presence of antibodies against $\alpha 5\beta 1$ and $\alpha v\beta 5$. Adhesion of $\alpha v\beta 3$ was the same for wild type nOPN and all mutants examined. In contrast, $\alpha v\beta 6$ adhered poorly to the D154A mutant and did not bind at all to the D154,157A double mutant. The $\alpha v\beta 5$ adhesion pattern was similar to $\alpha v\beta 6$, but $\alpha v\beta 5$-mediated adhesion was less sensitive to these mutations. $\alpha 5\beta 1$-mediated adhesion was only minimally affected by mutation of D154 and D157 and $\alpha 9\beta 1$ mediated adhesion was not affected at all by these mutations. However the, Y165A mutation dramatically inhibited $\alpha 9\beta 1$- and $\alpha 5\beta 1$-mediated adhesion, with no effect on adhesion mediated by $\alpha v\beta 3$, $\alpha v\beta 5$ or $\alpha v\beta 6$ (Fig. 6). These results suggest that these integrins have different structural requirements for interaction with osteopontin. $\alpha v\beta 3$-mediated adhesion appears to depend principally on the RGD sequence itself, $\alpha v\beta 6$- and $\alpha v\beta 5$-mediated adhesion is also sensitive to amino acids upstream of the RGD site, $\alpha 5\beta 1$–mediated adhesion depends on the RGD site and is sensitive to amino acids downstream, and $\alpha 9\beta 1$-mediated adhesion is completely dependent on the SVVYGLR sequence downstream of RGD.

Differential effects of thrombin- or MMP-3, 7-cleavage on cell adhesion mediated by $\alpha v\beta 6$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 5\beta 1$ and $a9\beta 1$

Since integrin mediated adhesion to osteopontin appeared to be affected by conformational changes close to or within the integrin binding site (Fig. 5), we next compared adhesion mediated by each integrin to full length osteopontin (fOPN) and

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recombinant forms mimicking two naturally occurring cleavage forms that are produced by cleavage by proteases, MMP-3 or MMP-7 (nOPN-dLR), or thrombin (the nOPN form used above) (Fig. 5). The MMP-3, 7-cleaved form (nOPN-dLR) was made by deletion of 2 residues, LR, of nOPN. fOPN was generated from full length cDNA. Integrins $\alpha v\beta 6$, $\alpha v\beta 3$ or $\alpha v\beta 5$ each bound equally well to nOPN, nOPN-dLR and fOPN, indicating that these 2 cleavages do not influence their binding to osteopontin. In contrast, $\alpha 5\beta 1$-mediated adhesion was dramatically affected by these cleavages. Although $\alpha 5\beta 1$ mediated robust adhesion to nOPN, $\alpha 5\beta 1$–mediated adhesion to fOPN or nOPN-dLR was substantially reduced (Fig. 7A), indicating that adhesion of $\alpha 5\beta 1$ to fOPN was enhanced by thrombin cleavage, but that osteopontin cleavage by MMPs would be inhibitory. To confirm these results, we enzymatically cleaved fOPN by thrombin or MMP-3 (Fig. 7C) and used these cleaved fragments for adhesion assays. As expected, thrombin cleaved osteopontin in two overlapping fragments of essentially the same molecular mass, which were also the same mass as nOPN. This band was separated in 15% polyacrylamide gel (data not shown). In addition to the 166G-167L cleavage site, MMP-3 cleaves two other sites in the C-terminal fragment of osteopontin (Agnihotri et al., 2001). MMP-3 cleavage was incomplete and generated several fragments, one of which was the same molecular mass as nOPN-dLR. Wells of cell adhesion plate were coated with these protease-treated fragments. Assays with these cleaved-fragments demonstrated the same findings as we observed for recombinant fragments (Fig. 7A, B).

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DISCUSSION

In the present study, we have identified a new osteopontin receptor, the integrin $\alpha v\beta 6$, that, like the integrins $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$ recognizes the RGD site in osteopontin. At least one other integrin, $\alpha 9\beta 1$ also binds to osteopontin, but recognizes a distinct sequence adjacent to the RGD site. By utilizing a variety of recombinant fragments of osteopontin, we were able to identify specific sequence requirements for each of these 5 integrin osteopontin receptors. $\alpha v\beta 3$ recognized all of the mutant fragments we generated, as long as the RGD sequence remained intact. $\alpha v\beta 5$ and $\alpha v\beta 6$ were both more sensitive to mutations in the sequence adjacent to the RGD site, but all

three of these αv-integrins could bind equally well to intact osteopontin or to the N-terminal fragments generated by thrombin or MMP-mediated cleavage. Interestingly, proteolytic cleavage had important effects on osteopontin binding of both α5β1, which recognizes the same RGD site as the αv-integrins and α9β1, which does not bind to this site. In both cases, adhesion was minimal to intact full-length osteopontin, was greatly enhanced by thrombin-mediated cleavage and was inhibited by MMP-mediated cleavage. These results suggest that proteolytic cleavage of osteopontin can substantially impact the specific integrin receptors that cells use to detect and respond to osteopontin.

The thrombin cleavage site is downstream from the RGD sequence by 7 residues, SVVYGLR (Fig. 4) (Senger, 1994). We have previously reported that either deletion of 2 residues, LR (nOPN-dLR), or alanine-replacement of the tyrosine in the SVVYGLR sequence (nOPN-Y165A) of nOPN abolishes integrin α9β1 mediated adhesion (Yokosaki et al., 1999). After we reported the SVVYGLR sequence, osteopontin was found to be a substrate for MMP-3 and MMP–7 (Agnihotri et al., 2001). A cleaved fragment of either MMP-3 or MMP-7 was identical to nOPN-dLR. Although this MMP-cleavage did not affect binding of αv-integrins, α5β1-mediated adhesion was inhibited either by the nOPN-dLR deletion or nOPN-Y165A, like α9β1. Thus the osteopontin SVVYGLR sequence appears to be required for optimal α5β1 binding. α5β1 has been most extensively characterized as a receptor for fibronectin (Mould et al., 2000; Obara et al., 1988; Pierschbacher and Ruoslahti, 1984). In that case, interaction with an RGD site is also necessary, but not sufficient for optimal α5β1-mediated adhesion. In addition to the classical synergy site, PHRSN (Aota et al., 1994), other sites that enhance α5β1-mediated binding, including TVRYYR (SVRYYR in mouse) (Redick et al., 2000) have been described. Interestingly, α5β1 mediated adhesion was reduced when fOPN was used as a substrate. This is consistent with a previous report that α5β1 on K562 cells binds to thrombin-cleaved but not to full length osteopontin (Barry et al., 2000). The integrin α9β1 recognizes the SVVYGLR sequence in osteopontin and we have previously reported that it does not bind to full length osteopontin, suggesting that the SVVYGLR site is cryptic in full length osteopontin and exposed upon thrombin cleavage (Yokasaki and Sheppard, 2000). The enhancement of α5β1 binding to osteopontin by thrombin-cleavage and reduction by MMP-cleavage within the SVVYGLR support this idea that the SVVYGLR sequence serves as a

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