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Retionic Acid and Its Receptors Are Required for Expression of Aryl Hydrocarbon Receptor mRNA and Embryonic Development of Blood Vessel and Bone in the Medaka Fish, *Oryzias latipes*

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ABSTRACT—Retinoic acid (RA), the active derivative of vitamin A, is essential for normal embryonic development of vertebrates because both the lack and excess of RA result in developmental malformations. We previously reported that aryl hydrocarbon receptor (AHR) is also required for vascular and bone formation by regulating cytochrome P450 expression. However, little is known about the roles of retinoic acid receptors (RAR) and retinoid X receptors (RXR) in the embryonic development of blood vessels and molecular cross-talk between RAR/RXR and AHR. We report for the first time that RA and RAR/RXR are required for expression of AHR mRNA and the embryonic development of blood vessel and bone. The embryonic organogenesis of medaka fish was specifically inhibited by an inhibitor of RA synthesis (diethylaminobenzaldehyde), antagonists of RAR (Ro41-5253) and RXR (Ro71-4595), agonist (β-naphthoflavone) and antagonist (α-naphthoflavone) of AHR, and excess RA. These reagents are useful for future studies to elucidate molecular mechanisms for vascular and bone formation in the medaka embryogenesis. Our results also show that medaka embryos may be useful for screening inhibitors of vascular formation for anti-cancer drugs.

Key words: retinoic acid, retinoic acid receptor, aryl hydrocarbon receptor, blood vessel, bone

INTRODUCTION

Vitamin A (retinol) and its derivatives (retinoids) are essential for both normal embryonic development and maintenance of differentiation in the adult organisms (Ross *et al*., 2000). Maternal insufficiency of vitamin A results in death of the fetus and congenital malformations. Excess dietary vitamin A and exogenous excess retinoic acid (RA) also cause teratogenesis. Vitamin A is metabolized to an active form, RA, through two sequential oxidative reactions mediated by retinol dehydrogenase and retinal dehydrogenase. RA exerts its biological effects by binding retinoic acid receptors (RAR) and retinoid X receptors (RXR), members of ligand-activated nuclear receptor superfamily for transcription. The RAR-RXR heterodimers are the functional units in transducing the retinoid signal *in vivo*.

An approach to the examination of molecular mechanisms of retinoid action in developmental regulation is the use of vitamin A-deficient embryos from mothers fed by vitamin A-free diets (Zile, 2001). Another *in vivo* approach to eliminate RA is to inhibit RA synthesis with inhibitors of aldehyde dehydrogenases, such as diethylaminobenzaldehyde (DEAB) (Perz-Edwards *et al*., 2001) and disulphiram (Stratford *et al*., 1996). These RA-deficient embryos from rats, chicken, quails, and fish develop gross abnormalities in the cardiovascular, ocular, and central nervous systems, limb, and trunk. A recent approach to elucidate developmental roles of RA is the use of knock-out (or knock-down with antisense oligos) mice with changes in retinol binding protein (Bavik *et al*., 1996) and enzymes involved in RA metabolism such as retinaldehyde dehydrogenase-2 (Niederreither *et al*., 1999) and a cytochrome P450-linked RA oxidase (Abu-Abed *et al*., 2001; Sakai *et al*., 2001). These mutant embryos also have many abnormalities similar to those of the RA-deficient and -excess embryos. The function of RA signaling in development has been addressed recently by the use of knock-out mice with changes in RAR/RXR gene structure (Kastner *et al*., 1995). Many of the abnormalities in these mutant mice resemble those observed in fetuses from the RA-deficient animals. These studies have demonstrated the critical roles of retinoid receptors in vertebrate ontogen-

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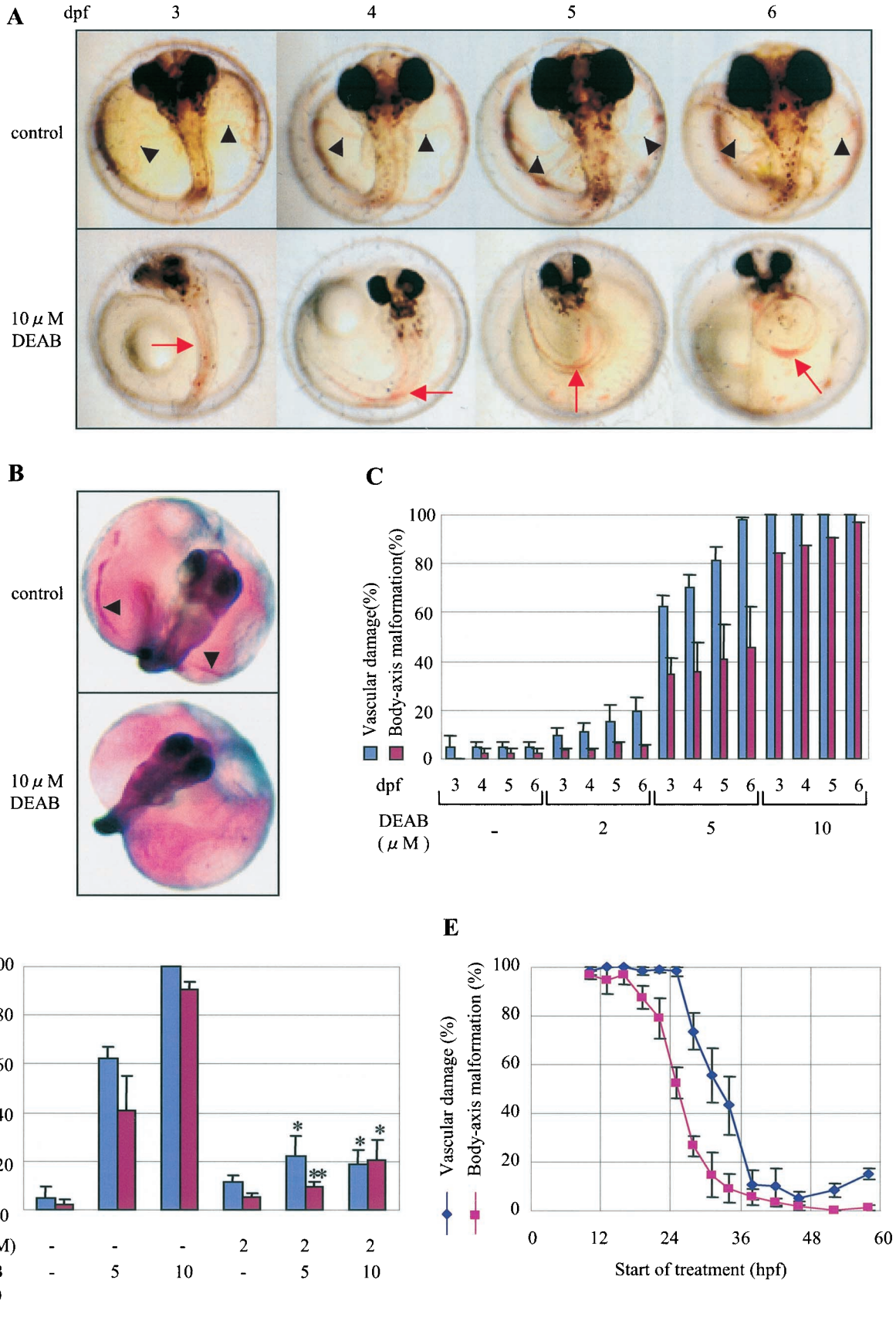


esis. However, defects in vasculogenesis have not been reported in these receptor knock-out mice.

Aryl hydrocarbon receptor (AHR), a ligand-dependent transcriptional factor, is also conserved among vertebrates and expressed in a variety of tissues and developmental time points (Gonzalez and F.-Salguero 1998). Although the role of AHR in detoxification of environmental aryl hydrocarbons through transcriptional activation of a battery of genes encoding various cytochromes P450 has been extensively studied *in vitro* (Hankinson, 1995) and *in vivo* (F.-Salguero *et al*., 1996; Mimura *et al*., 1997), there is only a limited knowledge of developmental and physiological functions of AHR in the mouse and fish. AHR-null mice have a number of abnormal phenotypes such as decreased accumulation of lymphocytes in the spleen and lymph nodes and reduction in liver size that are associated with accelerated rates of apoptosis (F.-Salguero *et al*., 1995; Gonzalez and F.-Salguero, 1998), and difficulties in reproduction (Abbott *et al*., 1999; Robles *et al*., 2000) and liver vascular remodeling (Lahvis *et al*., 2000). We previously reported that the treatment of medaka fish embryos with AHR antagonist and cytochrome P450 inhibitor inhibits the development of blood vessels and bone (Kawamura and Yamashita, 2002). Thus, AHR plays an important role in the development of bone, liver, and immune, reproductive, and vascular systems, although *in vivo* ligands have not been identified.

Although RAR/RXR and AHR may have common roles in the embryonic development, molecular interactions between them have not been reported. In experimental animals exposed to dioxin, a highly toxic environmental pollutant which binds to and activates AHR, decreased hepatic retinoid levels are a well-characterized effect (Nilsson and Hakansson, 2002). Mice lacking a functional AHR have greatly increased liver levels of retinoic acid (Andreola *et al*., 1997). Consistent with this, liver microsomes from the mutant mice have a decreased ability to metabolize retinoic acid (Andreola *et al*., 1997). These results indicate that AHR is involved in retinoid metabolism in animals. However, little is known about the interaction between retinoid signaling and AHR in early embryonic development. Here we examined whether RA and RAR/RXR play key roles in the development of medaka fish, especially in the vascular and bone formation, because there have been no pharmacological studies in fish using retinoid receptor antagonists. We also examined for molecular and physiological interactions between retinoid signaling and AHR. We found that RA and RAR/RXR are required for AHR gene expression and essential for vascular and bone formation.

MATERIALS AND METHODS

Fish and embryo culture

We used the d-rR strain of medaka fish, *O. latipes* (Kawahara and Yamashita, 2000). The fish were maintained at 25–26°C under artificial photo-period of 14L:10D, and fed by powdered Tetramin (Tetra). Eggs were collected within 10-h postfertilization (hpf), rinsed with tap water, and immersed in Yamamoto's salt solution (Yamamoto, 1969). Incubation with reagents started using 10-hpf eggs unless otherwise indicated. At least 30 eggs were used in each experiment. Retinoid receptor antagonists (Ro41-5253 and Ro71-4595) were provided by E.-M. Gutknecht (F. Hoffmann-La Roche Ltd, Basel). Other reagents were purchased from Sigma. These reagents were dissolved in dimethyl sulfoxide. The stock solutions were diluted over 1,000-fold with Yamamoto's solution. The solvent was added to the mock-treated eggs as a control. Eggs were incubated under the same condition as above except for the treatment with *all-trans* RA under shading, and inspected for yolk vein and body axis under a dissecting microscope. We also observed the development of yolk veins after staining with hematoxylin as described (Kawamura *et al*., 2002). Eggs with vascular damages (agenesis and degeneration of yolk vein, and blood clotting) (Kawamura *et al*., 2002) and body-axis malformation were counted. Data are presented as an average of two experiments or mean±SEM.

Isolation of complementary DNAs

Complementary DNAs for vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), RARα, and RARγ were synthesized from RNA in 3-day postfertilization (dpf) medaka embryos by RT-PCR and successive 5'- and 3'-RACE, essentially as described (Kawamura and Yamashita, 2002). Nucleotide sequences were deposited in DDBJ (accession no. AB121074, AB121073, AB154174, and AB154175, respectively).

Expression of VEGFR and PDGFR mRNAs

In order to observe the expression of blood vessel-associated marker genes, *in situ* hybridization was done essentially as described (Kawahara *et al*., 2000) using as probes the medaka cDNA clones described above encoding VEGFR and PDGFR for endothelial (Conway *et al*., 2001; Shalaby *et al*., 1995) and smooth muscle (Betsholtz *et al*., 2001; Hellstrom *et al*, 1999) cell-specific markers, respectively.

Bone staining

Eggs (10 hpf) were treated with reagents as described, and the incubation continued after hatching without feed. In order to

Fig. 1. Vascular damages and body-axis malformations caused by the treatment with RA synthesis inhibitor (DEAB). (**A**) Fertilized eggs (10 hpf) were incubated in medaka saline (control) and the same containing 2, 5, or 10 μM DEAB for 6 days. Photographs of control and 10 μM DEAB-treated embryos were taken at the indicated times. Yolk veins (arrowhead) form at 3 dpf in the control embryos. Note the following defects in the DEAB-treated embryos: agenesis of yolk veins, blood clotting (red arrow), bending (4 and 5 dpf) and coiling (6 dpf) tails, and small head. (**B**) The control and DEAB-treated embryos of 4 dpf stained with hematoxylin. Yolk veins are marked by arrowhead in the control embryo. Note the absence of yolk veins in the DEAB-treated embryo. (**C**) Rates of embryos with the vascular damage or the body-axis malformation. (**D**) Fertilized eggs were incubated in the saline containing both DEAB and RA at the indicated concentrations until 3 dpf (for vascular damages) and 5 dpf (for body-axis malformations), and counted for the defects. $*P<0.001$, $**P<0.01$. (**E**) Staged embryos (from 10 to 58 hpf) were treated with 10 μM DEAB before counting for the vascular damages at 3 dpf and body-axis malformation at 6 dpf. Rates of embryos with each damage are plotted at the starting times (hpf) of the treatment.

observe the bone development in the fry, calcified bone was stained with alizarin S as described (Kawamura and Yamashita, 2002).

RT-PCR

Total RNA was extracted from embryos and analyzed by RT-PCR as described (Kawahara *et al.*, 2000). Gene-specific primers (forward, F; and reverse, R) and PCR conditions were as follows: AHR, F 5'-CCAGCAGGAGTTCAGGAGGA-3' and R 5'-ATTTTAC-CCTTTGCGTCACA-3' at an annealing temperature of 60°C for 35 to 40 cycles; RARα, F 5'-AAGCAGGAGTGCACG-3' and R 5'-GGT-CAATGTCCAAGGAA-3' at an annealing temperature of 50°C for 30 cycles; and RARγ, F 5'-GAGATGGTGCTTCCC-3' and R 5'-CAGCTGAACACGGTGG-3' at an annealing temperature of 50°C for 30 cycles.

RESULTS

RA and retinoid receptors are required for vascular, body-axis, and bone formation

We examined the effects of an inhibitor of RA synthesis, diethylaminobenzaldehyde (DEAB), on vascular and body-axis formation. Embryos (10 hpf) were incubated in medaka saline containing 2, 5, or 10 μM DEAB for a week, and inspected for vascular and body-axis formation under a dissecting microscope (Fig. 1A). The embryos were also stained with hematoxylin (Fig. 1B). Yolk veins (arrowhead) form at 3 dpf in control embryos but did not in the embryos treated with 5 and 10 μM DEAB. In the treated embryos, blood clotting occurred at 3 dpf and continued during the treatment (shown by red arrow), body axis became to bend at 4 dpf and coiled by 6 dpf, and heads were smaller in size. Embryos with the vascular damage or the body-axis malformation were counted and the rates are shown in Fig. 1C. These results suggest that RA is required for vascular and body-axis formation. To test the specific activity of DEAB to inhibit RA synthesis, we examined whether a subteratogenic concentration of RA would rescue the defects by DEAB. The addition of RA restored normal vascular and body-axis formation (Fig. 1D), demonstrating that the DEAB-induced defects resulted from RA-deficiency.

We examined for the developmental period during which RA is required for vascular and body-axis formation. If RA were required at specific developmental times, embryos of later stages would no longer respond to DEAB while those of earlier stages would be sensitive to the reagent. Staged embryos were treated with 10 μM DEAB and examined for the vascular damages and body-axis malformation at 3 and 6 dpf, respectively (Fig. 1E). Half of the embryos did not undergo body-axis malformation or vascular damages at 25 or 31 hpf, respectively. These results indicate that RA is required for body-axis and vascular formation around 25 (early neurula stage) and 31 (early somite stage) hpf, respectively.

We next examined the effects of antagonists for RAR and RXR, Ro41-5253 and Ro71-4595, respectively. The treated embryos had abnormalities similar to those of the RA-deficient embryos: agenesis of yolk vein, blood clotting, body-axis malformation including a coiled body, and small head (Fig. 2A). If the antagonists competed with retinoids synthesized in the embryo for binding to receptors, reducing *in vivo* retinoid levels by the treatment with DEAB would act synergistically with the antagonist to worsen developmental defects. We examined the synergy between low concentrations of DEAB (2 μM) and the RAR antagonist (1 μM) that alone did not show considerable effects. Combination of these reagents clearly increased the rate of developmental defects (Fig. 2B). We therefore conclude that RA and RAR/RXR are required for proper development of vasculature and body axis.

In vivo RA levels are required to be strictly controlled and excess RA is also known to cause developmental malformations. We thus examined the effects of exogenously added RA on the development of medaka embryos. The

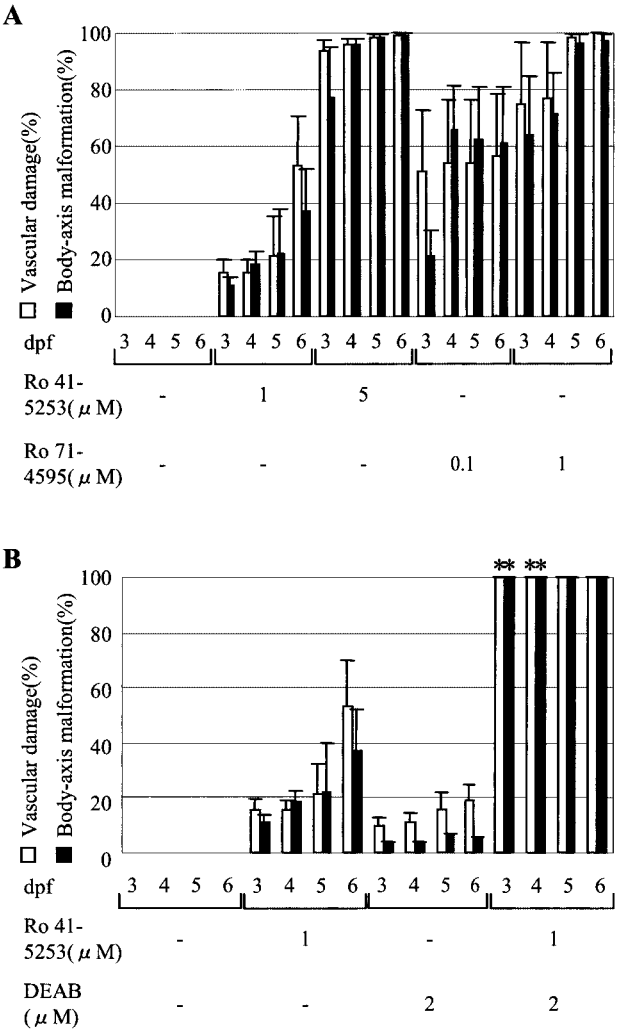


Fig. 2. Effects of retinoid receptor antagonists on vascular and body-axis formation, and synergistic inhibitory effects of the antagonist and RA synthesis inhibitor (DEAB). (**A**) Eggs were treated with RAR antagonist (Ro41-5253) and RXR antagonist (Ro71-4595) at the indicated concentrations, and counted for the vascular damages and the body-axis malformation. (**B**) Eggs were co-treated with the RAR antagonist and DEAB, and counted as described. *P<0.001.

embryos treated with more than 5 nM RA showed abnormalities similar to those of the embryos treated with DEAB and retinoid receptor antagonists such as agenesis of yolk veins, blood clotting (open arrowhead), a curved structure of tail (arrow), and small head (Fig. 3A). Embryos with these malformations were counted and the rates are shown in Fig. 3B. We further examined for a sensitive period in the development against excess RA according to the rationale described in Fig. 1E. Staged embryos were treated with 20 nM RA and examined for the vascular damages at 3 dpf (Fig. 3C). The result indicated a narrow window of sensitivity to the excess RA around 26 hpf (early neurula stage). These results indicate that a proper control of RA action is essential for early development of medaka embryos.

In order to examine whether RA and retinoid receptors are required for the expression of mRNAs encoding VEGFR and PDGFR known to be required for the development of blood vessel-forming cells (endothelial and smooth muscle cells, respectively) in mice, *in situ* hybridization was done using as probes the VEGFR and PDGFR cDNAs cloned in this study after treating medaka embryos with the RA synthesis inhibitor, retinoid receptor antagonists, and a teratogenic concentration of RA. In control embryos, signals for these mRNAs first appeared around 36 hpf lining along the embryonic axis, migrated to reach the tail region by 60 hpf, and were localized at or near the axial vessels (red arrowhead) shown in the cross sections at posterior regions of 4-dpf embryos (Fig. 4A). We noted that yolk veins do not express either VEGFR or PDGFR mRNA except VEGFR mRNA expressed in a limited region of yolk vein connecting to the embryo body (blue arrow). Treatments with the RA synthesis inhibitor (DEAB), retinoid receptor antagonists (Ro41-5253 and Ro71-4595), and excess RA inhibited the expression of VEGFR and PDGFR mRNAs (Fig. 4B). These results indicate that RA and retinoid receptors are required and their actions have to be strictly regulated for normal expression of VEGFR and PDGFR mRNAs.

We next examined the roles of RA and retinoid receptors in the bone formation. Embryos were treated with the same reagents as above but at lower concentrations allowing the development of embryos until a week after hatching, and stained for bones with alizarin S. Control fry developed calcified bones well at 3 days after hatching (dah) (Fig. 5A). Inhibition of RA synthesis with DEAB resulted in a gross inhibition of bone formation at a vertebral column and a caudal fin (Fig. 5B), while addition of RA rescued the defect (Fig. 5C). Treatments with the retinoid receptor antagonists partially inhibited the bone development at the posterior region of vertebral column and the caudal fin (marked in Figs. 5D and E). Excess RA also inhibited the development of bones at a vertebral column and a caudal fin (Fig. 5F). These results indicate that RA and retinoid receptors are essential for bone formation.

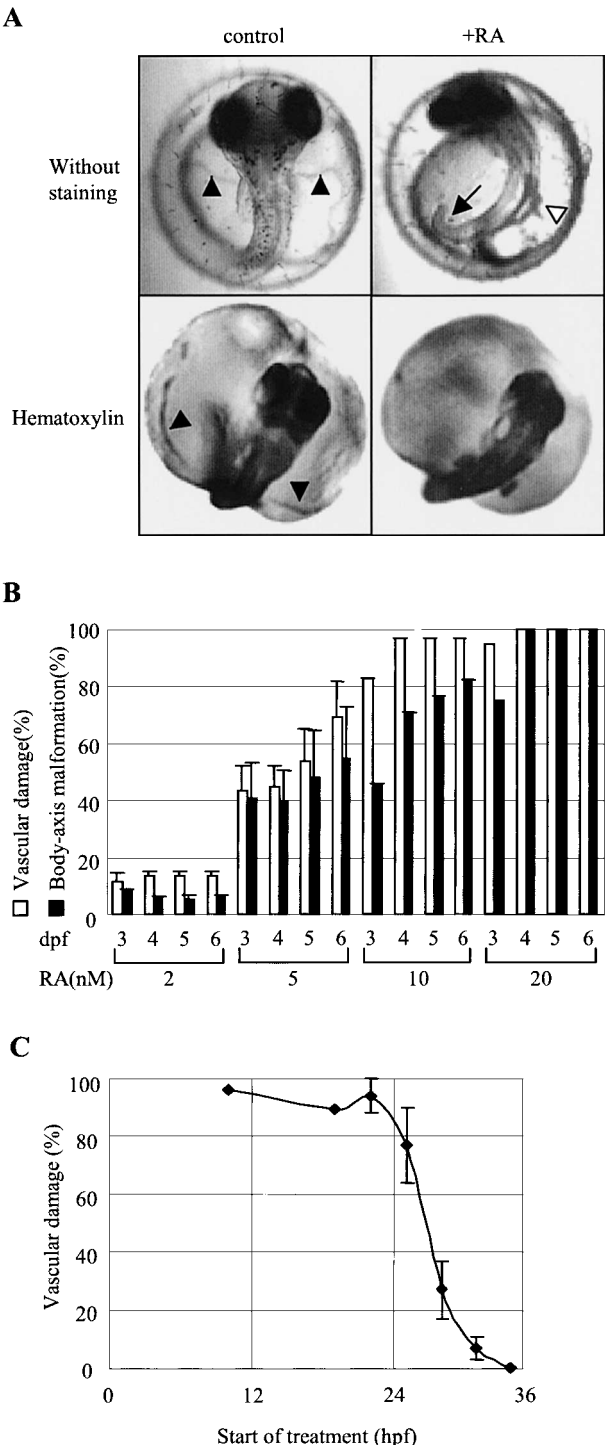


Fig. 3. Effects of excess RA on vascular and body-axis formation. (**A**) Eggs were treated with 10 nM RA until 4 dpf, stained with hematoxylin, and photographed. Yolk veins are marked by closed arrowheads. Note the following defects in the RA-treated embryos: agenesis of yolk veins, blood clotting (open arrowhead), a curved structure of tail (arrow), and small head. (**B**) Eggs were treated with RA at the indicated concentrations, and counted for the vascular damages and the body-axis malformation. (**C**) Staged embryos (from 10 to 34 hpf) were treated with 20 nM RA before counting for the vascular damages at 3dpf. Rates of embryos with vascular damages are plotted at the starting times (hpf) of the treatment.

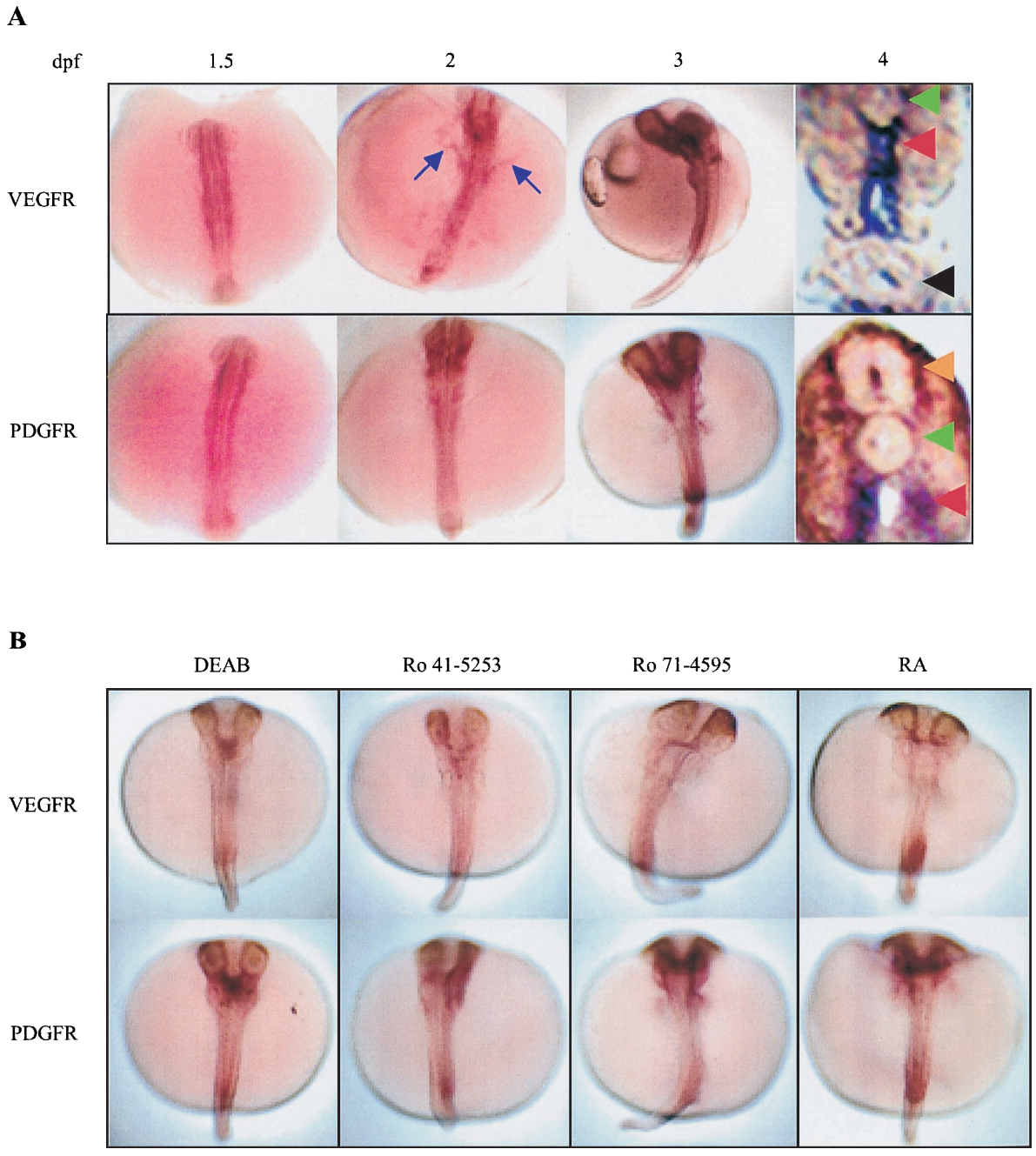


Fig. 4. *In situ* hybridization using probes for VEGFR and PDGFR mRNAs. (**A**) Whole-mount *in situ* hybridization of the embryos at the indicated stage, and cross sections at posterior regions of 4-dpf whole-mount samples. A region of yolk vein connecting to the embryo body (blue arrow), axial vessels (red arrowhead), neural tube (orange arrowhead), notochord (green arrowhead), and intestinal tract (black arrowhead) are indicated. (**B**) Effects of RA synthesis inhibitor DEAB (10 μM), RAR antagonist Ro41-5253 (5 μM), RXR antagonist Ro71-4595 (0.5 μM), and RA (10 nM) on the expression of VEGFR and PDGFR mRNAs after incubation until 3 dpf.

RA and retinoid receptors are required for expression of AHR mRNA

We examined the embryonic expression of AHR and retinoid receptor mRNAs until 6.5 dpf. Total RNA was extracted from embryos and analyzed for the steady-state level of mRNA by RT-PCR. Amplified cDNA fragments from AHR mRNA appeared after 5.5 dpf in the PCR of 35 cycles (Fig. 6A), and at 36 hpf in 40 cycles (Fig. 6B). These results indicate that AHR mRNA expression increased around 5.5 dpf and are consistent with the previous observation that dioxin-treated embryos show clear signs of vascular damages after 5 dpf (Kawamura and Yamashita, 2002). Expression of mRNAs for retinoid receptors (RARα and RARγ) were found to be essentially constant during the incubation (Fig. 6A).

Since RA signaling and AHR play key common roles in

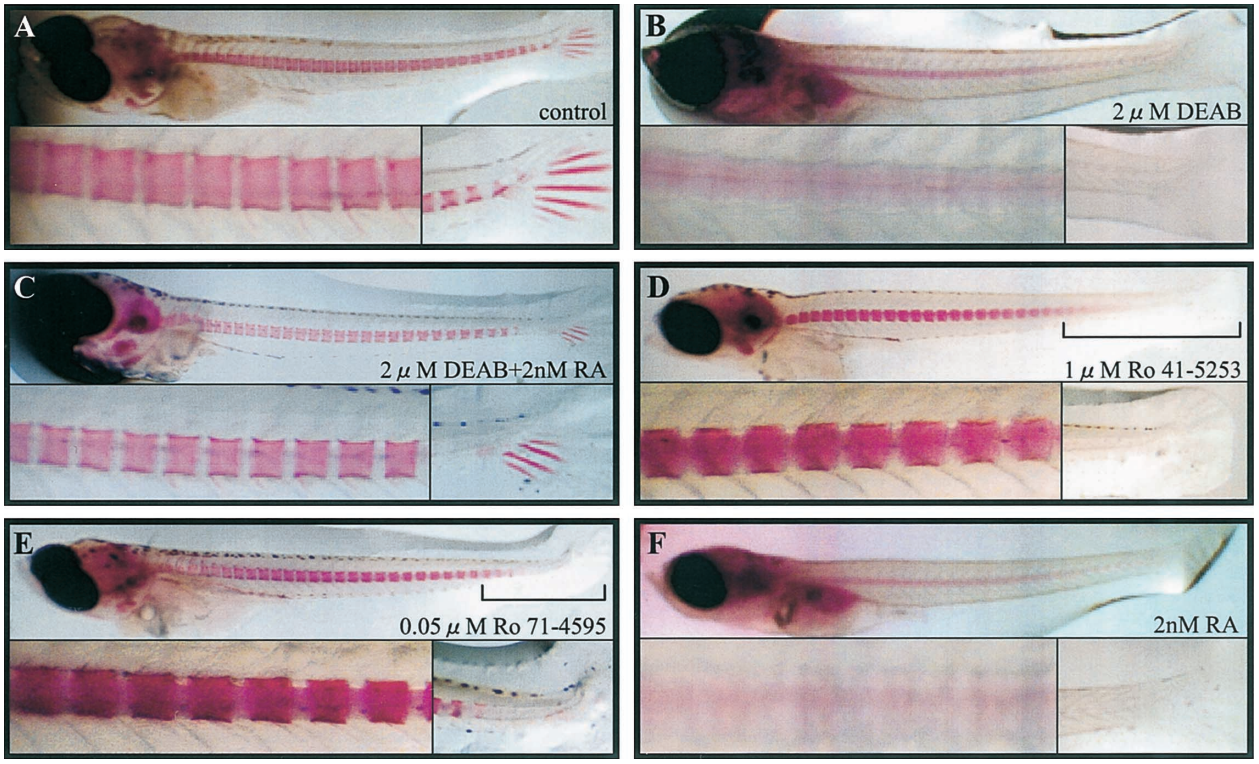


Fig. 5. RA and retinoid receptors are essential for bone formation. Eggs (10 hpf) were treated with the following reagents until 3 days after hatching, and the fry were stained for bones with alizarin S: (**A**) control; (**B**) 2 μM DEAB; (**C**) 2 μM DEAB plus 2 nM RA; (**D**) 1 μM Ro41-5253; (**E**) 0.05 μM Ro71-4595; and (**F**) 2 nM RA. Marked in (**D**) and (**E**) are regions where bone formation was inhibited.

the medaka embryogenesis, we examined whether AHR mRNA levels are controlled by RA and retinoid receptors. In embryos treated with the RA synthesis inhibitor, DEAB (Fig. 6B), and retinoid receptor antagonists (Fig. 6C), expression of AHR mRNA was greatly inhibited. In contrast, exogenous RA increased the expression (Fig. 6D). These results indicate that the expression of AHR mRNA is regulated by RA and retinoid receptors.

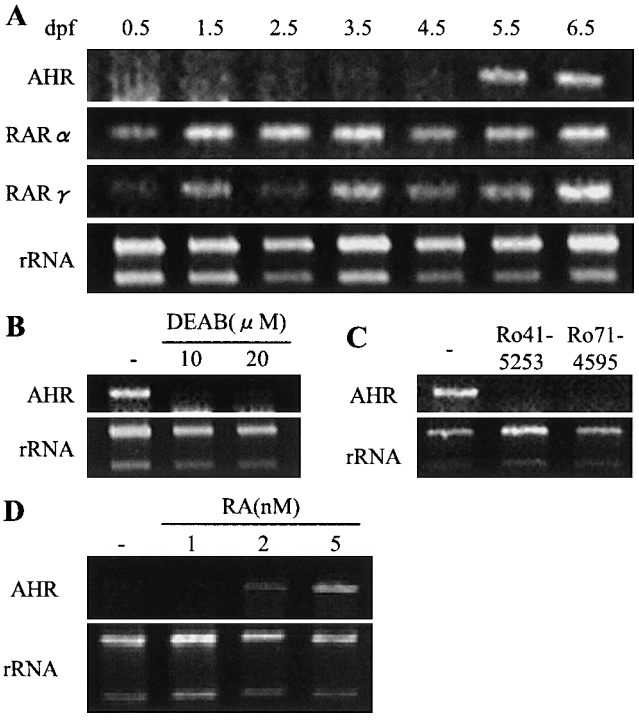


Fig. 6. Control of AHR mRNA levels by RA and retinoid receptors. Total RNA was extracted from embryos and analyzed for mRNA expression by RT-PCR. Ribosomal RNA (rRNA) is also shown as a normalizing marker for the total RNA used. (**A**) Expression of mRNAs for AHR, RARα, and RARγ at the indicated developmental time. AHR mRNA was analyzed at 35 cycles of PCR. (**B**) Inhibition of AHR mRNA expression by the treatment with RA synthesis inhibitor DEAB (10 and 20 μM) in the 36-hpf embryos. PCR was done at 40 cycles. (**C**) Inhibition of AHR mRNA expression by the treatment with RAR antagonist Ro41-5253 (5 μM) and RXR antagonist Ro71-4595 (2 μM) in the 36-hpf embryos, analyzed at 40 cycles. (**D**) Activation of AHR mRNA expression by the addition of RA (1, 2, and 5 nM) in the 36-hpf embryos, analyzed by two successive rounds of PCR reactions at 35 and 25 cycles.

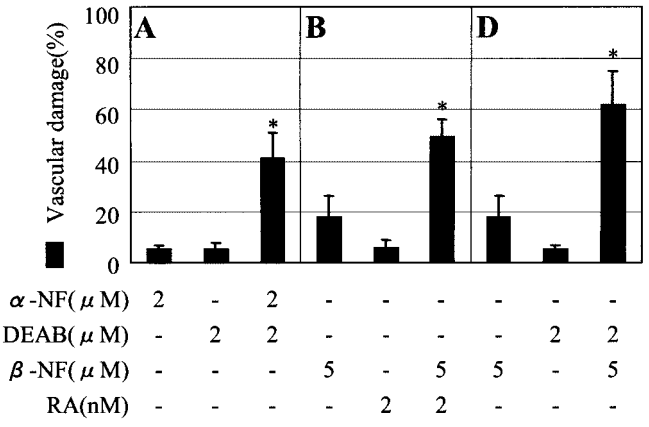
Physiological significance of the retinoid receptor-mediated expression of AHR mRNA

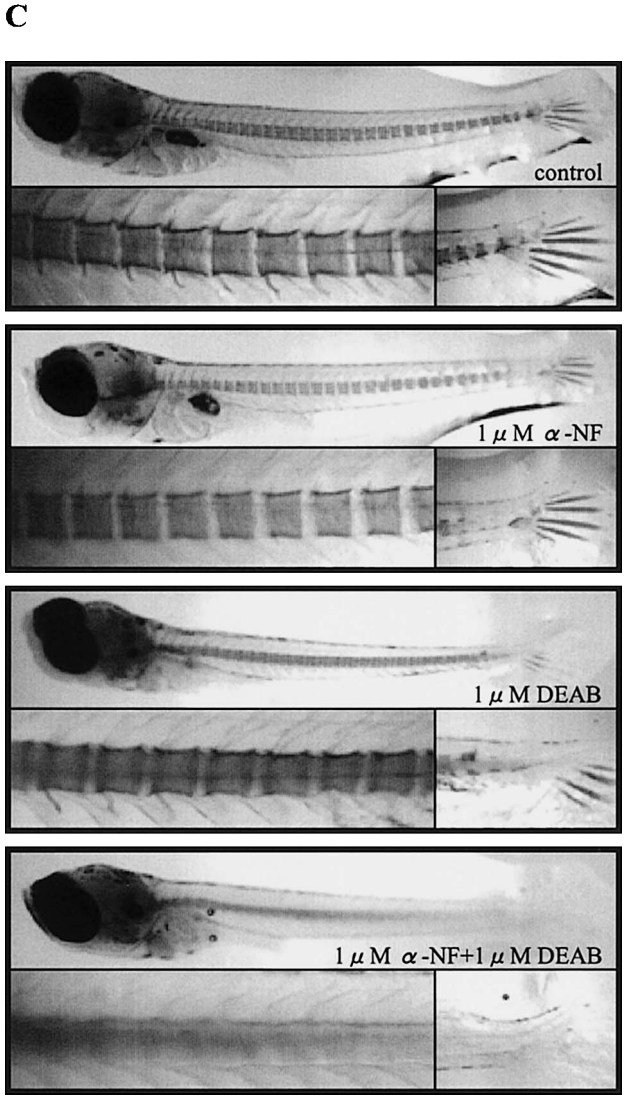
To test If the RA/retinoid receptor-mediated expression of AHR mRNA has a physiological role in the vascular development, embryos were treated simultaneously with doses of the RA synthesis inhibitor (DEAB) and the AHR antagonist (α-naphthoflavone, α-NF) that alone did not show any effect. The co-treatment did not apparently affect

the formation of yolk veins at 3 dpf (data not shown) but enhanced the rate of blood clotting at 5 dpf (Fig. 7A), which is a sign to the vascular damage by the inhibition or hyperactivation of AHR (Kawamura and Yamashita, 2002). For a reverse experiment, embryos were co-treated with RA and the AHR agonist (β-naphthoflavone, β-NF), also resulting in the enhanced vascular damages at 5 dpf (Fig. 7B). These results are consistent with the conclusion that RA signaling for expression of AHR mRNA plays a key role in the vascular homeostasis.

We also examined for the synergistic action in the bone formation. The combination of DEAB and α-NF worsened the bone formation (Fig. 7C), supporting the significance of the regulatory route in the bone formation.

Finally we examined for the well-known role of AHR in RA metabolism (Nilsson and Hakansson, 2002). If activation of AHR by agonist (β-NF) reduced *in vivo* RA level, the combination of β-NF and DEAB would further reduce it, then increase the rate of vascular abnormalities. This was true (Fig. 7D), suggesting that AHR is involved in RA metabolism also in medaka embryos.





DISCUSSION

RA and retinoid receptors are required for medaka embryogenesis

Vitamin A and its active form, RA, are essential for embryogenesis, and their actions must be strictly controlled. High dietary intake of vitamin A in the form of supplements (Rothman *et al.*, 1995) and therapeutic doses of synthetic retinoids for the treatment of severe acne (Rosa, 1983) cause congenital fetal malformations generally called "RA embryopathy". Total vitamin A deficiency is lethal to the embryo, while less severe deficiency results in a large number of developmental malformations including those similar to prevalent congenital defects called "caudal regression syndrome" whose environmental and genetic causes are unclear (Duhamel, 1961). Several clinical and numerous experimental studies have attempted to clarify some of the issues of mechanisms. A vascular etiology has been suggested but no definitive pathogenetic mechanism has been established (Van Allen, 1981). Genetic studies using knockout mice have demonstrated the critical roles of retinoid receptors in vertebrate ontogenesis (Kastner *et al.*, 1995). However, defects in vasculogenesis have not been reported in these receptor knock-out mice.

Fig. 7. Synergistic effects of the reagents for controlling RA level and AHR activity on the vascular and bone formation. (**A**) Effects of the co-treatment with RA synthesis inhibitor (DEAB) and AHR antagonist (α-NF) on vascular formation. Eggs were treated with DEAB (2 μM), α-NF (2 μM), or combination of both until 5 dpf and counted for blood clotting. (**B**) Effects of the co-treatment with RA and AHR agonist (β-NF) on vascular formation. Eggs were treated with RA (2 nM), β-NF (5 μM), or combination of both until 5 dpf and counted for blood clotting. (**C**) Effects of the co-treatment with DEAB and α-NF on bone formation. Eggs were treated with DEAB (1 μM), α-NF (1 μM), or combination of both, and the hatching fry were incubated successively until 5 dah. The fry were stained for bones with alizarin S, and photographed. (**D**) Effects of the co-treatment with DEAB and β-NF on vascular formation. Eggs were treated with DEAB (2 μM), β-NF (5 μM), or combination of both until 5 dpf and counted for blood clotting. *P<0.05

RA treatment also leads to progressive deletions of anterior structures in lower vertebrates such as *Xenopus* (Durston *et al*., 1989; Sive *et al*., 1990), zebrafish (Holder and Hill, 1991; Stainier and Fishman, 1992), and medaka (Inohaya *et al*., 1995). In the medaka embryos, treatment with 5 μM RA during an early development also results in the complete absence of yolk veins (Inohaya *et al*., 1995). Zebrafish embryos treated with 10 μM DEAB prior to 65% epiboly lack circulation and display cardiac edema and enlarged blood islands, which are indicative of vascular damages, and curved body axis (Perz-Edwards *et al*., 2001). Furthermore, zebrafish embryos homozygous for *raldh2* mutation (retinaldehyde dehydrogenase 2, allelic to *no-fin*) lack pectoral fins and cartilaginous gill arches and display an edema of the heart (Grandel *et al*., 2002). These studies indicate that RA is essential for normal development of vasculature, bone, and body axis also in lower vertebrates. However, molecular mechanisms by which RA signaling acts are not well understood.

In the present study, we examined the effects of the lack and excess of RA signaling on the medaka embryogenesis and found that RA and retinoid receptors are required for vascular, body-axis, and bone formation. Our findings are consistent with the previous conclusion that RA signaling plays important roles in many aspects of vertebrate development, and for the first time support a critical role of retinoid receptors in vasculogenesis. We found that DEAB can be used for specific inhibition of RA synthesis in medaka embryos. Retinoid receptor antagonists were also used successfully to inhibit vascular formation only with mild damages in the embryonic growth. These reagents are useful to future studies on vasculogenesis in medaka.

A proposal for the role of RA signaling and AHR in the vascular and bone formation and their involvement in dioxin toxicity

AHR is conserved among vertebrates, and ubiquitously expressed in embryos. Previously we found that activation of AHR by an endogenous ligand and subsequent induction of cytochromes P450 are specifically required for the development of blood vessels and bone (Kawamura and Yamashita, 2002). However, it has not been known how AHR expression is controlled at an early stage of development. Here we demonstrated for the first time that RA and retinoid receptors are required for the expression of AHR mRNA. Furthermore, the transcriptional cascade was found to be physiologically important for the development of vascular and bone systems. Taken together with the role of AHR in RA metabolism that is generally accepted and suggested in our experiment, we propose a feedback mechanism regulating *in vivo* RA levels, in which excessive synthesis of RA activates AHR mRNA expression, then, increased activity of AHR in turn stimulates conversion of RA to inactive metabolites (Fig. 8). Planar halogenated hydrocarbons such as dioxin are notorious environmental pollutants that are extremely toxic to early stages of vertebrate development (Peterson *et al*., 1993). Our model suggests two routes through which hyperactivation of AHR by binding to dioxin abrogates vascular and bone formation: one giving rise to RA-deficiency and another acting independently of RA. This model, although not verified at molecular levels, may provide a basis for future studies on chemoprevention of dioxin toxicity. It remains to be clarified what kinds of cytochrome P450 genes are transcriptionally induced by dioxin and involved in each route of the AHR signaling cascade.

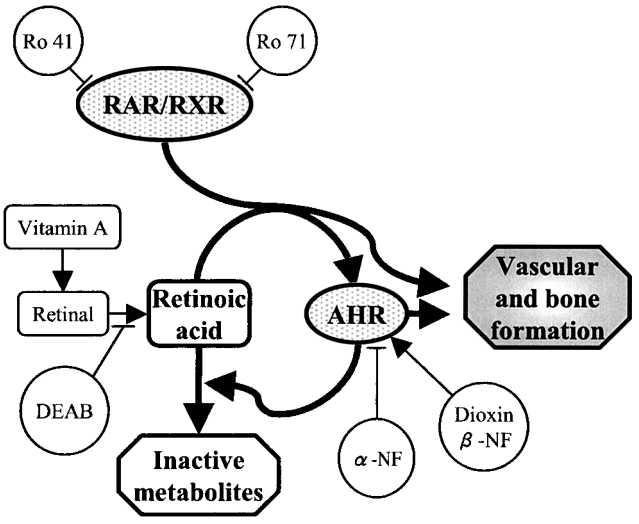


Fig. 8. A proposed role of RA, retinoid receptors, and AHR in the development of vasculature and bone. RA-bound retinoid receptors (RAR/RXR) are required for the vascular and bone formation and activate AHR mRNA expression. AHR acts for vascular and bone formation independently of retinoid receptors, and also for conversion of RA to inactive metabolites forming a negative feedback loop. Hyperactivation of AHR by dioxin abrogates vascular and bone formation through excess activation of the two routes. The sites at which the following reagents act are also shown: RA synthesis inhibitor (DEAB), antagonists for retinoid receptors (Ro41-5253 and Ro71-4595), and agonists (dioxin and β-NF) and antagonist (α-NF) for AHR.

Screening anti-cancer drugs using medaka embryos

The medaka embryo possesses a unique combination of features that make it particularly well suited for experimental and genetic analysis of vertebrate vascular development: transparency, extrauterus development, large yolk veins, survival in the complete absence of blood circulation. Our studies add to these features that medaka embryos are useful for screening anti-cancer drugs, because retinoids are effective chemopreventive agents against many forms of cancer (Hansen *et al*., 2000; Zusi *et al*., 2002), and because several anti-angiogenic drugs (inhibitory to blood vessel formation) are currently undergoing clinical trials for the treatment of cancer (Fan *et al*., 1995; Tosetti *et al*., 2002). Blood vessels form through a complex process involving multiple steps and several cell types. *In vitro* models, although useful in elucidating parts of this process, may not be representative of what occurs *in vivo*. Two useful *in vivo* systems that

have been used extensively in angiogenesis research are chorioallantoic membranes of chicken embryos (Hazel, 2003) and mouse cornea (Cao and Cao, 1999). However, these methods can be difficult and time-consuming to quantify, not very reproducible, and expensive. One can easily collect many numbers of medaka fertilized eggs all the year round, and can assay for vessel-inhibiting chemicals in high cost-performance. Furthermore, recent studies that endothelial progenitor cells involved in vasculogenesis (embryonic vascular formation) also play key roles in angiogenesis (new blood vessel formation from pre-existing vessels in adult animals) (Asahara *et al*., 1999; Zhang *et al*., 2002) support a rationale for screening anti-cancer drugs using embryonic vascular formation as an index. We therefore recommend medaka eggs for screening candidate drugs and, in addition, for assessment of medicinal activity in food, beverage, herbal plant, and crop.

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