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Induction of Bent Cartilaginous Skeletons and Undulating Notochord in Flounder Embryos by Disulfiram and $\alpha, \alpha'$-Dipyridyl

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ABSTRACT—Disulfiram causes undulation of the notochord and bending of pharyngeal cartilages in fish embryos. Using flounder embryos, this study aimed to elucidate which process of pharyngeal arch development was affected by the drug. Since disulfiram is known to block the synthesis of retinoic acid (RA) in vivo, we first examined whether the drug suppresses the expression of Hoxd-4 and shh, RA responsive genes related with the pharyngeal arch development. Disulfiram at a concentration which induces undulation of the notochord and bending of cartilage elements did not affect the expression of these genes. On the other hand, similar phenotypes of anomalies were found to be reproduced both in the notochord and pharyngeal cartilages by $\alpha, \alpha'$-dipyridyl which reduces the mechanical stability of collagen. Thus, we suppose that disulfiram causes anomalies by decreasing the mechanical stability of collagen and not by suppressing the expression of RA-responsive genes. Since disulfiram blocks ascorbate dehydrogenase, it is our hypothesis that this drug inhibits the maturation of collagen by affecting ascorbic acid metabolism.

INTRODUCTION

In teleosts, cartilaginous skeletons develop in the pharyngeal arches, neurocranium, and pectoral fin and around the eyes at the embryonic stage (Scilling et al., 1996). Comparing the skeletal malformations induced in flounder embryos by teratogens, we found that severe bending of pharyngeal and pectoral fin cartilage elements is caused by disulfiram (Suzuki et al., 2000). There are several reports of the teratogenic effects of disulfiram on the skeletons of chick and human, the drug having been used as a fungicide for grains and a treatment for alcoholism. Tibial dyschondroplasia is caused by disulfiram added to the starter rations of chick (Vargas et al., 1983; Edwards, 1987). Limb-reduction anomalies occurred in infants born to disulfiram-treated alcoholic mothers (Nora et al., 1977). The mechanism by which disulfiram causes such bone anomalies is unclear. The zebrafish mutant braK (brk) is characterized by bent cartilage elements in the pharyngeal arches (Neuhauss et al., 1996), showing a similar phenotype of anomaly as that induced by disulfiram in flounder embryos. Detailed analysis of the effects of disulfiram on embryogenesis may provide information on the mechanisms that cause bending in pharyngeal cartilages as well as the limb reduction reported in tetrapods.

The cartilaginous skeletons in the pharyngeal arches develop from the cranial neural crest through complex processes. In the early embryonic stage, the following processes occur: hindbrain segmentation, migration of neural crest cells from the hindbrain into the pharyngeal arches, and differentiation of cartilage precursor cells from neural crest cells (Langille and Hall, 1988; Schilling and Kimmel, 1994; Kimmel et al., 1995). In the late embryonic stage, the cartilage precursor cells rapidly proliferate and then mature into chondrocytes, and the cartilage matrix is synthesized (Hall and Miyake, 1992; Heusseune and Sire, 1992; Suzuki and Kurokawa, 1996; Kimmel et al., 1998). Various transcriptional and growth factors and their receptors, as well as extracellular matrices, function in these processes. Recent mutational analyses of zebrafish indicate that various phenotypes of pharyngeal cartilage anomalies are caused by the disruption of genes which function in the above processes (Neuhauss et al., 1996; Schilling et al., 1996).

Disulfiram inhibits two groups of enzymes, class-1 dehydrogenases and Cu$^{2+}$-dependent enzymes. This drug reduces RA content in vitro by inhibiting retinaldehyde dehydrogenase (RALDH), a class-1 dehydrogenase, which synthesizes RA from retinal (Marsh-Armstrong et al., 1995; Costaridis et al., 1996; Vallari and Pietruszko, 1982). RA is required for the initiation of Hox and sonic hedgehog (shh) genes, which func-
tion in the development of pharyngeal arch and pectoral fin (Sasaki et al., 1990; Simeone et al., 1991; Langston and Gudas, 1992; Ogura and Evans, 1995; Chang et al., 1997). In addition, disulfiram inhibits ascorbate dehydrogenase, a Cu²⁺-dependent enzyme that functions in the metabolism of ascorbic acid (Wimalasena and Dharmasena, 1994). Procollagen prolyl hydroxylase, which provides mechanical stability to collagen, needs ascorbic acid for catalytic activity (Hurych and Chvapil, 1965). Thus, disulfiram may affect the expression of RA responsive genes and the maturation of collagen, both of which are essential for skeletal formation. To address which process in the development of cartilaginous skeletons is affected by disulfiram, the present study first examined the effects of disulfiram on Hoxd-4 and shh expression in flounder embryos. Then, we compared the phenotype of anomalies induced by α, α'-dipyridyl, which is known to reduce the mechanical stability of collagen (Fiedler-Nagy et al., 1981), with those by disulfiram.

MATERIALS AND METHODS

Embryos

Japanese flounder (Paralichthys olivaceus) embryos at the two cell stage were collected from a hatchery tank at the National Research Institute of Aquaculture, Mie, Japan. They were kept in an incubator set at 20°C.

Disulfiram and α, α'-dipyridyl treatment

A stock solution of disulfiram (5 mM) was prepared in DMSO and added to seawater to give the final concentrations used in the experiments. α, α'-dipyridyl was directly dissolved in seawater. Fifty embryos were cultured in 20 ml of filtered seawater supplemented with the drugs.

Cartilage staining

Cartilages were visualized with 0.1% Alcian blue 8GX dissolved in 1% HCl/70% ethanol. Some of the stained embryos were bleached in 2% H₂O₂ in 1% KOH to remove pigments. Samples were mounted in 80% glycerol.

In situ hybridization

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.0, overnight, and kept in methanol at –20°C. The preparation of antisense riboprobes of flounder Hoxd-4 and shh and whole-mount in situ hybridization were conducted as described (Suzuki et al., 1988, 1989).

RESULTS

Effects of disulfiram on Hoxd-4 and shh expressions

The development of pharyngeal cartilage and the expression pattern of Hoxd-4 and shh in flounder embryos are briefly summarized as follows. At the prim-5 stage, 60 hours post-fertilization (hpf) in flounder, cartilage precursor cells have finished their migration from the hindbrain into the pharyngeal arches, and the Hoxd-4 expression domain shows clear anterior borders in the hindbrain and pharyngeal area (Fig. 1A, B). After this stage, cartilage precursor cells actively divide for 36 h, during which period shh expression begins as spots and widely expands on the pharyngeal endoderm (Fig. 1H). Then, the cartilage matrix begins to be synthesized, and cartilage elements form in the mandibular, hyoid, and 1st–4th gill arches by the open-mouth stage, 130 hpf (Figs. 2A and 3A, B).

Pulse incubation with RA for 1 hr at the shield stage, 22 hpf, shifts the Hoxd-4 expression border anteriorly, indicating sensitivity of the Hoxd-4 gene to RA at this stage (Suzuki et al., 1998). The inhibitory effect of disulfiram on RA synthesis in vivo appears at a concentration of 1 µM in zebrafish (Marsh-Armstrong et al., 1995). Based on the data, we first incubated flounder embryos with 5 µM disulfiram for 2 hr at the shield stage and examined the Hoxd-4 expression during the hatching period. The pulse exposure did not affect the position of the anterior borders of the Hoxd-4 expression domain in the hindbrain and pharyngeal area, even though the trunk was slightly shortened by the drug (Fig. 1C). When the disulfiram-treated embryos were further developed in normal seawater until the open-mouth stage, the pharyngeal cartilages developed normally (data not shown). Pulse exposure to a high dose (50 µM) of disulfiram strongly truncated the trunk (Fig. 1D, E). In these embryos, the anterior border of the Hoxd-4 expression domain was unclear, but the expression in the pharyngeal area and pectoral fin bud could still be recognized. Since the embryos treated with this concentration of the drug did not survive later than the prim-5 stage, the phenotype of cartilage anomaly could not be observed.

Treatment with 1 µM disulfiram from the 2–3 somite stage to the prim-22 stage induces truncation of the trunk and undulation of the notochord in zebrafish embryos (Marsh-Armstrong et al., 1995). Then, to reveal the effect of continuous exposure to disulfiram during the period from the initiation to the establishment of the Hoxd-4 expression domain, we incubated flounder embryos with 1 µM disulfiram from the shield (22 hpf) to prim-5 stage (60 hpf). As reported in zebrafish, the trunk of the treated embryos was truncated, and the notochord was undulated (Fig. 4B). The anterior borders of the Hoxd-4 expression domain were normally established both in the hindbrain and pharyngeal area (Fig. 1F, G). Hoxd-4 expression at the pectoral fin buds also began normally. Thus, Hoxd-4 expression in flounder embryos was insensitive to disulfiram at a concentration which causes anomaly in the notochord. Some of the treated embryos were transferred to normal seawater and developed further, and the structure of cartilages was observed at the open-mouth stage (130 hpf). The trunk was kept truncated, but cartilage elements formed in the mandibular, hyoid, and 1st - 4th gill arches, as in normal embryos (Figs. 2B and 3C, D). We could not detect structural anomalies in the cartilage elements. The pectoral fin cartilages also formed normally. Thus, it was concluded that disulfiram does not affect the establishment of the Hoxd-4 expression domain or cartilage development when given before the prim-5 stage, even though it affects notochord development.

To observe the effect of disulfiram on shh expression in the pharyngeal area and pectoral fin buds, embryos were incubated with 1 µM disulfiram for 36 hr from the prim-5 stage. In the treated embryos, the shh expression domain in the pha-
Anomalies Induced by Disulfiram and α, α'-Dipyridyl

Fig. 1. *Hoxd-4* and *shh* expression in normal and disulfiram treated flounder embryos. Embryos in A–G were hybridized with *Hoxd-4* antisense riboprobe at 60 hpf when normal embryos reached the prim-5 stage. The anterior borders of the *Hoxd-4* expression domain in the hindbrain (h) and pharyngeal area (p) are marked by arrows and arrowheads, respectively. The expression in the pectoral fin (f) is marked by asterisks. A, B: normal embryos. C, D–E: embryos incubated at 22 - 24 hpf (shield stage) with 5 and 50 µM disulfiram, respectively. F, G: embryos incubated with 1 µM disulfiram at 22–60 hpf. The anterior border of the *Hoxd 4* expression domain is unclear only in embryos in D, E. O: otic vesicle. Embryos in H and I are hybridized with the *shh* probe (96 hpf). H: normal embryo. I: embryo incubated with 1 µM disulfiram at 60–96 hpf. *shh* is expressed in the pharyngeal endoderm (arrows) and pectoral fin buds (arrowheads) at the same level in H and I. Bar, 500 µm in A, C, and E; 500 µm in D and F; 250 µm in B and G; 200 µm in H and I.

The pharyngeal endoderm was established normally, and the *shh* expression at the pectoral fin also started normally (Fig. 1).

**Effects of disulfiram and α, α'-dipyridyl on embryogenesis**

Because data could not be obtained to support the idea that disulfiram suppresses *Hoxd-4* and *shh* expression in the pharyngeal area, we decided to examine whether disulfiram affects the mechanical stability of cartilage.

In the embryos incubated with 1 µM disulfiram from the prim-5 to the open mouth stage, cartilage elements in the pharyngeal arches were obviously bent (Fig. 2C), as previously reported (Suzuki *et al.*, 2000). Detailed observation showed that cartilage elements existed in the mandibular, hyoid, and four gill bars, as in normal embryos (Fig. 3 E, F). Large cartilaginous elements were all severely bent, including the Meckel’s (m) and palatoquadrate (pq) cartilages in the mandibular arches, the ceratohyal (ch) and hyosymphycotic (hs) cartilages in the hyoid arches, and the ceratobrancial (cb) and basibranchial (bb) cartilages in the gill arches. Small cartilage elements, such as the basihyal (bh) in the hyoid arch, were hard to identify due to severe malformation. Other than the pharyngeal cartilages, the pectoral fin plate (pf) cartilages were deeply undulated. The trabeculae cranii (t) of the neurocranium was also bent, and the sclerotic cartilage(sc) around the eyes was undulated. Thus, all cartilage elements in the embryos, whether of neural or mesodermal origin, were affected by disulfiram. In spite of these strong anomalies, major cartilage elements of the pharyngeal arches could be identified. The notochord was not undulated by disulfiram at this stage (Fig. 3C). When embryos were treated with disulfiram from the shield to the open-mouth stage, both pharyngeal cartilages and the notochord were malformed, showing a combination of truncation of the trunk and bending of cartilages (Fig. 3D).

In the embryos treated with 5 µM α, α'-dipyridyl from the prim-5 to the open-mouth stage, large cartilage elements...
specific teratogenic effects on the notochord at the early embryonic stage and on the cartilaginous skeleton at the organogenesis stage.

DISCUSSION

Unsusceptibility of Hoxd-4 and shh expression to disulfiram treatment

Deficiency of vitamin A, a precursor of RA, in rat embryos causes loss of Hoxd-4 expression in the hindbrain and reduces the posterior rhombomeric segments of hindbrain and related posterior pharyngeal arches (White et al., 2000). The phenotype of the pharyngeal skeletal anomaly induced by RA-deficiency is, however, unclear. Since disulfiram decreases the RA content of embryos (Vallari and Pietruszko 1982; Marsh-Armstrong et al., 1995), we expected disulfiram to suppress Hoxd-4 and shh expression in the pharyngeal area and to induce skeletal anomalies via loss of the expression. Contrary to our expectations, loss of gene expression was not achieved by disulfiram at a concentration which caused anomalies in the notochord. An extremely high dose of disulfiram was needed to affect Hoxd-4 expression. However, disulfiram exhibits various side effects, particularly on the central nervous system (Peachey et al., 1981), and it is ambiguous whether the effect on Hoxd-4 expression is caused by suppression of RALDH. Even when embryos were exposed to disulfiram throughout early embryogenesis until the prim-5 stage, cartilages formed normally when the embryos were transferred to normal seawater. This supports the idea that the processes of hindbrain and pharyngeal arch development at the early embryonic stage are unsusceptible to the effect of disulfiram.

The RA content of embryos and localization of RALDH mRNA were recently analyzed in detail in zebrafish and chick. The level of RALDH-2 mRNA expression is high in trunk somites and low in the hindbrain (Swindell et al., 1999). Accordingly, the level of RA in the hindbrain region is particularly low (Marsh-Armstrong et al., 1995; Maden et al., 1998). Thus, RA is synthesized de novo in embryos to control levels regionally. However, since RA is contained in 8-cell-stage zebrafish embryos at the same level as in the shield to the prim-5 stage (Costaridis et al., 1996), RA seems to exist also as a maternal factor in the yolk of teleosts. Therefore, to explain why disulfiram did not suppress the Hoxd-4 and shh expressions, we speculate that RA is present in yolk at a concentration that is high enough to activate RA-responsive genes and that complete RA-deficiency could not be reproduced by disulfiram. When a relatively high dose of disulfiram was administered locally to the chick limb buds, loss of shh expression at the zone of polarizing activity was successfully induced (Stratford et al., 1996). On the other hand, when whole embryos were exposed to the drug, as in our experiments, strong side effects seemed to appear in various tissues without suppressing the expression of RA-responsive genes.

Effects of disulfiram on cartilages

Disulfiram induced severe bending in all cartilaginous

Fig. 2. Normal, disulfiram-, and α, α’-dipyridyl-treated embryos after cartilage staining. Embryos were stained at 130 hpf when normal embryos reached the open-mouth stage. A: normal embryo. B, C, D: embryos incubated with 1 μM disulfiram at 22–60, 60–130, and 22–130 hpf, respectively. 22, 60, and 130 hfp correspond with the shield, prim-5, and open-mouth stage, respectively. E: embryo incubated with 5 μM α, α’-dipyridyl at 60–130 hpf. Anomaly of the cartilaginous skeleton is obvious in C, D, and E. Anomaly of the notochord is obvious in B and D. Bar, 500 μm

formed in the mandibular to the 4th gill arches were severely bent (Figs. 2E and 3G, H). Anomalies were also observed in the pectoral fin plates, trabeculae, and sclerotic cartilage. In general, the degree of anomaly induced by α, α‘-dipyridyl was more severe than that by disulfiram. The phenotype of pectoral fin malformation induced by the two drugs was similar. Thus, the bending of cartilage elements was a common anomaly induced by disulfiram and α, α‘-dipyridyl.

Treatment with 5 μM α, α’-dipyridyl after the prim-5 stage did not affect the notochord (Fig. 2E), but treatment at the shield to the prim-5 stage caused severe truncation of the trunk and undulation of the notochord (Fig. 4C). Thus, it was indicated that disulfiram and α, α’-dipyridyl exert similar stage-specific teratogenic effects on the notochord at the early
skeletons irrespective of their origin, including pharyngeal arches, neurocranium, and pectoral fin. Mechanically unstable type II collagen is known to cause dyschondroplasia in craniofacial and limb skeletons (Metsäranta et al., 1992). Examining the effect of α, α'-dipyridyl, which reduces the mechanical stability of collagen (Fiedler-Nagy et al., 1981), on embryogenesis, we found that severe bending of cartilaginous skeletons is also induced by this drug. From the similarity of phenotype, we infer that disulfiram induces bent cartilages by affecting the mechanical stability of collagen, as does α, α'-dipyridyl. Our hypothesis on the mechanism of the teratogenic effect is that disulfiram inhibits ascorbate oxidase by Cu^{2+}-chelating activity, and the resultant disruption of ascorbic acid metabolism causes suppression of the hydroxylation of collagen.

Zebrafish mutant Brk is characterized by bending of the pharyngeal cartilaginous skeletons, so the loss of mechanical stability of the cartilages is attributed to mutation (Neuhauss...
Another character of Brk is the reduction of melanocytes. Disulfiram inhibits the pigmentation of skin by inhibiting a Cu²⁺-dependent enzyme, tyrosinase (Marsh-Armstrong et al., 1995). Reduction of melanocytes could also be realized in flounder embryos incubated with disulfiram (Fig. 1 B, G). Thus, the phenotype of anomaly in Brk and disulfiram-treated embryos is closely related with both cartilage and melanocyte development. Therefore, a gene coding for a Cu²⁺-dependent enzyme which metabolizes tyrosine and ascorbic acid (Goldstein et al., 1964; Menniti et al., 1986) might be mutated in Brk.

Undulation of the notochord is a remarkable anomaly of zebrafish embryos treated with disulfiram (Costaridis et al., 1996; Marsh-Armstrong et al., 1995). A similar anomaly was reproduced in flounder embryos by not only disulfiram but also α, α’-dipyridyl. Although the notochord is a non-chondrogenic tissue, cells of the notochord express type II collagen mRNA at the early embryonic stage (Bieker and Yazdani-Buicky, 1992; Yan et al., 1995). Though the undulation is attributed to the ability of disulfiram to suppress RA synthesis (Costaridis et al., 1996; Marsh-Armstrong et al., 1995), it is also possible that undulation of the notochord is another phenotype of the anomaly caused by the inhibition of collagen maturation. 

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**REFERENCES**


Chang B-E, Blader P, Fischer N, Ingham PW, Strähle U (1997) Axial (HNF3β) and retinoic acid receptors are regulators of the zebrafish sonic hedgehog promoter. EMBO J 16: 3955–3964


Suzuki T, Oohara I, Kurokawa T (1999) Retinoic acid given at the late embryonic stage depresses sonic hedgehog and Hoxd-4 expression in the pharyngeal area and induces skeletal malformation in flounder (Paralichthys olivaceus) embryos. Dev Growth Different 41: 143–152

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