Cadmium Affects Muscle Type Development and Axon Growth in Zebrafish Embryonic Somitogenesis

Elly Suk Hen Chow and Shuk Han Cheng

Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong

Received November 13, 2002; accepted January 21, 2003

We have previously reported that exposure to cadmium during zebrafish embryonic development caused morphological malformations of organs and ectopic expression of genes involved in regulating developmental process. One of the most common developmental defects observed was altered axial curvature resulting from defects in the myotomes of the somites. In this study, we investigated the mechanisms of cadmium-induced toxicity in zebrafish somitogenesis. We showed that the critical period of exposure was the gastrulation period, which actually preceded the formation of the first morphologically distinct somites. The somites thus formed lost the typical chevron V-shape and are packed disorderly. The myogenic lineage commitment of the axial mesodermal cells was not affected, as the myogenic regulatory transcription factors were expressed normally. There were, however, losses of fast and slow muscle fibers in the myotomes. The innervation of the muscle blocks by spinal motoneurons is an important process of the somitogenesis. Both primary and secondary motoneurons appear to form normally while the axon growth is affected in cadmium-treated embryos. The notochord, which is essential in the patterning of the somites and the central nervous system, showed abnormal morphological features and failed to extend to the tail region. Taken together, it appears that cadmium exposure led to abnormal somite patterning of the muscle fibers and defects in axonogenesis.

Key Words: zebrafish embryos; cadmium; somite patterning; muscle cell type; axon growth; myogenic regulatory factors.

Cadmium is a well-known developmental toxicant causing embryotoxic and teratogenic effects (reviewed by Webster, 1990; Domingo, 1994). Exposure to cadmium is suspected of causing developmental defects in humans (Chisolm, 1974). Cadmium exposure gives rise to a variety of developmental defects in chicks, mice, rats and frogs (Chen and Hales, 1994, De et al., 1993; Gilani and Alibhai, 1990, Sunderman et al., 1991). More recently, the model organism zebrafish has been employed to investigate the molecular and cellular basis of cadmium-induced developmental toxicity (Blechinger et al., 2002; Cheng et al., 2000). Trunk abnormalities resulting in distortions of body axis and altered axial curvatures were one of the predominant effects when zebrafish were exposed to cadmium, either during the first 24 h of embryonic development or from 3–7 days of age in hatched larvae. Indeed, trunk abnormalities are commonly identified as one of the major pathological traits found in fish dwelling in areas polluted with toxic chemicals (Kocan, 1996). There is, however, very little information on how trunk abnormalities arise as a consequence of toxicant exposure. This is in part due to the fact that trunk development in teleosts involves multiple processes that are highly regulated and present multiple targets for toxicant actions.

The most prominent feature of teleost trunk development is the process of segmentation. The fish trunk is composed of reiterated blocks of muscles, cartilage, bone, and nerves. These serially homologous blocks are derived from mesodermal segments called somites, which are formed during embryonic development. The zebrafish has provided a wealth of information on the molecular and genetic basis of somitogenesis (reviewed in Holley and Nüsslein-Volhard, 2000). The transparency of the embryos enables easy observation of cells while they differentiate and migrate. The availability of genetic mutants, on the other hand, enables identification of genes involved in various aspects of somitogenesis. We now have an overview of somite development in zebrafish, even though some of the key developmental regulatory factors are yet to be identified (reviewed in Stickney, 2000). The patterning of the mesoderm occurs during the gastrulation period, from 6 h post fertilization (hpf) to 10.5 hpf. During this period of development, the notochord, which is derived from the axial mesoderm, is formed in the midline, and is an important signaling center that plays pivotal roles in future patterning of the somites and neural differentiation. The first morphologically distinct somite pair appears at approximately 10.5 hpf. During the period of segmentation from 10.5 to 24 hpf, a total of about 30 pairs of somites are formed, and they bracket the notochord. With the formation of somites, the trunk begins to lift off the yolk sac and the tails extend. At 24 hpf, when segmentation completes, the somites have further developed into the myotome and the sclerotome. The myotomes will develop into skeletal muscles in the adult fish. Precursors to adult slow and fast muscles can be identified at 24 hpf. The embryonic fast
muscle fibers are the major constituents of the myotome and they are found in the deep portion surrounding the notochord. Embryonic slow muscle fibers, on the other hand, form a superficial monolayer on the surface of the myotome. The myotomes are separated into muscle blocks by myosepta in the anterior/posterior boundaries and by horizontal myosepta dorsoventrally.

Each muscle block is connected to specific motor neurons situated in their central nervous system by axon outgrowth (reviewed in Eisen, 1994). The primary motoneurons consist of three types, namely the caudal primary (CaP), middle (MiP), and rostral (RoP). Their axons pioneer the nerve path connecting the segmental motor nerve to the muscle blocks. Each type of primary motoneuron has a stereotypic axonal trajectory connecting them to their specific axial muscle targets in the somatic hemisegment, precisely. The secondary motoneurons develop later, and their axons extend along the path of the primary motoneurons. These processes of axon growth by which different motoneurons send their axons to reach respective muscle targets along an initially shared common axonal path, and then along divergent cell-specific pathways, are highly regulated events.

In our previous study, we reported that reduced myotome formation was observed in cadmium-exposed embryos with altered axial curvatures (Cheng et al., 2000). We have also reported normal expression of the gene, sonic hedgehog (shh), which is known to act upstream of muscle-specific genes. Our previous work suggests that cadmium might interfere with events subsequent to the inductive signals of shh and before the formation of myotomes, during somitogenesis. In the current study, we investigated the landmark events in somitogenesis in order to find out when and where cadmium might exert its toxicity in this important process of development. We first investigated whether the critical period of exposure occurred preceding or simultaneously with the morphological formation of somites. We then investigated whether the commitment of somitic cells to myogenic lineage and then to specific muscle cell types was affected. The commitment to myogenic lineage was investigated by the expression of two myogenic regulatory factors, myoD and myogenin, which regulate muscle cell differentiation (Weinberg et al., 1996). myoD expression marks the specification of myogenic precursor cells and the transcripts are expressed in the middle of each chevron-shaped somite at 24 h post fertilization (hpf). Myogenin expression occurs later than myoD during development, and the transcripts are expressed in a subset of myoD-expressing myogenic cells. The differentiation to fast muscle and slow muscle was investigated by staining for the fast skeletal muscle myosin heavy chain (myh5) and the slow myosin-binding protein C (smbpc) (Xu et al., 2000). We also examined the process of motoneuron innervation in the myotomes in order to find out whether this last step of somitogenesis was affected. We investigated the formation of primary motor neurons and the projection of their axons with the antibodies Isl-1 (Isl-1) and Znp-1, respectively (Inoue et al., 1994, Trevarrow et al., 1990). The antibody Isl-1 is specific for two types of primary motor neurons, the MiP and the RoP, while the antibody Znp-1 recognizes primary motor neurons and their axon outgrowths. We then investigated the projection of axons by the secondary motor neurons using the antibody Zn-5, which recognizes DM-GRASP, a cell adhesion molecule found on secondary motor neurons (Fashena and Westerfield, 1999). Finally, we used the antibody Zn-t-1, which labeled the notochord in zebrafish embryos, to investigate whether cadmium affected the formation of the notochord, the central organ in providing inductive signals for somite patterning (Trevarrow et al., 1990).

**MATERIALS AND METHODS**

**Chemicals.** Cadmium chloride (CdCl2·2H2O) was purchased from Sigma Chemical Company (St. Louis, MO). All chemicals were reagent grade.

**Zebrafish maintenance and embryo treatment.** Zebrafish were maintained and embryos were collected as previously described (Cheng et al., 2000). Embryos were exposed to 100 μM of cadmium chloride in embryo media at different developmental stages categorized in three groups: (1) exposure to cadmium during the gastrulation period (4–10.5 hpf); (2) exposure to cadmium during the segmentation period (10.5–24 hpf); (3) exposure to cadmium during both gastrulation and segmentation periods (4–24 hpf). The untreated controls were embryos maintained in culture medium only. Thirty replicas, consisting of 20 embryos in a 60-mm diameter Petri dish, were set up for each treatment group (i.e., n = 30 × 20 = 600). At 24 hpf, embryos were dechorionated by using a pair of fine forceps under a dissecting stereomicroscope. Embryos were examined for abnormalities in body flexure according to previous reported descriptions and method (Cheng et al., 2000).

**In situ hybridization.** Embryos at 24 hpf were dechorionated and fixed in buffers with 4% paraformaldehyde (PFA) at 4°C overnight. Whole-mount in situ hybridization was carried out as described by Westerfield (1994) with modifications (Cheng et al., 2000). Briefly, antisense RNA was synthesized by linearizing the plasmid and transcribing with T7 polymerase and Digoxigenin-11-UTP (Roche, Basel, Switzerland). Embryos were transferred to methanol and stored at −20°C to increase permeability. They were then washed and digested with 10 μg/ml proteinase K in PBS (phosphate-buffered saline) with 0.1% Tween 20 before incubating with the antisense probes at 55°C overnight. Following hybridization, probes were removed with high-stringency washes, 2 × SSC and 0.2 × SSC each twice for 30 min, at 62°C. Embryos were subsequently incubated with preabsorbed sheep anti-digoxigenin-alkaline phosphatase Fab fragments (Roche, Basel, Switzerland) for 2 h at room temperature on a nutorator. After washing, 5-bromo-4-chloroindolyl phosphate was added as substrate and nitro blue tetrazolium as coupler (Roche, Basel, Switzerland). We constructed plasmids containing cDNA probes for myoD (AF318503) and myogenin (AF202639) from the published nucleotide sequences in GenBank. We obtained plasmids containing cDNA probes for smbpc and myh5 from Dr. Z. Gong (Xu et al., 2000).

**Immunohistochemistry.** Embryos were collected at the end of treatment (at 24 hpf), washed three times, and transferred to fresh culture medium without cadmium for further incubation. At 30 or 48 hpf, embryos were dechorionated and fixed in buffers with 4% pfa at 4°C overnight.

Whole-mount immunostaining with antibodies was performed according to the method described (Westerfield, 1994) and (Cheng et al., 2000). Embryos were frozen at −20°C in acetone to permeabilize the tissues before treating with 2% goat serum in PBS/BSA/dimethyl sulfoxide (DMSO) buffer for 30 min to block non-specific binding sites. Specifically, the PBS/BSA/DMSO buffer was PBS (0.1% NaCl, 0.02% KCl, 0.02M PO4 [pH 7.3]) with 1% BSA and 1% DMSO. Embryos were then incubated in primary antibodies at 4°C overnight. After thorough washing, embryos were incubated for 30 min at room temper-
nature in biotinylated goat antimouse secondary antibody that was pre-blocked with zebrafish embryo powder (DAKO, Carpenteria, CA). Horseradish peroxidase–Streptavidin was added before incubation of embryos with chromogenic substrate (diaminobenzidine and hydrogen peroxide). Staining reaction was stopped by PBT (PBS, 0.1% Tween 20).

Culture supernatants against the following marker genes were obtained from the Development Studies Hybridoma Bank (University of Iowa, IA): 39.4D5 for Isl-1 (1:200; Inoue et al., 1994), Znt-1 (1:100; Trevarrow et al., 1990), and Znp-1 (1:200; Trevarrow et al., 1990); and from the Oregon Monoclonal Antibody Centre: Zn-5 (1:100; Fashena and Westerfield, 1999).

**Statistical analysis.** All data were presented as the mean ± SEM, which is indicated by bars in the figures. Significant difference between control and different cadmium-exposed groups was determined by one-way ANOVA with post hoc analysis using Dunnett’s multiple comparison tests (p < 0.05).

**RESULTS**

Exposure to cadmium during gastrulation causes trunk malformations. In order to investigate the timing of the critical period of exposure that would cause trunk malformations, we exposed embryos to cadmium at 100 μM during the gastrulation period only, during the segmentation period only, or during both periods. At 24 hpf when we scored for trunk malformations, untreated control embryos presented straight trunks while cadmium-exposed embryos presented distorted body axes with sideways curvatures of the trunk (Fig. 1). We termed this trunk malformation specifically as “altered axial curvature” here. The somites in the cadmium-treatment embryos lost the typical chevron v-shape and were packed in a disorderly manner.

It appeared that exposure to cadmium during the gastrulation period, before the formation of the first morphologically distinct somite, was critical in the induction of trunk malformations in the form of altered axial curvatures (Fig. 2). There were 11.92 ± 2.53% of embryos developed altered axial curvatures when they were exposed to cadmium from 4 hpf to 10.5 hpf only. These embryos were placed in culture medium devoid of cadmium during the segmentation period from 10.5 to 24 hpf, when formation of distinct somites took place and yet they still developed trunk malformations. Exposure to cadmium during the segmentation period, from 10.5 to 24 hpf when somites were formed, also induced altered axial curvatures albeit at lower percentages at 4.23 ± 1.87%. Exposure to cadmium during both gastrulation and segmentation periods induced the highest incidence of trunk malformations at 15.00 ± 2.88%. Nonetheless, exposure to cadmium caused significant increases in incidence of altered axial curvatures in the three treatment regimens tested when compared to the controls where only 2.69 ± 2.59% displayed trunk malformations.

**FIG. 1.** Lateral views of control (A) and cadmium (Cd)-exposed (B) embryos at 24 hpf. Viewed with DIC optics, anterior to the left; nt, notochord; s, somites. Scale bar, 250 μm; in inset, 1000 μm.

**FIG. 2.** Frequency (%) of altered axial curvature in zebrafish embryos after exposure to cadmium. Data are mean ± SEM (vertical bars); **p < 0.01; *p < 0.05.
Expression of myogenic regulatory factors (MRF) in malformed trunks. In zebrafish, the major part of the somites is the myotome. We examined the expression patterns of two myogenic basic helix-loop-helix transcription factors involved in regulating muscle cell differentiation, namely, myoD and myogenin, by in situ hybridization (Weinberg et al., 1996). In untreated embryos without trunk malformations, both genes were expressed as prominent bands in the middle of each chevron-shaped somitic hemisegment, with strongest expression detected in the tail region (Figs. 3A and 3E). The notochords were seen clearly at the midline under the apex of the somite arrows. Dorsal views of these embryos showed the orderly patterns of myoD and myogenin expression as parallel lines of myotomes flanking the notochord along the trunk (Figs. 3C and 3G). In cadmium-treated embryos with altered axial curvature, expressions of both genes were detected in the somites, with the tail region showing the highest levels of expression (Figs. 3B and 3F). The staining patterns in these lateral views revealed that the somites had lost the typical chevron shape and become closely packed. Nonetheless, all the somites had clear anterior-posterior boundaries and no fusion of somites was detected. The notochords, however, were not readily visible in the midline of the body. Dorsal views of these embryos showed that the myogenic tissues expressing these two genes flanking the notochord in the trunk region and merged when the notochord did not extend to the tip of the tails (Figs. 3D and 3H). Taken together, it appears that exposure to cadmium does not alter the dynamic patterns of expression of the MRF genes nor the specification of muscle-cell fate. The expression of the MRF in cadmium-treated embryos, hence, accounts for the successful somite formation even though the somites are formed in an irregular pattern.

FIG. 3. Localization of myoD and myogenin (mgn) transcripts in control and cadmium (Cd)-exposed embryos at 24 hpf. Lateral views (A, B, E, F) and dorsal views (C, D, G, H) with anterior to the left. Arrow heads indicate somites in typical chevron shape (black) or in atypical shape (white). Black arrows indicate strips of myogenic tissues flanking the notochord in control embryos. White arrows indicate merging of myogenic tissues in the caudal region where the notochord fail to extend in Cd-exposed embryos; nt, notochord. Scale bar, 500 μm.
Cadmium affects muscle-type development. Myotome in zebrafish consists mainly of two classes of muscle fibers: namely, the fast and the slow muscle fibers. We investigated the formation of muscle fiber types using markers specific for the fast muscle (myhz) and the slow muscle (smbpc), respectively (Xu et al., 2000). In untreated control embryos, the majority of the somite was composed of fast muscle fibers, which were found in the deep portion of the myotome. Expression of the fast muscle marker myhz gene revealed the typical chevron-shaped myotome hemisegments in the lateral view (Fig. 4A) and the longitudinal myotomes flanking the notochord in the dorsal view (Fig. 4C). The fast muscles appeared as continuous blocks along the straight trunk and tapered near the tail region. In cadmium-treated embryos with altered axial curvature, disorganized patterns of fast muscle formation could be observed in the lateral and dorsal views (Figs. 4B and 4D). More notably, however, were the gaps of staining denoting loss of fast muscle fibers in the posterior region of the trunk. Loss of fast muscle was detected in 65.7 ± 7.0% of embryos showing altered axial curvature. This observation reveals that in these regions of the trunk, blocks of fast muscle fail to form. Moreover, the region lacking fast muscle formation correlates to the curvature of the distorted body axis.

The slow muscles form as a superficial monolayer on the surface of the myotome and surround the fast muscle fibers. Absence of slow muscle in regions of the trunk was also detected in cadmium-exposed embryos (Fig. 5). Expression of the slow muscle marker, smbpc, revealed a staining pattern resembling 2 parallel thin lines along the trunks of the untreated control embryos in the dorsal view (Fig. 5A). In cadmium-treated embryos with trunk malformations, unilateral or bilateral loss of slow muscles was detected (Figs. 5B and 5C). Loss of slow muscle could be detected in 85.8 ± 9.0% of embryos displaying altered axial curvature. Interestingly, the regions lacking slow muscle did not necessarily correlate to the abnormal curvature in these embryos. For example, loss of slow muscle could occur at the posterior region of the trunk (Fig. 5B) or the anterior region of the trunk (Fig. 5C).

Cadmium causes motor axon defects. We examined whether cadmium exposure would interfere with the innervation of somitic muscle blocks by distinct motor neurons using immunostaining with antibodies specific for antigens found on different motor neurons. The antibody Islet-1 (Is1-1) recognized two types of primary motor neurons, namely, the MiP and the RoP situated at the ventral part of the spinal cord as
well as the dorsally located Rohon-Beard (RB) neurons (Figs. 6A, 6B, and 7A). Cadmium did not affect the development of primary motor neurons as the distribution and the abundance of these cells in the spinal cord of cadmium treated embryos with normal trunk formation (Fig. 6B) and with altered axial curvatures (Fig. 7A) appeared to be similar to that in the untreated

**FIG. 6.** Whole-mount immunohistochemistry showing axon growth in control and cadmium (Cd)-exposed embryos. The antibodies Isl-1 stains for primary motoneurons (A,B); Znp-1 for primary motoneurons and their axons (D,E); Zn-5 for secondary motoneurons and their axons (G,H). Lateral views with anterior to the left. White asterisks indicate both ends of a bifurcated primary motor axon in a cadmium treated embryo at 30 hpf (F). White arrows indicate stalled secondary motor axons in a cadmium-treated embryo at 48 hpf (H). A schematic diagram of the corresponding trunk segments showing primary motoneurons (C); primary and secondary motor axon pathways (F, I). The spinal cord is shaded light blue, the notochord yellow. The layer in between is the floor plate. Primary motoneurons are the small brown cells situated ventrally near the notochord while the large ones are the dorsally located Rohon-Beard neurons (C). CaP (green), MiP (orange) and RoP (blue) cell bodies reside in the spinal cord (F). Their axons leave the spinal cord at the same ventral root and transverse along a common path. At the horizontal myoseptum (dashed line), they pause at the trajectory point and then diverge along axon-specific pathways. Three populations of secondary motoneurons are the CaP-like (light green), the MiP-like (yellow) and the RoP-like (light blue) reside in the spinal cord, and their axons follow the common path pioneered by the primary motoneurons before diverging out to their own axonal pathway at the trajectory point located at the myoseptum (I); mn, primary motoneurons; rb, Rohon-Beard neurons. Scale bar, 200 μm.

**FIG. 7.** Whole-mount immunohistochemistry showing axon growth in cadmium (Cd)-exposed embryos with altered axial curvatures. The antibodies Isl-1 stains for primary motoneurons (A); Znp-1 for primary motoneurons and their axons (B); Zn-5 for secondary motoneurons and their axons (C). Lateral views with anterior to the left. White arrows denote loss of primary motor axon outgrowth (B) and loss of secondary motor axons (C) in the caudal body where altered axial curvatures occurred. Black arrows denote normal extension of motor neuron axons. Scale bar, 200 μm.
control (Fig. 6A) when the embryos reached 30 hpf. At this stage of development, the primary motor neurons have completed the axonogenesis to their respective axial muscle targets. Briefly, motor growth cones from 3 types of primary motoneurons migrate along a common path from the spinal cord to the nascent horizontal myoseptum, termed the trajectory point, before continuing on divergent paths to ventral, dorsal, and medial myotomal regions of the somite. The antibody Znp-1 labeled the primary motoneurons and revealed the common axonal path as well as the projections into ventral and dorsal somitic muscle blocks in embryos at 30 hpf (Figs. 6D, 6E, and 7B). In untreated controls, the common path of axon outgrowths from the spinal cord and the projection of the CaP axon into the ventral muscle block are revealed against the clear background of the notochord (Fig. 6D). The dorsal projection of the MiP and the rostral projection of the RoP were not clearly visible on this focal plane. Cadmium exposure injected the MiP and the rostral projection of the RoP were revealed against the clear background of the notochord (Fig. 6D). The dorsal projection of the MiP and the rostral projection of the RoP were not clearly visible on this focal plane. Cadmium exposure appeared to disrupt this process as some of the CaP axons stalled at the distal end of the cell-type-specific path, before continuing on divergent paths to ventral, dorsal, and medial myotomal regions of the somite. The antibody Znp-1 labeled the primary motoneurons and revealed the common axonal path as well as the projections into ventral and dorsal somitic muscle blocks in embryos at 30 hpf (Figs. 6D, 6E, and 7B). In untreated controls, the common path of axon outgrowths from the spinal cord and the projection of the CaP axon into the ventral muscle block are revealed against the clear background of the notochord (Fig. 6D). The dorsal projection of the MiP and the rostral projection of the RoP were not clearly visible on this focal plane. Cadmium exposure appeared to disrupt this process as some of the CaP axons stalled at the distal end of the cell-type-specific path, became stunted, and bifurcated in the ventral muscle block in embryos with normal trunk development (Fig. 6E). In cadmium-treated embryos with altered axial curvatures, the primary motor neuron axons showed very limited extension into the myotome in regions of trunk malformation (Fig. 7B). There were 10.67 ± 0.58% of all cadmium-treated embryos, regardless of their trunk curvatures, displaying stunted or bifurcated primary motor axons. The secondary motoneurons develop later and there are 30–40 of them in each somatic hemisegment. Their axons enter and complete the common path set out by the primary neurons as one nerve. When primary motor neurons are ablated, the extension of secondary motor axons to their targets is delayed (Pike et al., 1992). At the trajectory point, the secondary motoneurons become three subpopulations and their axons migrate along divergent axonal paths ventrally, dorsally, or rostrally, just like their primary motoneuron counterparts. The antibody Zn-5 labeled secondary motoneurons specifically (Figs. 6G, 6H, and 7C). At 48 hpf, secondary motoneurons completed their axonal migration along the common path and the axons of the ventral nerve extending to the ventral myotome could be clearly seen in this focal plane (Fig. 6G). In cadmium-treated embryos with normal trunk development, axons of the secondary motoneurons either stalled at the distal point of the common path or stalled at the trajectory point, failing to reach the ventral myotome at 48 hpf (Fig. 6H). In cadmium-treated embryos with altered axial curvatures, secondary motor neuron axonal growth was not observed at 48 hpf either (Fig. 7C). Of all the cadmium-treated embryos, 12.67 ± 2.08% displayed stalled growth of their secondary motor axons.

In both classes of motor neurons, axon growth inhibitions were detected in all cadmium-treated embryos, i.e., even in embryos without trunk malformations. Cadmium-treated embryos with normal trunk formation typically showed one to two abnormal axonal growths per embryo (Figs. 6E and 6H) while embryos with severe trunk malformation showed stunted axonal growth in all the affected somites (Figs. 7B and 7C).

Taken together, it appears that exposure to cadmium caused defects in axon growth but not in the cell fate specification nor in the differentiation of motoneurons.

Cadmium affects notochord development. The notochord plays a crucial role in somite patterning (reviewed by Bumcrot and McMahon, 1995). In order to find out whether the observed defects in somitogenesis arose from notochord defects, we examined the morphological features of the notochord in cadmium-treated embryos by in situ hybridization with probes against the myogenic tissues flanking the notochord. In untreated embryos at 24 hpf, the notochord appeared as a straight and rigid rod-like notochord (black dashed lines) extending into the caudal trunk in the control embryo (G) and the failure of the notochord (white dashed lines) to extend to the caudal region in Cd-exposed embryos (H). Scale bar, 200 μm; in inset, 150 μm.
embryos with altered curvature, a variety of notochord malformations could be detected. For example, looping of the notochord in the caudal region caused it to end abruptly and fail to extend to the tail tip (Fig. 8B). Flexure of the notochord was observed in another embryo with disarrayed and interrupted fast muscle fibers (Fig. 8D). Bulging of the notochord was observed when unilateral absence of fast muscle occurred (Fig. 8F). Another common malformation was the bent and undulated notochord forming a knob-like structure in the dorsal region of the trunk (Figs. 3F and 3H).

We performed immunohistochemistry with the antibody Znt-1 to stain for the notochord, specifically (Figs. 8G and 8H). In untreated control embryos at 24 hpf, the notochord can be detected as a rod-like structure throughout the trunk and extended into the tail region with a descending intensity of Znt-1 staining (Fig. 8G). In cadmium-treated embryos with curvature, the notochord looped off and failed to extend into the tail region, as shown by the abrupt loss of Znt-1 staining in the caudal regions (Fig. 8H).

The fate of axial mesodermal cells destined to become notochord was investigated. Where the notochord failed to form in the caudal region, the somitic cells simply committed to the myogenic lineage as they expressed the myoD and myogenin genes instead (Figs. 3D and 3H). Infringement by differentiated muscle cells in the notochord area could also be found in some cadmium-treated embryos (Fig. 4). In untreated controls, the fast muscle fibers appeared as flanking notochord (Figs. 4A and 4C). In cadmium-treated embryos, the fast muscle fibers could be found in the midline mesoderm (Figs. 4B and 4D). Taken together, it appears that the cell fate decision of axial mesodermal cells to notochord may be affected by cadmium and hence some of these cells take up the default pathway and become muscle cells.

DISCUSSION

The morphologically defective trunk in fish embryos is one of the most frequently reported malformations in developmental toxicity of metals (reviewed in Weis and Weis, 1990). In particular, trunk malformations following exposure to cadmium have been reported in fish species such as bluegill, medaka, flathead minnow, rainbow trout, salmon, garpike, carp, and zebrafish. Our previous report has indicated that loss of myosin heavy chain was found in regions of altered axial curvature, implicating the developing somites as targets of cadmium toxicity (Cheng et al., 2000). In this report, we made a catalogue of the events being affected by cadmium during somitogenesis and studied the possible mechanisms of cadmium-induced developmental toxicity. Our study here represents one of the first reports on when and where cadmium may exert its toxic effects on the developing somites. In summary, we showed that the critical period of exposure was the gastrulation period, which actually preceded the formation of the first morphologically distinct somites. The somites thus formed lose the typical chevron V-shape and are packed disorderly. The myogenic lineage commitment of the axial mesodermal cells was not affected as the myogenic regulatory transcription factors were expressed normally. There were, however, losses of fast and slow muscle fibers in the myotomes. We also investigated the innervation of the muscle blocks by spinal motoneurons, an important process of the somitogenesis. Both primary and secondary motoneurons appeared to form normally while the axon growth was affected in cadmium-treated embryos. The notochord, an organ essential in the patterning of the somites and the central nervous system, showed abnormal morphological features and failed to extend to the tail region.

We are, however, aware that the observed effects could either be direct actions of cadmium in interfering with various cellular mechanisms, possibly by replacing other divalent ions such as Ca$^{2+}$ or Mg$^{2+}$, or indirect actions of cadmium on inductive signaling molecules important in embryonic patternning and cell-fate specification.

Myogenic commitment and muscle type diversification. Myotomes are the major components of each somite. The process of myogenesis in embryonic development is a multi-step event involving the specification, differentiation, and migration of different cell types. We examined two landmark events in this study: namely, the commitment and the diversification of myogenic cells.

We found that the commitment to myogenic lineage by the axial mesodermal cells was not affected (Fig. 3). Axial mesodermal cells commit to the muscle lineage when they express the myogenic regulatory factors (reviewed in Currie and Ingham, 1998). These transcription factors are specific to skeletal muscle and bind to DNA. These factors regulate the expression of muscle structural genes such as myosins. Our data shows unchanged expression of myoD, which involves early muscle differentiation, and of myogenin, which is expressed more broadly at a later stage (Fig. 3).

The formation of fast and slow fibers, however, was affected (Figs. 4 and 5). The bulk of the myotome is composed of fast muscle fibers and the slow muscle forms only a monolayer surrounding the fast muscles in zebrafish. Myosins are hexameric molecules composed of two heavy chains and two light chains. They can be found in both fast and slow muscles. We showed gaps of missing fast muscle fibers in regions of altered curvatures when we labeled the fast skeletal muscle myosin heavy chain with the marker myhz (Fig. 4). Taken together, the cause of the loss of myosin heavy chain observed in cadmium-treated embryos in our previous study might be attributed mainly to the loss of fast muscle fibers but not to failures in myogenic commitment.

Axon growth. Zebrafish motoneurons follow stereotyped pathways and project to appropriate regions of the myotome by integrating various cues (reviewed in Eisen, 1994 and in Beatte, 2000). The primary motor neurons are born at the end of gastrulation at around 10 hpf and the axonal growth takes place
from 18 to 24 hpf. In this study, we showed that exposure to cadmium when these processes were taking place only affected the axonal growth, but not the formation of primary motor neurons (Figs. 6 and 7). Axons of the secondary motor neurons start to extend at approximately 26 hpf. They enter the common pathway pioneered by the primary motor axons, suggesting that the growth cones of secondary motor neurons might require the axons of the primary motor neurons to facilitate path finding (reviewed in Eisen, 1994). Ablation of primary motor neurons caused delayed axonal growth of the secondary motor neurons (Pike et al., 1992). In this study, we observed stalling of the axonal extension of secondary motor neurons in cadmium exposed embryos at 48 hpf, even though the process of axonal extension occurred when the embryos were no longer exposed to cadmium (Figs. 6 and 7).

The growth cone at the tip of an embryonic axon samples its local environment and makes decisions on where to navigate by motile responses. Calcium is recognized as the key second messenger in growth cone extension and in growth cone turning (reviewed in Doherty et al., 2000). Specifically, changes in the intracellular and extracellular Ca$^{2+}$ concentrations induce different turning responses of the growth cones (Zheng, 2000). We could only speculate here that the presence of cadmium might cause imbalances of the Ca$^{2+}$ concentrations, which in turn lead to failures of some motoneuron axons to reach the designated targets in the myotome (Fig. 6).

Molecular cues, which can be repulsive or attractive and act at short or long ranges, are also crucial in proper axon growth (reviewed in Beattie, 2000). Numerous classes of molecules have been identified and they include cell adhesion molecules, semaphorins, netrins, receptor phosphatases and Eph receptor protein tyrosine kinases. The divergent nature of cadmium ion could enable it to replace Mg$^{2+}$ in events involving phosphatases and kinases.

On the other hand, zebrafish mutants stumpy, unplugged, and diwanka show defects such as stalled or bifurcated axons (reviewed in Beattie, 2000). The functional roles of these genes have not been fully revealed. Similar defects are also observed in cadmium-treated embryos (Figs. 6E and 6H). We cannot postulate whether cadmium interacts with them directly to inhibit their functions or whether these genes utilize calcium as the messenger to mediate their effects on axon growth. Our report, however, provides a tentative link to the toxic effects of cadmium to embryonic axon growth in vivo. Other toxicants, such as ethanol, have been shown to disrupt central neuron migration in vitro (Liesi, 1997).

**Cadmium affects induction of somitogenesis.** Exposure to cadmium during the induction, rather than the formation, of the somites is more critical (Fig. 2). The gastrulation period denotes the formation of the notochord and the notochord precursor cells begin to express signaling molecules that exert patterning influences on the surrounding mesoderm. The notochord is the central organ in the induction of somitogenesis as analyses of zebrafish mutants defective in notochord formation invariably have disrupted mesoderm development, such as formation of U-shaped somite, lack of horizontal myosepta, and see review in Currie and Ingham (1998). These mutant studies reveal that a stepwise series of inductive events originating from the notochord act to pattern the somite. We observed malformed notochord with a variety of phenotypes such as looping, bulging, and flexures in cadmium-treated embryos (Figs. 1, 3, and 8).

It is also of interest to note that analyses of the zebrafish mutants reveal that cells fated to become notochord would only do so when muscle differentiation of these cells is suppressed and that the muscle development pathway is the default route for these midline cells (Amacher and Kimmel, 1998; Halpern, et al., 1995). In our observed data, exposure to cadmium caused some of the notochord cells to become muscle cells (Figs. 3D, 3H, 4B, and 4D).

Taken together, it appears that cadmium may exert its actions on the notochord during the gastrulation period, skews the developmental pathways of this organ resulting in morphological changes, and consequently, perturbs the inductive activities of this organ. Exposure to cadmium during the segmentation period, on the other hand, might affect other developmental mechanisms involved in formation of embryonic axis. For example, the convergence and extension cell movements during segmentation continue to shape the embryonic body by narrowing and lengthening the body axis (reviewed in Myers et al., 2002). This process involves the noncanonical Wnt pathway which utilizes the intracellular calcium as the second messenger (Slusarski et al., 1997). The additional effect on trunk malformation when the embryos were exposed during the segmentation period might due to perturbations to the Wnt signaling pathway mediated by the intracellular calcium.

**Somitogenesis and motility.** Our data showed that muscle cell type differentiation and motor axonal growth were affected in cadmium-treated embryos. We have also examined the physiological consequences in terms of locomotor activities. We touched the heads or tails of 24 hpf and 48 hpf embryos. Untreated controls displayed vigorous rapid coiling at 24 hpf and swam away swiftly at 48 hpf, as described in Saint-Amant and Drapeau (1998). Cadmium-treated embryos with normal trunk curvature displayed much less vigorous coiling at 24 hpf and swam away slowly at 48 hpf. On the other hand, cadmium-treated embryos with altered axial curvatures did not respond to touching at all. In other words, they did not coil nor swim...
away upon touching. It appears that the combination of losing muscle fibers and abnormal axon growth result in defects in locomotion and might have consequence to the long-term survival of these cadmium-treated embryos.

Signaling molecules. A number of molecules secreted by the axial structures, in particular the notochord and the neural tube, have been implicated in the control of myogenesis (reviewed in Currie and Ingham, 1998). Many of them belong to the Hedgehog and WNT families. In our previous report, we found that the expression of the signaling molecule shh was not perturbed in the trunks of embryos with altered axial curvature (Cheng et al., 2000). In this report, we provide evidence that some of developmental processes mediated by the shh molecule are not affected by cadmium while others are.

The formation of primary motoneurons required signaling of shh and its related members (Lewis and Eisen, 2001). We observed unchanged numbers of 2 subsets of primary neurons in cadmium-treated embryos, denoting lack of perturbation of shh-induced processes (Fig. 6B). On the other hand, signaling from shh and other Hedgehog family members were essential for slow muscle development (Blagden et al., 1997; Lewis et al., 1999). We observed bilateral and unilateral losses of slow muscle fibers in cadmium-treated embryos (Fig. 5B and 5C). Therefore, the loss of slow muscle fibers could be attributed partly to ectopic functions of the shh and its related members.

The shh signaling pathway is comprised of many mediators and quite a number of them are known to be involved in specific processes of somitogenesis. The presence of cadmium, although it did not influence shh expression, might perturb the functions of these mediators by various mechanisms. We can only postulate on some of the possibilities here. For example, most of Hedgehog signaling leads to the activation of transcriptional effectors that are zinc finger-containing proteins of the Gli family. The metal binding sites in other zinc finger binding proteins have been shown to be attractive targets for cadmium to bind in competition with Zn\(^{2+}\) (Makowski and Sunderland, 1992).

Tissue body burden. In this study, we set the exposure concentration at 100 μM cadmium as this concentration was found to induce total adverse effects in just under 40% of the embryos exposed to cadmium from 4.5 to 28 hpf (Cheng et al., 2000). We needed to gather enough embryos with malformations before we could proceed to determine the molecular mechanisms using in situ hybridization and immunohistochemistry. While this concentration of cadmium can hardly be found in nature, our experimental setting is in line with other tissue culture studies on the molecular mechanisms of cadmium toxicity at μM ranges (Alvarez-Barrientos et al., 2001; Bagchi et al., 2000; Chao and Yang, 2001; Dong et al., 2001; Hader et al., 1996).

We performed chemical analyses on the embryos to determine the cadmium concentrations. We found that the tissue body burden of cadmium was 6.867 ± 0.332 ppm from 700 embryos exposed to 100 μM cadmium from 4 to 24 hpf. In contrast, the tissue body burden of control embryos was 0.554 ± 0.012 ppm from the same number of embryos. The embryos examined were wet and we did not obtain an estimate of the dry weight from each batch of 700 embryos.

Zebrafish as a model organism of developmental toxicology. Fish embryos from different species have been used successfully in screening tests for aquatic toxicity assessment. The obvious advantages include the large numbers of embryos that can be obtained for better data analysis and the transparency of the fish embryos for morphological and functioning examinations. This report, however, illustrates additional advantages afforded by studying zebrafish embryos. The availability of marker gene probes and antibodies has enabled us to identify the affected morphological components, such as the muscle fibers, the axons, and the notochords. The wealth of information on the events in somitogenesis obtained from mutant analyses helps us to postulate on the respective molecular cues being affected by cadmium. The trunk malformation is among the most prominent deformities observed in fish embryos. The techniques employed in this study could well be used to study the developmental toxicology of other pollutants. It will be interesting to compare whether other toxicants perturb similar or different events in somitogenesis. With that, we will be able to devise read-out systems on morphological and molecular changes to assess toxicant-specific and -nonspecific effects.

ACKNOWLEDGMENTS

We thank Dr. Z. Gong, National University of Singapore, for providing us generously with muscle type-specific probes. The work described here was fully supported by a grant from the Research Grants Council of the Hong Kong SAR, China (Project # CityU 110901M).

REFERENCES


CADMIUM AFFECTS ZEBRAFISH SOMITOGENESIS


