Correlation of 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Apoptotic Cell Death in the Embryonic Vasculature with Embryotoxicity

Susannah M. Cantrell,*† Jennifer Joy-Schlezinger,‡ John J. Stegeman,‡ Donald E. Tillitt,† and Mark Hannink*†  

*Department of Biochemistry, University of Missouri–Columbia, Columbia, Missouri 65212; †Midwest Science Center, National Biological Service, Department of the Interior, Columbia, Missouri, 65201; and ‡Department of Biology, Woods Hole Oceanographic Institute, Woods Hole, Massachusetts 02543

Received April 14, 1997; accepted September 15, 1997

Vertebrate embryos are particularly sensitive to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Identification of tissues that are susceptible to the adverse effects of TCDD is requisite for understanding the embryo toxic effects of TCDD. The objective of the present study was to quantitate the temporal appearance of and dose dependence of apoptosis in TCDD-exposed medaka embryos (Oryzias latipes). A fluorescent-based DNA end-labeling assay provided a sensitive method for detection of TCDD-induced apoptosis in tissue sections of medaka embryos. Apoptotic cells were readily apparent in the medial yolk vein at all observed embryonic stages in TCDD-exposed embryos. Slope-comparison analysis indicated that TCDD-induced programmed cell death in the embryonic medial yolk vein was mechanistically linked to embryo mortality. These data are consistent with the hypothesis that vascular damage contributes to the acute embryo toxic effects of TCDD. However, as sublethal concentrations of dioxin-like compounds are more typical of environmental exposures, tissue damage was also assessed in medaka fry that were exposed to low doses of TCDD during embryonic development. Cell death was detected in gill and digestive tissues in visibly healthy medaka fry that had been exposed to low doses of TCDD during embryonic development. Cell death was also assessed in medaka fry that were exposed to low doses of TCDD during embryonic development. Cell death was detected in gill and digestive tissues in visibly healthy medaka fry that had been exposed to low doses of TCDD during embryonic development. Increased expression of cytochrome P450 1A 1 is a major biochemical consequence of TCDD exposure and is often used as a biomarker for exposure to dioxin-like compounds. Therefore, we compared the tissue distribution of TCDD-induced P450 1A expression and TCDD-induced programmed cell death. TCDD-induced programmed cell death co-localized with TCDD-induced P450 1A expression in both embryos and in visibly healthy post-hatch fry. Our results suggest that aberrant programmed cell death may be a suitable marker for exposure of feral organisms to dioxin-like compounds.

TCDD is the prototypic member of a group of widespread environmental contaminants generally described as planar heteroatomated hydrocarbons (PHHs). Most of the toxic effects of TCDD are mediated by a cytosolic receptor complex, the Aryl hydrocarbon Receptor (AhR) (Fernandez-Salguero et al., 1996). The presence of PHHs in the environment is of concern, as PHHs are toxic to a wide range of vertebrate species (Lindstrom et al., 1995; Peterson et al., 1993; Olson et al., 1990; Kleeman et al., 1988; McConnell, 1980). Although there are species differences in the degree of susceptibility to TCDD exposure, the embryonic stage of development is the most sensitive life stage for most species to the toxic effects of TCDD (Zabel et al., 1995; Peterson et al., 1993; Spitsbergen et al., 1991; Wisk and Cooper, 1990). Vascular damage is the most pronounced adverse effect of TCDD exposure during embryonic development of fish and avian species (Peterson et al., 1993). Persistent vascular hemorrhaging and pericardial edema are key morphologic indicators that vascular function is compromised in the developing embryo (Henry et al., 1997; Qinney et al., 1997; Spitsbergen et al., 1991; Wisk and Cooper, 1990). In fishes, TCDD-induced vascular lesions increase in severity as the embryo develops, ultimately resulting in decreased blood flow and concurrent regression of the vascular tissue (Henry et al., 1997; Qinney et al., 1997). Supporting the contention that vascular damage is a key physiological mediator of the embryo toxicity of TCDD, vascular damage, as assessed by TCDD-induced apoptotic cell death in the medial yolk vein, has been demonstrated in medaka embryos prior to the appearance of visible vascular lesions (Cantrell et al., 1996). Taken together, these data suggest the hypothesis that TCDD-induced vascular damage is causally linked to the embryo toxicity of TCDD. In the present study we further address this hypothesis by establishing dose-response curves for TCDD-induced apoptotic cell death in the medial yolk vein of TCDD-exposed medaka embryos.

Although embryo mortality is often the most sensitive of the observable adverse effects of TCDD exposure in fish, sublethal effects are almost certain to occur. For example, rainbow trout eggs exposed to graded doses of a complex mixture of PHHs developed yolk sac edema and subcutaneous hemorrhaging in the surviving organisms (Walker and Peterson, 1994; Walker et al., 1992; Spitsbergen et al., 1991; Walker et al., 1991).
Additionally, behavioral alterations, such as abnormal predator avoidance, have been observed in newly hatched fry that were exposed to low sublethal doses of dioxin-like compounds during early life stages (Mehrle et al., 1988). Behavioral alterations may not be directly lethal to the individual organism. However, the inability to avoid predation or to reproduce successfully would affect the overall success of a feral population. Concentrations of PHHs in the environment are often below the threshold for acute embryo mortality, yet the potential for sublethal doses of these chemicals to cause population level effects is almost wholly unknown. Therefore, a further objective of these studies was to determine the tissue specificity of programmed cell death in posthatch fry that were exposed to low doses of TCDD as embryos.

The final objective of these studies was to determine the tissue responsiveness of P450 1A expression in TCDD-exposed medaka embryos. The expression of P450 1A is regulated through the AhR and is often used as a biomarker of PHH exposure (Hahn et al., 1996). Furthermore, as elevated P450 1A expression has been implicated in oxidative stress (Park et al., 1996) and increased oxidative stress has been linked to the embryo toxicity of TCDD towards medaka embryos (Cantrell et al., 1996), it was important to determine the relationship between induction of P450 1A expression and the induction of programmed cell death in TCDD-exposed medaka embryos.

**EXPERIMENTAL PROCEDURES**

**Maintenance of medaka breeding cultures and egg rearing.** The medaka (Oryzias latipes) breeding stocks were housed in 10-gallon flow-through glass tanks with a male:female ratio of 4:1. The water temperature was maintained at 25–28°C and artificial light was provided with a photoperiod of 16 h light:8 h dark. All adults were fed twice daily with Wardly Spectra IV artificial flake food and freshly hatched brine shrimp (Artemia salina). The eggs were stripped daily from each female within 2 h postfertilization, separated, and placed into a petri dish containing tank water. Eggs were examined individually under a microscope and unfertilized eggs were discarded.

A water-bath dosing method was used to expose the medaka eggs to TCDD. The eggs were individually placed into wells of a 12-well culture slide containing 10 μl of tank water per well. The culture slide was placed inside a 100-mm petri dish containing a water-saturated filter paper to minimize evaporation. One microliter of iso-octane containing TCDD was added to the

![FIG. 1. Mortality curves for Medaka embryos and fry following exposure to TCDD. Newly fertilized medaka embryos were exposed to TCDD with waterbath concentrations of TCDD that resulted in total egg uptake of; 0, 1.5, 2.9, 5.8, 11.7, and 17.8 ng TCDD/g egg. Cumulative mortalities were quantitated at embryonic stage 33 (A), at hatch (B), and at 3 days posthatch (C). The quantitative data for development of pericardial sac edema and vascular hemorrhaging at 3 days posthatch is also shown (D). The mean mortalities and lesions from three separate experiments (N = 12 for each experiment) are indicated by the rectangles. The standard deviation from the mean is indicated by the thick bars in A–C and by the numbers above the bars in D. Student’s t tests were used to determine if each dosing group was statistically distinct from the untreated sample group. The confidence interval was set to 95. *Treatment groups with a p value of <0.05 relative to the untreated group.](image-url)
individual wells. The embryos were incubated in the TCDD exposure solution for 2 h at 29°C and then were removed and placed into a 100-mm petri dish containing 20 ml of tank water at a density of ~20 eggs per dish. The exposure concentrations of TCDD were 20, 10, 5, 2.5, 1.25, and 0 pg of TCDD/μl of tank water. The dose of TCDD in each egg from these water concentrations was estimated from experiments in which the incorporation of [3 H]-TCDD into medaka eggs by the water bath dosing method was measured (P. J. Wright and D. E. Tillitt, unpublished data). As the incorporation of [3 H]-TCDD into medaka eggs is too low to be measured for a single egg, 12 embryos that were individually exposed to each dose of [3 H]-TCDD were pooled and total [3 H]-TCDD incorporation in the pool was measured by liquid scintillation. The means ± SD of [3 H]-TCDD into medaka eggs were 17.8 ± 8.3 pg/g egg for the 20-pg/μl dose; 11.7 ± 6.9 pg/g egg for the 10-pg/μl dose; 5.8 ± 3.5 pg/g egg for the 5-pg/μl dose; 2.9 ± 1.7 pg/g egg for the 2.5-pg/μl dose; and 1.5 ± 0.9 pg/g egg for the 1.25-pg/μl dose. Using this method, the actual exposure of the embryo to TCDD presumably occurs during utilization of the yolk sac reserves during development.

Four distinct stages of medaka development were chosen for selection and analysis of the developing embryo. The named stages are according to the staging suggested by Iwamatsu, (1994). The first developmental stage selected for analysis was stage 26. This stage is identified by development of vitelline vasculature. Our previous studies demonstrated that stage 26 was the earliest developmental stage in which microscopic vascular lesions could be identified in TCDD-exposed medaka embryos (Cantrell et al., 1996). Embryos at developmental stage 33 are characterized by development of the gill and digestive organs. Stage 33 marks the onset of macroscopic lesions, including hemorrhaging and pericardial sac edema, in TCDD-exposed embryos. Embryos at stage 40 are characterized by hatching of the embryo from the chorionic membrane and by a marked increase in utilization of yolk sac reserves. Embryonic development is essentially complete at hatch. Three days posthatch was selected as the final stage of development for this study, as there is a marked increase in mortality of TCDD-exposed embryos at 3 days posthatch (Cantrell et al., 1996). Furthermore, the yolk sac reserves are typically depleted by 3 days posthatch and exogenous feeding is required to sustain the fry beyond 3 days posthatch.

The presence of a heartbeat, the presence of an intact pericardial sac, and the presence of circulating blood were used to assess the viability of TCDD-exposed embryos and fry. Embryos that did not meet all three criteria were scored as nonviable and were not utilized for subsequent histochemical analysis. Medaka eggs and fry were collected and preserved in 4% paraformaldehyde at developmental stages 26, 33, hatch, and 3-days posthatch for histochemical analysis. Following tissue fixation in 4% paraformaldehyde, the eggs were washed in 10 mM Hepes (pH 7.4) and were dehydrated by immersion in solutions containing increasing concentrations of ethanol from 50 to 100% ethanol followed by immersion in xylene and infiltration with paraffin. The paraffin-embedded samples were placed into plastic molding cassettes, sectioned into 10-μm sections, placed onto silanized slides, and stored at room temperature until analysis. All samples were used within 2 weeks of collection.

**FIG. 2.** Localization of programmed cell death in medaka embryo tissue sections at developmental stage 26. Paraffin-embedded tissue sections were assayed for programmed cell death and representative images were obtained using confocal laser scanning microscopy (CFLSM). Three dosing groups are represented: untreated (A and B), 2.9 ng/g (C and D), and 11.7 ng/g (E and F). Transmitted light images (A, C, and E) and the corresponding confocal images (B, D, and F) are shown. Apoptotic cells in the medial yolk vein are indicated by the arrows in both the transmitted light and confocal images. N, neural tissue; myv, medial yolk vein; Y, yolk.
Paraffin-embedded tissue sections of the medaka were dewaxed and rehydrated by immersion in solutions containing decreasing concentrations of ethanol (100–0%). The rehydrated tissue sections were analyzed for the presence of apoptotic cells by utilizing a terminal transferase-based assay which tags 3’OH DNA strand breaks with a fluorescein-conjugated antibody. As the presence of numerous 3’OH DNA strand breaks is a hallmark of apoptosis (Compton, 1992), positively stained cells were scored as apoptotic. The reagents for the apoptosis detection system were purchased in the form of a kit (Oncor, Gaithersburg, MD). As outlined in the Oncor protocol, the rehydrated tissue sections were washed in phosphate-buffered saline (PBS) and digested with 20 \( \mu \text{g/ml} \) solution of proteinase K (Sigma, St. Louis, MO). The slides were washed in PBS and incubated with a digoxigenin-conjugated

![FIG. 3. Localization of cell death in the anterior portion of the medaka embryo at developmental stage 33. Paraffin-embedded tissue sections were assayed for programmed cell death and representative images were obtained using confocal laser scanning microscopy (CFLSM). Three dosing groups are shown: untreated (A and B), 2.9 ng/g (C and D), and 11.7 ng/g (E and F). Transmitted light images (A, C, and E) and the corresponding confocal images (B, D, and F) are indicated. N, neural tissue; D, digestive tissue. The digestive tissue is indicated by the arrows.](image)

![FIG. 4. Stage-specific quantitation of programmed cell death in the medial yolk vein of medaka embryos treated with TCDD. Newly fertilized medaka embryos were exposed to water bath concentrations of TCDD that resulted in total egg uptake of; 0, 1.5, 2.9, 5.8, and 11.7 ng/g egg. Paraffin-embedded sections were assayed for the presence of apoptotic cells at stage 26 (A) and stage 33 (B). The number of positive apoptotic cells in the medial yolk vein was determined as a percent of the total cells in the medial yolk vein for sections obtained from three separate organisms. The standard deviations are indicated by the thick lines. Student’s \( t \) test was used to determine if each dosing group was statistically distinct from the untreated sample group. The confidence interval was set to 95. *Treatment groups with a \( p \) value of <0.05 relative to the untreated group.](image)
nucleotide in the presence of terminal deoxynucleotidyl transferase (TdT) followed by incubation with a fluorescein conjugated anti-digoxigenin antibody (Compton, 1992; Gorzyca, 1992; Gavioli et al., 1992).

Immunodetection of P450 1A in the paraffin-embedded tissue sections was accomplished using an indirect peroxidase-labeling method. The tissue sections were deparaffinized and hydrated in 1% bovine serum albumin/PBS containing 1% bovine serum albumin. The hydrated slides were incubated in 0.5% H2O2 in methanol for 45 min to block endogenous peroxidase. The hydrated tissue sections were immunochemically stained using monoclonal antibody (mAb) 1-12-3 made against scup P450E as the primary antibody (Park et al., 1986). The tissue samples were observed for peroxidase staining (red-brown deposit) using light microscopy. Companion sections were incubated with a nonspecific monoclonal immunoglobulin G2 (Smolowitz et al., 1991). All sections were counterstained with Mayer’s hematoxylin.

**Image analysis.** The image analysis system consists of a Nikon inverted Diaphot-TMD microscope along with a PT/LPS-220/250 DC power supply and Xenon arc lamp. The microscope was equipped with a Motion Analysis Inc., Dark Invader Night Vision system, designed for low-light microscopy. Optimus software was used for image analysis and data analysis was performed using EXCEL spreadsheet. The fluorescein-based DNA labeling assay and the low-light amplification system allowed for sensitive quantitative analysis of individual cells in the tissue sections of medaka embryos. Images for publication were obtained using Confocal Laser Scanning Microscopy (CFSM) imaging system equipped with a 20× objective lens (N.A. = 0.75). The confocal imaging software was COMOS (BioRad, Fremont, CA). The images were imported into Adobe Photoshop and printed on a Codonics dye sublimation printer. Images for quantitative analysis were obtained using the Optimus 4.0 image grabbing system. There was no image enhancement performed on the analyzed images.

To quantitate apoptotic cell death in the medial yolk vein, the total number of apoptotic cells in a defined area of the medial yolk vein was counted from each image. The total number of apoptotic cells was expressed as percent of the total cells in the defined section of the medial yolk vein. Three separate embryos from at least two independent dosing experiments were used to obtain a mean ± SD for each dose of TCDD.

The slopes for the apoptosis-response curve in the medial yolk vein were visually compared with the mortality response curves. Based on these comparisons, the dose response of apoptotic cell death in the medial yolk vein at stage 33 was selected for statistical comparison with the dose response of mortality at hatch. Analysis of covariance with interaction of the data curves was performed using SAS (Cary, NC) to determine if slopes were statistically similar. The confidence level was set to 95%.

**RESULTS**

Detection and Quantitation of Tissue-Specific Apoptotic Cell Death in Medaka Embryos

Edema, hemorrhaging, regression of vitelline vascular, and loss of circulation are consistent phenotypic markers of exposure to dioxin-like compounds for developing fish embryos (Henry et al., 1997; Cantrell et al., 1996; Walker et al., 1994; Peterson et al., 1993; Spitsbergen et al., 1991; Walker et al., 1991; Helder, 1981). The consistent appearance of vascular effects in TCDD-exposed embryos suggests that vascular damage may contribute to the potent embryo toxicity of dioxin-like compounds.

We have previously shown that programmed cell death in the medial yolk vein, a measure of vascular damage, correlates with microscopic hemorrhaging in the embryonic vasculature (Cantrell et al., 1996). In this study, we further investigated the relationship between cell death, vascular damage, and embryo mortality in TCDD-exposed medaka embryos. First, dose-response curves for vascular lesions and mortality were developed at specified times, relative to fertilization, to quantify lesion development and embryo toxicity. Medaka embryos were treated with a single 2-h waterbath exposure of TCDD within 5 h postfertilization. The egg concentrations of TCDD were 0, 1.5, 2.9, 5.8, 11.7, and 17.8 ng TCDD/g egg. Cumulative mortality was measured at stage 33, at hatch, and at 3 days posthatch. Lack of a heartbeat, a nonintact pericardial sac, and the absence of circulating blood were used to determine mortality. Embryos that did not meet all three criteria were scored as nonviable. Embryo mortalities at developmental stage 33 ranged from 0 to 70% (Fig. 1A). For the highest treatment group (17.8 ng TCDD/g egg), embryo mortality reached 75% at hatch (Fig. 1B) and 100% at 3 days posthatch (Fig. 1C). Pericardial sac edema and vascular hemorrhaging were the predominant dose-dependent morphologically visible lesions at all of the developmental stages examined (Fig. 1D).

The presence of tissue-specific cell death in TCDD-exposed medaka embryos was determined. An increase in the number of apoptotic cells was first detected in the medial yolk vein of TCDD-exposed embryos at developmental stage 26 (Figs. 2D and 2F). Increased numbers of apoptotic cells were also detected in other tissues of TCDD-exposed embryos, such as digestive tissue (Figs. 3D and 3F), although not until the later developmental stage 33. Quantitative analysis of programmed cell death in the medial yolk vein demonstrated a statistically...
A significant ($p = 0.02$) increase in apoptotic cells in the medial yolk vein of stage 33 embryos exposed to 11.7 ng TCDD/g egg (Fig. 4).

**Statistical Data Support a Causal Link Between Vascular Damage and Embryo Mortality of TCDD**

Analysis of covariance was used to characterize the mechanistic link between programmed cell death in the medial yolk vein and mortality. The dose–response curves for apoptosis in the medial yolk vein at stages 26 and 33 were plotted against the dose–response curve for mortality at each of the developmental stages (data not shown). The response curve for apoptosis in the medial yolk vein at stage 33 was nearly parallel to that of the mortality dose–response curve at hatch. Analysis of covariance determined that the slope for apoptosis in the medial yolk vein at stage 33 and the dose–response curve for mortality at hatch were not statistically different ($p = 0.26$; Fig. 5). Importantly, the slope for apoptosis in the medial yolk vein was shifted to the left of the mortality dose–response curve, consistent with the hypothesis that apoptosis in the medial yolk vein is a causal precursor for embryo mortality.

**Tissue Specificity of TCDD-Induced P450 1A Expression in Medaka**

The monoclonal antibody 1-12-3 was used to localize P450 1A in tissue sections from TCDD-treated medaka embryos to determine if cell death and P450 1A co-localized in the medaka embryo (Fig. 6). P450 1A expression in the medial yolk vein was detected in the TCDD-treated medaka embryos as early as stage 26 (Figs. 6B and 6C). Thus, TCDD-induced P450 1A expression co-localized with cell death in the medial yolk vein during early development of the medaka embryo.
TCDD-Induced Apoptosis and P450 1A Expression in Visibly Healthy Medaka Fry That Were Exposed to Low Doses of TCDD During Embryonic Development

We also investigated tissue damage and P450 1A expression in posthatch fry that were exposed to relatively low doses of TCDD during embryonic development. Although toxicity was observed at low doses of TCDD (1.5 and 2.9 ng/g egg), significant survival of TCDD-exposed organisms at 3 days posthatch is also observed (Fig. 1C), along with a concomitant reduction in the number of organisms that displayed visible vascular lesions (Fig. 1D). For the following experiments, only those embryos that did not display visible lesions at the 3 days posthatch stage were selected for histological analysis. Therefore, the embryos selected for analysis in these experiments clearly represent embryos that were exposed to a sublethal dose of TCDD during embryonic development.

The predominant locations of increased numbers of apoptotic cells in these TCDD-exposed but visibly healthy fry were in the digestive tissues (Figs. 7D and 7F) and gill tissues (Figs. 8D and 8F).

P450 1A expression was also analyzed in the sublethal dosing groups at 3 days posthatch. TCDD-induced P450 1A expression co-localized with cell death in the gill tissue (Figs. 6E and 6F) and digestive tissue (Figs. 6H and 6F) of these visibly healthy fry. TCDD-induced P450 1A staining, but not TCDD-induced cell death, was also detected in the heart (data not shown).

DISCUSSION

Vascular Tissue Is Particularly Sensitive to the Embryo-Toxic Effects of TCDD

Vascular hemorrhaging, pericardial sac edema, and reduced circulation are hallmark indicators of embryo exposure to dioxin-like compounds (Peterson et al., 1993; Walker et al., 1991; Helder, 1981; Wisk and Cooper, 1990). In the present

![FIG. 7. Detection of programmed cell death in the digestive tissue of visibly healthy posthatch fry exposed to low doses of TCDD during embryonic development. Medaka embryos were exposed to TCDD to give the following effective doses: (A and B) untreated; (B and C) 1.5 ng/g egg; and (D and E) 2.9 ng/g egg. Surviving fry that were visibly healthy were preserved, paraffin embedded, sectioned and assayed for cell death. The transmitted light images and the corresponding fluorescence images are shown. Apoptotic cell death was not detected in the tissue sections from untreated embryos (B). Apoptotic cell death was detected in the digestive tissues of the 1.5-ng/g (D) and 2.9-ng/g (F) dosing groups. Arrows denote the location of the digestive tissue (Int) in each image. The location of the retina (R) is indicated in the visible light images (upper right corner).](image-url)
study, a dose-dependent survey of tissue-specific apoptotic cell death and P450 1A expression revealed that the embryonic vasculature is highly responsive to TCDD. The medial yolk vein was the earliest site of TCDD-induced apoptotic cell death and P450 1A expression in developing medaka embryos. The medial yolk vein was also the most sensitive tissue to TCDD-induced cell death and P4501A expression during embryonic development. Our observation that the embryonic vasculature is highly responsive to TCDD is consistent with findings in lake trout studies. In lake trout embryos exposed to TCDD by microinjection, the vasculature was the most sensitive to the adverse effects of TCDD and the first tissue to show P450 1A staining (Guiney et al., 1997). The unique sensitivity of the embryonic vasculature to TCDD-induced P450 1A expression and cell death is consistent with the hypothesis that the vasculature is a physiological target for the embryo toxic effects of TCDD (Cantrell et al., 1996; Stegeman et al., 1995; Peterson et al., 1993).

Vascular Damage and Embryo Toxicity

The profound vascular effects in TCDD-exposed fish embryos suggest that disruption of the embryonic vasculature may contribute to the toxicity of TCDD toward fish embryos. In the present study, we addressed the hypothesis that TCDD-induced vascular damage is causally linked to the embryo toxicity of TCDD. Cell death in the medial yolk vein was used as a measure of vascular damage in tissue sections of the TCDD-exposed medaka embryos. The slope of the dose–response curve for apoptosis in the medial yolk vein was compared to mortality at hatch. The slope of the dose–response curve for apoptosis in the medial yolk vein was not statistically different from the slope of the dose–response curve for mortality at hatch. Furthermore, the dose–response curve for the apoptosis in the medial yolk vein at the developmental stage 33 was shifted to the left of the mortality curve. The relative position of the response curves suggests a causal link between vascular changes and the embryo toxicity of dioxin-like compounds.

<table>
<thead>
<tr>
<th>TCDD (ng/g)</th>
<th>Transmitted Light Image</th>
<th>Epifluorescent Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="A" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
</tr>
<tr>
<td>1.5</td>
<td><img src="C" alt="Image" /></td>
<td><img src="D" alt="Image" /></td>
</tr>
<tr>
<td>2.9</td>
<td><img src="E" alt="Image" /></td>
<td><img src="F" alt="Image" /></td>
</tr>
</tbody>
</table>

**FIG. 8.** Detection of programmed cell death in the gill tissue of visibly healthy posthatch fry exposed to low doses of TCDD during embryonic development. Medaka embryos were exposed to TCDD to give the following effective doses: (A and B) untreated; (B and C) 1.5 ng/g egg; and (D and E) 2.9 ng/g egg. Surviving fry that were visibly healthy were preserved, paraffin embedded, sectioned, and assayed for cell death. The transmitted light images and the corresponding fluorescence images are shown. Apoptotic cell death was not detected in the tissue sections from untreated embryos (B). Apoptotic cell death was detected in the gill tissues of the 1.5 ng/g (D) and 2.9 ng/g (F) dosing groups. Arrows denote the location of the gill arches in each image. The location of the retina (R) is also indicated in the visible light images (upper right corner).

*VASCULAR CELL DEATH AND EMBRYOTOXICITY OF TCDD*
These data are consistent with observations in other teleost species where vascular changes, including induction of P450 1A expression in vascular tissues, correlated with TCDD toxicity (Guiney et al., 1997).

A functional vasculature is required for proper early development, growth, and survival of fish embryos. The developing embryo utilizes the endogenous yolk reserves for nutrients (Heming and Buddington, 1988). A key indicator for decreased nutrient utilization is the presence of a large yolk sac at hatch. A large yolk sac at hatch is a common observation in embryos exposed to dioxin-like compounds (Walker et al., 1991; Wisk and Cooper, 1990). The large yolk sac in TCDD-exposed organisms, relative to the size of the yolk sac in untreated organisms, suggests that TCDD exposure interferes with proper absorption and utilization of the yolk sac. As the medial yolk vein is one of three veins in the medaka embryo that form the vitelline circulation, apoptotic cell death in the medial yolk vein, with consequent disruption of blood circulation (Cantrell et al., 1996), is likely to be responsible for decreased absorption of the yolk sac in TCDD-exposed organisms. The causal relationship between apoptotic cell death in the medial yolk vein and the embryo toxicity of TCDD suggests that the embryo toxicity of TCDD may result from defective transport of nutrients from the yolk sac to the embryo.

Relationship Between P450 1A Expression and Apoptotic Cell Death in the Embryonic Vasculature of TCDD-Exposed Embryos

The mechanism by which TCDD elicits vascular cell death in the early embryo is poorly understood. TCDD manifests its adverse effects through the AhR, a transcription factor belonging to the bHLH-PAS transcription factor family which includes the dimeric partner of AhR (Arnt), a hypoxia-inducible partner of Arnt (HIF-1α), and several Drosophila proteins (Per, Sim, and Tracheless) (Schmidt and Bradfield, 1996; Wang et al., 1995; Reyes et al., 1992). Once activated by TCDD, the AhR:Arnt complex initiates expression of several genes, including phase I (Cyp1 a1) and phase II (GST-Ya, NOQ1 [NADP(H) oxidoreductase]) genes, whose protein products are involved in oxidative metabolism of xenobiotic compounds (Schmidt and Bradfield, 1996; Hankinson, 1995; Poland and Knutson, 1982). In addition to these phase I and phase II genes that are direct transcriptional targets of the TCDD-activated AhR:Arnt complex, elevated expression of several other genes that are not direct transcriptional targets of the AhR:Arnt complex is also observed in TCDD-treated cells (Hankinson, 1995).

In the present study, we have co-localized P450 1A expression with sites of apoptotic cell death in TCDD-exposed embryos. The co-localization of P450 1A expression with apoptotic cell death is consistent with an AhR-mediated mechanism of action for TCDD-induced cell death. Induction of P450 1A in the embryonic vasculature simply may reflect the presence of a TCDD-activated AhR:Arnt complex in endothelial cells of the medial yolk vein. However, although TCDD is not likely to be metabolized by P450 1A to a chemically reactive product, induction of P450 1A may nevertheless contribute to TCDD-induced cell death. Exposure of cells and tissues to TCDD can markedly perturb cellular redox homeostasis towards oxidative stress (Park et al., 1996; Alsharif et al., 1994; Bagchi and Stohs, 1993; Berghard et al., 1993; Stohs et al., 1990). Cytochrome P450 enzymes are an efficient source of reactive oxygen molecules (Paller and Jacob, 1994), and P450 1A has been implicated as a source of reactive oxygen molecules in TCDD-treated cells (Park et al., 1996). Oxidative stress is known to alter the cellular signaling pathways that control cell proliferation, differentiation, and survival. In particular, oxidative stress can activate an intrinsic cellular signaling pathway that leads to apoptotic cell death (Hockenberry et al., 1993; Kane et al., 1993). We suggest that upregulation of P450 1A by the TCDD-activated AhR:Arnt complex may shift cellular redox homeostasis toward oxidative stress and thereby may perturb cellular signal transduction pathways that control apoptosis. The ability of a well-characterized antioxidant, N-acetyl cysteine, to provide significant protection against the embryo toxicity of TCDD in medaka is consistent with the involvement of oxidative stress in the embryo toxicity of TCDD (Cantrell et al., 1996). It will be important to determine the contribution of altered redox homeostasis towards the embryo toxicity of TCDD.

TCDD-Induced Cell Death as a Marker for Sublethal Exposures of Dioxin-Like Compounds

Sublethal doses of TCDD are more typical of the exposure concentrations of dioxin-like compounds in the environment (Devault et al., 1989). Therefore, we examined tissue sections from posthatch medaka fry that were exposed to low doses of TCDD as embryos. Gill and digestive tissues were the predominant locations of TCDD-induced cell death and P450 1A expression in posthatch fry that were treated with low doses of TCDD as embryos. As the embryos selected for these experiments were visibly healthy and did not display any phenotypic signs of TCDD exposure, our results suggest that tissue-specific cell death may be a suitable marker for sublethal exposure of organisms to dioxin-like compounds. Indeed, preliminary analysis of feral organisms exposed to dioxin-like compounds suggests that tissue-specific cell death can be detected in these organisms (S. Cantrell, P. J. Wright, and D. E. Tillitt, unpublished data).

Adverse effects in organisms exposed to sublethal doses of dioxin-like compounds are more subtle than the pervasive vascular failure observed in organisms exposed to acutely lethal doses of TCDD (Helder, 1981). The presence of TCDD-induced cell death in the gill and digestive tissues of medaka embryos exposed to sublethal doses of dioxin suggests that the function of these tissues may be compromised. As sublethal exposures of dioxin-
like compounds are environmentally relevant, it will also be important to assess the physiological and behavioral consequences of gill and digestive tissue damage for feral fish.

ACKNOWLEDGMENTS

We thank Ed Callahan for statistical analysis, Elizabeth Norton at the Cytology Core for confocal microscopy and digital imaging, Diana Papoulas for assistance with microscopy and histology, and Diana Papoulas and Eugene Greer for medaka maintenance and culturing. We thank Susan B. Jones and Erik Zabel for their critical comments on the manuscript. This work was supported by the University of Missouri Molecular Biology Program (M.H.), by the University of Missouri Research Board (M.H.), by Environmental Protection Agency grant R823890 (J.J.S.), by the Lyons Fellowship (J.J.S.), and by the National Biological Service, U.S. Department of the Interior (D.E.T.)

REFERENCES


