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Use of fish liver PLHC-1 cells and zebrafish embryos in cytotoxicity assays☆

P.H. Krone[a,b], S.R. Blechinger[a], T.G. Evans[a], J.A. Ryan[c,1], E.J. Noonan[c], L.E. Hightower[c,*]

[a] *Department of Anatomy and Cell Biology, University of Saskatchewan, 107 Wiggins Road, Saskatoon, Sask., Canada S7N 5E5*
[b] *Toxicology Group, University of Saskatchewan, 107 Wiggins Road, Saskatoon, Sask., Canada S7N 5E5*
[c] *Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269-3125, USA*

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Abstract

Heat shock proteins (HSPs) indicate exposure to cellular stress and adverse cellular effects, thus serving as biomarkers of these effects. The highly conserved Hsp70 proteins are expressed under proteotoxic conditions, whereas small HSPs are expressed in response to stressors acting on the cytoskeleton and cell signaling pathways. *Poeciliopsis lucida* hepatocellular carcinoma line 1 (PLHC-1) cells have been used extensively for studying effects of cytotoxicity. A number of assays have been developed to examine DNA levels, protein levels, growth rate, morphological changes, and viability. The boundary between sub-lethal and lethal effects of particular stressors has been determined. The methodology and analytical framework for these techniques along with sample assays using cadmium stressed PLHC-1 cells are described. A range of methodologies have been developed in the past decade that allow the analysis and interpretation of gene expression and function in vivo in zebrafish embryos, and many of these are now being applied to the development of embryotoxicity assays. Here we provide the theoretical background and methodology for utilizing Hsp70 expression as an indicator of toxicity in the zebrafish embryo. Hsp70 expression is activated in a tissue-specific manner in zebrafish larvae following exposure to a number of different toxicants, including cadmium. This has allowed the development of an *hsp70/eGFP* reporter gene system in stable transgenic zebrafish that serves as a reliable yet extremely quick indicator of cell-specific toxicity in the context of the multicellular, living embryo.

Keywords: Heat shock proteins; Cytotoxicity assays; PLHC-1 cells; Zebrafish; Embryos; Cadmium; Cellular stress response

1. Introduction

Many measurements of cellular molecules have been proposed or used as environmental biomarkers. These include, but are not limited to, measuring inhibition of enzymes such as acetylcholinesterases for studying pesticide exposures or inhibition of aminolevulinic acid dehydratase activity as an indicator of lead exposure; determining energy levels and reserves by measuring mitochondrial activity, ATP, glycogen, or lipid levels, and cellular oxygen : nitrogen or redox ratios; blood chemistry studies to determine stressor-induced changes in serum protein, glucose, and ion levels or porphyrin profiles; and measuring increases in stress-inducible protein levels or activities (reviewed in [1–3]). Stress-inducible proteins known as heat shock proteins (HSPs)[2]

☆ The work on zebrafish was from Krone, Blechinger and Evans and the studies of PLHC-1 cells were from Ryan, Noonan and Hightower.

* Corresponding author. Fax: +1 860 486 5709.
E-mail address: hightower@uconnvm.uconn.edu (L.E. Hightower).

[1] Presently Scientific Affairs Manager, Scientific Products Division, Corning, Inc.

[2] *Abbreviations used:* HSP, Heat shock protein; PLHC-1, *Poeciliopsis lucida* hepatocellular carcinoma line 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CIM, CO_2-independent medium; LDH, lactate dehydrogenase, NR, neutral red; SRB, sulphorhodamine blue; PDT, population doubling time; ZFIN, Zebrafish information network; eGFP, enhanced green fluorescent protein.

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appear to have good potential as biomarkers because they can be directly linked to cellular stress [4,5]. They are the focus of this article.

Because they are part of the cellular stress response, HSPs should be directly linked with the physiological state of the cell [6]. Because of these links, HSPs should then be useful as environmental biomarkers, which are defined as "biochemical, physiological, or histological indicators of either exposure to, or effects of, xenobiotic chemicals at the suborganismal or organismal level" [2]. At the cellular level, sub-lethal damage results when basal or constitutive cellular defenses are insufficient or incapable of handling the source of stress. Cellular systems for mediating, repairing, or removing the stressor-induced damage then respond so that vital cellular functions are not impaired.

In general HSPs are highly conserved and tightly regulated, and cells respond to heat shock by increasing their levels. Investigators have also found that this response could be induced by many other seemingly unrelated stressors, including hyperosmotic stress [7], amino acid analogues (*p*-fluorophenylalanine and canavanine), heavy metal ions (Cd^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, etc.), thiol-reactive agents (arsenite, iodoacetamide), alcohols, oxidative stress, and inhibitors of energy metabolism (dinitrophenol, azide) or proteolysis (ammonium chloride) [8]. Because HSPs are induced by so many different stressors, they are more accurately described as cellular stress proteins [9].

To explain the ability of many different stressors to invoke this heat shock or cellular stress response, Hightower [10] put forth the abnormal protein hypothesis: induction of HSPs is an adaptive cellular response to the presence of abnormal proteins. This hypothesis gained support from subsequent studies from Voellmy's laboratory showing that microinjection of denatured proteins into *Xenopus* oocytes results in induction of HSPs [11] and from the Hightower laboratory showing that the protein protectants D_2O and glycerol inhibit induction of HSPs by heat [12]. The acceptance of this abnormal protein hypothesis has led to the use of the term proteotoxicity to describe "damage to proteins caused by chemical and physical agents" [13]. This damage is usually in the form of unfolded, malfolded, or aggregated proteins [14]. Thus the induction of HSPs is directly linked to protein damage at the molecular level caused by a variety of stressors. Because they are the best understood and characterized HSP family, are highly conserved in both form and function, and their expression is closely (mechanistically) linked to proteotoxicity, Hsp70 family members are currently considered the best candidates for biomarkers of effect.

The small HSPs also are strongly induced by environmental stressors in a variety of vertebrate cells [15–18] and may have good potential as biomarkers for the effects of stressors on the cytoskeleton and cell signaling pathways. Overexpression of Hsp27 increases thermotolerance in mouse, monkey, human, and Chinese hamster cell lines. It also increases their resistance to cytotoxic drugs, TNF, and oxidative stress in a dose-dependent manner [19]. Overexpression of Hsp27 decreases cell proliferation in some cell lines but not others. The mechanisms by which Hsp27 helps protect against cytotoxicity during stress are not clear, but its elevated accumulation has been correlated with increased levels of glutathione [20]. Hsp27 may act as a chaperone to facilitate refolding of damaged proteins or protect the actin microfilament network and thus help stabilize the cytoskeleton. There is evidence that phosphorylation of Hsp27 modulates its ability to stabilize the microfilament network against heat-induced fragmentation [21,22].

We chose fish cell cultures to model initially the whole animal stress response for several reasons: First, whole fish have already proven useful in both field and laboratory studies for assessing water quality [23], and several standardized toxicity tests (acute toxicity LC50 assays) using rainbow trout (*Oncorhynchus mykiss*), bluegill sunfish (*Lepomis macrochirus*), and fathead minnow (*Pimephales promelas*) are in current use for environmental monitoring. Second, fish cell cultures have been successfully used in a variety of cytotoxicity tests to screen chemicals initially for their potential as hazards in aquatic environments and to correlate their toxicity with their physicochemical parameters (reviewed in [24–26]. Third, the cellular stress response has been previously studied (primarily by measuring induction of stress proteins) using fish cell lines derived from the Chinook salmon (*Oncorhynchus tshawytscha*), fathead minnow (*P. promelas*), winter flounder (*Pleuronectes americanus*), and desert topminnow (*Poeciliopsis lucida*) (reviewed in [27–29]).

We chose to detect potential HSP biomarkers by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) combined with silver staining to measure stressor-induced changes in protein levels rather than transient stimulation of mRNA and protein synthesis. Scans of silver-stained, one-dimensional SDS–PAGE gels, although not highly quantitative, are suitable for evaluating both acute and chronic changes in stress protein levels if done carefully, and they can be used to study wild fish populations. Ultimately, it is the amount and activity of stress proteins in the cell that determine their physiological impact. This is a practical approach for initially screening cultures for biomarkers, especially when suitable antibodies are unavailable. While many antibodies to HSPs are available, most were made using mammalian HSPs as the antigens, and although some cross react over a wide range of species including fish, others do not.

The cytotoxicity assays used for linking stress-induced changes in protein levels with corresponding changes in the physiological state of the cells were

chosen for their ability to separate stressor-induced sub-lethal cellular effects from lethal effects. This information is critical to understanding the nature of a stressor-induced response and its usefulness as a biomarker. Sample data from cadmium cytotoxicity assays have been included in Appendix A.

In addition to a cell culture assay system, it is valuable to have a HSP-based intact fish assay for cytotoxicity. The zebrafish (*Danio rerio*) is now widely used as a model system for examination of embryonic development but also shows great promise for in vivo cytotoxicity testing. The major strengths of the zebrafish are centered around the accessibility and manipulability of the embryo, which allow a wide range of experimental approaches carried out at the molecular and cellular levels to be interpreted in the context of the intact, multicellular embryo. Basic information about HSP expression and function in this system has increased substantially in the past decade. In particular, a number of heat shock genes are expressed in specific spatial and temporal patterns during early embryogenesis, and this information is being integrated into the mechanistic framework of developmental pathways [30,31]. Here we discuss the features of the zebrafish that make it attractive for the study of heat shock gene expression in vertebrate embryos, and some of the resources available to the zebrafish research community. We will then provide an overview of the experimental approaches behind the development and use of an *hsp70* promoter-based fluorescent reporter gene expression system in transgenic fish embryos. This system has proven to be an accurate and reproducible indicator of cell-specific induction of *hsp70* expression following toxicant exposure. Furthermore, the transgene responds in a dose-dependent manner at concentrations similar to those observed for classical morphological indicators of early life stage toxicity. An example for cadmium exposure is presented in Appendix B.

2. Description of methods

2.1. Cell cultures

The major cell line used in these experiments is the fish cell line *Poeciliopsis lucida* hepatocellular carcinoma line 1 (PLHC-1), initiated from a hepatocellular carcinoma, that has now been through over 200 passages in vitro. Its origin traces back to hepatic tumors that were induced in 1982 in *P. lucida*, a desert topminnow, using multiple doses of 7,12-dimethylbenz(a)anthracene. One of these tumors, a highly differentiated hepatocellular carcinoma, was serially transplanted approximately 11 times by Dr. M. Schultz [32]. Tissue from this transplantable tumor was removed in 1985 and subjected to five 1 h dissociations in a trypsin–ethylenediamine tetraacetic acid solution by Dr. L.E. Hightower. The dissociated cells were pooled, cultured at room temperature in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS), and used to establish the PLHC-1 cell line [27,33].

PLHC-1 cells have been used by several investigators for studying the cytotoxic effects of a variety of compounds, especially those that require activation by cytochrome P4501A1 [34–41]. The PLHC-1 cell line is available at the American Type Culture Collection (ATCC #CRL-2406).

2.2. Growth conditions

The requirements for growing PLHC-1 cells are similar to those for growing mammalian cells as reviewed in [42–44]. The established fish cell lines were propagated either in Eagle's minimal essential medium with Earle's salts (GIBCO, Grand Island, NY) plus 5% (v/v) FBS (Hyclone, Logan, UT) at a pH of 7.2–7.4 in a humidified incubator with 5% CO_2, or in CO_2 independent medium, (CIM; GIBCO) plus 5% FBS at a pH of 7.2–7.4 in sealed vessels. No antibiotics were used. This cell line was grown at 30 °C. Although earlier experiments were done using Eagles's minimal essential medium, all the experiments performed in the Hightower laboratory since 1993 have used CIM. This switch was made for several reasons: first, CIM eliminated the need for incubators with a regulated CO_2 supply (which were not available for growing cells at temperatures lower than 30 °C; second, it allowed better growth of the fish cell line at lower serum concentrations; third, through careful adaptation it eventually allowed the serum-free growth of PLHC-1 cells (see also [45,46]; and last, its buffering system permits better control of medium pH than traditional CO_2/bicarbonate-based buffering systems, which is especially important when running metabolic or functional cytotoxicity assays.

PLHC-1 cells were usually split every 7–10 days by dissociating with 0.05% (w/v) trypsin and 0.5 mM ethylenediamine tetraacetic acid in calcium- and magnesium-free phosphate buffered saline. As most fish cell lines cell grow poorly at low dilutions, they were usually subcultured at split ratios of 1 : 10 or less.

The PLHC-1 cells used in these experiments were from cryogenically preserved (stored in liquid nitrogen) stock cultures that have been tested for mycoplasma using fluorescent DNA staining with both positive and negative controls [47], and have always tested negative.

2.3. Preparation of test agents and solutions

All stock solutions unless otherwise stated were prepared in ultrapure water (18+ Mohms resistivity, prepared using a Barnstead Nanopure system) and then filter sterilized. The cadmium stock solution (2.5 mg/ml cadmium II) was prepared from reagent grade

$CdCl_2 \cdot 2 \cdot 5H_2O$ (Baker, Catalog #1208). All solutions were diluted into appropriate culture media for testing.

2.4. Cytotoxicity assays

For a typical assay 200 μl of cell suspension (approximately 1×10^5 cells/well) were added to each well of a 96-well plate using an eight channel pipettor with wide orifice tips, except the top row, which received water, and the bottom row, which received medium. Confluent cultures were used for all experiments. For experiments requiring protein analysis using SDS–PAGE, identical cells from the cultures used for the 96-well plate inoculations were usually plated at the same time into 12- or 24-well cell culture vessels. In order to maintain equivalent cell number to surface area (density) to volume ratios for these different multiwell plates, both the number of cells and the medium volume were increased proportionally (six-fold for 24-well plates, and 12-fold for 12-well plates). Test agent volumes were also proportionally altered for the larger surface area multiwell plates. All the cultures were then incubated for a minimum of two to three days before use in a cytotoxicity experiment to allow the cells to repair any damage that may have occurred during processing and handling and to allow the cultures to become confluent.

The lactate dehydrogenase (LDH) release assay estimates the number of dead cells and was performed using a purchased kit (Promega Catalog #G1780) with the procedure modified to allow other cytotoxicity assays to be run subsequently on the same cell cultures.

The neutral red (NR) uptake assay was a slight modification [33] of the method described by Borenfreund [48]. It measures lysosomal uptake of vital dye neutral red as an estimate for viable cell number.

The sulforhodamine Blue (SRB) protein staining assay was based on a modification of the procedure of Skehan [49]. It measures protein levels as an estimate of total cell number.

The DNA staining assay was a slight modification of the method developed by Schirmer and colleagues [50]. It measures DNA levels as an estimate of total cell number. This assay could not be run on the same cultures as the NR uptake or SRB staining assays since it required lysing the cultures as part of the assay, which is incompatible with the other assays.

2.5. Separation of lethal and sublethal cytotoxic effects

Several in vitro cytotoxicity assays were linked together in order to economically develop reasonable estimates of total cell numbers and their viability, thus obtaining an accurate picture of the cytotoxic effects of stressors on the cultures. The results from these assays were also used to determine the range of stressor concentrations resulting in sub-lethal effects (inhibition of growth) and to locate the approximate boundary separating lethal effects (cell lysis or death) from these sub-lethal effects.

Two different approaches were developed for locating this lethal–sub-lethal cytotoxic boundary. For Method 1, the LDH release assay was used to determine the lowest stressor concentration that induced an increase (relative to control levels) in the release of LDH, a cytosolic enzyme. Since this increase is a marker for cell lysis or death, it provided direct evidence of a lethal cytotoxic effect and defined the lower end of the zone of lethal effects. This in turn determined the approximate location of the lethal–sub-lethal cytotoxic effects boundary.

The second approach (Method 2) locates the lethal–sub-lethal cytotoxic effects boundary by using an assay that estimates the cell number (DNA staining, NR uptake, or SRB staining) to determine the initial stressor concentration at which the cell number first dropped below the initial cell number ($T = 0$ cell number) originally determined at the start of the experiment. A cell number lower than the initial cell number provides direct evidence that some of this cell loss was due to cell death, a lethal effect, and not inhibition of growth. This approach requires that the original cell number be either known or can be estimated, and is based on the assumption that sub-lethal cytotoxic effects precede lethal cytotoxic effects.

In experiments where the initial $T = 0$ cell number was not directly determined at the start of the experiment as above, it was estimated either by using the average $T = 0$ values determined from other studies done with the same cultures and performed under similar culture conditions, or by using the population doubling time of the cultures to extrapolate back to the initial cell number using the following equation for calculating population doubling times (PDTs) [51]:

$$\begin{aligned}\text{PDT(hours)} = {} & \text{growth period(hours)} \\ & \div \{\log_2(\text{final cell protein level}) \\ & - \log_2(\text{initial cell protein level})\},\end{aligned} \quad (1)$$

which, substituting $\log_{10}$ in place of $\log_2$ converts to

$$\begin{aligned}\text{PDT(hours)} = {} & \text{growth period(hours)} \\ & \div \{3.322(\log_{10}\text{final cell number} \\ & - \log_{10}\text{initial cell number})\}\end{aligned} \quad (2)$$

If 100% is substituted as the final cell number (representing the control at the end of the experiment), then solving this equation for the initial cell number will give the result as a percent of the final cell number:

$$\begin{aligned}\text{Initial cell number}(\%) = {} & \text{antilog of } 2 \\ & - \{\text{growth period(in hours)} \\ & \div 3.322(\text{PDT})\}\end{aligned} \quad (3)$$

The PDT was also calculated directly for experiments where more than one exposure period was used. The control cell number from the end of the longer exposure period was used as the final cell number; the control cell number from the end of the shorter exposure was used as the initial cell number, with growth period calculated as the difference (in hours) between the length of the two exposure periods. Once the PDT was known, it was substituted in the above formula to estimate the starting cell number. This approach assumes there was no major change in the growth rate over the course of the experiment.

2.6. Morphological examination and photomicrographs

Cells that had been fixed for the NR uptake or protein staining assays were stained for microscopic observation by adding 100 μl of 0.5% (w/v) aqueous crystal violet stain (Fisher) to each well for approximately 5 min. The plates were rinsed thoroughly with distilled water and allowed to air dry to allow direct microscopic observation of both the morphology and degree of cell loss of the fixed and stained cells.

2.7. Calculations

For most of the cytotoxicity experiments, the assay results were calculated as arithmetic means with their standard deviations, then normalized to their appropriate unexposed controls and plotted as a percentage of their control values. Each of the stressor exposure concentrations usually consisted of six to eight replicates with 14–24 replicates for the controls.

Coefficients of determination (r^2), which show the proportion of the total variation in the dependent variable (in these experiments usually either cytotoxicity or increases in HSP levels) accounted for by the independent variable (stressor concentration), were used to estimate the dose-dependency. Correlation coefficients (r) were used to determine if there was a linear relationship (correlation) between the Hsp70 levels and the sub-lethal cytotoxicity. The significance of the correlation coefficient (r), showing whether or not there exists a relationship between two variables, was determined using the following correlation test statistic [52]:

$$T = r \div \sqrt{\{(1 - r) \div (n - 2)\}}, \quad (4)$$

where $(n - 2) =$ degrees of freedom.

Whenever a coefficient of determination (r^2) value is used, the p value given is for its corresponding coefficient of correlation (r) value since r^2 values are not directly tested for significance.

For the cytotoxicity assays, Welch's separate-variance t test was used for the statistical comparisons of individual test values to control values since there was usually a large difference between the sample sizes of the control (typically $n = 14$–24) and test samples ($n = 6$–8). Under these conditions this t test is more conservative and reliable than standard t tests [53]. A simple Student's t test was used for the gel protein band induction studies.

2.8. Gel electrophoresis

Analytical SDS–PAGE was performed using a modified procedure of Laemmli as described in [33]. The test and control media were removed from the 12- or 24-well plates and each well was rinsed twice with cold wash buffer (62.5 mM Tris–HCl, pH 6.8). Sample buffer (2.35% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) ß-mercaptoethanol, and 62.5 mM Tris–HCl, pH 6.8) was added to each well to solubilize the cells. Cell lysates were collected from individual wells and stored at −20 °C. Test samples were heated to 90 °C for several minutes and equal volumes loaded in 9 or 10% (w/v) acrylamide: 0.27% (w/v) bis-acrylamide slab gels (approximately 12 cm × 16 cm, 0.75 mm thick) and run at 20 mA constant current for each gel for approximately 4 h to resolve individual bands.

The gels were fixed and stained using a modification of AG-25 silver staining kit procedure of Sigma Chemical: the reducer was added prior to the developer and silver solution (instead of after) to enhance the staining of Hsp70. The gels were fixed in 30% (v/v) ethanol, 10% (v/v) acetic acid for 30 min, rinsed in three changes distilled water, and incubated in reducer solution for 20–30 s. Gels were rinsed twice for 15 s each in distilled water and incubated in silver solution for 30 min. The gels were rinsed briefly in distilled water and placed in developer solution for several minutes until the bands were clearly visible. The gels were then immediately transferred to stop solution to prevent further development, rinsed in distilled water, and dried.

The dried gels were scanned on an LKB Ultroscan XL laser densitometer to quantify the density of the protein bands that visually showed dose-dependent accumulations greater than the control. Changes in the accumulation of each selected protein were quantified by measuring the density (area) under its individual peaks (at each test concentration) and the density of its control; normalizing both areas to the density of actin; and then expressing the normalized density of the protein as a fold increase relative to the control density. In addition one or more protein bands, whose staining intensities did not appear to be altered by the test agent treatments, were selected from each gel section scanned; these bands were also quantified and their densities used as internal quality control references to determine the inherent variability in the gel. A band was considered to be induced if its normalized density was significantly different (Student's t test; $p < 0.05$) from the average normalized density of the internal reference bands.

Those proteins showing visible increases had their relative molecular weights estimated by comparing their relative migration distances against a standard curve prepared using protein calibration standards (SDS-6H, Sigma Chemical) run on the same gel.

2.9. Basic features, care, and breeding of zebrafish

Zebrafish are small, schooling fish and thus have relatively low space requirements on a per animal basis. A colony of adult zebrafish can be maintained in a fraction of the space required for a comparable colony of mammals or larger fish models such as trout or salmon, and high density aquarium housing systems have been developed by several suppliers such as Aquatic Habitats, Apopka, FL, and Allentown Caging, Allentown, NJ. Adults are kept on a regular spawning schedule by maintaining breeding stock on a 14 h photoperiod, and supplementing flaked food with live and/or frozen foods such as brine shrimp or bloodworms. Under these conditions, fish spawn within the first hour of turning on of lights in the morning, allowing users to obtain a large number of embryos for experimental manipulation without any seasonal limitations. The embryos themselves develop rapidly (somitogenesis is completed within the first day following fertilization) and manipulations of environmental conditions are easily carried out when raising embryos in small disposable Petri dishes. Zebrafish eggs are relatively large, allowing the microinjection of molecules such as reporter genes, in vitro synthesized sense mRNA, and morpholino-modified antisense oligonucleotides. The last has proved to be a very effective method of gene-specific targeting in zebrafish embryos [54]. Internal morphological changes and transgene expression in both living and fixed whole embryos are easily monitored because of the translucency of the zebrafish embryo. This feature also makes the embryos amenable to the technique of whole mount in situ hybridization analysis for rapid examination the three-dimensional patterns of tissue-specific gene expression. Finally, the zebrafish has been the focus of successful broad-scale and smaller, targeted mutagenesis screens [55,56] as well as insertional mutagenesis screens [57] in an effort to identify genes that regulate embryonic development. These have provided literally thousands of mutant strains of fish that show defects in the development of virtually all tissue types of the embryo.

One of the biggest limitations of the zebrafish is the inability to create targeted gene knockouts, but the recent report of zebrafish cells with embryonic stem cell-like characteristics suggests that it may be a possibility in the future [58]. Additionally, there are only a limited number of zebrafish cell lines available, and even fewer that have been well characterized. Furthermore, wild populations of zebrafish are relatively inaccessible, making direct extrapolations to native conditions more difficult.

2.10. Resources available to the zebrafish community

Many resources are available at the University of Oregon through the Zebrafish International Resource Centre. A standardized set of guidelines on embryo care and experimental manipulations has been published [59], as have volumes of more specific research techniques [60,61] and a comprehensive staging series [62]. In addition, an extensive website known as the The Zebrafish Information Network (ZFIN) is maintained at the University of Oregon (www.zfin.org). ZFIN has information on numerous available resources such as anatomical and histological atlases, wild-type and mutant stocks, monoclonal antibody libraries, companies supplying zebrafish equipment and reagents, and individual zebrafish laboratories. A number of genomic resources are also available, including expressed sequence tags (www.genetics.wustl.edu), mapping tools, and the zebrafish genome sequencing project (www.sanger.ac.uk). The recent launch of the new journal *Zebrafish* promises to provide an exciting forum for new research on this and other aquarium fish models.

2.11. Transgenic zebrafish carrying an hsp70/eGFP reporter gene

While heat shock genes have long been of interest as potential markers of stress exposure in toxicological studies, including vertebrate developmental and reproductive toxicology, current knowledge of the potential impact of toxicants at the level of individual cells within the intact embryo is quite limited. Furthermore, many of the assays used for in vivo studies, such as in situ hybridization or immunohistochemistry, are time-consuming and laborious. This makes them difficult to apply to larger scale screening projects, and when carrying out mechanistic studies that examine multiple stages of embryonic and larval development. In contrast, the assay of enhanced green fluorescent protein (eGFP) activity requires only a few minutes under a fluorescent microscope, and offers greater sensitivity in detecting individual cell populations that express the transgene.

Our laboratory collaborated with the laboratories of J. Kuwada (University of Michigan) and J. Warren (Penn State) on the development of a transgenic line of *hsp70* promoter-driven eGFP zebrafish [63]. This *hsp70*-eGFP reporter gene has been stably integrated into the germ-line of the transgenic fish, and the line has now been maintained for over six years. The transgenic fish containing this construct appear to be biologically normal and do not exhibit any adverse effects as a result of the integrated transgene. This technology has allowed us to directly observe *hsp70* expression in living embryos and larvae. We previously reported that eGFP expression in this strain of fish serves as an accurate and reproducible indicator of cell-specific *hsp70* gene

activation, and responds in a predictable manner following exposure of embryos to heat shock. Thus, eGFP activity is seen only in the lens in embryos developing under non-stress conditions but is widely expressed following heat shock [64] (Fig. 2).

2.12. Acute zebrafish embryo/larval exposure assays

Acute embryonic and larval toxicity tests based on classical morphological indicators were first performed in order to determine median lethal (LC_{50}) and multiple effect (EC_{50}) values for different toxicants. Adult zebrafish were maintained in 37 l tanks on a 14 h photoperiod. Fertilized eggs were collected within 30 min of turning on of lights in the morning, and divided into batches of 50 eggs in sterile 25 ml Petri dishes. Eggs were rinsed of fecal matter and other contaminating material, and maintained in dechlorinized system water at 28 °C on a 14 h photoperiod. Water was changed twice daily. Any non-fertilized or dead eggs were removed during the first 24 h after spawning. Embryos began hatching on the third day of development and by 72 h post-fertilization (hpf) nearly 100% normally hatched. Shed chorions were removed at each water changing to reduce growth of bacteria and mold. Post-hatch larvae normally began to swim to the surface by the fifth day of development and began feeding between the sixth and eighth days [59]. The larval yolk sac is almost completely resorbed by this time and maintaining larvae beyond this age requires feeding. For the purpose of acute toxicity testing in larvae, the complication of food particles in the water was avoided by having the larval 96 h acute toxicity test run from the third day to the eighth day (72–168 hpf) without feeding during this period.

To determine which developmental period was the most sensitive, an initial range-finding 96 h exposure was performed on two distinct stages of development, embryonic and larval. The embryonic exposure began at the mid-blastula period (4 hpf) to post-hatched larvae (100 hpf), while the larval exposure began on the third day of development (72 hpf), when larvae have freshly hatched, and continued until the morning of the eighth day (168 hpf). These range-finding study showed that larvae were more sensitive to cadmium, arsenic, and other toxicants than embryos. The prevalent endpoints actually observed for any one compound must be determined empirically. For example, observed endpoints for cadmium and arsenic in the larvae were death, edema, skeletal deformities, and immobility (arsenic only), and therefore detailed acute larval toxicity tests were carried out with these endpoints. Post-hatch larvae were continuously exposed to a range of toxicant concentrations for 96 h beginning at approximately 72 hpf. Three replicate treatments (3×20 larvae) were carried out for each dose. Two times per day observations for effects were assessed and treatment solutions were changed; any dead larvae were counted before being removed. After the 96 h exposure was complete, final observations were made and larvae exhibiting any endpoint were tabulated for each treatment. The average number of larvae per treatment (average of three replicate treatments per exposure dose) showing mortality and multiple endpoints were calculated along with the standard error of the mean (SEM). The LC_{50} and EC_{50} concentrations and 95% confidence intervals were calculated from a linear regression of log-Probit transformations of the dose–response data using procedures described in [65,66].

2.13. Treatment of embryos for hsp70 and hsp70/eGFP expression analysis

Based on the 96 h acute toxicity dose-response relationship, doses for the *hsp70* expression experiments were selected in order to represent a low (no observed adverse effect concentration (NOAEC)), mid-range (near the EC_{50}), and high dose (at or greater than the LC_{50}). Single treatments for wild-type ($n = 50$ per dose pooled from three Petri dishes for each dose) and transgenic ($n = 50$ per dose only, each in a single petri dish) larve were used for each of the three doses plus a water control. An additional three treatments each of 15 wild-type larvae per dose were also collected for two hybridization negative controls and a a *hsp70* positive control (1 h 38 °C heat shock). This treatment is known to strongly induce *hsp70* mRNA expression throughout the embryo [30]. In order to detect a strong mRNA signal with in situ hybridization using wild-type larvae, an exposure duration of 3 h beginning at 80 hpf was chosen, and embryos were fixed in 4% paraformaldehyde immediately afterwards. Transgenic larvae for eGFP analysis were washed with several changes of clean water and allowed to develop for an additional period of 24 h. Other experimental models investigating hsp-reporter genes have used a recovery period to allow ample translation of reporter mRNA into protein signals [67], and we have found that a similar regime optimizes the eGFP signal in zebrafish. After 24 h recovery, live transgenic larvae were assessed for eGFP fluorescence and any tissues positive for *hsp70/eGFP* expression were counted.

2.14. Analysis of endogenous hsp70 gene expression following toxicant exposure

Endogenous *hsp70* mRNA expression was assessed in wild-type larvae using whole mount in situ hybridization as described in [59] and the technique will not be described here. The probe utilized represented a 642 bp PCR-amplified section of the zebrafish *hsp70-4* gene [68]. Following the 3 h exposure, wild-type larvae were immediately fixed for purposes of performing the in situ hybridization assay, Post-hybridized wild-type larvae to

be used for tissue counts were mounted, sectioned, and coverslipped. Serial sections were viewed under a Nikon E-600 microscope using Nomarski optics and the number of larvae per treatment positive for expression in a given tissue were counted. The proportion of larvae expressing *hsp70* in a given tissue was calculated for each treatment dose, and differences between control and treatment dose groups were tested for significance using fisher's exact test as described by Zar [66] and Gad [65]. Additionally, 15 larvae per treatment dose were cleared in a solution of benzyl alchohol/benzyl benzoate (1 : 2) for photography only and were not included in the counts for tissue expression.

2.15. Detection of eGFP activity

The detection of eGFP activity in living embryos and larvae is straightforward. The eGFP protein has an excitation energy of 488 nm and an emission energy of 507 nm, and is detectable in living embryos/larvae via fluorescent microscopy with the appropriate filter set. Embryos up to 48 h of age were viewed without an anaesthetic, whereas older embryos and larvae required incubation in a dilute solution of Tricaine to minimize movement. Any embryos that had not emerged from their chorions were first manually dechorionated using Dumont #5 forceps. For viewing, live embryos were placed in a depression slide, as this allows them to remain immersed during viewing. Epifluorescence microscopy of the live embryos was carried out on a Nikon E-600 compound epifluorescent microscope equipped with low power objectives and a Nikon Coolpix 990 digital camera. Water immersion objectives are preferable since they avoid any problems with image distortion at the water–air interface. Following observation and image capture, embryos were rinsed of Tricaine and returned to system water. They normally recover from the effects of the anaesthetic within a few minutes. Viewing was repeated as often as desired during longer time course experiments. Alternatively, embryos can be mounted in low melting point agarose and viewed in real time using time-lapse digital photography. Embryo counts and statistical analysis were carried out as described for in situ hybridization above.

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Appendix A

A.1. An example of an in vitro cytotoxicity assay system: effects of cadmium ions on PLHC-1 cells

The experiment shown in Fig. 1 used SDS–PAGE to evaluate changes in PLHC-1 protein levels induced by a 24 h exposure to cadmium while monitoring corresponding changes in cytotoxicity. LDH release,

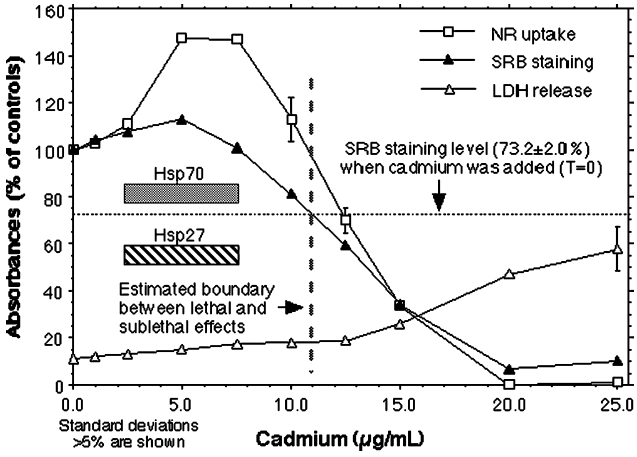

Fig. 1. Relationship between cadmium-induced cytotoxicity and changes in stress protein levels in PLHC-1 cells. Dose–response cytotoxicity curve of PLHC-1 cells to cadmium as determined by LDH release, NR uptake, and SRB staining assays. A 96-well plate culture was grown at 30 °C in CIM + 5% FBS for 72 h and then exposed to nine cadmium concentrations for 24 h. Media samples were then removed for an LDH release assay followed by a 3 h incubation with NR. SRB staining was done on the fixed cells after the NR uptake assay was complete. A second 96-well plate, set up under identical conditions, was processed for SRB staining at the beginning of the cadmium exposure period to generate the $T = 0$ SRB value ($73.2 \pm 2.0\%$). Data points are the arithmetic means of well OD ($n = 6$) normalized against the control wells ($n = 14$). The boxes labelled Hsp70 and Hsp27/Band 1 indicate the range of cadmium concentrations that resulted in detectable increases in these HSP protein levels (relative to controls) as determined by SDS–PAGE and densitometry.

NR uptake, and SRB staining assays were used to determine the cytotoxic effects of cadmium and whether these effects were lethal or sub-lethal.

Significant stimulatory increases were induced by low cadmium concentrations in both NR uptake ($p < 0.001$ at 2.5, 5, and 7.5 μg/ml, $p < 0.025$ at 10 μg/ml) and SRB staining levels ($p < 0.001$ at 1, 2.5, and 5 μg/ml) relative to their control levels, with the increases in NR uptake substantially higher than those in SRB staining from 5 to 10 μg/ml cadmium. The first direct evidence of cytotoxicity was the significant decreases relative to controls first measured at 12.5 μg/ml cadmium for NR uptake and at 10 μg/ml for the SRB staining ($p < 0.001$). However, since stimulatory increases in NR uptake have been demonstrated to be markers for sub-lethal cytotoxic effects, these adverse effects actually began at 2.5 μg/ml cadmium. Both the NR uptake and SRB staining results were dose-dependent over part of the range of cadmium concentrations tested. The NR uptake was linear ($r^2 = 0.998$; $p < 0.001$) and inversely related to increasing dose from 7.5 to 15 μg/ml cadmium while the SRB staining was linear ($r^2 = 0.990$; $p < 0.001$) and inversely related to increasing dose from 5 to 20 μg/ml.

The zero point ($T = 0$) SRB staining (protein) level was 73.2% relative to the 24 h control staining level. Since cadmium concentrations of 12.5 μg/ml and higher resulted in SRB staining and NR uptake levels lower than this starting SRB staining level, this indicates there was a decrease in total cell numbers relative to the original starting number, direct evidence of lethal effects. Thus, based on both the SRB staining and NR uptake data (Method 2), the cadmium-induced lethal–sub-lethal boundary was between 10 and 12.5 μg/ml cadmium. Additional support for placing the boundary between these cadmium doses was provided by the photomicrographs of the fixed cells that remained in the the 96-well plate after the cytotoxicity assays shown in Fig. 1 were completed (not shown). At both 7.5 and 10 μg/ml cadmium there were changes in cell morphology: many cells developed a more rounded or spherical appearance compared with controls, indicating cytoskeletal disturbances. These disturbances have been observed in cells exposed to heavy metal ions by others (reviewed in [69]) and may be an important component of the cytotoxic effects of cadmium and other heavy metal ions. These cytoskeletal disturbances have also been shown to be reversible [70], indicating they are only sub-lethal effects and thus consistent with the findings reported here. Due to these morphological changes, it is difficult to visually determine if there was a corresponding decrease in cell number (as indicated by the SRB staining) at these two cadmium concentrations. However, at 12.5 μg/ml cadmium, the empty patches clearly show cell detachment has occurred, indicating a decrease in cell number relative to the starting cell number; therefore, since the cultures were confluent when the cadmium was added, this decrease must be due to a net loss of cells. Our experience, as well as that of others [71,72], has shown that cells that lose their ability to remain attached to the substrate as a result of exposure to a toxicant are dead or dying, and thus represent a lethal effect.

At higher cadmium concentrations (above 12.5 μg/ml) the amount of LDH released increased in a linear dose-dependent relationship ($r^2 = 0.980$; $p < 0.001$), reaching a final level that was 47% above the control level. Based solely on this information, the boundary between lethal and sub-lethal effects is between 12.5 and 15 μg/ml cadmium, slightly higher than that determined by the rest of the data. However, in light of the data from the other assays, use of the lower range of cadmium concentrations is appropriate.

For the second part of this experiment, samples were taken from duplicate PLHC-1 cultures that had been treated to identical culture conditions, cadmium doses, and exposure duration, and then run on 10% polyacrylamide gels (not shown). Cell lysates for SDS–PAGE samples could not be prepared from the 10 μg/ml (or higher cadmium concentrations) because, as a result of the cytotoxic effects of the cadmium on cell attachment, too many cells were lost from the wells during processing. The protein bands from these silver-stained gels that showed visible changes were quantitated by scanning densitometry and their relative molecular masses determined: five cadmium-induced protein bands were observed. Two major bands with approximate relative molecular masses of 70 and 27 kDa had the largest relative increases that started at 2.5 μg/ml cadmium, while three other minor bands of 25, 32, and 35 kDa showed less intense increases.

The protein gel results are representative of three similar experiments evaluating 24 h exposures of PLHC-1 cells to cadmium. The average increases in the 70 kDa protein bands at 5 and 7.5 μg/ml cadmium were 1.68 ± 0.13-fold and 1.86 ± 0.16-fold, respectively, and both were significant ($p < 0.001$). In addition, the combined average increases in the 70 kDa protein were dose-dependent and linear ($r^2 = 0.908$; $p < 0.001$). For the 27 kDa protein band, the average increases for this band at 5 and 7.5 μg/ml cadmium were 2.98 ± 0.49-fold and 2.69 ± 0.56-fold, respectively, and these were also both significant ($p < 0.005$ and $p < 0.025$). At lower cadmium doses (1–7.5μg/ml), the increases in the 27 kDa protein band levels were dose-dependent ($r^2 = 0.644$; $p < 0.001$), but at higher levels they decreased. All these increases were initially observed at cadmium concentrations that did not induce cytotoxic effects.

A comparison of these cadmium-induced proteins with heat-inducible proteins from PLHC-1 cells indicates that the two major cadmium-inducible protein bands, with relative molecular masses of 70 and

27 kDa, appear to be HSPs. Western blots (both one- and two-dimensional) prepared from SDS–PAGE studies using monoclonal antibodies specific for the Hsp70 family have confirmed that the 70 kDa protein band consists of several proteins, all of which are members of the Hsp70 protein family. Almost all the protein in the band from the control cells consisted of the constitutively expressed (Hsc70) member of the Hsp70 family while most of the cadmium-induced increases in this band were due to increases in several isoforms of Hsp70, the inducible member of the Hsp70 family (data not shown). The two rectangular boxes in the left panel of Fig. 1 indicate the range of cadmium concentrations that showed increases in the levels of these two heat shock proteins in the PLHC-1 cells.

Appendix B. Assessment of cadmium toxicity in zebrafish larvae using hsp70 gene expression

B.1. Toxicity in larvae exposed to cadmium chloride

In preliminary work, we found that the larval period appeared more sensitive to cadmium toxicity. Thus, only this developmental stage was chosen to derive dose–response relationships for cadmium. Edema, trunk abnormalities, and mortality were the endpoints that exhibited the clearest dose dependency, were consistently observed at each dose, and required the least amount of subjectivity to score. The endpoint category edema was defined as an abnormal swelling in any one of the head, pericardial region, or thorax. Trunk abnormalities were defined as curvature in the anterior–posterior body axis as viewed laterally (sloped back or hunched back) or from a dorsal or ventral position (bent anterior trunk). Mortality was readily identified in larvae lacking a heartbeat and that displayed obvious white necrotic tissue.

These endpoints were used for establishing the dose–response relationships for larvae exposed to cadmium chloride and used in the calculation of LC_{50} and EC_{50} values. Graphs representing the percent response for mortality alone and for all endpoints grouped together (any of trunk abnormalities, edema, immobility, and/or mortality) were prepared, and linear regression of their Probit–log transformation was carried out. For cadmium chloride, the median lethal and multiple endpoint effect doses along with their 95% confidence intervals were calculated to be $LC_{50} = 18.8$ μM (4.8–73.8 μM) and $EC_{50} = 1.7$ μM (0.7–4.1 μM) [73].

B.2. Induction of endogenous hsp70 following cadmium exposure

Whole mount photos of cadmium exposed wild-type larvae revealed a dose-dependent increase in *hsp70* mRNA detected in the gill and head regions. Serial sections of cadmium chloride-treated (3 h pulse exposure) larvae identified *hsp70* transcripts in the developing gill, skin, olfactory epithelium, lateral line organ, digestive tract, liver, and pronephros, while no expression was detected in controls at this stage of development. Expression of *hsp70* showed a dose-dependent increase in the number of tissue types expressing. The 125 μM Cd dose showed expression in skin, gill, olfactory, digestive tract, liver, and pronephros (primitive kidney). The 5 μM dose revealed *hsp70* expression in the olfactory tissue, gill, and skin while only the gill and skin expressed at 0.2 μM Cd. The proportions of larvae per treatment dose expressing *hsp70* in a given tissue were then calculated from counts of the serial sections expressed as a percent. The differences in proportions between the Cd treatments and controls expressing *hsp70* in a given tissue were highly significant for all tissue types ($p < 0.00001$) except for the digestive tract at 5 μM Cd which was not significantly different from the control ($p > 0.05$) [73].

B.3. Induction of reporter hsp70-eGFP following cadmium exposure

Post-hatch transgenic larvae (80 hpf) were exposed to a 3 h pulse of cadmium chloride to determine if expres-

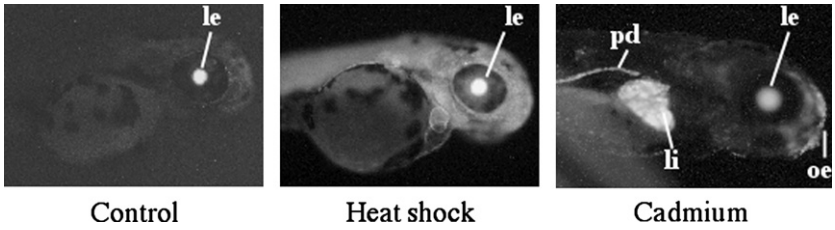

Fig. 2. Expression of eGFP in living zebrafish larvae carrying a stably integrated *hsp70/eGFP* transgene. The transgene is expressed in the lens during normal development at control temperature (28 °C), but is strongly activated throughout the embryo following a 1 h heat shock at 37 °C. In contrast, eGFP activity is evident in the liver, pronephric ducts (primitive kidney), olfactory epithelium, and scattered cells of the skin following a 3 h exposure of transgenic larvae to cadmium choride. The endogenous *hsp70* gene is expressed in similar tissue-specific patterns [64,73]. All larvae are four days of age, and were viewed using a Nikon E600 epifluorescence microscope equipped with a Nikon Coolpix digital 990 camera. le, lens; pd, pronephric ducts; li, liver; oe, olfactory epithelium. (Adapted and reprinted from [64] and [73], with permission).

sion of the *hsp70*-eGFP reporter gene mimicked Cd-induced patterns of endogenous *hsp70* expression observed in wild-type larvae. Cadmium-induced expression of the *hsp70*-eGFP reporter gene in live larvae 24 h after exposure followed a dose-dependent pattern in terms of the number and types of different tissues expressing, and was clearly similar to that observed for endogenous *hsp70* in wild-type larvae. The 125 μM dose caused GFP fluorescence in olfactory epithelium, gill, skin, liver, pronephros, and lateral line (see Fig. 2). Olfactory, gill, and skin expression was also seen at 5 μM, while only gill and skin expressed the *hsp70*-eGFP reporter gene at the lowest (0.2 μM) Cd dose. The proportion of larvae per treatment expressing *hsp70*-eGFP in a given tissue was significantly different from the control ($p < 0.0025$) for all treatment doses [73].

The appearance of GFP in transgenic larvae was also monitored in real time following Cd exposure in order to investigate the temporal pattern of *hsp70* activation. Larvae from a 3 h pulse exposure to 125 μM Cd were observed during recovery in water. Expression of *hsp70*/*eGFP* was observed first in olfactory epithelium and gill (8 h post-exposure), followed by liver (16 h) and subsequently the pronephros (24 h) [73].

In subsequent studies, we have found that the olfactory receptor neurons that strongly express eGFP at and below EC_{50} range concentrations also activate cell death pathways (Blechinger and Krone, unpublished data). Interestingly, olfaction-mediated predator avoidance behaviors are disrupted in cadmium-exposed rainbow trout [74], and this correlates closely with cadmium uptake in the olfactory system. Thus, reproductive and other behavioral effects that rely on proper olfaction can be disrupted following cadmium exposure. Recent work carried out in collaboration with D. Chivers and R. Kusch, University of Saskatchewan, suggests that this is also true for 60 day old juvenile zebrafish exposed to cadmium as 5 day old larvae (Blechinger et al., unpublished data). This work relies on one of the major strengths of using eGFP transgenes in transgenic zebrafish for whole animal assays, that is, the assay is carried out in living embryos without the addition of any assay reagents. As a result, direct correlations between conditions that enhance *hsp70* promoter activation and subsequent morphological and behavioral effects can be made hours, days, or even weeks later in the same individual animals.

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