Aquatic Toxicology 89 (2008) 103–112

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Cytotoxicities and induction of metallothionein (MT) and metal regulatory element (MRE)-binding transcription factor-1 (MTF-1) messenger RNA levels in the zebrafish (*Danio rerio*) ZFL and SJD cell lines after exposure to various metal ions

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ARTICLE INFO

Article history:
Received 19 January 2008
Received in revised form 5 June 2008
Accepted 6 June 2008

Keywords:
LC_{50} values of metal ions
Real-time PCR
Gene expression
Molecular mechanism
In vitro toxicity

ABSTRACT

Using zebrafish liver (ZFL) and caudal fin (SJD) cell-line models, the induction of metallothionein (MT) and metal regulatory element-binding transcription factor-1 (MTF-1) mRNA levels by various metal ions (Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{+}, As^{3+}, As^{5+}, Cr^{3+} and Cr^{6+}) were studied using the real-time PCR. The LC_{50}-24 h values of the metal ions were determined for the two cell lines prior to their exposure to different concentrations (10%, 25%, 50%, 75% and 100% LC_{50} values) of the heavy metal ions for RNA assay. The two cell lines were sensitive to As^{3+}, Cd^{2+} and Hg^{+}. $Zn^{2+}$and Cu^{2+} were moderately toxic, and As^{5+} and Cr^{3+} were less toxic to both cell lines. Each of the metal ions tested was found to cause significant induction of the mRNA levels in the SJD cells. Only Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and As^{3+} caused significant induction of MT mRNA levels in ZFL cells. $Zn^{2+}$and Cd^{2+} were efficient MT inducers in the two cell lines, but As^{3+} and As^{5+} are strong inducers only in the SJD cell line. In both cell lines, Cu^{2+} and Hg^{2+} are moderate inducers, and Cr^{3+} and Cr^{6+} were weak inducers of MT mRNA. MTF-1 induction is believed to be insufficient to cause MT gene induction, but As^{3+} and Cd^{2+} induced MTF-1 in ZFL cell line. Cd^{2+} was the most efficient inducer of MT in the cell lines, and also induced MTF-1 levels with clear dose-responsiveness in SJD cell line. These results indicated that MT induction can occur without MTF-1 induction for many metal ions such as Zn^{2+} and Cu^{2+}.

1. Introduction

Metallothionein (MT) is a low molecular weight cysteine-rich intracellular metal-binding protein for the homeostasis of essential metals and detoxification of non-essential metal ions (Andrews, 1990, 2000; Kägi, 1991; Palmiter, 1998). MT gene expression is inducible by heavy metal ions and has been shown to play an important role in the sequestration and detoxification of various heavy metal ions (Hogstrand and Haux, 1990; Chan, 1994, 1995; Adams and Freedman, 2000; Tom and Auslander, 2005; Amiard et al., 2006; Thirumoorthy et al., 2007). However, the induction of MT mRNA by various metal ions remains to be tested for each fish species (Chan et al., 1989, 2006; Eller-Jessen and Crivello, 1998; Cheung et al., 2004, 2005; Tom and Auslander, 2005; Chan and Chan, 2008).

The molecular mechanism of the metal induction of MT gene expression can be influenced by *cis*-acting elements in all MT genes, which comprises metal response elements (MREs) (Searle et al., 1984, 1987; Stuart et al., 1984, 1985; Olsson et al., 1995, 1998). The metal response element (MRE) is a *cis*-acting element in target genes for the binding of a *trans*-acting factor, called MRE-binding transcription factor-1 (MTF-1) (Westin and Schaffner, 1988; Andrews, 2001), which mediates the induction of the MT gene by various metal ions. However, the molecular mechanism of how various metal ions induce MT genes remains to be investigated (Palmiter, 1994; Bittel et al., 1998; Bourdineaud et al., 2006; Marr et al., 2006).

MTF-1 is a ubiquitously expressed zinc finger protein of around 80 kDa essential for basal and metal-induced MT gene transcription (Heuchel et al., 1994; Samson and Gedamu, 1995; Bittel et al., 2000; Lichtlen and Schaffner, 2001; Wang et al., 2004; Li et al., 2006). It was identified later in a wide range of species including mice, humans, mice, chickens, fruit flies (*Drosophila melanogaster*), pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*), trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis* spp.) (Radtke

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doi:10.1016/j.aquatox.2008.06.006

et al., 1993; Brugnera et al., 1994; Otsuka et al., 1994; Auf der Maur et al., 1999, 2000; Carvan et al., 2000; Dalton et al., 2000; Zhang et al., 2001; Chen et al., 2002; Cheung, 2003). Similar to an MRE, MTF-1 is conserved in evolution: human, mouse and fish MTF-1s are highly similar, with a 93% amino acid identity (Brugnera et al., 1994; Lichtlen and Schaffner, 2001) in their zinc finger DNA-binding domains (Brugnera et al., 1994; Cheung, 2003).

Unlike other zinc finger transcription factors, which appear to be constitutively active to bind DNA under normal physiological conditions, MTF-1 is reversibly bound to its target DNA, and the binding is dependent on changes of the availability of free Zn^{2+} in the cytoplasm (Westin and Schaffner, 1988; Radtke et al., 1993; Dalton et al., 1997; Koizumi et al., 1999; Bittel et al., 2000; Laity and Andrews, 2007). That is, the binding of MTF-1 to the MREs requires Zn^{2+} activation, and the Zn^{2+} can enter the nuclei from a dietary source to activate MT gene expression (Cousins and Lee-Ambrose, 1992; Laity and Andrews, 2007). Once the MTF-1 senses free Zn^{2+}, it adopts a reversible DNA-binding conformation. This allosteric change causes exposure of zinc fingers, and the MTF-1 can move to the nucleus and activate gene expression by associating with the gene promoters carrying MREs (Andrews, 2001; Lichtlen and Schaffner, 2001; Jiang et al., 2003). Recent studies have also confirmed that the mediation of MT gene expression starts by the translocation of MTF-1 protein from the cytoplasm to the nucleus and bind with MREs in target genes (Smirnova et al., 2000; Chen et al., 2007). However, whether metal ions can induce the production of MTF-1 in the cytoplasm and how other metal ions induce MT via MTF-1 remains to be elucidated.

The induction of the MT gene by other heavy metal ions, such as arsenic and chromium, has not been investigated. For example, one study found that Cr(VI) could depress the induced human MT-IIA gene by interfering with MTF-1 (Majumder et al., 2003). In fact, MTF-1 serves only as a zinc sensor (Palmiter, 1994) and mediator of hypoxia to induce MT genes (Murphy et al., 1999), and not as a sensor of Cd^{2+} or oxidative stress (Daniels et al., 2002). However, it is also possible that Zn^{2+} can function to release MTF-1 from another inhibitor (Palmiter, 1994), and it was demonstrated that MTF-1 bound to other "mediators" to recruit TFIID for activation of MT gene transcription (Marr et al., 2006).

Zebrafish are an ideal in vivo platform to study the relationship between MT gene expression and metal ions. Preliminary studies report that the zebrafish embryo/larva is a sensitive model for exposure experiments and accumulates metal ions (Li et al., 2004; Chen et al., 2004; Chan et al., 2006). A zebrafish MT gene (zMT-II) and its promoter have been previously characterized (Yan and Chan, 2002, 2004). Activation of the zMT-II promoter was observed after transfected into SJD cells (zebrafish caudal fin fibroblast cells) (Yan and Chan, 2002), and HepG2 cells (human hepatic cell line) (Yan and Chan, 2004) after exposure to different metal ions. Induction of zMT-II promoter after exposure to Zn^{2+}, Cd^{2+}, Cu^{2+} and Hg^{2+} was confirmed, with decreasing potency, whereas hydrogen peroxide, Ni^{2+}, Pb^{2+} and Co^{2+} did not cause a significant induction of the zMT gene promoter in either the HepG2 or SJD cells (Yan and Chan, 2002, 2004). The goal of this study was to evaluate the effects of 8 metals on the cytotoxicity and expression of MT and MTF-1 in two zebrafish cell lines. Specifically this study focused on the impact of MTF-1 expression and its relation to MT expression and toxicity.

2. Material and methods

2.1. Cell culture

The ZFL cells are zebrafish liver cells obtained from the American Type Culture Collection (ATCC CRL-2643). They were cultured at 28 °C in an incubator with 50% Leibovitz's L-15 medium containing 2 mM L-glutamine (Vitacell 30-2008), 35% Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and 4 mM L-glutamine (Gibco 12100), 15% Ham's F12 with 1 mM L-glutamine (Gibco 21700), 15 mM HEPES, 0.01 mg/ml insulin (Sigma I-1882), 50 ng/ml epidermal growth factor (EGF), 0.15 g/l sodium bicarbonate and 5% heat-inactivated fetal bovine serum (FBS, freshly added). The cells were sub-cultured by removing the medium, rinsing it with PBS (phosphate buffer saline) and then adding 1–2 ml of trypsin (0.25%)–EDTA (0.03%)–polyvinylpyrrolidone (0.5%) solution.

The SJD cells are zebrafish caudal fin SJD.1 cells obtained from the American Type Culture Collection (ATCC CRL-2296). They were cultured at 28 °C in an incubator with 5% CO_2 in DMEM medium containing 4 mM L-glutamine and 4.5 g/l glucose (Gibco #12100), and 15% heat-inactivated FBS. The cells were sub-cultured by removing the medium, rinsing it with PBS and then adding 1–2 ml of trypsin solution.

2.2. Alarmar blue™ assay

To obtain toxicity reference data for metal exposure study, alarmar blue™ assay was conducted to determine the LC_{50} values of different metal ions of the two zebrafish cell lines. Viable cell counting was performed by using trypan blue exclusion assay to determine the cell numbers in the culture. Cells (1×10^5) were seeded in each well of a 96-well flat-bottomed plate and incubated for one night. Metal solutions were diluted serially with the culture medium to different concentrations and added to the cultured cells. Six replicate treatments were done on the same plate for each dose of a metal, and the treated cells were incubated in the incubator as described above. After 24 h, the medium was replaced with a fresh medium containing alarmar blue™ amounting to 10% of the total medium volume, and incubated for 5 h. The fluorescent readings from each well were measured using the CytoFlour™ 2350 fluorescent plate reader system (Millopore) with an excitation wavelength of 485 nm and emission wavelength of 595 nm. The LC_{50} values of the heavy metal ions of the cells were determined using GraphPad Prism® analysis software using non-linear regression (curve fit) analysis.

2.3. Metal treatment, RNA preparation and reverse transcription

Cells (8×10^5, viable cells were counted using trypan blue exclusion assay) were seeded in each well of a 96-well culture plate. After incubation overnight, the media in the plate were replaced with different concentrations of metal solution diluted serially with the culture medium to 10%, 25%, 50%, 75% and 100% of their 24 h-LC_{50} values. Six replicate treatments were done on six plates for each dose of a metal, and the treated cells were incubated in the incubator for 12 h to determine the MTF-1 mRNA levels and for 24 h for MTF-1 study, presuming that MTF-1 should be induced prior to the induction of MT if MTF-1 is involved to MT induction.

The cells treated with metal ions were washed with PBS and homogenized with 0.6 ml of Tri-Pure Isolation Reagent (Roche, Indianapolis, IN, USA) on ice; after homogenization, 100 μl 1-bromo-3-chloropropane (BCP) was added and mixed in thoroughly. After centrifugation at 12,000 × *g* for 15 min at 4 °C to separate the organic phase and aqueous phase, the upper aqueous phase was transferred into a fresh tube, and 500 μl isopropanol was added to precipitate total RNA. The tubes were then centrifuged at 12,000 × *g* for 15 min at 4 °C to form RNA pellets. The RNA pellets were washed with 500 μl 70% ethanol and allowed to air dry. Finally, the RNA pellets were dissolved in 40 μl DEPC-treated ddH_2O at 70 °C for 10 min, and the dissolved RNA samples were stored at −80 °C.

Two μl of the RNA samples was added to 498 μl dH_2O and mixed in thoroughly. The absorbance of the diluted RNA samples was