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Regulation of metallothioneins and ZnT-1 transporter expression in human hepatoma cells HepG2 exposed to zinc and cadmium

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ABSTRACT

Essential and non-essential metals can affect vital cellular processes, when over-accumulated within the cells. For this reason, cells have evolved multiple protein sensors, transporters, and other type of proteins to regulate and control free metal homeostasis. Among these, metallothioneins (MT) and ZnT-1 transporter play a key role in the regulation of free Zn concentrations.

Herewith, MT expression in Zn (170 μM) and Cd (0.1 and 10 μM) exposed HepG2 cells is analyzed and compared. In addition, the modulation and localization of the membrane transporter ZnT-1 has been investigated. MT-I and MT-II were up-regulated in response to both Zn and Cd exposure and, as expected, Cd represented the most potent inducer. Namely, 0.1 μM Cd was able to up-regulate MT-I, and -II in a way comparable to 170 μM Zn. This is in agreement with MT general function of metal-chelating protein, acting with higher tolerance to essential metals than to non-essential ones. ZnT-1 protein, a plasma membrane specific Zn transporter, was up-regulated as well by both Zn and Cd, although in the same way. Immunofluorescence technique provided evidence that high levels of ZnT-1 measured by biochemical techniques, are related to an increased localization of the transporter at the plasma membrane.

1. Introduction

Zinc (Zn) is an essential nutrient and component of a wide variety of metalloenzymes, transcription factors and other proteins. It plays a critical role in biochemical processes such as cell proliferation, differentiation and programmed cell death, in the regulation of DNA synthesis and mitosis, and it is involved at the level of cellular signal transduction (Beyersmann and Haase, 2001). However, overaccumulation of this metal can generate reactive oxygen species which interfere with vital cellular processes leading to cell death.

Cadmium (Cd) is a toxic transition metal of great environmental and occupational concern, classified as a human carcinogen (IARC, 1993). Cd exposure principally occurs through consumption of food and drinking water, inhalation, and cigarette smoke. This metal is poorly excreted and cannot undergo metabolic degradation to less toxic species, leading to an extremely long biological half life (>20 years). Cd accumulates primarily in the liver and kidney, reaching values in the higher μM range even in not occupationally exposed population (Friberg and Vahter, 1983). The central nervous system white matter, female and male reproductive systems represent other prominent Cd targets (Waalkes, 2003; Ohana et al., 2006). At molecular levels, Cd at low, non-cytotoxic concentrations, interferes with Zn-finger protein domains by Zn displacement and consequently inactivation of protein functions (Hartwig et al., 2002).

In a wide variety of tissues and cells, the major route for Zn^{2+} and Cd^{2+} permeation has been shown to be via the L-type calcium channel (LTCC) (Ohana et al., 2006). Once crossed the membrane barrier, the intracellular homeostasis of Zn and Cd is primarily regulated by two proteins, ZnT-1 and metallothioneins (MT).

ZnT family consists of ten members responsible for Zn homeostasis by either lowering cytoplasmic levels or by sequestering it into secretory and synaptic vesicles. ZnT-1 is the first mammalian zinc transporter described among ZnT family members and the most ubiquitously expressed (Palmiter and Findley, 1995). All the other members of the ZnT family studied so far are located on intracellular organelles. ZnT-1 protein, responsible for zinc efflux, is the only member of the family located on the plasma membrane of many cell types with a structure displaying a six membrane-spanning domain (McMahon and Cousins, 1998). The presence of ZnT-1 protein confers resistance against Zn toxicity. However, its physiological functions remain unknown.

MT are ubiquitous low molecular weight, cysteine-rich proteins. In higher eukaryotes *MT* are the best understood

Abbreviations: BSA, bovine serum albumin; $CdCl_2 \cdot xH_2O$, Cd; FBS, foetal bovine serum; MT, metallothioneins; MTF-1, metal transcription factor 1; OD, optical density; PBS, phosphate buffered saline; PMSF, phenyl-methylsulphonyl fluoride; SDS, sodium dodecyl sulphate; $ZnSO_4 \cdot x7H_2O$, Zn.

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metal-regulated genes. Four functional *MT* genes have been identified (*MT-I* to *MT-IV*), but *MT-I* and *-II* are the most widely distributed isoforms. MT proteins play a role in Zn homeostasis *via* the metallothionein(MT)/thionein(T) system to control the concentration of ready available Zn (Tapiero and Tew, 2003). In addition, MT are stress inducible proteins which provide protection against oxidative stress, and against toxic metal ions (e.g. Cd, Hg and Co), or essential trace elements by their chelation, thus reducing the biological interaction of reactive species.

Both ZnT-1 and MT expression is modulated by Zn *via* the metal responsive element (MRE) of the transcription factor MTF-1. The MTF-1 is a metalloregulatory protein that serves as an intracellular Zn sensor to trigger gene expression and restore homeostasis, since an increase in free zinc in cells serves as a second messenger to activate the DNA-binding activity of MTF-1. However, it is less clear how Cd senses MTF-1 activity. Actually, MTF-1 is a cellular protein located in the cytoplasm of unstressed cells that rapidly translocates to the nucleus for DNA-binding and gene activation (Andrews, 2000; Smirnova et al., 2000).

The study of MT and metal ion transporter is of great importance since an increasing number of human diseases are thought to be related to metal homeostasis disturbance (Rolfs and Hediger, 1999).

The aim of the present study was to assess and compare the expression of the two metal-regulated proteins, MT and ZnT-1, in a cell line (human hepatoblastoma cell line HepG2) derived from a target organ exposed to essential trace element Zn, and to toxic metal Cd.

2. Materials and methods

2.1. Cell cultures

Human hepatoma cells HepG2 were routinely grown in a monolayer culture in the presence of Opti-MEM medium (Invitrogen, San Giuliano Milanese, MI, Italy) supplemented with 10% heat inactivated foetal bovine serum (Invitrogen) and 1% antibiotics at 37 °C in a humidified atmosphere of 5% CO_2. The medium was replaced twice a week and cells were trypsinised and diluted every 7 days at 1:3 ratio. The cells were trypsinised and transferred either into 165 cm^2 flasks (Costar) for biochemical preparations or into 8 cm^2 plastic plates (Costar), for fluorescence assays.

2.2. Metallothionein extraction and biochemical analysis

HepG2 cells were exposed 24 h after seeding to non-cytotoxic (Urani et al., 2005) Cd (0.1 and 10 μM) and Zn (170 μM) concentrations for 24 h. Stock solutions of 1 mM and 0.01 mM $CdCl_2{\cdot}xH_2O$ (BDH Laboratory, MI, Italy, cat No. 104784R), and of 7 mM $ZnSO_4{\cdot}x7H_2O$ (Sigma–Aldrich, MI, Italy, cat. No. Z-4750) in ultrapure Milli Q water (Millipore) were sterilized by filtration (0.22 μm), and stored at 4 °C. Cells grown in complete culture medium represented negative controls. At the end of treatments, the cells were harvested, and washed by centrifugation (200*g*, 10 min, 4 °C), with PBS to remove Cd excess. Harvested cells were resuspended in 10 mM Tris–HCl (pH 7), 5 mM EDTA, 1 mM protease inhibitor PMSF and immediately frozen for homogenization. Defrosted samples were clarified by centrifugation (20,000*g*, 45 min 4 °C). Low molecular weight proteins (20 μg) were separated electrophoretically by 12% NuPage gels (invitrogen) and processed according to Mizzen et al. (1996) to enhance membrane transfer and retention of small size proteins. Mouse anti-metallothionein (MT-I, -II) was used as a primary antibody (Zymed Laboratories, San Francisco, CA, USA cat. No. 18-0133). Anti-mouse alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA) was used as secondary antibody, and protein binding visualized by the colorimetric substrate BCIP/NBT (Sigma–Aldrich, Milano, Italy). MT expression was quantified by the optical densitometric analysis and compared to untreated negative controls.

2.3. Membrane protein extraction and biochemical analysis

Twenty four hours after seeding, HepG2 cells were treated with non-cytotoxic Cd (0.1 and 10 μM) and Zn (170 μM) concentrations for 24 h. After treatment, membrane protein preparation was performed according to McMahon and Cousins (1998) with minor modifications previously reported (Urani et al., 2003). Briefly, samples were harvested, washed by centrifugation (200*g*, 10 min, 4 °C) in PBS, resuspended in HES buffer (20 mM Hepes pH 7.4, 1 mM EDTA, 250 mM sucrose, protease inhibitor cocktail, Sigma), homogenated by sonication (Soniprep 150, MSE) for 30 s at amplitude 6 μm, and centrifuged (100,000*g* for 30 min at 4 °C) to obtain the crude membrane fraction. Proteins (100 μg) were resolved on 10% NuPage gels (invitrogen), and were blotted (1 h, room temperature, 30 V constant current) to a nitrocellulose membrane. Blocked membranes (Tween buffer: PBS, 0.1% Tween-20, 8 mM NaN_3, containing 5% BSA) were incubated overnight at room temperature with rabbit antiserum raised against rat ZnT-1 (provided by R.J. Cousins, Food Science and Human Nutrition Department, University of Florida, USA). The appropriate secondary antibody (anti-rabbit Ig alkaline phosphatase conjugate, Sigma) and the colorimetric substrate BCIP/NBT allowed the protein visualization. The estimate of protein levels was performed by densitometry analysis of protein bands, and ZnT-1 levels expressed as percentage of negative controls.

2.4. ZnT-1 immunolocalization

HepG2 cells were plated on sterile glass coverslips (9000 cells/cm^2) and left in complete medium until sub-confluence was reached. After 24 h of treatments (170 μM Zn, and 0.1 and 10 μM Cd), the cells were methanol fixed (5 min on ice), washed in PBS, and membranes permeabilized in Triton X-100 0.1% for 3 min. The cells were blocked in PBS + 1%BSA, and incubated with ZnT-1 antiserum at 37 °C in humid atmosphere. After 1 h of incubation, the samples were washed in PBS + 1%BSA and probed with an appropriate Alexa Fluor 488 (Molecular Probes, Invitrogen) secondary antibody. The coverslips were mounted and viewed (at least 500 cells/slide) under a Zeiss Axioplan microscope (Karl Zeiss, Arese, Italy) equipped with epifluorescent optics and digital camera (CoolSnap-ProColors Media Cybernetics, Bethesda, MA, USA), and pictures taken and stored by Image Proplus software (Media Cybernetics).

2.5. Optical densitometry and statistical analysis

The relative protein expression of immunochemical results was measured by the Gel Doc image system (Bio-Rad Laboratories, Milano, Italy) followed by quantitative analysis with Quantity One dedicated software. Optical densities (OD) are the arbitrary units that allow the quantification of protein levels. Data are expressed as OD or as percentage of increment (Δ%) vs. control levels of at least three independent experiments (mean ± S.D.). Student's *t*-test and ANOVA (Multiple Range Test) were used for sample comparisons (Statgraphics Plus version 5.0).

3. Results

3.1. Metallothionein expression

Accumulation over physiological levels of essential elements, or exposure to toxic metals usually triggers the activation of specific