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Attenuation of cadmium-induced testicular injury in metallothionein-III null mice

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ABSTRACT

Aims: In order to evaluate the role of metallothionein (MT)-III in cadmium (Cd)-induced testicular toxicity, we examined the sensitivity of MT-III null mice to severe testicular injury caused by Cd.
Main methods: Male MT-III null mice, MT-I/II null mice and wild-type mice were given a subcutaneous injection of $CdCl_2$ (15 μmol/kg). The testis was collected from each mouse at 6, 12 and 24 h after Cd administration.
Key findings: Testicular hemorrhages by evaluating morphology, hemoglobin content and histological parameters in the 3 types of mice were elevated by Cd injection in a time-dependent manner. The degree of hemorrhage in Cd-injected MT-I/II null mice was similar to that in the wild-type mice. In contrast, hemorrhage in the MT-III null mice was attenuated compared with that in wild-type mice and MT-I/II null mice. Cd levels, MT-I and MT-II mRNA levels and Cd-binding molecules in the testis were similar between MT-III null mice and wild-type mice. In microarray analysis, high expression of purine-nucleoside phosphorylase 2 (Pnp2), retinal degeneration 3 (Rd3), and cadherin-like 24 (Cdh24) was revealed in the testis of MT-III null mice under normal or Cd-treated conditions.
Significance: MT-III null mice were found to show attenuation of Cd-induced severe testicular toxicity. These results suggest the lack of MT-III contributes to protection of testis from Cd. In addition, regulation of Pnp2, Rd3, and Cdh24 mRNA levels may involve the sensitivity of MT-III null mice to Cd.

Introduction

Cadmium (Cd) is an environmental contaminant that can adversely affect a number of tissues including the liver, testis, kidney, lung and bone. Various studies have demonstrated that Cd causes disruption of the vascular system in the testis and blood–testis barrier, and acute testicular damage leads to edema, hemorrhage and necrosis (Parizek and Zahor 1956; Prozialeck et al. 2008; Siu et al. 2009).

Metallothionein (MT) is a cysteine-rich low-molecular weight protein with high affinity for metals such as Cd and mercury (Klaassen et al. 1999). MT-I and MT-II, major isoforms, are expressed ubiquitously in tissues and are induced by various stimuli including some metals. Moreover, these proteins have been shown to be protective against metal toxicity and oxidative stress (Cai et al. 1999; Klaassen et al. 1999; Shibuya et al. 2008). MT-I/II null mice with disrupted MT-I and MT-II genes exhibit high sensitivity to Cd toxicity, such as hepatotoxicity, nephrotoxicity and bone toxicity (Klaassen et al. 2009). However, Liu et al. (2001) have shown that MT-I and MT-II do not have a protective effect on Cd-induced testicular toxicity using MT-I/II null mice, because Cd causes severe testicular damage in both MT-I/II null mice and wild-type mice.

MT-III was discovered as a growth inhibitory factor that was deficient in the brain of patients with Alzheimer's disease, and mainly occurs in the brain (Uchida et al. 1991; Palmiter et al. 1992; West et al. 2008). The MT-III has also been identified in the testis, epididymis, prostate, uterus, ovary, kidney, intestine and tongue (Moffatt and Séguin 1998; Hozumi et al. 2008). Despite the fact that MT-III is found in various tissues, most studies have focused on understanding the role of MT-III in the brain and have indicated the protective effects of MT-III against brain injury and oxidative stress (Hozumi et al. 1998; Uchida et al. 2002).

On the other hand, a recent study from our laboratory has found that sensitivity of MT-III null mice to Cd-induced acute hepatotoxicity is less than that of wild-type mice, although MT-III is not expressed in the liver of wild-type mice (Honda et al. 2010a). The lack of MT-III was tolerant to Cd-induced hepatotoxicity. Although the mechanism of

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resistance is not clear, MT-III may influence not only hepatotoxicity, but also Cd-induced other toxicity. Therefore, in the present study we examined the susceptibility of MT-III null mice to Cd-induced acute testicular injury by evaluating morphology, hemoglobin content and histological parameters. In addition, to elucidate contribution of other MTs (mainly MT-I and MT-II) in the same experimental condition, we also examined response to Cd testicular injury in MT-I/II null mice.

Materials and methods

Chemicals

$CdCl_2 \cdot 2.5H_2O$, 10% neutral buffered formalin solution and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals

MT-I/II null mice (Masters et al. 1994), MT-III null mice (Erickson et al. 1997) and wild-type mice, which are of 129/Sv genetic background, were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were routinely bred in the laboratory animal facility of Aichi Gakuin University. All strains of mice were housed in cages in ventilated animal rooms at 23 ± 1 °C with relative humidity. They were maintained on standard laboratory food (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water ad libitum and received humane care throughout the experiment according to the guidelines established by Aichi Gakuin University.

Treatments

Ten- to twelve-week-old male MT-I/II null mice, MT-III null mice and wild-type mice were randomly assigned to control and experimental groups, with 5 to 6 mice per group. Each experimental group was given a single subcutaneous injection of $CdCl_2$ at a dose of 15 μmol/kg, and then mice were sacrificed under diethyl ether anesthesia at 6, 12 and 24 h after administration. The testis was collected from each mouse to evaluate testicular injury.

Hemoglobin concentration

The testis was homogenized in 9 volumes of saline, and the homogenates were centrifuged at 4 °C for 15 min at $16000 \times g$. Testicular hemoglobin (Hb) content in 20 μl supernatant or Hb standard solution was determined by the addition of 180 μl color reagent in the Hemoglobin B Kit (Wako, Osaka, Japan).

Histopathology

The testis was fixed in 10% neutral buffered formalin solution and embedded in paraffin. Deparaffinized tissue sections (5 μm thick) were stained with hematoxylin and eosin.

Cd concentration and HPLC analysis

Prior to analysis of the Cd concentration, the testis was digested with nitric acid and hydrogen peroxide. After digestion, inorganic residues were dissolved in ultrapure water, and metal analysis was carried out using inductively coupled plasma-mass spectrometry (ICP-MS, ICP model 4500; Agilent Technologies, Santa Clara, CA, USA).

The testis was homogenized in 9 volumes of saline, and the homogenates were ultracentrifuged at 4 °C for 60 min at $105000 \times g$ to separate supernatant and pellet. The ratio of Cd content in supernatant and pellet was determined. In addition, the filtered supernatant (200 μl) was applied to TSK-Gel G 3000 SW column (TOSOH, Tokyo, Japan) and was eluted with 50 mM bicine ammonia (pH 8.2) at a flow rate of 0.8 ml/min. Cd in the eluted sample was detected continuously by ICP-MS connected to the high-performance liquid chromatography (HPLC) column.

MT-I, MT-II and MT-III mRNA levels

Total RNA was isolated from the testis of MT-III null mice and wild-type mice using QuickGene RNA tissue kit S II (Fujifilm, Tokyo, Japan). Real time RT-PCR was performed to determine the gene expression of MT-I, MT-II and MT-III using a reaction mixture containing the primers for a total of 30 cycles. As the reference standard, the level of β-actin mRNA was determined, and the relative mRNA levels of the selected gene compared with that of β-actin were calculated. The PCR primers were as follows: MT-I gene, forward 5′-TCTAAGCGTCACCACGACTTCA-3′ and reverse 5′-GTGCACTTGCAGTTCTTGCAG-3′; MT-II gene, forward 5′-CCTGTGCCTCCGATGGAT-3′ and reverse 5′-ACTTGTCGGAAGCCT-CTTTG-3′; MT-III gene, forward 5′-CTGAGACCTGCCCCTGTC-3′ and reverse 5′-TTCTCGGCCTCTGCCTTG-3′; and β-actin gene, forward 5′-GATCTGGCACCACACCTTCT-3′ and reverse 5′-GGGGTGTTGAAGGTCT-CAAA-3′.

DNA microarray analysis

DNA microarray analysis was performed as described previously (Honda et al. 2010b). Total RNA (5 μg) was applied to an OpArray™ Mouse V4.0 slide that had 35,852 genes registered (Operon Technologies, Alameda, CA, USA). A Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies Inc., Santa Clara, CA, USA) was used to synthesize complementary RNA (cRNA) from the double-stranded cDNA template. A primer containing poly dT and the T7 polymerase promoter was annealed to the poly A^+ RNA. Reverse transcriptase was then added to the reaction to synthesize the first and second strands of cDNA. Next, the double-stranded cDNA from wild-type mice and MT-III null mice was transcribed in the presence of cyanine (Cy) 3 and Cy5-labeled nucleotide, respectively. These two sets of fluorescently-labeled cRNA were mixed and hybridized to an OpArray™ slide for 16 h at 42 °C using a Lucidea SlidePro Hybridizer (GE Healthcare UK Ltd., Buckinghamshire, England). A fluorescent image of the OpArray slide was recorded with CRBIO (Hitachi Software Engineering, Tokyo, Japan). The digitized image data were processed with DNASIS Array software (Hitachi Software Engineering). After global normalization, the data were filtered to exclude genes with low expression levels. The ratios of the intensity of Cy5 (MT-III null mice injected with Cd or control) to that of Cy3 (wild-type mice injected with Cd or control) were calculated, and a difference of 2-fold change was used to select up-regulated and down-regulated genes. Information on each gene on the slide was obtained from the National Center for Biotechnology Information (NCBI) database.

Real time RT-PCR was performed for the selected genes to verify the microarray data. The PCR primers were as follows: purine-nucleoside phosphorylase 2 (Pnp2) gene, forward 5′-GCCGGGAAAGCAGCTG-CACA-3′ and reverse 5′-TGGCCCCACCTCAGTGGCAT-3′; retinal degeneration 3 (Rd3) gene, forward 5′-TGGAGCTGGCTGGGCAGATGA-3′ and reverse 5′-ATAGGTGGGCCTGGGCGTGT-3′; cadherin-like 24 (Cdh24) gene, forward 5′-GCAGCACTGCCACGGTGACT-3′ and reverse 5′-TGCTGAGACCGGTGGGCGAT-3′; RIKEN cDNA 2010204K13 gene, forward 5′-CCAGTCGGCGAAATCCTGGCG-3′ and reverse 5′-AGGTCA-GAGGGGGAAAGGGCG-3′; and olfactory receptor (Olfr) 272 gene, forward 5′-CGCTGGGGCAGGCCATCAAA-3′ and reverse 5′-TCAACG-TTTTCCTTCCCAGCATGT-3′.

Statistical analysis

The data are presented as mean ± standard deviation (S.D.) for each experimental group ($n = 3$ to 6). Statistical analyses were performed using one-way or two-way analysis of variance