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Original Article

Role of metallothionein-like cadmium-binding protein (MTLCdBP) in the protective mechanism against cadmium toxicity in the testis

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Abstract: The role of metallothionein (MT)-like cadmium (Cd) binding protein (MTLCdBP) in protecting the testes against Cd toxicity was examined. In the acute Cd exposure treatment, cadmium chloride was intraperitoneally injected at 2 mg Cd/kg to Wistar male rats. In the chronic Cd toxicity treatments, 20 mg Cd/kg/d was orally administered for 5 d a week for 5, 10, and 15 wk. MT (-I,-II) and MTLCdBP were measured using ELISA and Cd-Hem methods, respectively. Testicular tissues were immunostained with antibodies of MT-I,-II, MT-III, and MTLCdBP. Expression of HO1, OGG, iNOS, COX2, and p53 was measured by RT-PCR. Cd concentration in the testis increased dose-dependently in response to Cd exposure. MTLCdBP concentration increased markedly with increasing Cd accumulation. Significant increases in expression of iNOS, HO1, COX2, and OGG1 were observed in the acute exposure treatment. In the chronic oral administration group, expression of MT-I, MT-II, MT-III, iNOS, HO1, and COX2 did not change. Positive immunostaining of MTLCdBP was observed in testicular interstitial tissue. In the testis protected from Cd toxicity, MTLCdBP induction increased significantly with increasing Cd accumulation. Our results suggest that MTLCdBP plays an important role in protecting the testis against Cd toxicity.

Key words: Cadmium, Testicular toxicity, Metallothionein (MT), metallothionein-like cadmium-binding protein (MTLCdBP), immunostaining, Hemorrhagic inflammation, Gene expression

Introduction

Cadmium (Cd) is an environmental pollutant and is well-known as the cause of itai-itai disease. In the industrial workplace, respiratory exposure to Cd is known to cause pulmonary edema and renal dysfunction. Even today, occupational Cd exposure occurs during mining for metals or in the manufacture of pigments and batteries utilizing Cd. The general population can be exposed to Cd through contaminated drinking water, food, and smoking

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habits. Industrial activities, such as metal smelting and refining, and municipal waste incineration also release Cd into the atmosphere as Cd oxide, chloride, or sulfide[1–3].

Cd is a highly toxic heavy metal and may cause severe damage to embryos and the reproductive organs of adults, including both the ovaries and the testes[4]. The testes are especially sensitive to Cd toxicity, with the metal causing hemorrhagic inflammation and edema, followed by necrosis and mortality. Testicular damage induced by acute Cd toxicity has been studied by Parizek[5], but the precise mechanisms underlying Cd toxicity to the testes remain unclear.

The toxicity of Cd can be mitigated by pre-administration of small doses of Cd before exposure to toxic doses and by simultaneous administration of Cd and selenium[6–8]. These protective mechanisms may involve the participation of metal-binding proteins, the high molecular selenium-cadmium complex, and metallothionein (MT)[9, 10]. MT in the testes is reduced by Cd injection, whereas in chronic oral administration of Cd, MT is induced and increases as a response to Cd accumulation in testicular organs. However, although the MT concentration in the testis is higher than that in the liver and the kidney, the testis is very sensitive to injected Cd[9–12]. Therefore, the question remains whether MT in the testis is sufficient to protect against Cd toxicity.

In addition, the presence of iso-MT-I,-II, and -III in the testis is reported from MT gene expression; however, the role of MT proteins and whether their induction is in response to Cd accumulation are not clear. The MT-like Cd-binding protein (MTLCdBP) is also present in the testis[9, 11, 12]. The heat-stable, metal-binding MTLCdBP is low in cysteine, in contrast to cysteine-rich MT, and is induced and synthesized in response to Cd accumulation in the testis[9, 11–20]. In rodents, MT-I/-II has been localized in spermatogenic, Sertoli, and interstitial cells, although there are discrepancies in their precise cellular distribution, and their role in protein induction is not clear[18, 20–28]. In addition, Cd toxicity in the testis was not reduced in MT-I transgenic mice even though the expression of MT-I was 5.2-fold higher than in the wild type[29]. Therefore, the high susceptibility of the testis to Cd toxicity may not be entirely related to the levels of MT-I/-II but is possibly related to the genetic background[30]. It remains unclear why the testis displays a relatively high basal level of MT-I/-II compared to other organs (e.g., the liver), and it has been suggested that it fails to detoxify Cd.

Tesmin, a metallothionein-like protein, has also been reported in the testis[17]. Although it has been suggested that tesmin participates in spermatogenesis, more recently, it has been classified as a member of the CXC-hinge CXC family[28, 31–33]. In normal mice, tesmin has been detected in the cytoplasm in pachytene spermatocytes, and is translocated to the nucleus in the late pachytene or diplotene spermatocytes. It is also suggested that tesmin may have some characteristics related to MT-I/-II, such as metal sensitivity, since the expression of tesmin appears to be responsive to Cd. Studies have found that tesmin in spermatocytes is affected by Cd treatment[31, 32]. Nonetheless, the role(s) of MTs and tesmin in the testis remains unclear.

In this study, we aimed to clarify the role of MTLCdBP in protecting against Cd toxicity. We measured the separation of MT and MTLCdBP in the testis by using a combination of the Cd-Hem and the ELISA methods. The former is a measurement method using physical properties (heat stability, Cd binding) of MT, and the latter uses MT-I,-II antibody. In addition, we examined the participation of MTLCdBP in the protective mechanism against Cd toxicity by comparing the distribution of these proteins by immunostaining MT-I,-II, and -III and MTLCdBP in testicular tissue. This was followed by an investigation of gene expression of MT-I, MT-II, and MT-III and tesmin in the testes.

Materials and Methods

Animals and Cd administration

The present study was approved by the animal ethics committee of Kitasato University, Japan. Five-week-old male Wistar rats were purchased from CLEA Japan, Inc. The animals were fed commercial pellets (CE-2, CLEA Japan, Inc.) and were given water *ad libitum*. They were kept in temperature- and humidity-controlled rooms on a 12 h light/dark cycle during the experimental period. After preliminary breeding for 1 wk, the rats were divided into experimental groups of five animals each and used in the experiment from the age of 6 wk.

Animals were divided into three experimental groups of five animals each, and distilled water was administered to the control group. In the acute Cd toxicity treatment (Cd (ip) group), cadmium chloride ($CdCl_2$) was intraperitoneally injected into rats (five per group) at a rate of 2 mg Cd/kg. In the Cd pretreatment group (Cd (ip-ip) group), 0.2 mg Cd/kg was injected as a pretreatment 24 h before injection of the toxic dose of 2 mg Cd/kg. In the chronic Cd oral administration experiment, 30 animals were divided into groups with five animals each, and distilled water was orally administered to the control group. In the chronic Cd toxicity treatments, 20 mg Cd/kg was orally administered

to rats for five days a week for five, 10, and 15 wk (groups Oral5W, Oral10W, and Oral15W, respectively). Twenty-four hours after the final administration of Cd, the animals were euthanized by cardiac blood collection under somnopentyl anesthesia. The animals were perfused with physiological saline from the heart to remove residual blood from the organs and the testes were collected immediately.

Measurement of MT and MTLCdBP concentration

MTLCdBP concentration was measured by the Cd-Hem method[34]. MT was measured by the ELISA method using the antibody of iso-MT (I, II)[35] (Metallothionein ELISA kit, Frontier Institute Co., Ltd., Hokkaido, Japan).

Gene expression of MT and tesmin and measurement of clinical biochemical indices

Gene expression levels of MT and tesmin[17, 31–33] and biochemical indices were measured by RT-PCR. Glutathione (GSH) and glutathione S-transferase (GST) in the testis were measured as biochemical indicators[11]. Total RNA was isolated from the testis with ISOGEN (NIPPON GENE Co. Ltd., Tokyo, Japan), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthetized from 1 μg of RNA using a PrimeScript® II 1st strand cDNA Synthesis kit (TaKaRa Bio Inc., Shiga, Japan). qPCR was performed using a Fast SYBR® Green Master Mix (Applied Biosystems) and a thermocycler (Applied Biosystems, Foster City, CA, USA).

Gene-specific primers used for amplification of rat MT-I (GenBank accession no. NM_138826), MT-II (M11794), MT-III (NM_053968)[36], MTL5 (NM_001012069), iNOS (NM_012611), COX-2 (NM_017232), HO 1 (NM_012580), OGG1 (NM_030870), p53 (NM_030989), β-ACTIN (BC063166) and GAPDH (AF106860) cDNAs were as follows: MT-I forward (F), 5′-GACTGCCTTCTTGTCGCTTA-3′; MT-I reverse (R),5′-AGCAGCACTGTTCGTCACTT-3′; MT-II-F5′ACTCTACAGCGATCTCTCGT-3′; MT-II-R 5′-GGCTAGGTTCCTTACGTTGT-3′; MT-III-F 5′-TTGCCTGGAGGAACTAAGCT-3′; MT-III-R 5′-TTCACATAGGCTGTGTGGGA-3′; MTL5-F 5′-AGGAATCCTGCTGCAAGTTC-3′; MTL5-R 5′-TCCGTTATCGACCTGTGTGA-3′; iNOS-F 5′-CACCTCGGATATCTCTTGCA-3′; iNOS-R 5′-ATGAGCTCATCCAGAGTGAG-3′; COX-2-F 5′-TACGTGTTGACGTCCAGATC-3′; COX-2-R 5′-TCCTCGCTTCTGATCTGTCT-3′; HO1-F AGGATTGCGCAGAAGGCCAT-3′; HO1-R 5′-GTCTCTGCAGAGGTAGTATC-3′; OGG1-F 5′-GCAGATCAAGTGTGGACACT-3′; OGG1-R 5′-CAGTGATGCGAGCAATGTTG-3′; p53-F 5′-CCAAGTCTGTTATGTGCACG-3′; p53-R 5′-TGTCGTCCAGATACTCAGCA-3′; beta-ACTIN-F 5′-TCCTCCCTGGAGAAGAGCTA-3′; beta-ACTIN-R 5′-ACTCCTGCTTGCTGATCCAC-3′; GAPDH-F 5′-AGCTGAACGGGAAGCTCACT-3′; GAPDH-R 5′-ATGAGGTCCACCACCCTGTT-3′.

Immunohistochemical staining

Testis tissues collected were fixed in formalin and made into paraffin blocks with a Sakura automatic packaging machine (VIP-5Jr, Sakura Finetek). Three consecutive sections, each three microns, were obtained with a Yamato microtome and stained with a VENTANA automatic immunostaining apparatus (Roche). The primary antibodies, anti-MT-I,-II (rabbit, polyclonal) and anti-MT-III (mouse, monoclonal), were obtained from Frontier Research Institute, Inc. The antibody of MTLCdBP (rabbit, polyclonal) used was the antiserum of MTLCdBP protein prepared and purified from the testis in our laboratory[9, 12, 37].

Image analysis of immunohistochemical-staining samples of testis tissue

The proportion of tissue positively stained for MT and MTLCdBP was observed using a light microscope (Olympus BH 2). The observation visual field was analyzed by setting it to 10 to 12 visual fields for testicular tissue and 3 to 5 visual fields for stromal cells. Images of the observed field were obtained with a CMOS camera (ARTCAM-130MI, ARTRAY, Inc.) and software (AU-UMMF, ARTRAY Inc.). The image was selected by using image processing software (Adobe Photoshop ver. 7.0.1), and image analysis software (Scion Image for Windows) was used to select the stained area ratio of the parenchymal tissue, excluding the vacuoles.

Measurement of elements

The concentrations of Cd, zinc (Zn), copper (Cu), and iron (Fe) in the testis were measured using flame and flameless methods with an atomic absorption photometer (Hitachi Z-5010) after wet-ashing the tissue with nitric acid.

Statistical analysis

Results are shown as mean and standard deviation. Data were analyzed using one-way analysis of variance (ANOVA), with Scheffé and Fisher's PLSD post hoc tests at significance level $p<0.05$. In addition, where necessary, binary data were analyzed using analysis of variance with Bonferroni correction.

Results

Cd concentrations in the testes increased with increasing Cd exposure under both acute exposure by injection and chronic exposure by oral administration. In the oral Cd administration group, Cd concentration in the testes was about 5.3 times higher than that in the Cd acute injection administration group. However, testicular disorders, like hemorrhagic inflammation or edema, were not found (Fig. 1). On the other hand, Fe concentration increased significantly in the acute exposure group, and Cu concentration decreased in the chronic exposure group against the rise in Cd concentration in the testes (Table 1). Hemorrhagic disorders and edema of the testes were observed with acute Cd exposure, but testicular disorder was reduced when administration of a large amount of Cd was preceded by small-dose Cd pretreatment, indicating a protective effect. Testicular weight (both uncorrected and corrected for body weight) increased due to hemorrhagic inflammation and edema in the acute Cd exposure group (Cd (ip)). In the chronic oral Cd administration group, testicular damage, such as that seen in the acute Cd exposure group, was not observed (Fig. 1, Table 1; similar results have been reported previously)[11].

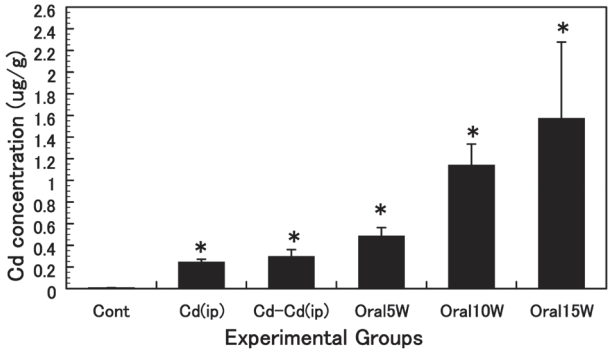

Fig. 1. Cadmium (Cd) concentration in the testis after Cd exposure by intraperitoneal injection (ip) or oral administration in Wistar rats.

Cont: control group injected with distilled water; Cd (ip): acute Cd toxicity treatment at 2 mg Cd/kg; Cd (ip-ip): Cd pretreatment at 0.2 mg Cd/kg 24 h prior to treatment at 2 mg Cd/kg; chronic toxicity treatments involving oral administration of 20 mg Cd/kg/d 5 days a week for 5 (Oral5W), 10 (Oral10W), and 15 (Oral15W) wk.

*Significantly different from the control at $p<0.05$. by ANOVA followed by the Scheffé test or Fisher's PLSD test.

GSH concentration and GST activity of detoxification-related indicators showed a decrease in the Cd (ip) group due to Cd toxicity (Table 2). Significant increases in the expression of iNOS, HO1, COX2, and OGG1 were observed in the Cd (ip) group. Increased expression of HO1 gene was observed in the Cd (ip-ip) group. There was a significant decrease in p53 expression in the Cd (ip) and Cd (ip-ip) groups. On the other hand, disorders caused by Cd in the chronic Cd oral administration group did not tend to change iNOS, HO1, or COX2 gene expression, although a significant increase was observed in iNOS at week 10 and COX2 at week 15. In addition, expression of the OGG1 gene decreased at 15 wk (Table 2).

MT concentrations in the testes measured by ELISA did not increase with increasing Cd concentration in both the acute and chronic exposure groups. However, MTLCdBP concentration in the testes, measured by the Cd-Hem method as Cd-bonded heat-stable protein, increased significantly with increasing Cd concentration (Fig. 2). In the Cd (ip) group, MT and MTLCdBP concentrations showed a decreasing trend, consistent with the results of a previous study11) when large doses of Cd were administered by injection. However, in the Cd (ip-ip) group, MT and MTLCdBP concentrations did not change compared to the control group (Fig. 2).

Expression of Mt-I, Mt-II, Mt-III, and Tesmin (MTL5) in the testis was examined for the chronic Cd-exposed group, whose MTLCdBP concentration increased with increasing Cd accumulation. However, all these genes did not show any change in expression corresponding to Cd

Table 1. Testis weight, the percentage of testis to body weight and the concentration of zinc (Zn), copper (Cu) and iron (Fe) in the testis after the acute (ip-injection) and chronic (oral administration) exposure of cadmium (Cd)

Experimental group	Control	Cd (ip)	Cd-Cd (ip-ip)	Oral control	5 wk Cd	Oral control	10 wk Cd	Oral control	15 wk Cd
Testis weight (g)	3.17 ± 0.28	3.56 ± 1.01*	3.36 ± 1.31	3.33 ± 0.13	3.09 ± 0.22	3.39 ± 0.33	2.70 ± 0.35	3.31 ± 0.22	3.12 ± 0.22
Testis weight to body weight (%)	0.91 ± 0.06	1.03 ± 0.06*	1.02 ± 0.11	0.94 ± 0.05	1.10 ± 0.06	0.88 ± 0.02	1.05 ± 0.10	0.78 ± 0.03	0.96 ± 0.06
Zn (μg/g)	20.4 ± 1.4	20.2 ± 2.8	25.1 ± 3.5	23.4 ± 2.6	19.9 ± 1.0	25.5 ± 2.8	23.2 ± 0.7	24.2 ± 1.2	22.8 ± 0.7
Cu (μg/g)	1.30 ± 0.54	1.03 ± 0.35	1.27 ± 0.27	1.75 ± 0.1	0.92 ± 0.26	1.77 ± 0.1	0.87 ± 0.25*	1.76 ± 0.2	0.48 ± 0.11*
Fe (μg/g)	16.3 ± 2.9	41.1 ± 25.8*	27.7 ± 5.8*	16.2 ± 1.9	12.5 ± 0.6	22.2 ± 1.8	14.4 ± 1.4	25.7 ± 2.6	18.0 ± 1.1

*Significantly different to the control group at $p<0.05$ by ANOVA-Bonferroni's Multiple comparison test and Scheffé test.

Table 2. Glutathione concentration, enzyme activity, and relative gene expression after cadmium exposure by ip-injection and oral administration

Experimental group	Control	Cd (ip)	Cd-Cd (ip-ip)	Oral 5 wk control	Oral 5 wk	Oral 10 wk control	Oral 10 wk	Oral 15 wk control	Oral 15 wk
GSH (μmol/g)	3.05 ± 0.24	1.62 ± 0.93*	3.05 ± 0.24	3.08 ± 0.40	3.28 ± 0.67	3.42 ± 0.32	3.06 ± 0.64	3.64 ± 0.18	3.50 ± 0.41
GST activity (nmol/min/g)	3.38 ± 1.52	1.92 ± 0.98*	2.11 ± 0.64	2.33 ± 0.51	2.57 ± 0.75	2.31 ± 0.47	2.78 ± 0.35	2.65 ± 0.28	2.78 ± 0.39
iNOS (/β-act)	45.7 ± 36.1	283.33 ± 0.04*	44.3 ± 69.5	45.7 ± 36.1	138.1 ± 88.2	41.3 ± 37.7	273.4 ± 388.8*	75.2 ± 41.9	43.9 ± 46.2
COX2 (β-act)	101.3 ± 78.8	224.5 ± 102.1*	63.4 ± 63.4	101.3 ± 78.8	95.6 ± 30.4	44.1 ± 19.6	73.5 ± 54.4	62.9 ± 27.1	286.4 ± 249.2*
HO1 (β-act)	11.9 ± 2.9	13.8 ± 2.6*	17.1 ± 6.0*	11.9 ± 2.9	8.9 ± 0.7	19.4 ± 5.2	11.5 ± 4.9	9.8 ± 0.8	14.7 ± 5.5
P53 (βact)	7.2 ± 1.9	1.8 ± 0.6*	2.3 ± 0.03*	7.2 ± 1.9	7.2 ± 0.5	5.2 ± 1.1	6.6 ± 1.1	6.8 ± 0.6	5.4 ± 0.8
OGG1 (βact)	182.2 ± 41.6	223.0 ± 31.0*	310 ± 65.0	182.2 ± 41.6	227.0 ± 19.5	201.7 ± 46.3	226.6 ± 28.4	236.3 ± 28.0	170.9 ± 29.3*

*Significantly different to the control group at $p<0.05$ by ANOVA-Bonferroni's Multiple comparison test or Scheffé-test.

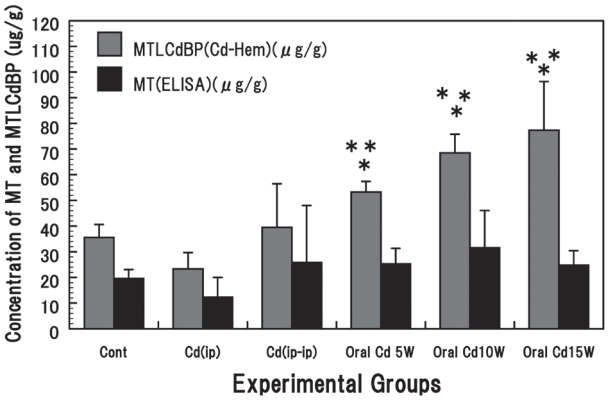

Fig. 2. Concentration of metallothionein and metallothionein-like Cd-binding protein in the testis after Cd exposure by ip injection and oral administration.

*Significantly different to the control group at $p<0.05$ by ANOVA-Scheffé-test.

**Significantly different to the MT-I,-II,-III group at $p<0.05$ by ANOVA- Scheffé-test.

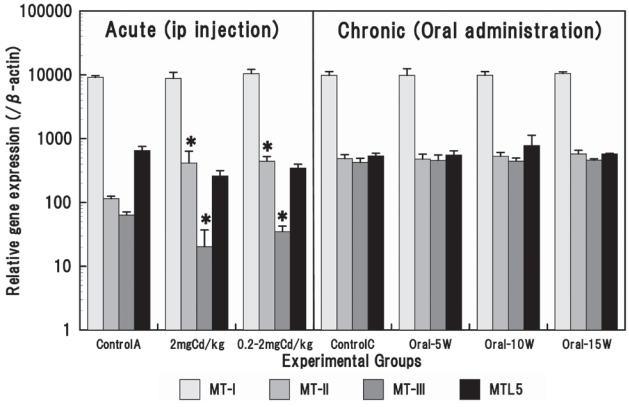

Fig. 3. Relative gene expression of MT-I,-II,-III and tesmin (MTL5) in testis after acute and chronic exposure of cadmium.

Cont: control group injected with distilled water; Cd (ip): acute Cd toxicity treatment at 2 mg Cd/kg; Cd (ip-ip): Cd pretreatment at 0.2 mg Cd/kg 24 h prior to treatment at 2 mg Cd/kg; chronic toxicity treatments involving oral administration of 20 mg Cd/kg/d 5 d a week for 5 (Oral5W), 10 (Oral10W), and 15 (Oral15W) wk.

Control group for chronic oral Cd administration was shown as the average value for each control group at 5, 10, and 15 wk.

*Significantly different from the control at $p<0.05$. by ANOVA followed by the Scheffé test or Fisher's PLSD test.

concentrations in the testes. Expression of MT-I gene was higher than the expression of MT-II and -III. MT protein concentration measured by the ELISA method did not increase (Figs. 2 and 3). In the acute exposure group, an increase in expression of the MT-II gene was observed, although it is not clear whether this was due to strong stimulation caused by Cd toxicity. Conversely, MT-III gene expression was decreased by Cd toxicity (Fig. 3).

In the immunostaining assay, positive staining of MT-I,-II, MT-III, and MTLCdBP showed an increase in response to increasing Cd concentration. In addition, the degree of positive staining was in descending order of MTLCdBP>MT-III>MT-I,-II. That is, the MTLCdBP antibody prepared by us showed more positive staining than MT-I,-II, and MT-III (Figs. 4–7). When comparing the distribution of positive immunostaining within testicular tissue, there was no difference in the degree of immunostaining among MT-I,-II, MT-III, and MTLCdBP in parenchymal tissues. MT-III and MTLCdBP showed clearer positive immunostaining than MT-I,-II but the difference was not statistically significant (Fig. 5). However, a significant difference in positive immunostaining was observed between MT-III and MTLCdBP in interstitial tissue. An increase in positive immunostaining of MT-I,-II and MT-III was observed at 15 wk after Cd administration. In particular, positive staining of MT-III and MTLCdBP showed a significant increase at 15 wk (Fig. 6).

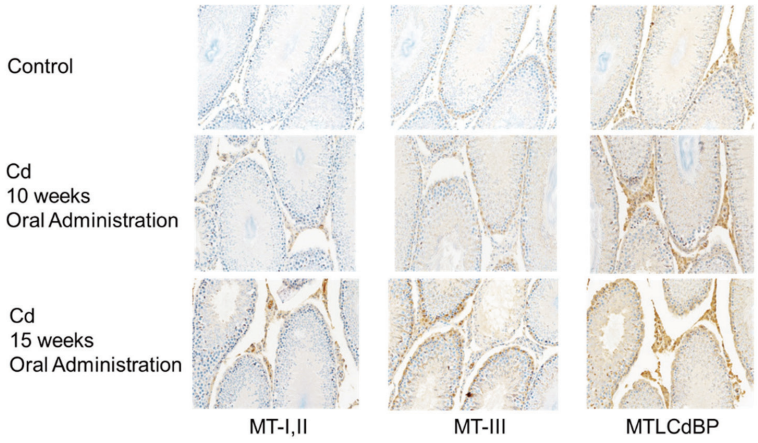

Fig. 4. Immunohistochemical staining of metallothionein-I,-II (MT-I,-II), -III (MT-III), and metallothionein-like cadmium-binding protein (MTLCdBP) in the testes of Wistar rats subjected to chronic cadmium (Cd) exposure.
Control: administered with distilled water.
Cd 10 wk and Cd 15 wk oral administration: oral administration of 20 mg Cd/kg/d 5 d a week for 10 (Oral10W), and 15 (Oral15W) wk.
Brown color in the tissue indicates positive staining.

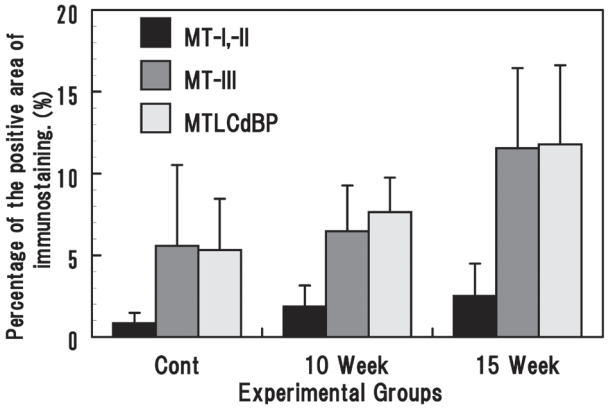

Fig. 5. Positive immunostaining of metallothionein (MT)-I,-II,-III and metallothionein-like cadmium-binding protein (MTLCdBP) in testicular parenchymal tissue after oral cadmium administration.
Cont: control. Cadmium (Cd) 10 wk and Cd 15 wk: oral administration of 20 mg Cd/kg/d 5 d a week for 10 and 15 wk.

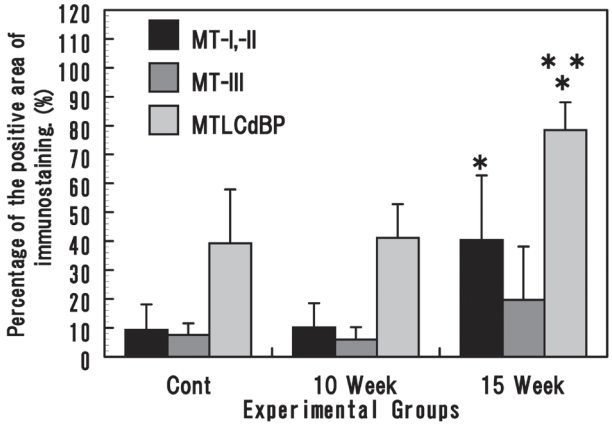

Fig. 6. Positive immunostaining of metallothionein (MT)-I,-II,-III and metallothionein-like cadmium-binding protein (MTLCdBP) in testicular interstitial tissue after oral cadmium administration.
Cont: control. Cadmium (Cd) 10 wk and Cd 15 wk: oral administration of 20 mg Cd/kg/day 5 d a week for 10 and 15 wk.
*Significantly different to the control group at $p<0.05$ by ANOVA-Scheffé-test.
**Significantly different to the MT-I,-II,-III group at $p<0.05$ by ANOVA- Scheffé-test.

Considering the possibility of cross-reaction between the MTLCdBP antibody and the MT-I,-II and MT-III antibodies, the intensity of immunostaining of MT-I,-II and MT-III was subtracted from the intensity of immunostaining of MTLCdBP before comparison. As a result, the positive staining of interstitial tissue was stronger than that of parenchymal tissue; in particular, it showed a significant increase at 15 wk after Cd administration (Fig. 7).

Furthermore, a positive relationship was found in between positive immunostaining of MTLCdBP protein in interstitial tissue and MTLCdBP protein concentration measured by Cd-Hem method and Cd concentration. Positive MTLCdBP immunostaining also increased significantly with increasing concentrations of MTLCdBP protein and Cd (Fig. 8).

Discussion

MTLCdBP (Cd binding, heat stable, low cysteine, low molecular weight protein), eluted in the MT fraction by Sephadex G-75 gel filtration, protects the testes from hemorrhagic inflammatory disorder caused by acute Cd toxicity. During chronic oral administration of Cd, this protein is inducibly synthesized in the testes, depending on the amount of Cd accumulation[11]. Therefore, it is important to clarify the presence and role of heat stable metal-binding protein in the testes since testicular tissue is susceptible to heat stimulation and heat action affects sperm production[38, 39]. In addition, since MT binds to essential elements such as Zn and Cu and is thought to be related to the cellular homeostasis of these two elements and fetal function and growth, understanding the role of MT and MTLCdBP is an important area of research. Although Mt-I, Mt-II, and Mt-III are present in the testes, induction of MT protein corresponding to Cd accumulation has so far not been reported. Furthermore, whether MTLCdBP is an MT protein is still not clear.

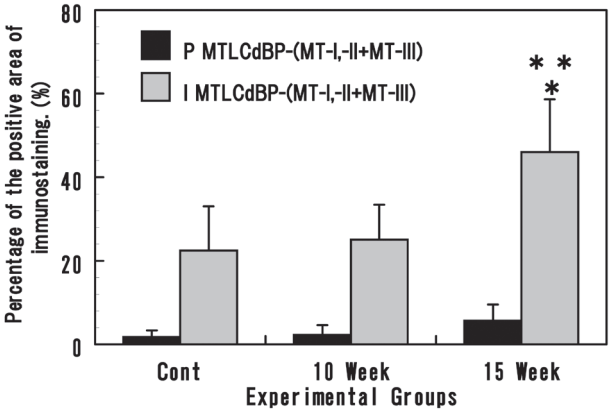

Fig. 7. Positive immunostaining of metallothionein (MT)-like cadmium-binding protein (MTLCdBP) in testis (testicular interstitial and parenchymal tissues) after oral cadmium administration.
P MTLCdBP: parenchymal MTLCdBP; I MTLCdBP: interstitial MTLCdBP.
*Significantly different to the control group at $p<0.05$ by ANOVA-Scheffé-test.
**Significantly different to the MT-I, II, III group at $p<0.05$ by ANOVA- Scheffé-test.

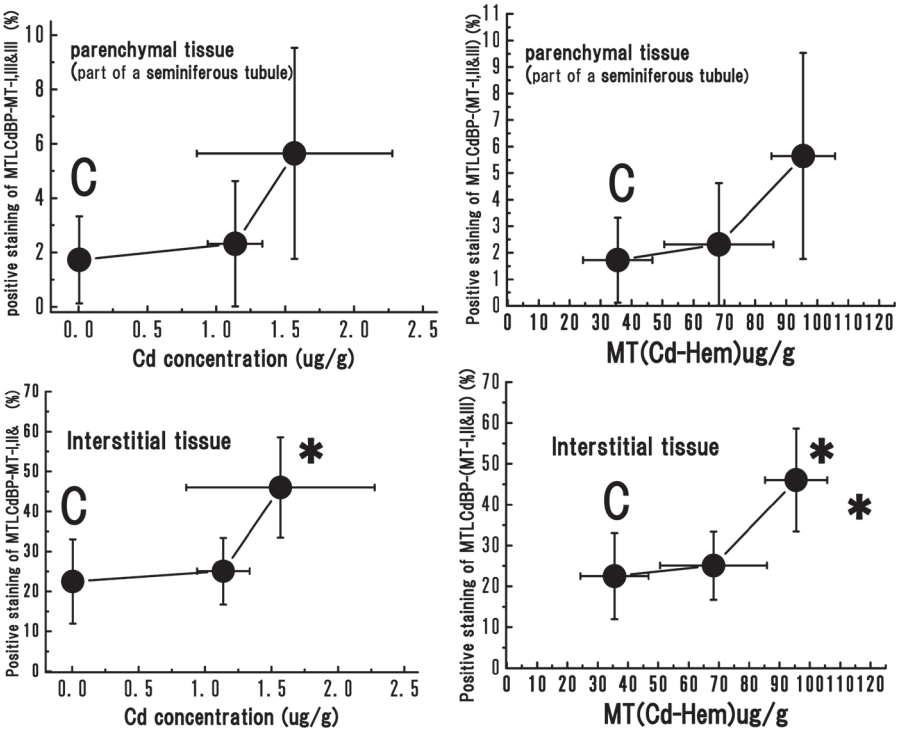

Fig. 8. Relationship between the positive immunostaining percentage of metallothionein-like cadmium-binding protein (MTLCdBP) and the concentration of Cd and MTLCdBP in the testicular interstitial tissue and parenchymal tissue after oral cadmium administration.
C: Control: administered with detailed water.
MTLCdBP: MTLCdBP protein measured by Cd-Hem method.
*Significantly different to the control group (C) at $p<0.05$ by ANOVA-Scheffé-test for positive immunostaining percentage of MTLCdBP-(MT-I,-II, and -III).
**Significantly different to the control group (C) at $p<0.05$ by ANOVA-Scheffé-test for MTLCdBP protein measured.

We found that acute Cd toxicity causing hemorrhagic inflammation and edema was alleviated by pretreatment with a small amount of Cd, confirming the results of a previous study[11] (Table 1; unreported results). On the other hand, Cd toxicity by chronic oral administration did not show significant toxicological effects, assessed through clinical biochemical examination and gene expression of indicator substances related to inflammation and toxicity protection, despite the elevated testicular concentration of Cd (Tables 1 and 2; Fig. 1).

Expression of Mt-I, Mt-II, and Mt-III in the testes did not increase with increasing Cd concentration (Figs 2 and 3). On the other hand, the increase in the concentration of MTLCdBP corresponded to the increase in Cd accumulation (Fig. 2). The expression of tesmin, which is thought to be involved in spermatogenesis, appears to be responsive to Cd, suggesting that it has some characteristics related to MT-I/-II, such as metal sensitivity. Nonetheless, the roles of testicular MT and tesmin have not been determined so far[31, 32]. In our study, expression of the tesmin gene did not increase, indicating that it may not be involved in alleviating Cd toxicity (Fig. 3), i.e., since there was no change in tesmin gene expression, it may not be directly involved in the Cd toxicity reduction mechanism. On the other hand, a lack of MT-III may contribute to protection against frost damage of the testes from Cd, as MT-III null mice showed reduced Cd-induced testicular toxicity. In addition, some studies using transgenic MT-I overexpressing mice and MT-I/-II null mice have shown that MT-I and MT-II may not have a protective effect against Cd-induced testicular injury[19, 20, 28, 29].

Although MT-III is known to be unresponsive to MT-I and MT-II inducers, there are several reports showing that MT-III expression is induced in the brain by testosterone, zinc, and mercury vapor[40–42]. Since MT-III mRNA levels decreased after orchiectomy, but the control levels were maintained by testosterone and MT was expressed mainly in basal cells with regulation by testosterone, it has been suggested that heavy metals affect and induce MT in the proximal region of the epididymis. However, MT-III mRNA levels were not affected by Cd treatment[41]. Although the MT concentration in the testes is higher than that in the liver and the kidney, induced synthesis of MT by Cd administration is not observed, and it is reported to decrease by acute Cd exposure[11]. On the other hand, MTLCdBP is inducibly synthesized by long-term oral administration of Cd, and the composition ratio of cysteine is markedly different from that of MT[11, 28].

The above studies and our results show that in Cd exposure by chronic oral administration under normal physiological conditions, unlike in the case of acute Cd exposure by injection, MTLCdBP is inducibly synthesized in the testis even if Cd accumulates in a dose-dependent manner. Cd may be bound by MTLCdBP, and thus Cd toxicity may be alleviated. We found that MTLCdBP showed more positive staining than MT-I,-II and MT-III, and its induction and distribution in testicular interstitial tissue were significantly increased compared to those of MT-I,-II and MT-III (Figs. 4–7). MTLCdBP in interstitial cells may be responsible for preventing Cd toxicity to Leydig cells[38]. We also found that immunostaining was more positive for MT-III than for MT-I,-II in testicular parenchyma. MT-III may have been present originally in testicular tissue and may be involved in reducing the toxicity of harmful elements such as Cd. However, the lack of change in expression of MT-I, MT-II, and Mt-III in our study suggests that MT may not be involved in Cd toxicity alleviation to the same extent as MTLCdBP. We found that MTLCdBP concentration measured by the Cd-Hem method increased with increasing testicular Cd concentration, and the degree of positive immunostaining with MTLCdBP antibody also increased. The degree of positive staining was in descending order of MTLCdBP>MT-III>MT-I,-II (Figs. 5–7). This suggests that Cd toxicity is alleviated by MTLCdBP rather than by MT.

There are different cells involved in spermatogenesis in the testis, and it is possible that different proteins and hormones may be involved at each stage[28]. MT is thought to bind to Cd and reduce its toxicity. However, despite the MT concentration in the testes being higher than that in the liver or the kidney, its induced synthesis is low in the testes in normal physiological state, suggesting that it does not play a significant role in alleviating Cd toxicity[11, 28].

In conclusion, in the present study, a comparison of immunostaining in testicular tissue by MTLCdBP polyclonal antibody and MT-I,-II and MT-III antibodies showed that intra-tissue distribution and staining of these proteins in testicular tissues were significantly different, and the tissue distribution of MT proteins (-I, -II, and -III) was also different. Increased gene expression and induced synthesis of MT were not observed in the testis (Figs. 1–3). In contrast, MTLCdBP, with an amino acid composition different from that of MT, was inducibly synthesized consistent with the increase in Cd concentration, suggesting that MTLCdBP has an important role in the protective mechanism against Cd toxicity in the testis (Figs. 5–8).

Future studies of MTLCdBP protein, particularly using microarray and MALDI-TOF-MS analysis, should investigate in detail its role in the testes where cells of different

reproductive developmental stages exist. In addition, our study found that the concentrations of Cu in the testes decreased with increasing accumulation of Cd (Table 1). Testicular toxicity of Cd may be reduced by its interaction with other metals such as Zn, Se, Cu, etc.[6–8, 10, 42, 43]. Therefore, in order to elucidate the mechanism of Cd toxicity in the testes in detail, it is necessary to investigate interactions related to testis uptake of Cd, Zn, and Cu, with specific consideration of metal transporters such as Zn[44–48], as well as searching for and identifying other candidate proteins involved in Cd detoxification.

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