Localization of Metallothionein (MT) and Expression of MT Isoforms Induced by Cadmium in Rat Dental Pulp

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ABSTRACT—We investigated the induction of metallothionein (MT) by cadmium (Cd) in the dental pulp of rat incisors. Time-course studies of MT mRNA expression after single Cd injection were observed by Northern-blot analysis. The isoform-specific expressions of MT mRNAs (MT-I, MT-II and MT-III) were observed using the reverse transcriptase-polymerase chain reaction (RT-PCR) method. Both MT-I and MT-II mRNA levels increased within 3 h, peaked at 3 h and then decreased. These findings demonstrated that MT-I and MT-II mRNA were rapidly induced by Cd in dental pulp. MT-III mRNA was constitutively expressed in rat dental pulp, but the expression level did not change by Cd treatment. The localization of MT protein in Cd-treated rat dental pulp was determined by immunohistochemical staining using anti-MT antibody against MT-I and MT-II. MT protein was localized in the specific cell type of odontoblasts (secretory odontoblasts and resting odontoblasts). In conclusion, it is likely that stained MT in the immunohistochemical study should be MT-I and/or MT-II. Furthermore, MT-I and/or MT-II in Cd-treated rat dental pulp was localized in odontoblasts, in which accumulation of Cd were reported. The cell-specific synthesis of MT may be associated with its metal storage and detoxification role in dental tissues.

Keywords: Metallothionein, Cadmium, Dental pulp, Odontoblast, Pulp cell

Metallothionein (MT) is characterized by its low molecular weight, high cysteine content, absence of aromatic amino acids and histidine and high affinity for heavy metals including cadmium (Cd) (1 – 3). MT is synthesized in many organs such as the liver, kidney, pancreas and spleen after administration of heavy metals (1 – 5). With regard to dental pulp, MT gene expression in rat dental pulp was found after the treatment of Cd using the reverse transcriptase-polymerase chain reaction (RT-PCR) method (6). In mammals, there are four major isoforms of MT, which are generally designated MT-I, MT-II, MT-III and MT-IV. MT-III expression is virtually brain-specific, MT-IV expression is squamous epithelium cell-specific, and MT-I and MT-II genes are expressed in many tissues including the liver, kidney and brain (3, 7 – 10). MT-I and MT-II expressions are enhanced by exposure to metal ions such as Cd and Zn, but MT-III failed to respond to metal ions in the brain (4, 5, 11 – 13). There is no information about the levels of isoforms of MT in dental pulp.

On the other hand, Cd causes some effects on dental tissues: a significant influence on caries susceptibility (14), bleaching of enamel pigmentation (15) and disappearance and deformation of odontoblasts (16). After Cd injection in rats, Cd accumulation mainly occurred in the cells of enamel organ and odontoblasts of dental pulp (17). Previous studies reported that MT-like protein was isolated from the dental pulp of Cd-treated rat incisor (6, 18), and the epithelial cells and ameloblasts of enamel organs induced MT protein after consecutive Cd injection (19). However, there is no data concerning the localization of MT in dental pulp, which includes odontoblasts and plays an important role in dentine formation (20).

Accordingly, we investigated the expression patterns of MT isoforms and the localization of MT protein in Cd-treated rat dental pulp using Northern-blot analysis, the RT-PCR method and immunohistochemical staining.

MATERIALS AND METHODS

Animals and treatments
Male Wistar rats (8- or 9-week-old) were obtained from
Japan SLC Co., Ltd. (Shizuoka). They were fed CE-II (Clea Japan, Inc., Osaka) and water ad libitum for at least 1 week. Lighting was maintained on a 12-h light/dark (light time: 6:00 A.M. – 6:00 P.M.) cycle and temperature was maintained at 24 ± 1°C. Rats were injected subcutaneously with CdCl₂ (4.5 Cd mg/kg) (Nacalai Tesque, Inc., Kyoto) dissolved in saline. The control rats were injected subcutaneously with saline. All injections were in a volume of 2 ml/kg for rats.

**Dental pulp and RNA isolation**

The dental pulps were scraped from maxillary and mandibular incisors for total RNA isolation. The tissue was composed of a layer of columnar odontoblasts and pulp cells. Total RNA was isolated from the dental pulp at 0, 1, 3, 6, 12 and 24 h following the injection using Trizol (Gibco BRL, Tokyo) as previously described (21). Approximately 50 mg dental pulp was homogenized in a glass homogenizer with 1 ml Trizol, according to the manufacturer’s directions. Isolated RNA was treated with DNase I (Takara Shuzo Co., Ltd., Shiga). The RNA yield and purity were determined by absorbance at 260 nm and A<sub>260</sub>/A<sub>280</sub>.

**Northern-blot analysis of RNA**

For the radiolabeled MT cDNA probe of Northern-blot analysis, the RT-PCR product amplified from Cd-treated rat liver RNA used a rat and mouse MT-I consensus primer set (sense: 5'-GGTACCCCTAATCGCTCTGC-3', antisense: 5'-AAGCTTTGCAGAC(A/G)CAGCCC-3'). These primers were constructed according to the known sequence (22) and included restriction sites in the 5' terminal. RT-PCR was performed using an RNA PCR kit (AMV) (Takara). DNase-treated total RNA was reverse transcribed to cDNA using random hexamers with AMV-derived reverse transcriptase for 30 min at 42°C and then RT-generated cDNA encoding MT genes were amplified with Taq DNA polymerase. PCR followed the manufacturer’s recommended procedures. The PCR product (9 μl) was separated by 1% agarose gel electrophoresis. The amplified MT cDNA fragment (156 bp) was purified using a DNA spin trap filter (SUPREC-02: Takara) and labeled with Klenow enzyme and [α-<sup>32</sup>P]dCTP (110 TBq/mmol) (Amersham Pharmacia Biotech Tokyo) using a random primer labeling kit (Takara). The nucleotide sequence of the MT cDNA fragment was almost identical to that of the reported rat MT-I cDNA, which had >80% homology to rat MT-II mRNA and 70% homology to rat MT-III mRNA. Therefore, there was the possibility that the hybridization to the MT cDNA probe detected rat MT-I, MT-II and MT-III mRNA in all samples. Fifteen micrograms of RNA was treated with 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 50% deionized formamide and 2.2 M deionized formaldehyde for 15 min at 55°C and then chilled on ice. These samples were applied to 1% denatured agarose gel and electrophoresed for about 3 h at 50 V. Following electrophoresis, the gel was soaked in ultrapure diethyl pyrocarbonate (DEPC) treated water and 0.1 M sodium acetate sequentially. To check for loading efficiency, ribosomal RNA bands were visualized by soaking the gel in 0.1 M sodium acetate, 0.1 M 2-mercaptoethanol and 0.5 μg/ml ethidium bromide solution for 30 min followed by viewing in UV. The gel was destained by soaking it in 0.1 M sodium acetate and 0.01 M 2-mercaptoethanol solution and DEPC-treated water sequentially. Then the gel was soaked 50 mM NaOH and 10 mM NaCl, 0.1 M Tris-HCl (pH 7.5) and 20 × SSC (1 × SSC: 0.15 M NaCl, 0.15 M sodium citrate buffer) sequentially prior to capillary transfer of RNA in 20 × SSC to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After the transfer, the membrane was baked for 2 h at 80°C and soaked in pre-hybridization solution (5 × SSC, 1% SDS and 1 × Denhardt’s solution) for 2 h at 65°C. Next, the membrane was soaked in hybridization solution (0.02 M Tris-HCl (pH 8.0) containing 0.75 M NaCl, 2.5 mM EDTA, 1 × Denhardt’s solution, 1% SDS, 50 μg/ml salmon sperm DNA and 5 μl of radiolabeled MT cDNA probe (MT-I and MT-II consensus probe)) for 16 h at 65°C. The membrane was washed at room temperature in 2 × SSC and 0.1% SDS for 5 min, washed three times at 65°C in 0.2 × SSC and 0.1% SDS for 15 min, and then finally washed at 65°C in 2 × SSC for 5 min. The membrane was exposed to imaging plates and analyzed using a Bioimaging Analyzer BAS2000 (Fuji Film Co., Ltd., Tokyo). Autoradiography was performed by exposure of the membrane to X-ray film (Fuji Film Co., Ltd.) using a high-plus intensifying screen at −80°C for the required time performed.

**Analysis of MT isoforms specific expression using the RT-PCR method**

The expression levels of MT-I, MT-II and MT-III mRNA in rat dental pulp were each measured using the RT-PCR method. In this method, the amount of PCR product derived from cDNA of each MT isoform was normalized against that from a housekeeping gene sequence such as glyceraldehyde 3-phosphate dehydrogenase (G3PDH) to compare each relative MT isoform mRNA expression in samples. In this method, the amount of PCR product derived from cDNA of each MT isoform was normalized against that from a housekeeping gene sequence such as glyceraldehyde 3-phosphate dehydrogenase (G3PDH) to compare each relative MT isoform mRNA expression in samples. The RT-PCR method was carried out as previously reported (23, 24), with minor modifications. Oligonucleotides primer sets were synthesized as the MT-I/II consensus sense primer: 5'-ACCCCAACTGCTCCTG(C/T)(T/G)CC-3', MT-I specific antisense primer: 5'-AGGTTGACGGCAAAACTCTG-3', MT-II specific antisense primer: 5'-ACACCATTTGAGGACGCC-3', MT-III specific sense primer: 5'-ACCTGACAGCCTCCCTG-3' and MT-III specific antisense primer: 5'-GGACACCAAGCAATTCAC-3' (Amersham Pharmacia). These primers were constructed.
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according to the known sequences (22, 25). G3PDH primers were the G3PDH control amplimer set (Clontech, Laboratories, Inc., Palo Alto, CA, USA). 0.03 µg DNase-pretreated total RNA was used with 20-cycle PCR for MT-I, MT-II and G3PDH and with 30-cycle PCR for MT-III. Tag DNA polymerase, each primer and [α-32P]dCTP for quantification of amplified DNA fragments were added and amplified in each reaction mixture of 25-µl final volume. The conditions were such that the amplification of the PCR products was linear with respect to the amount of RNA. Each PCR reaction mixture for MT isoforms and G3PDH in each total RNA were simultaneously electrophoresed on an 8% polyacrylamide gel. The gels were stained with 0.5 g/ml ethidium bromide solution followed by viewing under UV illumination and then dried, exposed to an imaging plate, and analyzed using BAS2000.

Immunohistochemistry

Twenty-four hours after CdCl₂ or saline injection, the rats were killed under pentobarbital (40 mg/kg) anesthesia and fixed with 4% paraformaldehyde in 0.08 M phosphate buffer (pH 7.3) by cardiac perfusion. The mandible containing the incisor were removed and further fixed for 2 h at 4°C. After demineralization with 10% formic acid at 4°C for 14 days, these samples were dehydrated through an ethanol and xylene series and embedded with paraffin. Sections of 5 µm in thickness were cut from the blocks and deparaffinized and placed in 3% hydrogen peroxide in distilled water for 3 min to extinguish endogenous peroxidase activity. To block any non-specific antibody binding, the sections were incubated in 5% normal rabbit serum for 30 min, followed by anti-MT monoclonal antibody (against horse MT-I and MT-II, 1:50; Dako Co., Carpinteria, CA, USA) for 2 h at room temperature. The antibody is specifically reactive to a conserved epitope common to several mammalian species of MT-I and MT-II (26). After washing with PBS-Tween 20 (0.05%) (PBS-T), the sections were incubated in biotinylated rabbit anti-mouse immunoglobulins (1:300, Dako) for 30 min at room temperature. After washing with PBS-T, the sections were incubated in AB-Complex/HRP (Dako) for 30 min at room temperature. The sections were washed with PBS-T and 0.05 M Tris-HCl (pH 7.6), followed by staining with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.3% hydrogen peroxide in 0.05 M Tris-HCl (pH 7.6). All slides were counterstained with hematoxylin, dehydrated and mounted in Entellan. Three rats were used in each group. The specificity of the staining reaction was checked by omitting the primary mouse anti-MT monoclonal antibody from the procedure.

Statistical analyses

Statistical values were expressed as the mean ± S.D. The effect of Cd on MT expression compared to that of control levels at time zero was assessed by one-factor ANOVA following Fisher’s post-hoc test.

RESULTS

Time-course study of MT mRNA expression in dental pulp

From Northern blot analysis, MT mRNA from the dental pulp of Cd-treated rats increased in a time-dependent manner. In the dental pulp, the MT mRNA level increased within 3 h and declined by 6 h. MT mRNA expression was rapidly induced in dental pulps (Fig. 1). Figure 2 shows the time-course study of the MT-I and MT-II mRNA expression levels in dental pulp in Cd-treated rats. Both MT-I

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**Fig. 1.** Analysis of MT mRNA induction in rat dental pulp using Northern-blot analysis. Autoradiographs (A) and semiquantitative analysis of MT mRNA induction (B). Fifteen micrograms of DNase I-pretreated total RNA was used. Hybridization was done with a radiolabeled MT cDNA probe (MT-I and MT-II consensus probe) for 16 h at 65°C. Autoradiography was performed by exposing to X-ray film. The membrane was exposed to imaging plates. The photostimulated luminescence (PSL) values of each band in panel B were measured with BSA2000. In rat dental pulp, MT mRNA increased within 3 h after Cd injection and declined by 6 h.
and MT-II mRNA levels increased within 3 h and declined by 6 h. This was similar to the findings by Northern-blot analysis. Each fold increase level of MT-I mRNA was higher than that of MT-II mRNA. Figure 3A shows that MT-III mRNA was present in not only brain but also dental pulp, but the expression level in the dental pulp was lower than that of the brain. In the time-course study, the MT-III mRNA expression level did not change after Cd injection in rat dental pulp (Fig. 3: B and C).

**Immunohistochemical localization of MT protein in dental pulp**

The localization of MT (MT-I and MT-II) protein in rat dental pulp was studied by the immunohistochemical method (Fig. 4). MT protein was detected in Cd-treated
rat odontoblasts at the late maturation stage. We classified odontoblasts into three types based on the morphology: preodontoblasts, which were located near the apical end of an incisor and showed a cylindrical shape with the nucleus localized at the proximal end; secretory odontoblasts, a cell type that became columnar and had abundant cytoplasm, and relatively thick dentin had been formed; resting odontoblasts, which were small, cuboidal in appearance, and had poor cytoplasm. MT-positive cells were secretory odontoblasts, which showed fairly wide increment of mineralized dentin, and resting odontoblasts (Fig. 4: B and C). Secretory odontoblasts beginning mineralization were not MT-positive (Fig. 4A). Staining for MT was observed in both the cytoplasm and the nucleus, and nucleus staining was prominent in some odontoblasts (Fig. 4: B and C). Furthermore, no staining was observed in the preodontoblasts and the pulp cells. In control rat tissue, each cell in the dental pulp was negative for MT immunostaining (Fig. 4D).

DISCUSSION

A previous study reported that MT-like protein was isolated from the dental pulp of Cd-treated rat incisor (6, 18). In a recent study, the localization of MT protein was investigated immunohistochemically in the enamel organ of rat incisors (19). That report showed that interperitoneal consecutive injections of Cd for 7 days (1.5 mg Cd/kg per day) induced the synthesis of MT in the papillary epithelial layer of the secretory zone, in a single layer of epithelial cells of the presecretory zone, and in ameloblasts of the postsecretory zone. Cd effects and accumulation were mainly
demonstrated in both the enamel organ and odontoblasts in incisors and developing teeth (15–17), but the distribution of MT has not been reported in the cells of dental pulp including odontoblasts.

In this study, we immunohistochemically investigated the intracellular localization of MT protein (MT-I and MT-II) in dental pulp of rat incisor after single Cd injection (4.5 mg Cd/kg). Interestingly, the intracellular localization of MT protein was not diffusely distributed in all pulp cells. It is notable that specific staining for MT protein was detected in the odontoblasts. Moreover, we classified odontoblasts based on the morphology and found that MT positive cells were secretory odontoblasts, which showed fairly wide increment of mineralized dentin, and resting odontoblasts. Secretory odontoblasts beginning mineralization were not MT-positive. We also investigated MT expression in the enamel organ using MT immunostaining (data not shown). The findings obtained were similar to those in a previous study (19).

Some previous studies reported that Cd caused lesions in dental tissues. The administration of 5 ppm Cd in drinking water produced cross-striations, gave a more uniform and regular pattern of pigmentation and bleaching similar to those observed with fluoride (14). After injection of Cd (1.7 mg Cd/kg per day for 3 weeks), odontoblasts in rat incisors had partially disappeared, or were deformed in most cases (16). Cd injections during molar development in rats showed strong caries promoting ability in female rats, most cases (17). Cd partially negated the cariostatic effects of fluoride. After injection of Cd into young rats, accumulation of Cd occurred in dental tissues of developing teeth (enamel organ and odontoblasts). As Cd effects and accumulation were mainly observed in enamel organs and odontoblasts in dental tissues, Cd may influence the mineralization of teeth. The cell-specific MT synthesis in dental tissues may be associated with Cd effects and detoxification. Furthermore, previous studies investigated the localization of MT in various organs and the ability of induction of MT in various cells after Cd treatment (27–31). Danielson et al. (28) showed the expression of MT in specific epithelial cells of different organs; and because of the secretory, absorptive, or nutritive properties of the MT-localization cells, they suggested that MT may be involved in metal storage or transport in addition to its commonly proposed detoxification role.

There are a few reports about MT expression in another cells, which form hard tissues such as bone and teeth. Miyahara et al. (32) reported that Cd showed dose-dependent effects on MT synthesis in an osteoblast cell line (MC3T3-E1) that had a high alkaline phosphatase (ALP) activity and formed an abundant bone matrix (33). In addition, we have previously reported that the expression level of MT mRNA increased in a dental pulp cell line (RPC-C2A) after Cd treatment (34), which had high ALP activity, but did not have fully differentiated mature odontoblasts (35). After RPC-C2A became confluent, MT mRNA levels increased after Cd treatment, but when the cells were sparse, MT mRNA level did not increase. Although the Cd content of confluent RPC-C2A cell was higher than that of the pre-confluent cell, the cell viability was higher than that of the pre-confluent cell (34). Thus, these in vitro studies showed that Cd directly synthesized MT in the cells, which regulated mineralization in hard tissues, and MT is associated with metal storage and detoxification.

Several reports showed that MT is localized in the cytoplasm, nucleus or both (27, 36, 37). Banerjee et al. (27) reported that the presence of MT predominantly in the cytoplasm in control rat hepatocytes and renal tubular epithelium and its localization in both the nucleus and cytoplasm following Cd treatment. The other report showed that Cd could enter the cell nucleus where it binds with an unknown nuclear component, within 1 h of Cd administration. Cd binding protein (MT-like) was detected in the cytoplasm within 2 – 3 h of Cd treatment. As the MT-like protein concentration increases, the nuclear Cd content decreases and Cd is found to be bound to MT-like protein in the cytoplasm (38). Many metal binding proteins, which bind Zn or Cu, are present in nucleus during differentiation. MT can bind Zn or Cu and transport them across the nuclear membrane (37). In rat odontoblasts, staining for MT was observed in both the cytoplasm and the nucleus, and the nuclear staining was prominent. We suppose that Cd may be sequestered in the nucleus instead of Zn or Cu, and MT may transport Cd outside the nucleus to detoxify in odontoblasts.

Notably, we could measure the levels of MT mRNAs in Cd-treated rat dental pulps using the RT-PCR method, which is specifically and quantitatively able to measure each MT mRNA isoform. As a result, both MT-I and MT-II mRNA levels increased within 3 h and declined by 6 h. However, these levels in livers were still considerably high at 6 h (data not shown). These findings were confirmed by our Northern-blot analysis. These results showed that high levels of MT-I and MT-II mRNA were maintained longer in liver than in dental pulp. Choudhuri et al. (31) reported that MT-I mRNA levels reached peak levels at 6 h after Cd administration in the liver of both mice and rats. Furthermore, Durnam and Palmiter (39) reported that MT-I mRNA levels reached peak levels within 4 h after Cd administration in mouse liver. High levels of MT mRNA were maintained longer in the liver than kidney. The manner of MT mRNA in the kidney was similar to that of dental pulp. We also found that MT-III mRNA was present in rat dental pulp, but the expression level in the dental pulp was lower than that of the brain. Interestingly, it is notable that the
MT-III mRNA expression level is not changeable, but both MT-I and MT-II mRNA levels are changeable after Cd injection in rat dental pulp.

Thus, it is likely that stained MT in the immunohistochemical study should be MT-I and/or MT-II, because MT-III mRNA the expression level is not changeable after Cd injection in rat dental pulp. Furthermore, MT-I and/or MT-II in Cd-treated rat dental pulp was localized in the specific cell type of odontoblasts. Taken together, the cell specific synthesis of MT may be associated with metal storage and the detoxification role of MT in dental tissues.

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