Metallothionein (MT) is a low molecular weight (6,000 7,000 Da), cystein-rich (about 33% amino acid), heat stable and metal binding protein. Mammalian MT has four major isoforms, MT-1, -2, -3 and -4. MT-1 and MT-2 are slightly different in their amino acid sequences and are identified in most organs (Kägi and Nordberg, 1979; Harmer, 1986; Kägi and Kojima, 1987). MT is thought to be involved in various physiological roles such as metabolism of essential metals, detoxification of toxic metals and scavenging of free radicals (Bauman et al., 1991; Satoh and Tohyama, 2000). Also, it was suggested that MT is involved in various diseases (Goulding et al., 1995; Apostlova et al., 1997; Nagamine et al., 2005; Hidalgo et al., 2006; Yamasaki et al., 2006; Miyazaki et al., 2007).

Many immunoassays, such as radioimmunoassays (Tohyama and Shaikh, 1981; Garvey et al., 1982; Mehra and Brenner, 1983) and enzyme-linked immunosorbent assays (ELISA) (Cousins, 1991; Akintola et al., 1995), have been developed to determine MT 1/2 for use in laboratory practice in humans and experimental animals. However, no standard method for MT 1/2 measurement for clinical or experimental studies has been established. Therefore, an MT 1/2 assay with adequate sensitivity,
specificity that is easy to use in human and experimental specimens routinely is required.

Recently, we raised anti-MT specific antibodies and used them for immunohistochemical staining (Nakazato et al., 2008). We also developed an easy and specific competitive ELISA to determine MT-1 and MT-2 (Nakajima et al., 2010). The antibody used for the studies was a polyclonal antibody which can recognize both MT-1 and MT-2 in humans and animals (mouse, rat, rabbit etc), and the lowest detection limit of this ELISA was 0.6 ng/ml. Using the ELISA, we conducted a feasibility study for MT-1/2 determination in wild type (WT) and MT knock-out (KO) mice and compared the results with those of immunohistochemical staining. To confirm the molecular weight (MW) of MT detected in organs by the ELISA, the homogenates were eluted by Sephadex G-75 and MT 1/2 MW was determined. Further, the MT-1/2 concentration in the urine of Long-Evans Cinnamon (LEC) rats, known as an experimental model of Wilson disease associated with genetic copper metabolism dysfunction and also as having a significantly increased MT concentration in the liver, was also determined by the ELISA.

The comparative results between the MT 1/2 ELISA and immunohistochemical staining in the liver, kidney and brain of experimental animals are described in this manuscript.

MATERIALS AND METHODS

Sample preparations
MT-1/2 KO mice (Masters et al., 1994), MT-3 KO mice (Erickson et al., 1997) and WT mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were routinely bred in the laboratory animal facility of Aichi Gakuin University. MT-1/2 and MT-3 KO mice were of the 129/Sv strain, and age- and sex-matched 129/Sv mice were used as WT controls. All strains were routinely bred in the laboratory animal facility of Aichi Gakuin University. MT-1/2 and MT-3 KO mice were intraperitoneally injected with CdCl₂ (5, 10 and 20 μmol), and their organs were excised after 6h. The specimens of liver, kidney and brain were used for ELISA, immunohistochemical staining and MT-1/2 molecular weight determination by Sephadex G-75 fractionation. One milliliter of PBS was added to 0.5 g tissues and homogenized and centrifuged at 5,000 × g for 30 min. The supernatant was diluted with PBS and used for ELISA and MT-1/2 molecular weight determination by Sephadex G-75. For immunohistochemical staining, the removed tissues were fixed in 10% formalin solution and embedded in paraffin, and 3 μm-thick sections of these tissues were used for staining.

The urine samples were collected from both LEC rats and Long-Evans Agouti (LEA) rats, which were one of genetic controls for them. The rat urine samples were collected at 5-, 6-, 8-, 10-, 15- and 30-weeks of age. Their urine samples were collected by loading weak fear on the rats from 9 am to 12 pm. The samples were centrifuged at 2,000 × g at 20°C for 10 min in order to avoid eluting abundant proteins, especially uromodulin, from the urinary cell debris. The supernatants were stored at -80°C until experiments. The urinary concentrations of MT-1/2 were determined by the ELISA and metals (Cu and Zn) were determined by a colorimetric method and an atomic absorption method, respectively, at SRL (Tokyo, Japan).

Competitive ELISA
The competitive ELISA (Frontier Institute, Hokkaido, Japan) was performed according to the method we developed (Nakajima et al., 2010). Briefly, 50 μl of sample specimen and 50 μl of MT antibody (primary antibody) were added per well and incubated for 1 hr at 4°C in a microtiter plate coated with rabbit MT-2A. After incubation, wells were washed 3 times with PBS with 0.01% Tween 20 (PBS-T). Then, 100 μl of HRP conjugated anti-rabbit IgG goat antibody (secondary antibody) was added per well and incubated for 1 hr at room temperature. After incubation, wells were washed 3 times with PBS-T, and 100 μl of color substrate 3,3',5,5'-tetramethylbenzidine were added and reacted for 10 min at room temperature. After the reaction, 50 μl of 1N H₂SO₄ was added to stop the reaction. Optical density was read at a 450 nm wavelength by a plate reader (MPRA4, Toso, Tokyo, Japan).

Determination of MT-1/2 molecular weight in the liver, kidney and brain
To adjust the similar MT concentration in homogenate samples, 0.1 ml (wild and MT 3KO) to 1 ml (MT 1/2 KO) of tissue homogenate supernatants were applied to a Sephadex G-75 column (1 × 45 cm, GE Healthcare (Utspsala, Sweden)) and eluted to determine the MT molecular weight (MW). PBS with 0.02% Tween was used for the elution of the MT homogenate with a flow rate of 0.6 ml/min and 1 ml fractions were collected for the MT-1/2 assay.

Immunohistochemical staining
After the sections were deparaffinized and treated
with methanol containing 0.3% H$_2$O$_2$ for 30 min at room temperature to eliminate endogenous peroxidase activity, they were washed with water. The sections were immersed in hot 0.01 mol/l citrate buffer (pH 6.0) and heated in a microwave oven for 5 min for antigen retrieval. The sections were incubated with polyclonal antibodies (1:50 dilution) (Nakazato et al., 2008) for 120 min at room temperature. After incubation, the sections were washed 3 times with PBS-T, and incubated with a secondary antibody (Histofine Simple Stain Mouse MAX-PO (R), Nichirei, Tokyo, Japan) for 60 min at room temperature. After incubation, the sections were washed 3 times with PBS-T, and incubated in a color reaction solution (0.05 mol/l Tris-HCl buffer (pH 7.6) 150 ml, diaminobenzidine 30 mg, H$_2$O$_2$ 10 μl) for 20-30 min at room temperature, then washed with tap water for 5 min. Finally, the sections were counter-stained with Mayer’s hematoxylin.

Statistical analysis

Qualitative data are expressed as means ± S.D. Statistical analysis was conducted using ANOVA (SPSS, Statistics 17.0). A value of p < 0.05 was considered to indicate statistical significance.

RESULTS

MT-1/2 concentration in mouse livers, kidneys and brains

The mean concentrations of MT-1/2 in the livers, kidneys and brains of WT and MT-KO mice are shown in Fig. 1. In all organs, the MT-1/2 concentrations in MT-1/2 KO mice were significantly lower than WT and MT-3 KO mice. The MT-1/2 concentrations in the liver and kidneys of WT mice were higher than those of MT-3 KO mice, while the MT-1/2 concentrations in the brains of MT-3 KO mice were higher than those of WT mice.

The livers in WT mice showed strong MT-1/2 expression by immunohistochemical staining, while the livers in MT-3 KO mice showed weak MT-1/2 expression. However, MT-1/2 KO mice showed significantly weak MT-1/2 expression (Figs. 2a-c). The urinary tubules in the kidneys of WT and MT-3 KO mice showed strong MT-1/2 expression, while those in the kidneys of MT-1/2 KO mice showed significantly weak MT-1/2 expression (Figs. 2d-f). The MT-1/2 expression in the brain was stronger in MT-3 KO and WT mice than MT-1/2 KO mice (Figs. 2e-i).

MT-1/2 concentration in mouse livers administered Cd

In WT mice, the MT-1/2 concentration in the liver was significantly increased with Cd administration dose dependently. However, the MT-1/2 concentrations in the livers of MT-1/2 KO mice did not increase with Cd administration (Fig. 3 (A)). By immunohistochemical staining, the MT-1/2 expression in the livers of WT and MT-3 KO mice increased significantly after Cd administration, while that in the livers of MT-1/2 KO mice showed only a slight increase after Cd administration (Fig. 3 (B)).

Two MW peaks of MT-1/2 in mouse liver, kidney and brain homogenates

MT with a molecular weight of (LMW) 10,000 (most
common MW of MT) was mostly depleted in all the organs (kidney (A), brain (B), liver (C)) of MT-1/2 KO mouse. However, MT with a higher molecular weight (HMW, over 100,000) remained significantly in the kidneys, brains and liver with the similar manner (Fig. 4).

**MT-1/2 concentration in the urine of LEC rats**

Urinary MT-1/2 concentration significantly increased in LEC rats with age (Fig. 5). Especially, at the stage of acute hepatitis, the urinary concentration markedly increased, but did not increase in LEA rats at 15-weeks of age (a control for acute hepatitis stage LEC rat). The MT-1/2 concentrations in LEC rats were significantly higher than in LEA rats after 8 weeks of age. The MT-1/2 concentration in LEC female rats during acute hepatitis was significantly higher than that in male LEC rats. The serum MT-1/2 concentration significantly increased in the acute hepatitis stage (15 weeks) compared to the earlier stage, similar to that in urine (data not shown).

Urinary Cu and Zn concentrations in LEC rats increased significantly in parallel with urinary MT 1/2 concentrations. Cu increased significantly more compared to Zn at the acute hepatitis stage (data not shown).

**DISCUSSION**

Many investigations suggested that MT is involved in various physiological phenomena and is associated with the occurrence and prognosis of various diseases (Harmer, 1986; Bauman et al., 1991; Satoh and Tohyama, 2000; Goulding et al., 1995; Apostlova et al., 1997; Nagamine et al., 2005; Hidalgo et al., 2006; Yamasaki et al., 2006; Miyazaki et al., 2007). Therefore, many analytical methods have been developed to determine MT concentrations in various specimens, including plasma and urine, for diagnostic purposes (Tohyama and Shaikh, 1981; Garvey et al., 1982; Mehra and Bremner, 1983; Cousins, 1991; Akintola et al., 1995). However, these assays have seldom been used for routine clinical and diagnostic use until recently. The major reason may be the difficulty in measuring the low plasma concentrations of MT which could diagnose MT deficiency associated with various diseases.
diseases. We developed an ELISA assay which could be a satisfactory tool for measuring plasma MT1/2 concentration as well as the tissue or organ MT1/2 concentration. In order to confirm the practical usefulness, we evaluated the MT 1/2 ELISA method in experimental animals using MT-1/2 KO mice with deficient MT expression and LEC rats which are known to have a high MT expression in the liver (Sakurai et al., 1992a, 1992b).

The MT-1/2 concentration determined by ELISA in organs of MT-1/2 KO mice was significantly low, and MT-1/2 expression by immunohistochemical staining of these organs was significantly weaker than that in WT and MT-3 KO mice, as shown in Fig. 1. The MT-1/2 concentration in the WT mouse liver significantly increased with Cd administration, while that in the MT-1/2 KO mouse liver did not increase at all with Cd administration. Similarly, MT-1/2 expression in the liver by immunohistochemical staining showed a significant increase in WT mice after Cd administration, but did not increase in MT-1/2 KO mice. The same reactivity was shown in the ELISA assay and by immunohistochemical staining. These results support the idea that this ELISA assay is a useful and specific tool to determine MT concentration in animal specimens.

Although it has been reported previously that MT has two peaks, namely high MW above MW 100,000 and small MW (approximately 10,000) (Nakajima et al., 1991a, 1991b), this is the first report to reveal the dis-
distribution change of MT 1/2 in KO mice. We observed a small amount of residual MT-1/2 in the livers, kidneys and brains in MT-1/2 KO mice by ELISA and immunohistochemical staining. The reason for this incomplete deletion of the MT-1/2 gene has been explained by the incomplete targeted gene disruption when Searle et al. (1984) first raised this KO mouse. The regular MW-MT (MW approximately 10,000) was depleted mostly in liver, kidney and brain of MT-1/2 KO mice. However, MT with HMW (above 100,000) remained significantly in all the organs of MT-1/2 KO mice compared to LMW MT-1/2. Therefore, it was suggested that the remaining MT at a small amount in KO mice is mostly HMW MT-1/2. HMW MT-1/2 may be either a precursor protein or an aggregated protein due to the SH moiety of MT-1/2 having a large protein structure. However, further studies are needed to clarify the mechanism of presence of MT at two molecular weights.

The LEC rat is a model rat of hepatitis and Wilson disease, and Cu levels in the liver become significantly high soon after birth and increase with age (Enomoto et al., 1992; Mori et al., 1994). Sakurai et al. (1992a, 1992b) previously reported a significant increase in MT-1/2 in LEC rat livers. In this study, we found a significant increase in serum and urine MT-1/2 concentration and urine Cu and Zn in LEC rats at the acute hepatitis stage. Cu increased significantly in parallel with the MT-1/2 concentration. Cu rather than Zn was more strongly associated with the increase in MT-1/2 concentration in LEC rats.

In conclusion, we evaluated the MT-1/2 concentration in WT, MT-1/2 KO, MT-3 KO mice and LEC rats using a newly developed MT-1/2 ELISA. The data showed theoretically acceptable results which reflected the physiological phenomenon. The MT concentration determined by this ELISA correlated well with the MT expression by immunohistochemical staining. Therefore, the new MT-1/2 ELISA and its antibody for immunohistochemical staining could provide a robust tool for physiological and toxicological studies in experimental animals, as well as for diagnostic use of human specimens.

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