Direct Visualization of Copper–Metallothionein in LEC Rat Kidneys: Application of Autofluorescence Signal of Copper–Thiolate Cluster

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We report on the histochemistry of copper–metallothionein (Cu-MT) in the kidneys of Long Evans Cinnamon (LEC) rats. We used the visualization principle of histochemistry based on the autofluorescence emission from the fluorophore of Cu-thiolate clusters in proteins. Intense autofluorescence signals were observed with a ring at the outer stripe of the outer medulla. Orange fluorescence signals were observed in the nuclei and cytoplasm of proximal straight tubular (PST) cells of segment 3 (S3) at the outer stripe of the outer medulla, and yellow-orange signals were detected in lysosome-like organelles in the proximal convoluted tubule (PCT) cells of segments 1 and 2 (S1 and S2) adjacent to the glomeruli in the cortex. These fluorescent materials were identified as Cu-MT because both signals were quenched by withdrawing Cu+ or by blocking cysteine residues, the distributions of cysteine residues and immunoreactive MT showed identical patterns to the localization of the fluorescence signals, and the fluorescent proteins containing Cu were eluted at the same Kd value of purified Cu-MT by gel filtration chromatography. However, a high level of MT mRNA was detected only in the outer stripe of the outer medulla where the orange fluorescence signals were detected, but not in the cortex. This difference in localization between the protein and the mRNA suggested that synthesis of renal MT occurs de novo in the outer stripe of the outer medulla. The yellow-orange fluorescent Cu-MT located in the lysosomal organelles at S1 and S2 of the PCT cells in the cortex could be Cu-MT of nonrenal origin, i.e., Cu-MT transported from other organs. (J Histochem Cytochem 44:865–873, 1996)

KEY WORDS: Metallothionein; Copper; Autofluorescence; LEC rat; Kidney; In situ blotting; mRNA distribution.

Introduction

Copper (Cu) is an essential trace element and plays remarkable roles (1). Although the toxicity associated with metabolic disorders of chronic Cu accumulation has been investigated (2–4), many uncertain points remained, and animals with the disorders were required for precise metabolic studies. The Long–Evans rat with a cinnamon-like coat color (LEC) is a mutant animal in which age-dependent Cu accumulates in the liver and kidneys, resulting in damage to the organs. These rats have been used as model animals to study Cu metabolic disorders (5–8). Metallothionein (MT) is associated with Cu metabolic disorders, since age-dependent accumulation of Cu binding MT (Cu-MT) was shown to occur in LEC rats (9–12). MT is a low molecular weight (~7 KD) protein with a high metal (7 to 20 atoms/molecule) and sulfur (~33% as cysteine residue) content. Its synthesis is induced by heavy metals and several other factors. The main functions of MT are detoxification of heavy metals and homeostasis of essential trace metals (13,14). With acute exposure to heavy metal ions, the ions induce new synthesis of MT in the liver and are captured by the protein. With prolonged chronic exposure, however, MT is released from the liver into the bloodstream owing to minor hepatic injury (15,16), and is subsequently reabsorbed by the brush border of the proximal convoluted tubule (PCT) cells after glomerular filtration in the kidneys (17,18). In contrast to the role of MT in the liver, the absorbed MT in the kidneys is toxic because of the free heavy metal ions that may be released.

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from the MT by lysosomal degradation and/or by oxidation in the PCT system (19). On the other hand, Squibb et al. (20) suggested that MT in the kidneys is not only taken up from blood but is also newly induced. Although the existence of two origins of MT in the kidneys, i.e., renal-induced MT and nonrenal MT, has been determined, the distribution of both kinds of MTs in the kidneys has not been studied. To clarify the relationship between transported Cu-MT and renal-induced Cu-MT, we designed this study to visualize Cu-MT and survey its mRNA. We used autofluorescence emission of Cu-MT to visualize the protein. This unique property of autofluorescence emission is based on the Cu\(^{2+}\)-cholate coordination in the protein, not only with Cu\(^{2+}\) but also with the cysteine residues in the protein (21–23). Moreover, the emissions may provide some information on the state of the protein, e.g., partial copper ion release or oxidation of the protein (21). In this study we demonstrated the simultaneous presence of MT with two origins in the kidneys of LEC rats: partially oxidized Cu-MT of nonrenal origin and newly synthesized MT in the kidneys. The importance of the specificity of the histochemistry and the classification of Cu-MTs by their origin in LEC rat kidneys is discussed.

Materials and Methods

**Chemicals**

Tins base, mercuric chloride, 1-[4-chloromercuiphenylazo]-2-naphthol (mercury orange; MO), ninotolblue tetaazonium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Sigma (St Louis, MO). Zeta probe membrane was obtained from Bio-Rad (Richmond, CA). Barhcopurine disulfonic acid (BCS) was obtained from Dojindo (Kumamoto, Japan). The nick-translation kit was obtained from Takara (Kyoto, Japan). Mouse anti-MT (clone E9; isotype IgG\(\lambda\)) was from Zymed (San Francisco, CA). Colloidal gold (10 nm)-conjugated streptavidin, biotinylated sheep anti-mouse Ig\(\alpha\)K, and [\(\alpha\)-\(^{32}\)P]-dCTP were obtained from Amersham International (Poole, UK). We prepared pure Cu\(_{12}\)-MT from commercially available Cd\(_{3}\)Zn\(_{2}\)-MT (Sigma) by the method previously described by Oikawa et al. (26) as the reference fluorescence signal for Cu-MT.

**Animals**

Two inbred strains of male rats (30 weeks old, 200–300 g body weight), Long-Evans Cinnamon (LEC) and Long-Evans Agouti (LEA; normal counterpart of LEC strain) were used in this study. Animals from the domestic line of Hokkaido University and/or from Charles River Japan (Tokyo, Japan) were available for study. They were kept on a laboratory diet and water ad libitum, and were housed in a facility maintained at 22°C with a 12-hr light–dark cycle.

**Histochemical Procedures**

All histochemical procedures were carried out according to our previous report (27), with slight modifications. The sections were mounted on glass slides or dry membranes in a cryostat box and thawed at 25°C (thickness of sections 5 µm for glass slides, 20 µm for blotting membranes). The slides were immediately immersed in acetone for 5 min. The sections were mounted with polymer plastic mixture (trimethylol propane trimethacrylate liquid and benzoin methyl ethyl ether crystal, 50:1 mixture) and polymerized by UV irradiation for 1 min according to technical file No. 230 of the Pharmacia PhastSystem (Uppsala, Sweden). Instead of plastic embedding, immersing the tissue on glass in 70% ethanol was also possible. Autofluorescence signals were observed with an epifluorescence microscope (BX-50-PLA; Olympus, Tokyo, Japan) using a modified U-MWU filter cube (400-nm dichroic mirror, 330–385-nm excitation filter, 420-nm or 530-nm barrier filter). Signals of Cu-MT on a Zeta probe membrane were also detected by illumination with a UV light (365 nm, without the barrier filter).

**Detection Specificity**

To assess the detection specificity of the autofluorescent histochemistry, several chemical properties of Cu-MT were studied, e.g., existence of a Cu\(^{2+}\)-cholate cluster in the protein, cysteine residue distribution, immunohistochemistry of MT, and distribution of the expressed MT mRNA in the tissue. These distribution patterns were compared with those by autofluorescence histochemistry.

**Quenching Test.** To determine whether Cu\(^{2+}\) is essential for autofluorescence emissions, the blot was treated with BCS as a specific Cu\(^{2+}\)-chelating agent. The blot was incubated in 10 mM BCS for 1 hr at 25°C. After incubation, the blot was washed five times for 5 min with distilled water.

A sulfur-containing amino acid cysteine is well recognized as a key residue of metal–cholate clusters in proteins. To determine whether the residue is also associated with autofluorescent emissions, the residue was blocked with Hg\(^{2+}\). The blot was soaked in 1 mM HgCl\(_2\) for 1 hr. After BCS or Hg treatment, the blot was observed under UV (365-nm) excitation.

**Cysteine Residue Distribution.** The distribution of the residues was visualized on the tissue blot with MO staining, since the molecule is a specific detector of cysteine residues (28). The blots were incubated with 0.1 mM MO in 70% ethanol for 3 hr and were washed with 70% ethanol three times for 5 min.

**Immunodetection of MT.** To prove the existence of MT on the tissue blot, the immunoreactivity for MT on the membrane blot was detected according to the method previously reported (27) and the instruction manual of AutoProbe (Amersham), with slight modification. The blot was processed with a blocking solution (3% polyvinylpyrrolidone, 3% nonfat dry milk) for 1 hr, a primary antibody (E9) (1:500 dilution) overnight, a biotin-tagged secondary antibody (1:200) for 1 hr, streptavidin–colloidal gold (1:200) for 1 hr, and silver enhancement for 30 min. Detection specificity of E9 against mammalian MT was previously reported by Jasani et al. (29, 30), and we also confirmed the detection specificity of this antibody by the primary antibody omission test and/or by E9 pretitrated with purified MT (not shown). To avoid nonspecific staining by endogenous peroxidases or alkaline phosphatase, we used colloidal gold–silver staining for visualization of MT.

**MT mRNA Hybridization Analysis**

To examine the genomic expression of the protein in the renal tissue, the distribution of mRNA-encoded MT was detected as follows. Rat MT-I cDNA obtained from the livers of Sprague-Dawley male rats (unpublished data, Kurasaki et al.) was used as a probe in this hybridization. About 450-BP cDNA fragments, including full-length rat MT-I mRNA, were purified by acrylamide gel electrophoresis, and the isolated cDNA fragments were labeled with [\(\alpha\)-\(^{32}\)P]-dCTP according to the nick translation system. Binding specificity of labeled cDNA was confirmed by a hybridization study of hepatic MT mRNA from MT-I-induced rats by Cd administration and/or by saline-injected rats (data not shown). The tissue-blotted membranes were soaked in 0.9 M NaCl, 50 mM trisodium citrate solution for 10 min and subsequently heated at 80°C for 2 hr before hybridization. Hybrid-
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Acid Phosphatase Histochemistry

We used the method of Gossrau (32) with BCIP as a substrate. Incubation was carried out for 1 hr at 25°C with 0.5 mM BCIP, 0.5 mM NBT, and 45 mM dimethylformamide in 40 mM CH3COOH, 100 mM CH3COONa. As a control experiment, the substrate was omitted.

In Vitro Study

The organs were homogenized with 2 volumes of a buffer solution (100 mM Tris, 25 mM HCl) with a Potter-Elvehjem homogenizer (250 rpm, 6 strokes/min) at 0°C, and the homogenate was centrifuged at 110,000 x g for 1 hr at 4°C (Beckmann TL-100; Palo Alto, CA). The supernatant was filtered with an Ultrafree C-3 (Millipore; Bedford, MA), subsequently applied to a Superdex 75 column HR 10/30 with extension adapter on a SMART system (Pharmacia), and then eluted with 100 mM Tris, 50 mM HCl. The absorbances of the eluate at 220 nm and 280 nm were monitored with a uPeak Monitor. The concentrations of Cu and Zn were measured with a flame atomic absorption spectrophotometer (Varian SpectrAA-300; Mulgrave, Victoria, Australia) and the fluorescence signals of each fraction were observed at the UV excitation of 365 nm at -80°C.

Results

Emission Properties

The autofluorescence signals in the renal section were detected at UV excitations of 250–385 nm. In the kidneys of LEC rats, orange fluorescence signals with a ring shape were observed predominantly in the outer stripe of outer medulla (Figure 1A). The localization of the signals on the tissue blots was identical to that of the tissue sections (Figure 1B). The autofluorescence signals of pure protein on nylon blots indicated an identical signal from Cu-MT in the tissue (Figure 2M-a and 2M-b). No fluorescence was found in the kidneys of LEA control rats (Figure 1C). Autofluorescence signals in the renal tissue section were detected in the green background by microscopic observation with the modified U-MWU filter cube (Figures 2A, 2C, 2H-a, and M-a) with a 530-nm barrier filter; by using a U-WBV filter cube (excitation range 400–440 nm), the signals in the tissue were detected weakly, and with a U-WB filter cube (450–480 nm) the signals were not detected at all (not shown).

To visualize Cu-MT in the kidneys, a barrier filter (cutoff filter against the emission) of 530 nm (cutoff range below 530 nm) was suitable, since the spectral range of the autofluorescence emission of Cu-MT in the tissue was 550–700 nm. On the other hand, nui-
sance autofluorescence signals from other biomolecules have been reported below 550 nm of excitation (23). With a 420-nm barrier filter (cutoff range below 420 nm), the background appeared bright blue and the autofluorescence signals of Cu-MT appeared red-orange. However, the intensity and contrast of the signals were re-
duced in the tissue and on the blot (Figures 2B, 2J, 2K, and 2M-b).

Detection Specificity

The existence of Cu-MT in LEC rat kidneys was confirmed by chromatography of the tissue extract (Figure 1I-a). The protein showed a low molecular weight, high Cu content, and a distribution coefficient (Kd) of 0.52 that agreed with purified rat liver MT (33). No Cu-MT was detected in the renal tissue of LEA rats (Figure 1I-b). Contributions of both Cu+ and thiole groups to the autofluorescence emissions were confirmed by withdrawing Cu+ and/or by blocking the cysteine residues.

The signals in LEC rat kidneys were quenched by removing Cu+ (Figure 1D) and by blocking the SH group of cysteine residues (Figures 1E and 2D). The results showed that the Cu+–thiolate cluster was an indispensable chemical factor for the autofluorescence emissions. The thiole group by itself was visualized with a MO coupling reaction to cysteine residues. The labeling pattern was found in the kidneys of LEC rats (Figure 1F) but was not observed in LEA rat kidneys (not shown). The localization of immunoreactive MT (Figure 1G) showed good analogy with those of autofluorescence emissions and MO staining. Strong MT immunoreactivity was observed in the outer stripe of the outer medulla of LEC rat kidneys which clearly demonstrates the existence of MT showing autofluorescence signals. All the histochemical data were co-localized in LEC rat kidneys and showed that the cells contained Cu-MT with fluorescence signals.

Genomic Expression of MT

The induction of new renal MT in LEC rat kidneys was confirmed by visualizing the mRNA of MT on the blot. Interestingly, the high level of MT mRNA was found predominantly in the outer stripe of the outer medulla (Figure 1H). This distribution pattern corresponded with the pattern of orange autofluorescence signals in the outer stripe of the outer medulla (Figures 1A and 1B). In the cortex, however, expression of MT mRNA was scarcely detected. No significant expression of MT mRNA was detected in the kidneys of LEA rats (not shown).

Detailed Localization of Autofluorescence Signals in the Tissue

Under microscopic analysis of epi-illumination with UV light (U-MWU filter cube) of sections of LEC rat kidneys, strong orange autofluorescence signals were observed in the outer stripe of the outer medulla (Figures 2A and 2B). A weak yellow-orange signal was also seen in the cortex (Figure 2C). Under high-power microscopic analysis with a U-MWU filter cube, the orange signals were detected at segment S3 of the PST (Figure 2E). Each tubule segment was classified by hematoxylin–eosin staining (the proximal tubules were more strongly stained than the medullary thin ascending limb of Henle’s loop) and by morphological appearance (the proximal tubules had a higher epithelium and a larger lumen). The orange autofluorescence signals of Cu-MT were predominantly localized in both the cytoplasm and nuclei of the tubular epithelium in the PST S3 in the outer stripe of the outer medulla (Figures 2E, 2F, and 2J). MT mRNA was also localized in the outer stripe of the outer medulla (Figure 1H). However, there were no signals in the capsular space.
surrounding the glomerulus, other segments, such as the collecting tubules, and in the distal convolution (Figures 2A–2C). The kidneys of LEA control rats did not show any fluorescent signals in the tissue (Figure 2G). Yellow-orange signals were located near the glomeruli of the PCT segments S1 and S2 in the cortex. Yellow-orange signals appeared mainly within the cytoplasmic granules (Figures 2H-a and 2I-a). It is interesting that they also exhibited acid phosphatase activity, which is a histochemical marker for lysosomes (Figure 2I-b). Moreover, the granules showed several signs of Cu-MT, e.g., the presence of cysteine residues (Figure 2H-b), antigenicity of MT (Figure 2L), and quenching by Hg²⁺ treatment (Figure 2D). Although in the cortex the histochemical aspects of the lysosome-like granules indicated the presence of Cu-MT, expression of MT mRNA was not found (Figure 2H).

Discussion

Our report describes the histochemistry of Cu-MT, which is a protein possessing Cu⁺-thiolate (Cu⁺-S) clusters. We confirmed the detection specificity of Cu-MT by the chemical properties of the protein, and we found different distributions of the protein, i.e., newly induced renal Cu-MT in the outer stripe of the outer medulla and accumulated Cu-MT in the cortex, transported from other organs.

Cu⁺-thiolate Fluorophore as a Specific Probe to Visualize Cu-MT

Two individual methods to visualize Cu-MT have been reported. One is an immunodetection assay to visualize MT in the tissues (29,30,34,35). The technique is based on specific recognition of the protein portion of MT, not on the recognition of metals bound to MT. The identity of the metals bound to MT, e.g., Cd-MT, Zn-MT, Cu-MT, and apo-MT, could not be determined by immunodetection of MT (29). The other methods showed the copper histochemistry using detector molecules or dyes bound to Cu ions, e.g., orcein, rhodamine, and rubenac acid staining. These histochemical techniques, however, show a broad specificity in selecting metals. Not only do these detector molecules recognize other divalent cations but they also sometimes fail to detect Cu ions despite the presence of high concentrations of Cu in the tissue (36–38). The histochemical methods for Cu staining do not always show a good correlation with analytical measurements of the Cu content by immunoreactive staining for MT (35), since only Cu⁺ bound to MT, not Cu²⁺ (21–25). The orange autofluorescence of Cu-MT from Neurospora crassa was observed by in vitro study (21). Fluorescent properties have been shown for purified Cu-MT where Cu⁺ is captured by cysteine residues. Since then, reports have shown that mammalian Cu-MT emits autofluorescence in the spectral region of 500–650 nm (from yellow to red in visible color), and its intensity reaches a maximum when 12 Cu⁺ are bound to the protein (24,25). Stillman et al. (23–25) have reported that emission spectral data are detected in vivo, similar to those observed from Cu-MT in vitro. However, the histochemistry of Cu-MT using autofluorescence signals in tissues has not been shown. In this study we successfully demonstrated the histochemical localization of Cu-MT in the kidneys of LEA rats by using its autofluorescence emissions. The kidneys were presumed to be a target of Cu toxicity (8,39). Cu-MT visualization using a fluorophore probe of the Cu⁺-thiolate cluster has several advantages over the immunoassay of MT and Cu-histochemistry. First, the procedure is simple and does not require fixation and related tissue treatments. Second, there is direct detection of Cu-MT, since the autofluorescence emission of the protein corresponds to its metal–protein coordination in the protein molecule. In animal tissues, however, several other biomolecules, e.g., vitamin A, lipofuscin, and porphyrins, emit similar autofluorescence under excitation (40). Therefore, to determine whether autofluorescence signals are due to Cu-MT, we compared the current histochemistry with the other indispensable reference markers related to the protein–chemical aspects of MT, e.g., the existence of Cu⁺-thiolate clusters, immunoreactivity, and cysteine histochemistry. The autofluorescence signals were located together with cysteine residue distribution and immunohistochemistry of MT (Figure 1B). Moreover, analyses by gel filtration chromatography showed no autofluorescent substance other than Cu-MT in the cytosol fraction of LEC rat kidneys (Figure 1I). These results show that the autofluorescence emissions in this study depend on the Cu⁺-thiolate bond of Cu-MT.

Localization of Cu-MT and MT mRNA

The interesting findings of the detailed Cu-MT and its mRNA localization are as follows: (a) orange fluorescent Cu-MT was located in the cytoplasm and nuclei of PST S3 of the outer stripe of the outer medulla (Figure 1B), and MT mRNA was also localized in the same stripe (Figure 1H); and (b) yellow-orange fluorescence signals were observed in the lysosome-like organelles of PCT S1 and S2 adjacent to the glomeruli in the cortex (Figure 2C), but MT mRNA was not detected in this region. Material emitting yellow-orange signals was also identified as Cu-MT, since the fluorescent granules exhibited all the properties of Cu-MT. However, the emission wavelengths of the protein in the granules were shorter than...
for Cu-MT located in the outer stripe of the outer medulla. The change in emission was presumed to be caused by the partial release of Cu ions and the resulting oxidation of Cu-MT in the cortex, because the emission of model complexes of Cu-MT changed from orange to yellow by decreasing the molar ratio of the Cu+-thiole group (21). The localization of Cu-MT at PCT S1 and S2 in the cortex corresponded well with the immunohistochemistry of the protein in the kidneys of Cu-loaded rodents (29,30,34,35). MT readily passes through the glomerulus in MT administration experiments (15–20). After glomerular filtration, this protein binds to the brush border of PCT cells and is taken up into these cells (18). Renal toxicity caused by experimentally administered MT was observed in S1 and S2 of the PCT cells of the cortex (18). Radio-isotope-labeled cysteine residues of MT are also primarily confined to these cells (19). In addition, in these cells of LEC rat kidney, morphological changes, e.g., vacuolar degeneration of nuclei and/or swelling, were observed (39). We demonstrated that the protein in the cortex is located in the lysosome-like organelles. These results suggest that the cortical Cu-MT is involved in the degradation process in these cells. We confirmed that the protein located in the cortex is transported from other organ(s), since no MT mRNA was observed in this region, suggesting no synthesis of MT in the cells (Figure 2F). MT mRNA was, however, successfully detected in the outer stripe of the outer medulla. In this area, the distribution of MT immunoreactivity and/or the orange signals showed an identical pattern to that of MT mRNA, indicating that the protein emitting the orange signals is biosynthesized in the outer stripe of the outer medulla. We postulate that the inducer of MT in the outer stripe of the outer medulla is Cu ions that were released from partially degraded Cu-MT in the lysosome-like granules in the cortex (Figure 3). Several reports have shown that bound metal ions are released from MT by lysosomal degradation (41–44). This "lysosomal theory" was based on studies showing that the protein is degraded when it is reabsorbed into the PCT system in the kidneys. The scheme is supported by experiments showing that subcellular fractionation of metals bound to MT is concentrated in the lysosomes 30 min after MT administration (43,45). The Cu ions released from lysosomes in the PCT S1 and S2 at the cortex are believed to be a logical inducing factor for de novo synthesis of Cu-MT in the outer stripe of the outer medulla.

In conclusion, we confirmed that the orange and yellow-orange fluorescence signals of LEC rat kidneys are evidence of Cu-S clusters in Cu-MT. Orange fluorescent Cu-MT was detected predominantly in the outer stripe of the outer medulla, as well as MT mRNA.
On the other hand, yellow-orange fluorescent signals of Cu-MT were located in the cortex, but genomic expression of MT mRNA was not detected in this region. This difference in distributions between the protein and its mRNA suggests the existence of two origins of Cu-MT in LEC rat kidneys: newly induced renal Cu-MT, showing orange signals in the outer stripe of the outer medulla, and yellow-orange fluorescent signals of Cu-MT of nonrenal origin in cortex.

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