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Biological significance of non-acetylated metallothionein

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Abstract

The biological significance of non-acetylated metallothionein (MT) was investigated from the viewpoint of N^{α}-acetylation after induction of MT synthesis by metallic and non-metallic inducers, by partial hepatectomy and under physiological conditions. N^{α}-Acetylated and non-acetylated forms of MT-2 in liver supernatants and plasma were detected by the tandem size-exclusion and anion-exchange HPLC columns with in-line detection by mass spectrometry. The non-acetylated isoform of MT-2 (MT-2′) was present at a comparable level to the N^{α}-acetylated form of MT-2 (MT-2) at an early stage after induction by not only zinc but also cadmium, and by partial hepatectomy in the livers of rats. Plasma MT-2 in neonatal rats was similar to liver MT-2 in the composition of N^{α}-acetylated and non-acetylated forms, suggesting that there are no differences in the roles of N^{α}-acetylation of MT in the extracellular trafficking of MT. The column switching HPLC method with in-line detection by inductively coupled argon plasma mass spectrometry (ICP-MS) was shown to be a sensitive and powerful method to detect MT proteins at not only isoform level but also at acetylated and non-acetylated form levels.

Keywords: Metallothionein

1. Introduction

Two biological roles of metallothionein (MT) have been proposed; one is in homeostatic regulation of essential heavy metals such as copper (Cu) and zinc (Zn) [1,2]. The other is in protection of cells from harmful chemicals, i.e., non-essential and excessive essential heavy metals, reactive oxygen species, radicals and alkylating agents [3–6]. MT was believed to be a cytosolic protein and functions primarily in the cytoplasm through the two biological roles mentioned above. However, MT is now known to undergo intracellular trafficking to and from the nucleus [7–9], and also extracellular trafficking from the liver to the bloodstream [6,10].

Although it is known that the two isoforms of MT, i.e., MT-1 and -2, behave differently in chemical reactions such as oxidative degradation [11], it is not known whether there are any differences in the biological roles mentioned above between the two isoforms. Further, it is not known how MT is trafficked or whether any special forms of MT are present for intra- and extracellular trafficking.

Although we observed the presence of a third isoform of MT in the livers of rats at an early stage after induction by Zn and named it MT-2′ based on its chromatographic behavior [12], it has not been paid much attention and has not been classified as one of the MT isoforms. Meanwhile, MT-2′ was

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shown to be also induced by epidermal growth factor (EGF) plus Zn [13].

Recently, we studied MT-2′, its possible biological role in the intracellular trafficking of MT because of its expression, and the coincidental localization of MT in the nucleus at an early stage after induction by Zn or partial hepatectomy [14,15]. Our investigation on the chemical structure of MT-2′ revealed that MT-2′ is quite similar in chromatographic behavior to MT-2 and is 42 Da smaller than MT-2 in molecular mass, as determined by mass spectrometry (MS), indicating that MT-2′ is a non-acetylated form of MT-2 [16].

Non-acetylated forms of MT have been demonstrated to be present as minor constituents in mammals [17–19] and also in invertebrates [20,21] during analysis with the use of new types of mass spectrometers. However, besides the detection and identification of non-acetylated forms of MT, there have been no further studies on the biological significance of non-acetylated forms of MT.

In the present study, the biological role of non-acetylated MTs was investigated by following the expression and turnover of MT-2′ in the liver after its induction under various conditions (essential and non-essential metals, non-metallic chemicals, partial hepatectomy and physiological conditions). We also analyzed MT in the plasma of neonatal rats with a special focus on the presence or absence of an acetyl group on the N-terminus.

Although non-acetylated form of MT-1 (MT-1′) was suggested to be present together with acetylated form of MT-1 by off-line MS [16], they were not separated under the chromatographic conditions employed. Therefore, acetylated and nonacetylated forms of only MT-2 were analyzed in the present study. MT-2 was separated into N^{α}-acetylated and non-acetylated forms on size-exclusion and anion-exchange high-performance liquid chromatography (HPLC) columns by the column switching method with in-line detection by inductively coupled argon plasma (ICP)-MS.

2. Materials and methods

2.1. Animals and reagents

Male Wistar rats (five weeks of age, approximately 110 g body mass) and neonatal Wistar rats (two days of age) with a lactating dam were purchased from Clea Japan, Tokyo, Japan. The adult rats were given a standard diet (CE-2, Clea Japan) and tap water freely in a conventional animal room maintained at 25°C and 55% relative humidity with a 12-h light period. Cadmium acetate (96.3% enriched ^{113}Cd) was purchased from Oak Ridge National Laboratory (Oak Ridge, TN, USA). Other reagents were of the highest grade available.

2.2. Animal treatments

For the time–course study, groups of three rats received a single subcutaneous (s.c.) injection of *n*-hexane at the dose of 2.0 ml/kg body mass, and were killed 1, 2, 4, 8, 16, 24 or 48 h after the injection under ether anesthesia. Three rats treated with saline served as controls.

Groups of three adult rats received a single s.c. injection of *n*-hexane at the dose of 0.5 ml/kg body mass or a single s.c. injection of cadmium acetate at the dose of 2 mg Cd/kg body mass, and were killed 10 h after the injection under ether anesthesia. For partial hepatectomy, the left lateral and median lobes (approximately 70%) of the liver were removed from three adult rats under light ether anesthesia [22].

For the re-induction study, groups of six rats received a single s.c. injection of cadmium acetate at the dose of 2 mg Cd/kg body mass. After 48 h, three of the six rats were killed under ether anesthesia, and the other three rats received a single intraperitoneal (i.p.) injection of zinc acetate at the dose of 15 mg Zn/kg body mass and were killed 10 h after the last injection under ether anesthesia. The livers of both groups were homogenized in four volumes of 10 m*M* Tris–HCl, pH 8.4, containing 250 m*M* glucose using a glass–PTFE homogenizer under an atmosphere of nitrogen. Each homogenate was ultracentrifuged at 170 000 *g* for 60 min at 4°C. A liver supernatant of female neonatal rats (four days of age) was also prepared under the same conditions. Plasma of the neonatal rats was prepared by centrifuging clotted blood at 1600 *g* for 10 min. As the substitution of Zn in MT by monoisotopic Cd enhances the detection limit for MT on ICP-MS, ^{113}Cd was added to the plasma (10 μg Cd/ml plasma), and the mixture was incubated for 10 min at 0°C.

2.3. Determination of metal distributions by the column switching HPLC–ICP-MS method

A 0.1-ml portion of a sample was applied to an SW column (TSKgel G3000SW, 600×7.5 mm, with a guard column of 75×7.5 mm; Tosoh, Tokyo, Japan), which was eluted with 25 m*M* Tris–HCl, pH 8.0, at the flow-rate of 1.0 ml/min. The MT fraction obtained with the SW column was introduced directly into an anion-exchange column (Shodex Asahipak ES-502N 7C, 100×7.6 mm; Showa Denko, Tokyo, Japan) through a four-way valve [23]. The anion-exchange column was eluted with a linear concentration gradient of Tris–HCl buffer solution (pH 7.2) from 2 to 50 m*M* at the flow-rate of 1.0 ml/min. The eluate was introduced into the nebulizer of an ICP-MS apparatus (HP 4500; Yokogawa Analytical Systems, Musashino, Japan), and the concentrations of Zn and Cd were continuously monitored at $m/z=$ 66 and 113, respectively. Data were processed with a laboratory-developed program.

3. Results

3.1. Changes in the ratio of non-acetylated to acetylated sub-forms of MT after induction with n-hexane

MT in the liver supernatant was separated into MT-1 (that comprises acetylated and non-acetylated forms [16]), MT-2′ (non-acetylated form of MT-2) and MT-2 (N^{α}-acetylated form of MT-2) on size-exclusion and then anion-exchange HPLC columns by the column switching method [12], and the amounts of Zn and Cd bound to each form (MT-1, -2′ and -2) were detected in-line by ICP-MS (HPLC–ICP-MS method) at retention times of 21.1, 40.8 and 43.2 min, respectively.

The induction of the synthesis of MT-2′ was examined with a non-metallic inducer, *n*-hexane [24], in the present study. The amounts of Zn bound to MT-1 and -2 (each isoform includes both N^{α}-acetylated and non-acetylated forms) were estimated for liver supernatants prepared from rats injected with *n*-hexane, as shown in Fig. 1. The amount of Zn bound to MT-1 increased with time after the injection and attained a plateau after 24 h, while MT-2 increased much more rapidly up to 24 h after the injection, and then started to decrease. The relative ratio of the non-acetylated to N^{α}-acetylated MT-2 (MT-2′/MT-2) decreased with time rapidly up to 24 h and slowly thereafter (Fig. 1). Although the amount of total MT induced by *n*-hexane was different from those induced by Cd and Zn, MT-2′ was shown to be induced by *n*-hexane at an early stage after the induction and to disappear accompanying a simultaneous increase in the MT-2 peak, suggesting that the rate of synthesis of MT-2, i.e., acetylation of the N-terminus, increases with time after induction.

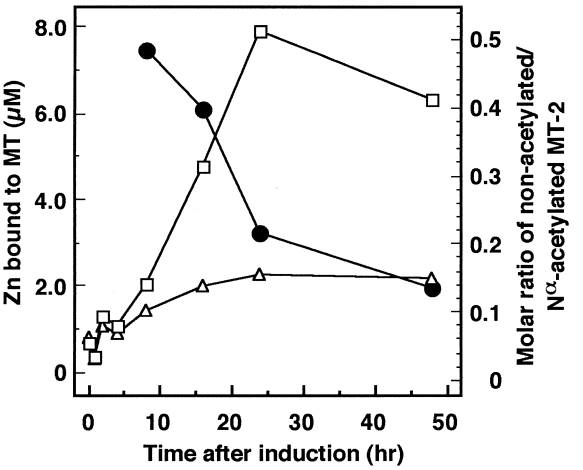

Fig. 1. Time-dependent changes in the concentration of Zn bound to MT and the amount of non-acetylated MT-2. Rats received a single s.c. injection of *n*-hexane at the dose of 2.0 ml/kg body mass, and were killed 0, 1, 2, 4, 8, 16, 24 or 48 h after the injection. Liver supernatants were subjected to HPLC–ICP-MS analysis by the column switching method. Zn in the eluate obtained on size-exclusion or anion-exchange HPLC was monitored by ICP-MS at $m/z=66$. The concentrations of Zn bound to MT-1 (open triangles) and MT-2 isoforms (open squares) were estimated from the elution profiles on size-exclusion HPLC. The ratio of non-acetylated to N^{α}-acetylated MT-2 (closed circles) was calculated from the elution profile on anion-exchange HPLC.

3.2. Comparison of the ratio of non-acetylated to N^{α}-acetylated forms of MT after induction with different inducers; Cd, n-hexane and partial hepatectomy

The relative amounts of non-acetylated MT-2 (MT-2′) induced by Cd (Fig. 2A), *n*-hexane (Fig. 2B), and partial hepatectomy (Fig. 2C) were compared to determine whether the presence of the

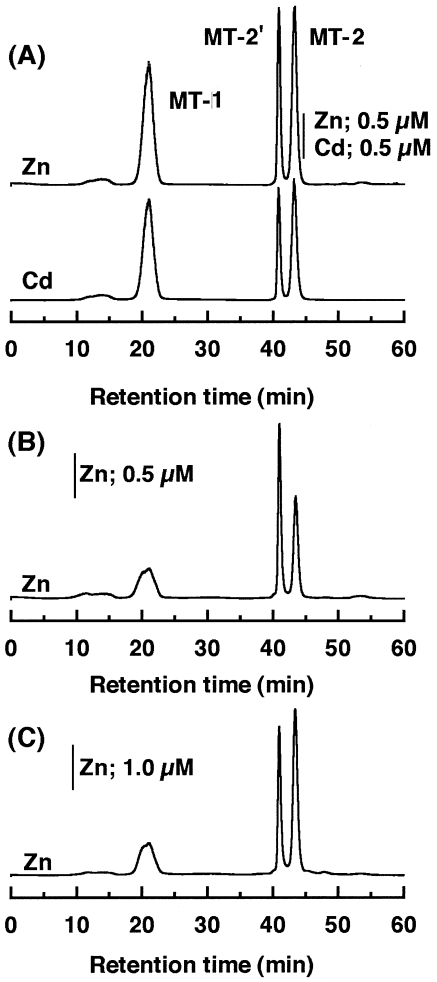

Fig. 2. Elution profiles of Zn and Cd in the livers of rats treated with Cd, *n*-hexane and partial hepatectomy. Rats received a single s.c. injection of Cd (2 mg Cd/kg body mass) (A) or *n*-hexane (0.5 ml/kg body mass) (B), or were partially hepatectomized (C), and then were killed 10 h after the treatment. Liver supernatants were subjected to HPLC–ICP-MS analysis by the column switching method. Zn and Cd in the eluate obtained on the anion-exchange column were monitored by ICP-MS at $m/z=66$ and 113, respectively. The vertical bars indicate the detection levels for Zn and Cd.

non-acetylated form is dependent on inducers for MT synthesis. MT-1 in Fig. 2 was suggested to comprise two form (non-acetylated MT-1′ and N^{α}-acetylated MT-1) in our previous study [16]. Although the ratio of MT-2′ to -2 was much lower on induction with Cd than with Zn in our previous study [12], this ratio in Fig. 2A is almost unity. This difference is not explainable except by the difference in the injection routes for Cd, i.e., intraperitoneal (i.p.) and subcutaneous (s.c.) in the previous and present experiments, respectively. The ratio in Fig. 2b is approximately 1.4 on the injection with the non-metallic inducer, *n*-hexane. This ratio was the highest we observed with various inducers. Hepatectomy also induced MT-2′ efficiently, as shown in Fig. 2C. The amounts of MT-1 were lower on induction with *n*-hexane and hepatectomy than with Cd.

3.3. Effect of MT synthetic capacity on the ratio of non-acetylated to N^{α}-acetylated forms

Non-acetylated forms of MT can occur for either positive reasons, such as trafficking, or passive reasons, such as limited capacity for co-translational acetylation or enhanced deacetylation. These possibilities were examined by comparing the relative ratios of MT-2′ to -2 under two different sets of conditions as to the MT synthetic capacity. As it is known that MT can be synthesized much faster and in a greater quantity when animals are pre-treated with small doses of MT inducers and then challenged with higher doses, rats were pre-treated s.c. with Cd at the low dose of 0.5 mg Cd/kg body mass once and then Zn was injected i.p. at the high dose of 15 mg Zn/kg body mass 48 h after the pre-treatment, as shown in Fig. 3.

Fig. 2A is presented again as Fig. 3A with the same vertical scale as the rest of Fig. 3 to demonstrate the relative amounts of MT induced under different conditions. The amounts of Cd and Zn bound to MT-1 and -2 are higher at 48 h (Fig. 3B) than that at 10 h (Fig. 3A). However, the relative ratio of MT-2′ to -2 decreased significantly with time from approximately 1.0 to 0.05, indicating that the MT-2 isoform increased about 20 times in the N^{α}-acetylated form from 10 to 48 h after the injection of Cd.

The second injection of Zn caused increases in the amounts of Zn bound to MT-1 and -2, and in the ratio of Zn bound to MT-2′ and -2 (Fig. 3C). The amounts of MT proteins synthesized after the second injection were estimated, as shown in Fig. 3D, by subtracting the MT peaks in Fig. 3B from those in Fig. 3C on the assumption that seven atoms of Cd and/or Zn are bound to each form. MT-2 and -2′

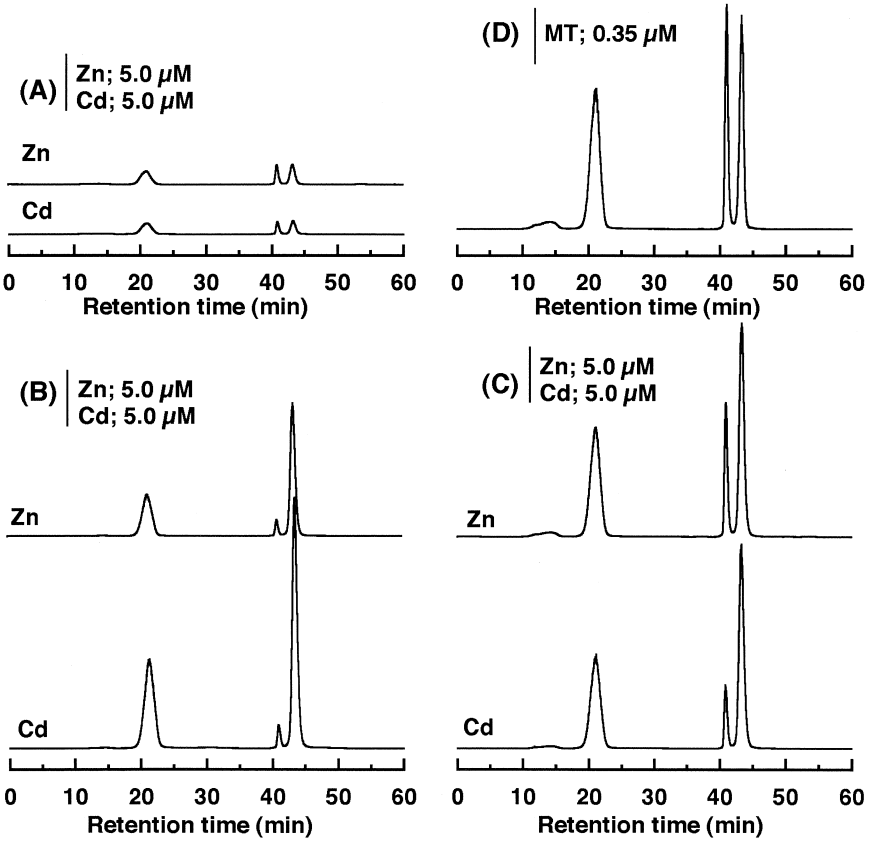

Fig. 3. Elution profiles of Zn and Cd in the livers of rats treated with Cd followed by Zn. Rats were injected s.c. with of Cd at the dose of 2.0 mg Cd/kg body mass, and then were divided into two groups; one group was killed 48 h after the Cd injection (B), and the other received a single i.p. injection of Zn at the dose of 15 mg Zn/kg body mass 48 h after the first injection and was killed 10 h after the Zn injection (C). The amounts of MT proteins (D) synthesized after the second injection were estimated by subtracting the Cd and Zn peaks of MT in (B) from those in (C) on the assumption that seven atoms of Cd and/or Zn are bound to each form of MT. Liver supernatants were subjected to HPLC–ICP-MS analysis by the column switching method. Zn and Cd in the eluate obtained on the anion-exchange column were monitored by ICP-MS at $m/z=66$ and 113, respectively. Fig. 2A is presented again as (A) with the same detection level as in (B) and (C) to show the relative amounts of MT. The amounts of MTs synthesized after the second Zn injection were estimated by subtracting (C) from (B) on the assumption that seven atoms of Zn and/or Cd are bound to MT. The vertical bars indicate the detection levels for Zn and Cd.

were shown to be synthesized in large quantities and in comparable amounts in the liver after the challenge of the pre-treated rats.

3.4. Roles of N^{α}-acetylation of MT in extracellular trafficking

Possible roles of N^{α}-acetylation of MT in extracellular trafficking were examined by comparing the composition of N^{α}-acetylated and non-acetylated forms of MT-2 between in the liver and plasma of neonatal rats. Female neonatal rats of four days old were used because of the high constitutive MT concentrations in the livers of neonates than in those of mature rats, and the higher plasma MT concentrations in female than in male pups [25]. Both isoforms of MT were detected in the form mostly binding Zn, as shown in Fig. 4A. Cu was also detected bound to MT and Cu,Zn-superoxide dismutase (SOD), although approximately 50-times less than in the case of Zn and was mostly bound to the MT-1 isoform. MT-2 in the liver was further sepa-

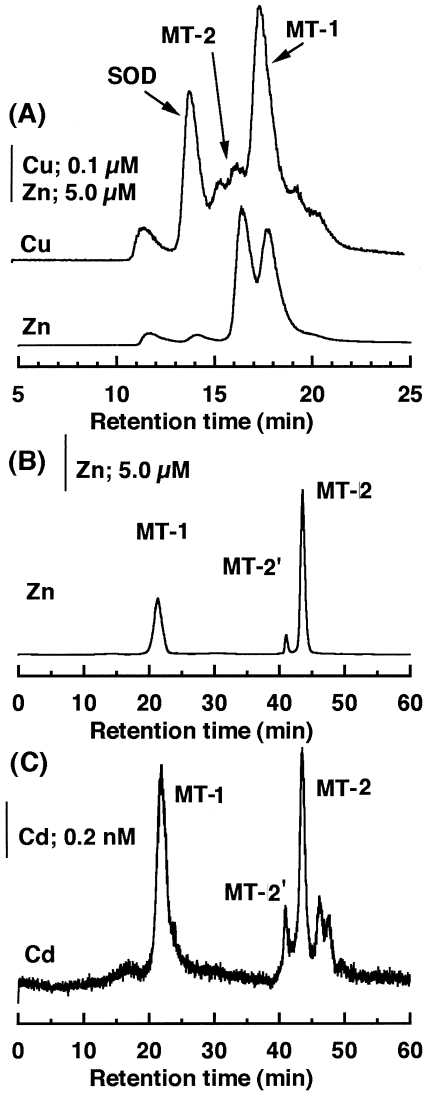

Fig. 4. Elution profiles of Zn and Cd in the liver supernatants and plasma of neonatal rats. Liver supernatants were subjected to size-exclusion HPLC–ICP-MS (A) and column switching HPLC–ICP-MS (B). The plasma of four-day-old neonatal rats was incubated with ^{113}Cd acetate (10 μg Cd/ml plasma) for 10 min at 0°C, and then was subjected to column switching HPLC–ICP-MS (C). Zn and Cd in the eluate were monitored at m/z=66 and 113, respectively. The vertical bars indicate the detection levels for Zn and Cd.

rated into N^{α}-acetylated and non-acetylated forms, as shown in Fig. 4B. Although the non-acetylated form of MT-2 was present in the neonatal liver, the relative ratio of MT-2′ to -2 was low (Fig. 4B).

Plasma MT in the neonatal rats was also analyzed on the composition of N^{α}-acetylated and non-acetylated forms. The concentrations of MT in the plasma are low comparing with that in the liver of neonates and plasma MT was detected only by radioimmunoassay with a high sensitivity [26,27]. Therefore, Zn in plasma MT was replaced with enriched Cd (96.3% enriched ^{113}Cd), and then MT was separated by HPLC, and Cd bound to MT was detected in-line by ICP-MS. Plasma MT was detected for the first time by a method other than radioimmunoassay, and was analyzed also for the composition of N^{α}-acetylated and non-acetylated forms, as shown in Fig. 4C. Although the relative ratios of MT-1 and -2, and MT-2′ and -2 were not exactly the same between MT in the liver and plasma, both isoforms and both sub-forms were present in the plasma, suggesting that there are no distinct differences on the roles of the non-acetylated and N^{α}-acetylated forms in extracellular trafficking. The Cd peaks that eluted slower than MT-2 in the plasma (Fig. 4C) were not characterized further.

4. Discussion

The non-acetylated form of MT, MT-2′, was shown to be present in the livers of rats after induction by various inducers for MT, i.e., not only by metallic (essential and non-essential metals) and non-metallic inducers (*n*-hexane), but also by partial hepatectomy and under physiological conditions (neonatal livers). Non-acetylated forms are known to be present in other mammals such as mice [13,28] and invertebrates [20,21]. These results suggest that non-acetylated MT is present commonly with different relative ratios of non-acetylated to N^{α}-acetylated MT depending on the physiological and experimental conditions.

Non-acetylated MT may have biological roles distinct from the usual N^{α}-acetylated MT or may be present simply as a result of a restricted or limited capacity for co-translational acetylation owing to the interference of the antepenultimate proline residue of MT in the N^{α}-acetylation [29]. N-Terminal processing of nascent proteins is thought to facilitate the turnover of proteins [30] or to regulate the activities of peptide hormones [31]. Likewise, non-acetylated

or deacetylated MT may be susceptible to biological degradation. Although the synthesis of non-acetylated MT reached a plateau earlier than that of N^{α}-acetylated MT under any of the present conditions, non-acetylated MT was retained at a lower level than N^{α}-acetylated MT at the later stage, suggesting that MT-2 is synthesized more than MT-2′ at the later stage of synthesis after induction.

Further, the non-acetylated sub-form, MT-2′, was always present in a higher amount at an early stage than a later stage after induction. In addition, non-acetylated forms of MT were shown to become dominant when MT synthesis was enhanced by the challenged dose after the pretreatment. These results indicate that more non-acetylated forms are present when the capacity for co-translational acetylation is relatively low.

Possible roles of non-acetylated MT in the extracellular trafficking of MT may be ruled out by the observation that non-acetylated and acetylated forms of MT were effluxed in a similar manner without discrimination into the bloodstream under physiological conditions as shown in neonates, suggesting that there are no special forms for extracellular trafficking.

Although the presence of both non-acetylated and N^{α}-acetylated forms of the same protein in mammalian cells would be unusual, MT induced under these conditions may be useful for research on protein acetylation at the N-terminus.

The column switching method was shown to be effective to separate MT not only into isoforms but also further into acetylated and non-acetylated forms. The use of ICP-MS as the detector for an HPLC was demonstrated to be highly sensitive and the use of an enriched stable isotope further enhance the detectability of MT. As the result, MT in the bloodstream was detected for the first time at the levels of not only isoforms but also acetylated and non-acetylated forms of MT.

Summarizing the present biological observations by the column switching HPLC–ICP-MS method, (i) non-acetylated MT-2 is induced by both metallic and non-metallic inducers and is present at an early stage of induction owing to the limited capacity for co-translational acetylation and (ii) the N^{α}-acetylated form is synthesized more than the non-acetylated form when the capacity becomes sufficient at a later stage of induction. Further, (iii) extracellular trafficking of MT was shown to be independent of the N^{α}-acetylation. However, (iv) the coincidental appearance of MT-2′ with the localization of MT in a nucleus at an early stage of induction has not been elucidated from the viewpoint of non-acetylated MT for intracellular trafficking.

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