Separation of metallothionein isoforms extracted from isoform-specific knockdown cells on two-dimensional micro high-performance liquid chromatography hyphenated with inductively coupled plasma-mass spectrometry

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A two-dimensional (2D) micro HPLC-inductively coupled plasma mass spectrometry (ICP-MS) system consisting of a gel filtration column and an anion-exchange column was constructed to separate two metallothionein (MT) isoforms. MT-I and MT-II genes were specifically knocked down with the RNA interference (RNAi) technique, and the effects of the knockdown were evaluated by the reverse transcription-polymerase chain reaction (RT-PCR) and the 2D micro HPLC-ICP-MS system. The minimum cell number required by our 2D micro HPLC-ICP-MS system was calculated to be 2.0 x 103, and the detection limit of MT protein was estimated to be 22.2 mg/L.

Introduction

The speciation analyses of such biological samples as blood plasma, tissue extract and urine using conventional HPLC require relatively large volumes of sample at the mL level. However, the volumes of samples including extracts of gene modified cells, digested spots from two-dimensional (2D) electrophoresis and tissue biopsy extracts are limited in recent metallome research. Thus, an analytical technique for samples having ultra small volumes, i.e., a micro/capillary HPLC-ICPMS, is needed in place of conventional HPLC.[1,2] It was recently reported that the introduction of small interfering RNA (siRNA) induces specific and potent gene silencing effects in many species.[3] This phenomenon, called RNA interference (RNAi), is employed in one of the most powerful and useful molecular biological techniques to show protein function, called gene knockdown. Mammals have at least four metallothionein (MT) isoforms located on an identical chromosome. Two of them are ubiquitously expressed in all tissues and cell types, and are called classical MTs, i.e., MT-I and MT-II.[4] The primary structures of these two MTs are highly homologous with each other compared with those of the other two isoforms, MT-III and MT-IV. Although an immunological assay using antibody raised against each classical MT isoform has been reported,[5] analytical techniques using the antibodies are not common because of difficulty of preparation. Therefore, anion-exchange HPLC, which separates MT-I and MT-II based on their isoelectric points, is the method of choice.[6] Although it is known that these MT isoforms behave differently in chemical reactions, such as oxidative degradation,[7] it is not known whether there are any differences in the biological roles mentioned above between the two isoforms. Two strains of MT knockout mouse are available, but both MT-I and MT-II are simultaneously knocked out in those strains.[8,9] Thus, they are inappropriate for use in the investigation of the difference in the biological function of classical MTs. In this study, we constructed a 2D micro HPLC-ICP-MS system that consists of a gel filtration column and an anion exchange column to separate two MT isoforms. To enhance the sensitivity of ICP-MS for the detection of the metal bound to MT, a low-volume spray chamber with sheath flow was newly designed and an enriched stable isotope was used. Furthermore, MT-I and MT-II genes were specifically knocked down with the siRNA technique. Although another gene-silencing technique with antisense oligonucleotides had been applied to knockdown MT genes,[10] their effects on isoform specificity at the protein level were not determined. Thus, the effects of the siRNAs were evaluated using the 2D micro HPLC-ICP-MS system.

Experimental

Materials and reagents

Cadmium acetate (96.3% enriched in 113Cd) was purchased from Oak Ridge National Laboratory (Oak Ridge, TN, USA). Tris(hydroxymethyl)aminomethane was purchased from Sigma (St. Louis, MO, USA). 1,3-Propanediamine HCl of analytical grade, standard solution of 1000 mg L□1 rhodium (Rh) dissolved as Rh(NO3)3, and other reagents of the highest grade were purchased from Wako Pure Chemical Industries Co., Ltd (Osaka, Japan).

Cell culture and gene knockdown with siRNAs

Mouse Hepa 1–6 cells were obtained from the RIKEN BioResource Center (Tsukuba, Japan). Cells were maintained as previously reported.11 Double-stranded RNAs (dsRNAs) were used as siRNAs (Stealth RNAi, Invitrogen). The targeted sequences of MT-I and MT-II were as follows: siRNA_MT-I, CTGCAAGAACTGCAAGTGCACCTCC and siRNA_MT-II, TGCAAAGAGGCTTCCGACAAGTGCA, respectively. Hepa 1–6 cells were seeded on a six-well plastic plate at 3.0 □ 105 cells/well, and were pre-incubated for 24 h. The pre-incubated cells were either transfected or not transfected with 100 nM siRNA targeting MT-I or MT-II in the medium optimized for siRNA transfection (Opti-MEMsI, Invitrogen), containing 1.0% Lipofectaminet2000 (Invitrogen), and exposed to 1.0 mM 113Cd-enriched Cd(CH3COO)2 1 h after the transfection. The cells were harvested 23 h after the Cd exposure and subjected to RT-PCR and 2D micro HPLC-ICP-MS, as mentioned below.

Isolation of RNA and mRNA determination by reverse transcription polymerase chain reaction

Because of the high homology between MT-I and MT-II, mRNAs, cDNA probes specifically hybridizing with MT-I or MT-II mRNA are not available. Thus, RT-PCR is the only method that can discriminate MT-I mRNA from MT-II mRNA. Hepa 1–6 cells were seeded on a 6 cm plastic dish at 3.0 x 105 cells per well, and treated with isoform-specific siRNA and 113Cd-enriched Cd(CH3COO)2 using the same protocol as that mentioned above. Total RNA was isolated from the cells with Isogens (Wako), following the protocol provided by the manufacturer. RT-PCR was performed in one tube with a Titaniums One-Step RT-PCR kit (BD Biosciences, Palo Alto, CA, USA). Gene-specific primers used for amplification of mouse MT-I, MT-II and b-actin cDNA were as follows: MT-I-F, 5-ATGGACCCCAACTGCTCCTGCTCCACC-3; MT-I-R,

5-GGGTGGAACTGTATAGGAAGACGCTGG-3; MT-II-F, 5-CAAACCGATCTCTCGTCGAT-3; MT-II-R, 5-GAGAACGGGTCAGGGTTGTA-3; b-actin-F, 5-GTGGGCCGCTCTAGGCACCAA-3; b-actin-R, 5-CTCTTTGATGTCACGCACGATTTC-3. RT-PCR was conducted under the following conditions: reverse transcription of cDNA at 50 ˚C for 60 min, denaturation of the reverse transcriptase at 94 1C for 5 min, and 24 (for b-actin) or 27 (for MT-I and MT-II) cycles of PCR, i.e., denaturation of cDNA at 94 1C for 30 s, annealing at 55 ˚C for 30 s and extension at 68 1C for 1 min. A 10 mL portion of the PCR products was loaded on a 5% non-denaturing polyacrylamide gel and electrophoresis was conducted at 100 V for 40 min. The PCR product sizes of MT-I, MT-II and b-actin were 259, 312 and 540 bp, respectively. After the run, the gel was soaked for 30 min in 10 000-fold diluted SYBR Safet (Molecular Probes, Eugene, OR, USA), a fluorescent reagent that stains DNA. The DNA bands were viewed and quantified with an image analyzer (LAS2000; Fuji, Tokyo, Japan).

Sample preparation and 2D micro HPLC-ICP-MS

Hepa 1–6 cells were seeded and treated using the same protocol as that mentioned above. The cells were collected and suspended to 2.0 □ 104 cells m/L with 10 mM Tris-HCl, pH 7.2. The suspended cells were disrupted with an ultrasonic homogenizer (Bioruptors UCD-200, Cosmo Bio Co., Ltd, Tokyo, Japan) on ice at 200 W, 20 kHz for 30 s three times at 30 s intervals. The cytosolic fraction for 2D micro HPLC-ICPMS analysis was obtained by ultracentrifugation of the homogenate at 105000g for 60 min at 4 ˚C. The on-line 2D micro HPLC system (Prominence, Shimadzu, Kyoto, Japan) used in this work is illustrated in Fig. 1a. A micro gel filtration column packed in a polyether ether ketone (PEEK) housing (Shodex Protein KW802.5-M8E, 0.8 mm i.d. x 250 mm) and a micro anion-exchange column packed in a PEEK housing (Shodex DEAE9A-M8B, 0.8 mm i.d. x 50 mm) were kindly provided by Showa Denko (Tokyo, Japan). A 100 nL aliquot of cell cytosol was applied to the first-dimension (1D) gel filtration column, and the column was eluted with 25 mM Tris-HCl, pH 7.2 at a flow rate of 4.0 mL/min. The eluate containing MT isoforms from the 1D column was pooled in a 10 mL loop via a six-port switching valve, and introduced into the second dimension (2D) anion-exchange column. The column was eluted with a concentration gradient of 2 mM (buffer A) and 100 mM (buffer B) 1,3-propanediamine HCl, pH 9.0 at a flow rate of 4.0 mL min□1 under the program depicted in Fig. 2b. The eluate from the 2D column was introduced into an ICP-MS (Agilent7500cs; Yokogawa Analytical Systems, Hachiouji, Japan) through the interface consisting of a total consumption nebulizer kindly provided by

Professor Andreas Prange (GKSS-Research Centre, Geesthacht, Germany) and a low-volume (4.5 mL) spray chamber with an in-house designed sheath flow (Fig. 1b). The metals in the eluate were monitored at m/z 65 (Cu), 66 (Zn), 111(Cd) and 113 (Cd). The operating conditions of the ICP-MS are the same as previously reported.2

Results and discussion

Hyphenation of 2D micro HPLC with ICP-MS

At the initial stages of hyphenation between the 1D micro HPLC and the ICP-MS, a conventional spray chamber designed for hyphenation of capillary electrophoresis to ICP-MS was used.12 It has a sheathless, single path and a 90° bent shape. However, as the bent shape was ineffective for the connection of a micro/capillary HPLC to an ICP-MS, a spray chamber was newly designed to improve signal sensitivity and stability of ICP-MS detection, as depicted in Fig. 1b. Whereas the maximum sensitivity of 100 mg/L Rh used as tuning solution was 2.26 x 105 cps when the conventional chamber was employed under our operating conditions, the maximum sensitivity was increased to 3.66 x 105 cps when the sheath flow spray chamber was used. The signal stability, which was expressed as the relative standard deviation (RSD) for 200 data points, was also improved from 2.77% to 1.59% by using the sheath flow spray chamber. Recently, it was reported that MT isoforms were one dimensionally separated on a capillary reversed-phase (C8) column.13 In that study, the MT isoforms applied to the column were purified proteins. Thus, as shown in the present study, 2D separation may be required to separate MT isoforms in the presence of matrices such as cell supernatant.

Separation of MT Isoforms on 2D micro HPLC-ICP-MS

In the present study, 113Cd-enriched Cd was used to enhance the detection of MT as a Cd peak by ICP-MS. Cd in biological samples is generally detected at m/z 111 or 113 to avoid isobaric interference. The isotope abundances of 111Cd and 113Cd are 12.8 and 12.2%, respectively. Since Cd enriched in 96.3% 113Cd was used in this study, the detection level of enriched Cd at m/z 113 was increased by 7.9 times compared with that of the non-enriched one. Indeed, both MT-I and MT-II, which bind to 113Cd, were clearly detected with the 2D micro HPLC-ICP-MS system (Fig. 2). It was reported that the detection limit of MT protein expressed in the 114Cd concentration was estimated as 11–23 mg/L according to 3s criterion by a capillary electrophoresis coupled with an ICP sector field MS (a high resolution type of ICP-MS).14 In the present study using a micro HPLC hyphenated with ICP quadrupole MS, the detection limit expressed in 113Cd was calculated as 22.2 mg L□1. When the cells are exposed to Cd, MT induced in the cells binds not only Cd but also Zn.15 However, Zn could not be detected on the second dimensional micro HPLC column since the detection limit of Zn is higher than that of Cd, furthermore the stable isotope enrichment was not applied to the Zn detection in

this study. Cu could not be also detected on the 2D column because the amount of Cu bound to MT was not increased by the Cd treatment. MT-I and MT-II were eluted at the retention times of 26.0 and 28.8 min, respectively, on the 2D anion-exchange column (Fig. 2). In our previous study, Tris buffer was used as an eluent to separate MT isoforms on an anion-exchange column. 16 However, volatile salts may be more suitable than nonvolatile salts, such as Tris, to prevent salt deposition on the spray chamber and salt clogging in the capillary tubing. Thus, 1,3-propanediamine HCl, a volatile salt, was used in place of Tris. This salt gave good separation under alkaline conditions because the MT isoforms were retained well on the micro anion-exchange column at the initial concentration.

RT-PCR evaluation of MT-Isoform-Specific knockdown

The fluorescence intensities of the reverse transcription polymerase chain reaction (RT-PCR) products of MT-I and MT-II mRNAs were increased by 3.57 ± 0.30 and 18.1 ± 2.94 times, respectively, by the Cd treatment (Fig. S1, ESIw). Treatment with MT-I siRNA inhibited the induction of MT-I (1.03 ± 0.46 compared with control). On the other hand, the expression of MT-II was not affected by the MT-I siRNA treatment, suggesting that MT-I siRNA specifically inhibited the induction of MT-I. The treatment with MT-II siRNA gave similar results to that of MT-I siRNA treatment, i.e., the Cd-induced MT-II expression was decreased from 18.1 ± 2.95 to 2.22 ± 1.27 times of the control, whereas that of MT-I was not apparently reduced. These observations suggest that siRNAs specifically designed for MT-I and MT-II are able to decrease the expression of their targets at the mRNA level in spite of high identity (75%) and homology (82%) between MT-I and MT-II in their amino acid sequences.4

2D micro HPLC-ICP-MS evaluation of MT-isoform-specific knockdown

As shown in Fig. 2, the expression of MT-I and MT-II proteins, which was expressed as 113Cd peak intensity, was 3800 and 4650 cps, respectively, on treatment with Cd. The expression of MT-I was decreased (to 2200 cps), whereas that of MT-II was not (4900 cps), on treatment with MT-I siRNA (Fig. 3a). On the other hand, the expression of MT-I and MT-II was 3430 and 860 cps, respectively, on treatment with MT-II siRNA (Fig. 3b). One of the advantages of 2D micro HPLC-ICP-MS over RT-PCR is that the protein expression of both isoforms can be directly compared in terms of 113Cd peak intensity. MT-II siRNA was found to more effectively and specifically knockdown its target gene than MT-I siRNA. Although MT-I mRNA expression was completely inhibited by MT-I siRNA treatment (Fig. S1w), MT-I protein actually existed, albeit in a small amount

(Fig. 3a). These results suggest that the results of RT-PCR do not always reflect MT protein expression. Therefore, 2D micro HPLC-ICP-MS may have an advantage over RT-PCR in evaluating the effects of siRNA and in estimating the protein expression of MT isoforms in gene-modified cells.

Conclusions

MT-isoform-specific knockdown was observed by RT-PCR and 2D micro HPLC-ICP-MS. 2D micro HPLC-ICP-MS, which consists of a gel filtration column and an anion-exchange column, was an effective tool to separate MT isoforms prepared from cultured cells. Indeed, a 100 nL portion of cell supernatant was sufficient for injection into the column, suggesting that the minimum cell numbers required for our 2D micro HPLC-ICP-MS system was 2.0 x 103. To our knowledge, this is the first report of the combination of nano-speciation with the RNAi technique. The techniques shown in this study are expected to contribute to clarifying the physiological and/or biological roles of MT isoforms. Moreover, since the RNAi technique has wide applications, the combination of nano-speciation with the RNAi technique may open new doors in the study of metallomics.

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Figures

Fig. 1 (a) Schematic diagram of the 2D micro HPLC-ICP-MS system. A, B and C: low-flow (micro) pumps, D: injector, E: mixer, F: system controller, G: valve switching unit, H: six-port switching valve, I: 1D micro column (gel filtration), J: 2D micro column (anion exchange), K: photodiode array spectrophotometer, L: total consumption nebulizer, M: low-volume sheath flow spray chamber, N: plasma torch. (b) Detailed view of the spray chamber.

Fig. 2 Elution profiles of 113Cd from the cytosolic fraction of 113Cdenriched Cd(CH3COO)2 treated Hepa 1-6 cells on the 1D gel filtration column (a) and the 2D anion-exchange column (b). The 113Cd distributions on the 1D and 2D columns were monitored by ICP-MS. The gradient program is depicted by a broken line.

Fig. 3 Effect of RNAi-mediated isoform-specific silencing on the protein expression of MT-I and MT-II.

Fig.1

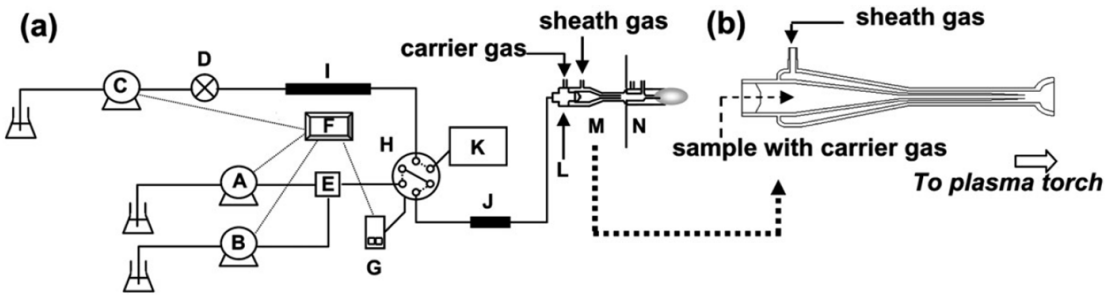

(a)
sheath gas
carrier gas
D
I
C
F
H
K
A
E
J
B
G
L
M
N
(b)
sheath gas
sample with carrier gas
To plasma torch

Fig.2

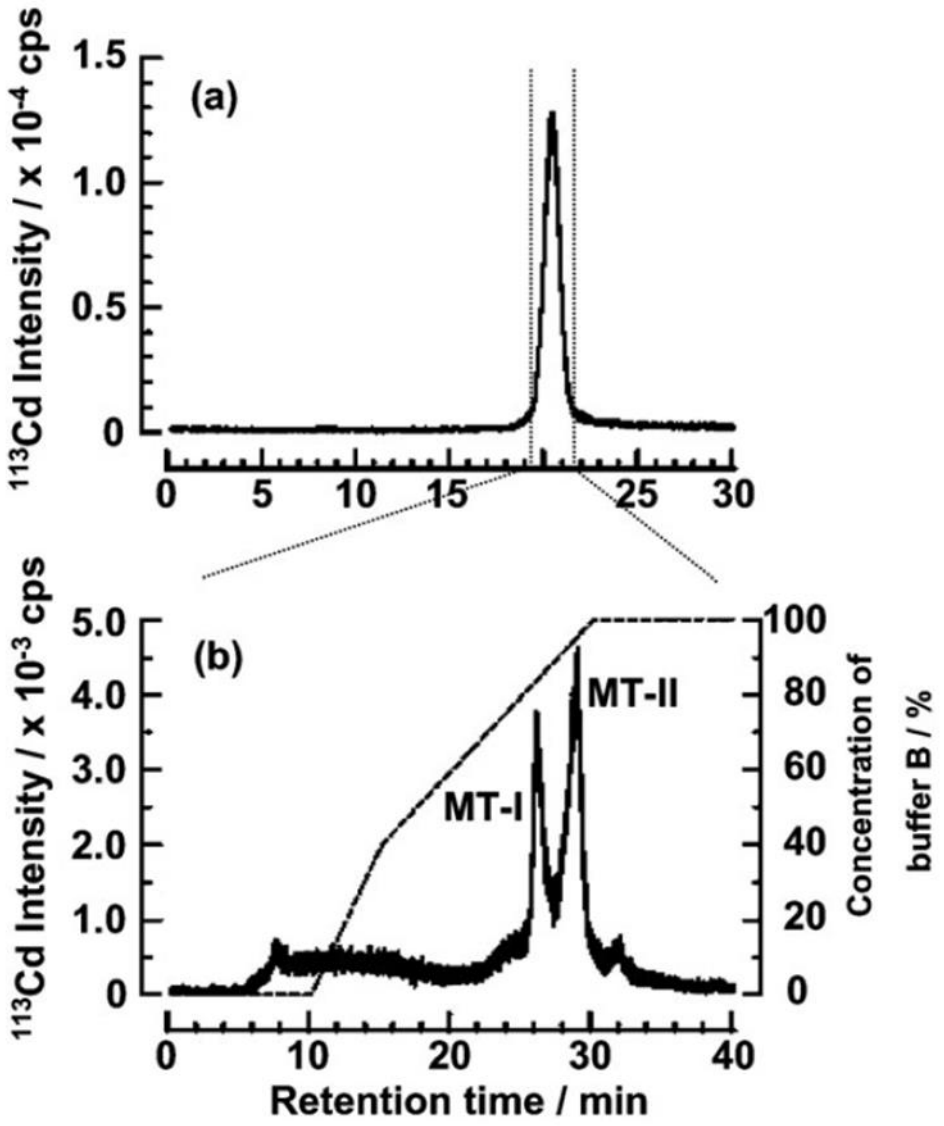
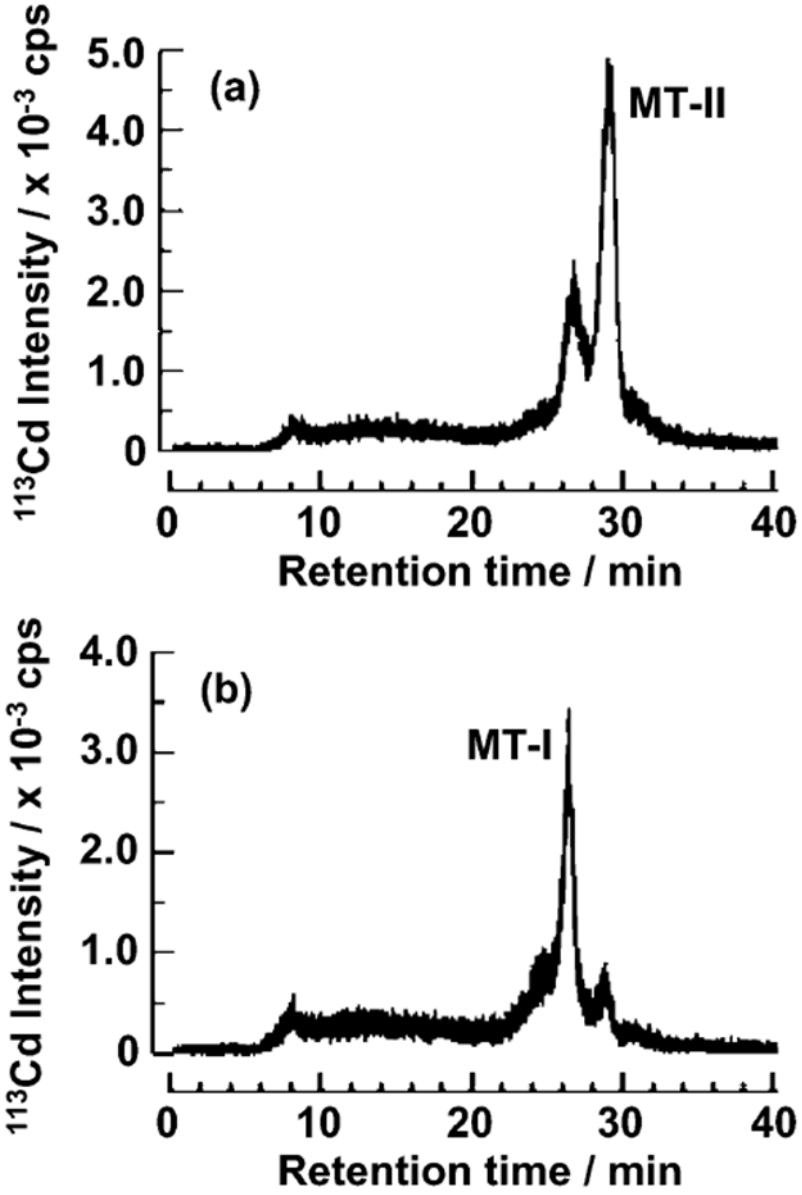

(a)
^{113}Cd Intensity / x 10^{-4} cps
1.5
1.0
0.5
0
0
5
10
15
25
30
(b)
^{113}Cd Intensity / x 10^{-3} cps
5.0
4.0
3.0
2.0
1.0
0
MT-I
MT-II
100
80
60
40
20
0
Concentration of buffer B / %
0
10
20
30
40
Retention time / min

Fig.3


(a)
MT-II
^{113}Cd Intensity / x 10^{-3} cps
5.0
4.0
3.0
2.0
1.0
0
0
10
20
30
40
Retention time / min
(b)
MT-I
^{113}Cd Intensity / x 10^{-3} cps
4.0
3.0
2.0
1.0
0
0
10
20
30
40
Retention time / min