Region-Dependent Differences and Alterations of Protective Thiol Compound Levels in Cultured Astrocytes and Brain Tissues

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We examined region-dependent differences and alterations in the levels of protective thiol compounds, glutathione (GSH) and metallothionein (MT)-I and -II, in cultured rat astrocytes under several culture conditions and in brain tissues of rats at postnatal and weaning periods. Regardless of culture conditions, both protein concentrations and mRNA expressions of MT-I and -II were much higher in the cerebral hemisphere than in cerebellar astrocytes, whereas no difference was observed in GSH concentration. In both astrocytes, the GSH concentrations did not change within 12 h but significantly increased 24 h after being maintained in a serum-free defined medium. At 24 h, protein concentrations and mRNA expressions of MT-I and -II also increased in the respective astrocytes, and were further enhanced when maintained in the presence of 50 μM Zn2+. In the brain tissues, the MT-I/-II protein concentrations were significantly higher in the cerebral cortex (a part of the cerebral hemisphere) than in the cerebellum, whereas the GSH concentration was similar at both postnatal day (P)1 and P35. In addition, the concentrations in the respective regions were significantly higher at P35 than at P1. These results suggest that region-dependent differences in the cellular levels of GSH and MTs in cultured astrocytes might reflect the in vivo differences, and that the levels of the respective thiol compounds in cultured astrocytes increase after serum elimination along with the region-dependent differences.

Key words glutathione; metallothionein; astrocyte; brain; regional difference

Astrocytes play many important roles such as maintaining neuronal survival and functions.1,2) Therefore, it has been suggested that an astrocyte dysfunction may indirectly reinforce the neurotoxicity induced by some chemicals including mercury compounds, methylmercury (MeHg) and Hg2+2, through disturbing ion homeostasis and inhibiting glutamate uptake into the astrocytes.3—5) Since MeHg neurotoxicity is observed at relatively local regions,6) we hypothesized that this might reflect brain region-specific susceptibility to mercury compounds in astrocytes. However, regardless of culture conditions, no differences between astrocytes from the cerebral hemisphere and cerebellum were observed not only in susceptibility but also in mercury accumulation for the first few hours after exposure to MeHg or Hg2+.7) In addition, we found that both susceptibility and mercury accumulation decreased with elapsed time after serum elimination until exposure.8) Although the differences in susceptibility to these compounds could, therefore, be explained by those in mercury accumulation, it might be possible that some factors related with cellular defense against their toxicity build up with time after serum elimination. It has been demonstrated that some thiol compounds such as glutathione (GSH), an ubiquitous thiol-containing tripeptide, and metallothioneins (MTs), cysteine-rich low molecular weight proteins, are pivotal factors in protecting against the toxicity of mercury compounds in cultured astrocytes. For example, GSH can protect against the acute cytotoxicity of both MeHg and Hg2+,8) and MTs induced by Zn2+ can also do so against that of MeHg.9) In addition, since MT-I and -II in cultured astrocytes are induced by metals including Hg2+ at higher doses than in cultured hepatocytes,10,11) these MT isoforms would be able to protect against the toxicity of Hg2+ as observed in cultured hepatocytes.12)

In the present study, we examined alterations after serum elimination and region-dependent differences in the cellular levels of the protective thiol compounds, GSH and MTs, in cultured astrocytes prepared from the cerebral hemisphere and cerebellum. In addition, to clarify the significance of these alterations in cultured astrocytes, we also investigated the changes and brain region-dependent differences in these levels in vivo.

MATERIALS AND METHODS

Animals Male and female Wistar rats obtained from CLEA Japan Co. (Osaka, Japan) were maintained at 23.5 ± 1.5 °C and 55 ± 10% relative humidity under a 12-h light cycle. The animals were given standard laboratory chow and tap water ad libitum. Pregnant rats were prepared as previously described7,13) and housed individually until birth. The animals received humane care throughout the experiment according to the Guidelines of the National Institute for Environmental Studies (NIES) and those of the National Institute for Minamata Disease (NIMD).

Cell Cultures Astrocyte cultures were prepared from the cerebral hemisphere and cerebellum of newborn rats (within 24 h after birth) as previously described.7) In brief, the two brain regions, with the meninges carefully dissected off, were separately washed in Ca2+-, Mg2+-free Hanks’ balanced salt solution and treated with 0.1% trypsin at 37 °C for 10 min. Cells obtained were suspended in Basal Medium Eagle’s with Earl’s salts supplemented with 15% fetal calf serum (FCS; © 2006 Pharmaceutical Society of Japan

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Twenty-four hours after the administration, the rat was perfused with ice-cold saline from the heart under ether anesthesia, and the liver was then excised. Total RNA was prepared from the rat liver using an RNaseasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Poly A⁺ RNA was reverse transcribed with oligo dT primer (Invitrogen Co.) and reverse transcriptase (Invitrogen Co.). The reverse transcribed product was used as a PCR template with the following various sets of PCR primers: MT-I: sense, CCTCTTGTGCTTACACGTTTGCTC, anti-sense, CATGCTC CGGTAGAAACCCGGGTATTAG, and MT-II: sense, CTT CAGAACTCCTACGGAATCTC, anti-sense, GAAAAA AAGTGTGGAACCCGGTCAGG. The PCR products were gel-purified and confirmed by direct sequencing.

Total RNA in cultured astrocytes was prepared as described above 24 h after being maintained in SFDM with or without 50 μM Zn²⁺, or without the medium change. RNA was separated by electrophoresis in a 1% gel containing 6.7% formaldehyde, and was transferred to a nylon membrane (Hybond-N+; Amersham Biosciences Co., Piscataway, NJ, U.S.A.) with 10×SSPE (0.15 M NaCl, 0.01 M Na₂HPO₄, and 1 mM EDTA, pH 7.4) as the transferring solution as previously described. The membrane was pre-hybridized at 68 °C for 2—3 h in Express hybridization solution (BD Bioscience Clontech, Palo Alto, CA, U.S.A.) containing 200 mg/ml denatured salmon sperm DNA. cDNA fragments of rat MT-I and -II were labeled with [α-³²P]dCTP (Amersham Biosciences Co.) and the random primer DNA labeling kit version 2 (Takara Bio Co., Shiga, Japan). The radioactive probe was added to the pre-hybridization solution and incubated for 12—16 h at 68 °C. The membrane was washed with 2×SSPE/0.1% sodium dodecyl sulfate (SDS) and 0.1×SSPE/0.1% SDS at 50 °C. The hybridized cDNA was located, and radioactive cDNA was quantified by exposure to a Bio-Image plate and analyzed with a FUJIX Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film, Tokyo, Japan).

Statistical Analysis Significant differences between individual means were determined by one-way analysis of variance (ANOVA) followed by Duncan’s new multiple range test or by Student’s t-test. Differences were considered significant at p<0.05.

RESULTS

Figures 1 and 2 show cellular levels of protective thiol compounds, GSH and MTs, respectively, in cultured astrocytes from the cerebral hemisphere and cerebellum under several culture conditions. In the presence of serum, no difference was observed in total GSH concentrations between astrocytes from both regions (Fig. 1). In both astrocytes, the concentrations did not change within 12 h, but significantly increased 24 h after serum elimination (Fig. 1). Even at that time, however, no region-dependent difference was observed (Fig. 1). In contrast to the GSH levels, MT-I/II protein concentrations were significantly higher in the cerebral hemisphere than in cerebellar astrocytes under all conditions examined (Fig. 2A). These protein concentrations more than doubled 24 h after serum elimination (Fig. 2A, nos. 3 and 4), compared with those in the respective astrocytes maintained in the 15% FCS-containing medium (Fig. 2A, nos. 1 and 2).
In the presence of serum, expression levels of MT-I and -II mRNA, which normalized the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, in cerebellar astrocytes were 17% and 13% of those in cerebral hemisphere astrocytes, respectively (Fig. 2B, lanes 1 and 4). Both MT-I and -II mRNA expressions increased approximately two-fold 24 h after serum elimination in cerebral hemisphere astrocytes (Fig. 2B, lanes 1 and 2), while increasing approximately 4 and 10 times in cerebellar astrocytes (Fig. 2B, lanes 4 and 5), respectively. In the astrocytes from the respective regions, protein concentrations (Fig. 2A, nos. 3—6) and mRNA expressions (Fig. 2B, lanes 2, 3, 5 and 6) of MT-I and -II were much higher with exposure to 50 μM Zn²⁺ than in those without it, suggesting that region-dependent differences in the MT levels might be maintained regardless of culture conditions, even after induction by Zn²⁺.

To clarify the significance in these alterations observed in cultured astrocytes, total GSH and MT-I/-II proteins were examined in the whole brain, cerebral cortex (a part of the cerebral hemisphere) and cerebellum of P1 and P35 rats. The concentrations of total GSH (Fig. 3A) and MT-I/-II proteins (Fig. 3B) in all of the respective brain regions examined were significantly higher at P35 (nos. 5—7) than at P1 (nos. 1—4). At both times, the total GSH concentration in the cerebral cortex (nos. 2, 3 and 6) was similar to that in the cerebellum (nos. 4, 7) as well as that in the whole brain (nos. 1 and 5) (Fig. 3A). In contrast, the MT-I/-II protein concentrations in the cerebral cortex (nos. 2, 3 and 6) were significantly higher than in the cerebellum (nos. 4 and 7) in both P1 and P35 rats, although they were similar to those in the whole brain (nos. 1 and 5) (Fig. 3B).

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Fig. 1. Time-Dependent Changes in Total GSH Concentrations in Cultured Astrocytes

Astrocytes prepared from the cerebral hemisphere (C) or cerebellum (●) were maintained in SFDM for 0—24 h. Values represent the mean ± S.D. obtained from 3 determinations. Values with different letters (a and b) indicate significant differences (p<0.05).

Fig. 2. Protein Concentrations and mRNA Expressions of MT-I and -II in Cultured Astrocytes

Astrocytes prepared from the cerebral hemisphere (CH) or cerebellum (CB) were maintained in SFDM with or without of 50 μM Zn²⁺ for 0 or 24 h. (A) MT-I/-II protein concentrations. Values represent the mean ± S.D. obtained from 3 determinations. Values with different letters (a—e) indicate significant differences (p<0.05). (B) mRNA expressions of MT-I and -II. MT-I and -II mRNA expressions were normalized to the expression of GAPDH mRNA, and expressed as percentages in relation to the ratio (MT-I/GAPDH or MT-II/GAPDH) in the cerebral hemisphere astrocytes in the presence of serum (lane 1).

Fig. 3. Concentrations of Total GSH and MT-I/-II Proteins in Brain Tissues of P1 and P35 Rats

Abbreviations of brain regions: WBr, whole brain; CC, cerebral cortex; CB, cerebellum. Values represent the mean ± S.D. obtained from 4 to 5 rats. (*) indicates significant differences between different brain regions at the specified ages (p<0.01). (#) indicates significant differences from P1 rats in the specified brain regions (p<0.01).
DISCUSSION

In the present study, there were region-dependent differences in concentrations of MT-I/-II proteins both in vivo and in cultured astrocytes. The MT-I/-II protein concentrations in vivo were higher in the cerebral cortex (a part of the cerebral hemisphere) than in the cerebellum at both P1 and P35 (Fig. 3B). In addition, the concentrations of these MT isoforms were higher in the cerebral hemisphere (including the cerebral cortex) than in cerebellar astrocytes (Fig. 2A). In contrast, no region-dependent difference in the GSH concentrations was observed either in vivo (Fig. 3A) or in cultured astrocytes (Fig. 1). It has been demonstrated that MT-I and -II are mainly expressed in glial cells such as astrocytes rather than in neurons. Moreover, GSH levels appear to be higher in astrocytes than in neurons both in vivo and in culture. Moreover, since GSH released by astrocytes is provided to neurons and used as a source of neuronal GSH, an alteration in the GSH metabolism in astrocytes would be reflected by the neuronal GSH concentration. Accordingly, region-dependent differences in these concentrations in cultured astrocytes may in part reflect those in the in vivo levels of astrocytes. We also found in this study that the concentrations of GSH and MT-I/-II proteins increased in the respective astrocytes after serum elimination (Figs. 1, 2A), and those in the respective brain regions in vivo were higher at the weaning than at the postnatal period (Figs. 3A, B). Although the reasons for the increases in cultured astrocytes after serum elimination remain uncertain, it is possible that cultured astrocytes maintained in SFDM for 24 h would undergo maturation, as demonstrated in some previous reports, in spite of some differences in culture conditions. Therefore, one possibility is that the increases in cultured astrocytes might result from their maturation. In addition, serum-deprivation stress might partly contribute to the increases. To clarify why these levels increase after serum elimination, further study in detail would be necessary.

We recently found that susceptibility was higher in cultured astrocytes exposed to MeHg or Hg^{2+} immediately after serum elimination rather than in those exposed 24 h after. Since concentrations of GSH and MT-I/-II proteins, which could preserve the toxicity of mercury compounds in cultured astrocytes, increased in the respective astrocytes at least 24 h after serum elimination (Figs. 1, 2A), such alterations might in part explain the lower susceptibility to mercury compounds in astrocytes maintained in SFDM for 24 h. We recently reported, on the other hand, that accumulation of Hg^{2+} for 24 h was higher in the cerebral hemisphere than in cerebellar astrocytes when it was exposed both immediately and 24 h after serum elimination, although that for 3 h was similar between astrocytes from both regions. In contrast, no region-dependent difference in MeHg accumulation for 3 or 24 h was observed, except for that for 24 h exposed 24 h after serum elimination. In the present study, the MT-I/-II protein concentrations were higher in the cerebral hemisphere than in cerebellar astrocytes regardless of culture conditions, even after being maintained in the presence of Zn^{2+} (Fig. 2A), although the region-dependent differences have not been determined after exposure to mercury compounds. It is well known that MTs bind to Hg^{2+} but hardly to MeHg. In addition, it is possible that Hg^{2+} binds mainly to high molecular weight proteins at 3 h, and the binding shifts to MTs at 24 h. Accordingly, the region-dependent differences in accumulation of Hg^{2+} but not MeHg for 24 h might reflect those in MT-I/-II protein concentrations. Thus, the alterations and differences in the cellular levels of these thiol compounds might in part explain those in the susceptibility to the toxic metals such as mercury compounds and in their retention in cultured astrocytes.

The present results suggest that region-dependent differences in the cellular levels of GSH and MTs in cultured astrocytes might reflect the in vivo differences, and be maintained regardless of culture conditions. It is also suggested that, in cultured astrocytes, the levels of these thiol compounds increase after serum elimination, and these alterations might modulate the metal toxicity.

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