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【Original Article】

Region-Dependent Differences in Morphological Changes Induced by Mercury Compounds and Lysophosphatidic Acid in Cultured Rat Astrocytes

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Region-dependent differences in morphological changes induced by an increment of cAMP, and an exposure with either mercury compounds or lysophosphatidic acid (LPA), a Rho activator, were examined in cultured astrocytes from the cerebral hemisphere and cerebellum of newborn rats. When the cells were maintained in a serum-containing medium, the morphology was a flat polygonal shape, and prominent actin stress fibers were observed in astrocytes from both regions. After changing the medium to a serum-free one containing 0.5 mM dibutyryl cyclic AMP (dbcAMP), the morphology changed to a process-bearing stellate shape together with a loss of stress fibers in both astrocytes. When methylmercury (MeHg) was exposed to the cells at 3 μM for 3 h immediately after the medium change, most cerebral hemisphere astrocytes showed a polygonal shape, together with the formation of stress fibers, whereas only a few cerebellar astrocytes did so. In contrast, inorganic mercury did not influence the morphology in either astrocyte at that concentration. Moreover, similar to MeHg, LPA also region-dependently induced spreading of astrocytes, suggesting that Rho proteins might be key molecules in the MeHg-induced alteration in astrocyte morphology. The present results suggest that morphologies in the presence of serum and dbcAMP-stimulated stellation are similar in the cerebral hemisphere and in cerebellar astrocytes in cultures, and that there are marked region-dependent differences in responses to LPA and mercury compounds, especially MeHg-induced shape changes (spreading) and actin reorganization.

1. INTRODUCTION

Astrocytes play many important roles, such as maintaining neuronal survival and functions, regulating neurotransmitters, and contributing axon guidance in the central nervous system (CNS).[1-3] It is well-known that astrocyte morphology changes during development and after injury,[4,5] and similar changes can be observed in cultured astrocytes as described below. Therefore, clarifying the mechanism and properties of the regulation and maintenance of cultured astrocyte morphology would help in understanding *in vivo* alterations. It has been well-established that the flat polygonal shape of cultured astrocytes is changed to a process-bearing stellate shape by the elevation of cellular cAMP levels, accompanied with an accelerated degree of astrocyte differentiation. For example, the expression levels of glial fibrillary acidic protein (GFAP),[6] a cytoskeletal protein of astrocytes, and the activity of glutamine synthetase,[7] both of which correlate with the degree of astrocyte differentiation,[8] are increased by stimulation with dibutyryl cyclic AMP (dbcAMP), which is a non-metabolized analogue of cAMP. In contrast, the stellate shape in cultured astrocytes induced by cAMP is changed to a polygonal one by lysophosphatidic acid (LPA),[9-11] thrombin[7,9] and endothelins.[10,12,13] It has been demonstrated that these changes involve the small GTP-binding protein RhoA[9-11] and cytoskeletal elements, such as rapid changes in actin organization.[9-11,13,14] Thus, the significance and mechanism of

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morphological changes in cultured astrocytes have been well investigated. However, not many reports examined the shape changes in cultured astrocytes induced by toxic chemicals, such as heavy metals, although some demonstrate that tributyltin[15] and manganese[16,17] stimulate stellation, and that cadmium and cobalt inhibit cAMP-induced stellation.[18] Although it has been revealed that there are brain region-dependent differences in astrocyte properties, including glutamate uptake activity and the regulation of its transporters responding to cytokines and hormones,[19-21] only a few reports have demonstrated region-dependent differences in shape changes.[20,22,23]

It is well-known that methylmercury (MeHg) and mercury vapor pass through the blood-brain barrier and damage the CNS.[24,25] It has been suggested that, in the case of an intoxication by mercury compounds, an astrocyte dysfunction may indirectly reinforce the neurotoxicity, since mercury compounds inhibit the uptake of glutamate (a major excitatory transmitter), which can induce neuronal cell damage,[26-28] into the astrocytes.[29,30] However, there is scant information about the shape changes induced by MeHg and mercuric ion (Hg^{2+}) in cultured astrocytes. We earlier revealed that the morphology of cerebellar astrocytes transiently changes to a stellate shape after serum elimination, and that although 3 μM of MeHg could alter it to a polygonal shape, Hg^{2+} could not do so, at least at the same concentration.[31] However, the stellation after serum elimination was transient in cerebellar astrocytes, and also brain region-dependent, because no stellation was observed in the cerebral hemisphere astrocytes.[31] Therefore, the effects of both mercury compounds on astrocyte morphology, such as spreading, must be studied under conditions in which the morphology of both astrocytes continue to be stellate, to clarify whether the responses are mercury compound- and region-dependent in cultured astrocytes.

In the present study, astrocytes prepared from the cerebral hemisphere and cerebellum were maintained in a 15% serum-containing medium or in a serum-free medium containing dbcAMP with or without an exposure to MeHg or Hg^{2+}, and the alterations in cell morphology and actin organization were investigated. In addition, to determine the reasons for the region-dependent difference in the changes in astrocyte morphology induced by mercury compounds, the spreading effect of LPA (a Rho activator) on stellate astrocytes stimulated by dbcAMP was also examined in astrocytes from both regions.

2. MATERIALS AND METHODS

2.1 Animals

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, and given standard laboratory chow and tap water *ad libitum*. Pregnant rats were prepared as previously described,[31,32] and housed individually until birth. The animals received humane care throughout the experiments according to the Guidelines of the National Institute for Minamata Disease (NIMD) and those of the Chiba Institute of Science.

2.2 Cell Culture

Astrocyte cultures were prepared from the cerebral hemisphere and cerebellum of newborn rats (within 24 h after birth) as previously described,[31] with minor modifications. In brief, cells were obtained from the respective regions by treatment with 0.1% trypsin at 37˚C for 10 min, and grown in Basal Medium Eagle's with Earl's salts supplemented with 15% fetal calf serum (FCS; Invitrogen Co., Carlsbad, CA, U.S.A.), 0.1% L-glutamine, 0.6% D-glucose, antibiotics (Penicillin-Streptomycin; Invitrogen Co.), and an antimycotic (Fungizon; Invitrogen Co.) in culture flasks (25 cm^2; BD Bioscience; Billerica, MA, U.S.A.) at 37˚C in 6% CO_2 in a humidified atmosphere. After cells reached confluence, culture flasks were vigorously shaken by hand to remove small cells on the protoplasmic cell layer. Monolayer cells, almost all of which were GFAP-positive astrocytes (see Fig. 1), were trypsinized and re-suspended with the fresh 15% FCS-containing medium. Cells were then plated on poly-L-lysine (PLL; Sigma, St. Louis, MO, U.S.A.)-coated glass coverslips (13 mm in diameter) or culture plates (BD Bioscience).

2.3 Treatment of Mercury Compounds and LPA

In some astrocyte cultures, after reaching 85-95% confluence, the 15% FCS-containing medium was changed to a serum-free defined medium (SFDM), the composition of which was previously described,[31,33] containing 0.5 mM dbcAMP (Sigma). Immediately or 3 h after the medium change, some cultures were exposed to methylmercuric chloride (Tokyo Chemical Industry Co., Tokyo, Japan) or mercuric chloride (Wako Pure Chemical Industry, Osaka, Japan) at a final concentration of 1 or 3 μM, according to the method previously described.[31] Other cultures were added to LPA, a Rho acti-

vator, at concentrations of 1 or 10 μM. Control cultures were added to solvent alone.

2.4 Immunofluorescence and Phalloidin Staining

Filamentous (F)-actin and GFAP expression patterns were examined using tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (Sigma) and antibody against GFAP (DakoCytomation; Carpinteria, CA, U.S.A.), respectively. Cells on glass coverslips were washed with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline [PBS(-)], and then fixed with 4% formaldehyde followed by permeation using 0.2% Triton X-100 in PBS (-). They were washed with PBS (-) and incubated with TRITC-conjugated phalloidin (100 ng/ml) for 30 min at room temperature. After washing with PBS (-), the cells were incubated with anti-GFAP (1:500) for 90 min at room temperature. They were then washed with PBS (-) containing 0.1% bovine serum albumin (BSA; Sigma), and incubated with anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugate (1:200) for 60 min at room temperature. After the cells were washed with PBS (-) containing 0.1% BSA, nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI; Sigma). TRITC-conjugated phalloidin were diluted in PBS (-), and the others in PBS (-) containing 10% horse serum (Sigma) and 1% BSA. The cells were washed and then mounted using PermaFluor Aqueous Mountant (Immunon Shandon; Pittsburgh, PA, U.S.A.). Stained samples were observed and photographed under a Nikon fluorescence microscope Eclipse E800.

2.5 Mercury Analysis

Cells on 6-well plates were rinsed 3 times with PBS (-) and then lysed in 0.1% sodium dodecyl sulfate (SDS) in PBS (-). The content of total mercury in each cell lysate was measured by an oxygen combustion-gold amalgamation method[34] using a Rigaku Mercury Analyzer MA-2 (Nippon Instruments Co., Tokyo, Japan). Whole protein content was measured using a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

2.6 Cell Viability Analysis

Cells on 24-well plates were fixed with glutaraldehyde (1% as a final concentration), and viable cell numbers were assessed as previously described.[32] In brief, fixed cells were stained with 0.1% crystal violet in 0.2 M 2-[N-morpholino]ethanesulfonic acid (pH 6.8) for 20 min. The dye incorporated into the cells was eluted into 10% acetic acid, and the absorbance at 595 nm was determined spectrophotometrically.

2.7 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 $p < 0.05$.

3. RESULTS

3.1 Astrocyte Morphology and Stress Fiber Formation After Exposures of Mercury Compounds

The morphology (Figs. 1, 2), GFAP expression (Fig. 1), actin stress fiber formation (Fig. 1), and nuclei (Fig. 1) in cultured astrocytes from the cerebral hemisphere and cerebellum were examined under several conditions. Astrocytes from both regions showed a flat polygonal shape with prominent stress fibers when they were maintained in the 15% FCS-containing medium (Fig. 1A). When the medium was changed to a SFDM containing 0.5 mM dbcAMP, the morphology of both astrocytes changed to a process-bearing stellate shape within 1 h (Fig. 2A). In addition to the shape change, stress fibers disappeared and nuclei sizes became smaller by at least 3 h after the medium change (Fig. 1B). When MeHg (3 μM) was exposed to the cells for 1-2 h immediately after the medium change, the morphology of astrocytes from both regions showed a stellate shape as observed in the absence of MeHg (Fig. 2A). However, 3 h after MeHg exposure, the morphology of most cerebral hemisphere astrocytes showed a polygonal shape, whereas only a few cerebellar astrocytes did so (Fig. 1C). It should be noted that almost all cells that showed a polygonal shape caused by MeHg exposure were accompanied by the formation of stress fibers and larger nuclei, regardless of the brain regions (Fig. 1C). Therefore, astrocyte shapes closely correlated not only with actin organization but also nuclei size, suggesting that both might be used as markers of changes in astrocyte morphology. Neither exposure to MeHg at 1 μM for 3 h nor to Hg^{2+}, even at 3 μM for 5 h, affected the shape in astrocytes from both regions (Fig. 2A). To see more clearly the spreading effect of MeHg on astrocyte morphology, an exposure with MeHg was performed 3 h after the medium change, at which time almost all cells showed a stellate shape (Fig. 1B). As expected, there was a region-dependent difference in the spread of astrocyte morphology induced

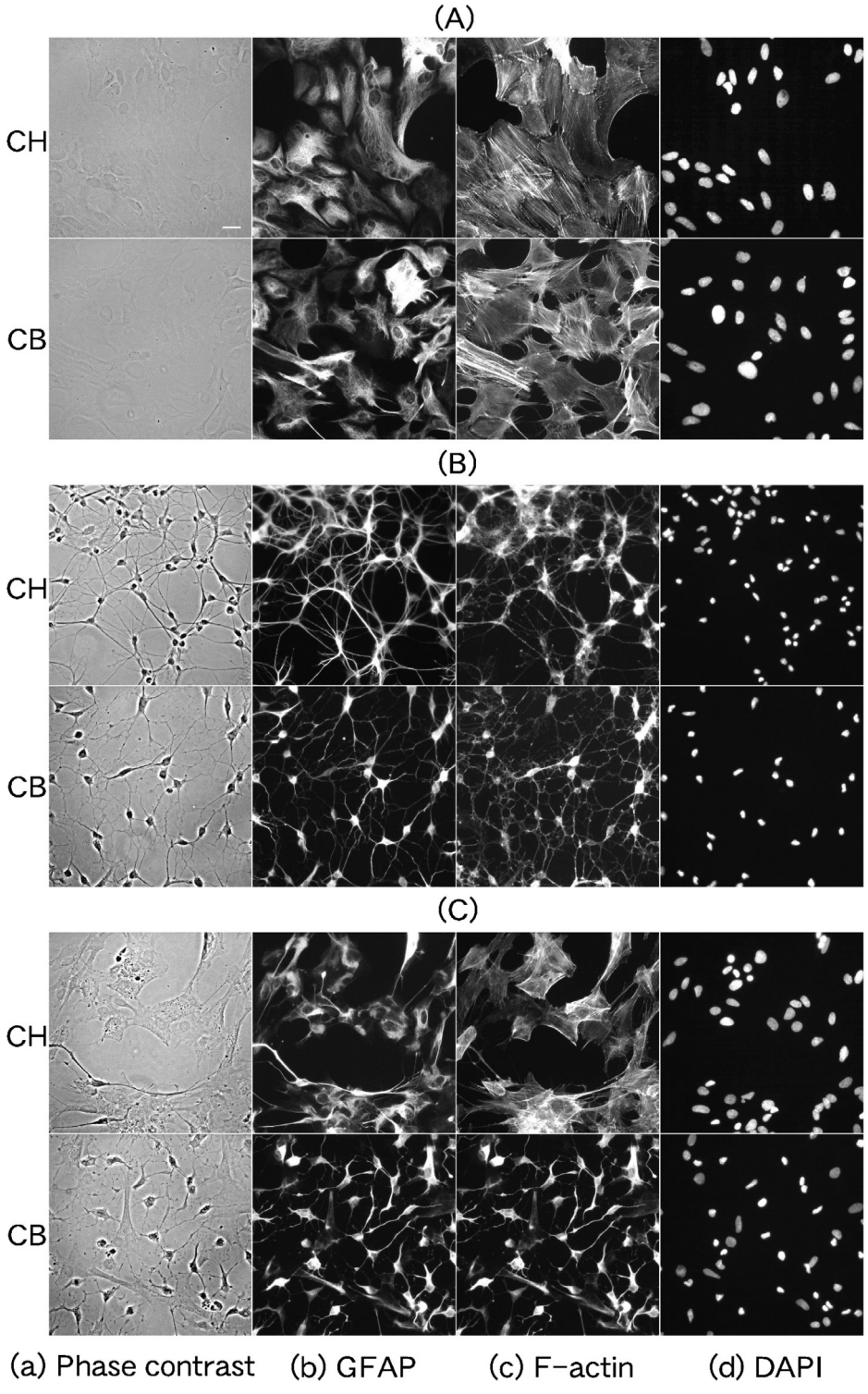

Fig. 1 Morphology, GFAP Expression, F-actin Organization and Nuclei of Cultured Astrocytes Prepared from Cerebral Hemisphere (CH) or Cerebellum (CB)

(A) Astrocytes maintained in a 15% FCS-containing medium. (B) Astrocytes maintained in a SFDM containing 0.5 mM dbcAMP for 3 h. (C) Astrocytes exposed to MeHg at 3 μM for 3 h immediately after changing a 15% FCS-containing medium to a SFDM containing 0.5 mM dbcAMP. Bar=25 μm.

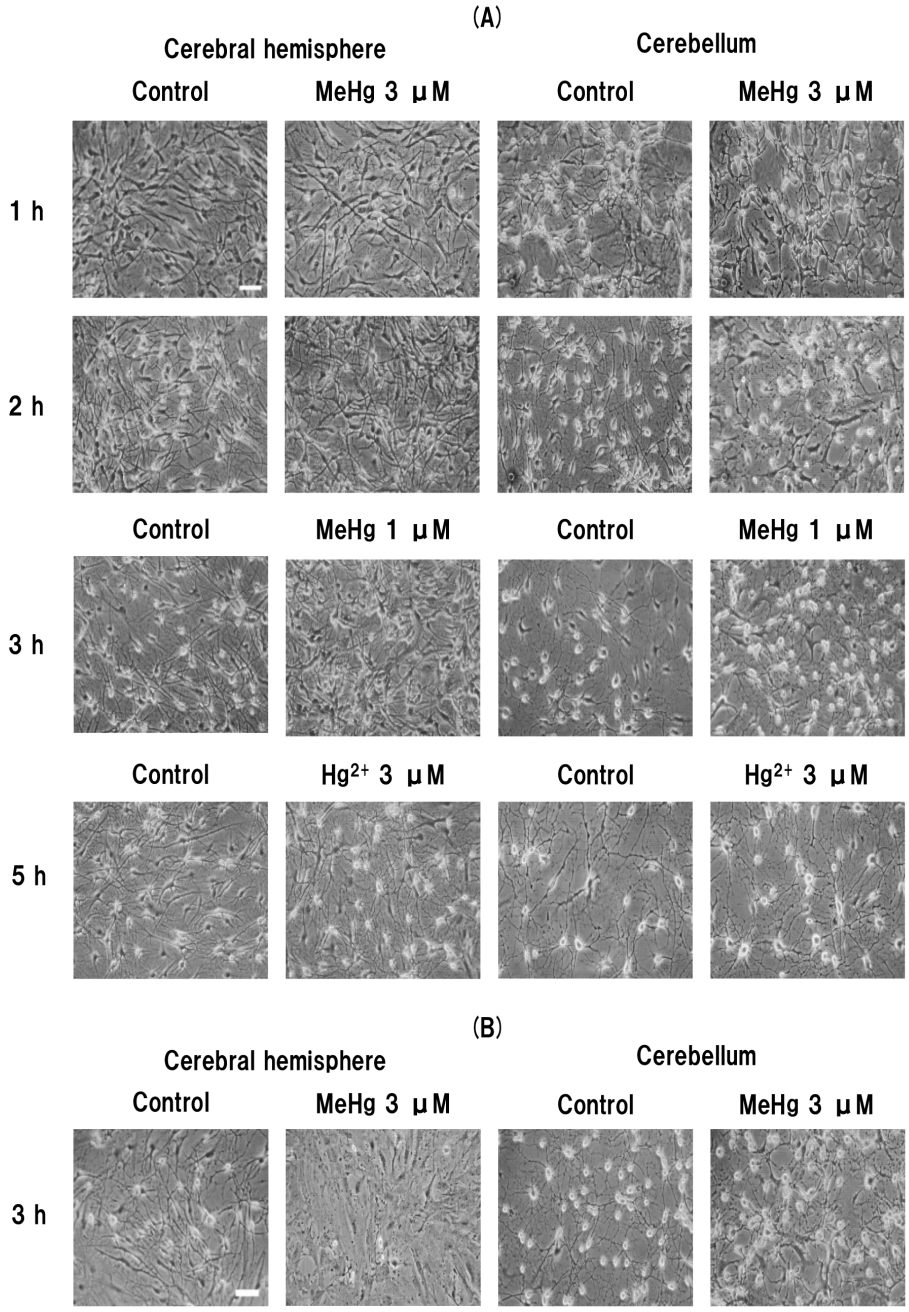

Fig. 2 Time-Dependent Changes in Cell Morphology after Exposure to Mercury Compounds in Cultured Astrocytes Prepared from Cerebral Hemisphere or Cerebellum

Mercury compounds were exposed at 0-3 μM immediately (A) or 3 h (B) after changing the 15% FCS-containing medium to a SFDM containing 0.5 mM dbcAMP. Bar=50 μm.

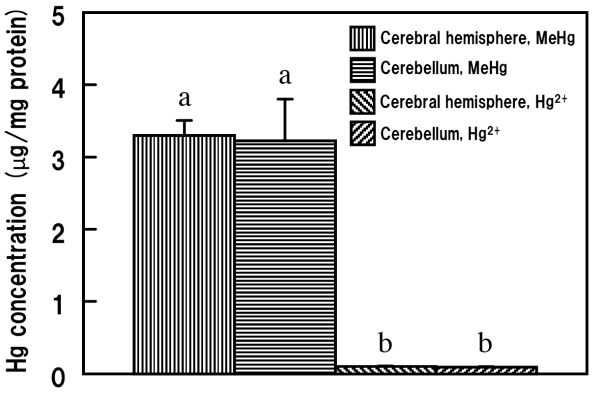

Fig. 3 Mercury Accumulation after Exposure to Mercury Compounds in Cultured Astrocytes Prepared from Cerebral Hemisphere or Cerebellum

Cultures were exposed to mercury compounds at 3 μM for 3 h immediately after changing the 15% FCS-containing medium to a SFDM containing 0.5 mM dbcAMP. Values represent the mean ± S.D. obtained from three determinations. Values with different letters (a, b) are significantly different ($p < 0.05$).

by the exposure of MeHg at 3 μM for 3 h (Fig. 2B), as was observed in the cells exposed to MeHg immediately after the medium change (Fig. 2A). This and the fact that astrocyte morphology was stellate 1-2 h after MeHg exposure performed immediately after the medium change to a SFDM containing dbcAMP (Fig. 1A) suggest that MeHg causes an alteration to a polygonal shape rather than an inhibition of stellation by dbcAMP stimulation.

Accumulations of the respective mercury compounds in astrocyte cultures exposed to them were examined to clarify the relationship between shape changes and mercury content in the cells. When astrocyte cultures were exposed to MeHg or Hg^{2+} at 3 μM for 3 h immediately after the medium change, mercury accumulation was more than 30 times higher in MeHg-treated than in Hg^{2+}-treated astrocyte cultures from the respective regions (Fig. 3). However, mercury accumulation was similar in the cerebral hemisphere and cerebellar astrocytes, regardless of whether exposed to MeHg or Hg^{2+} (Fig. 3).

Viable cell numbers were not influenced by the 3 h-exposures of either mercury compound at 3 μM, compared to those in the respective control cultures exposed to solvent alone, regardless of the elapsed time from the medium change to their exposures (data not shown). Similar results were previously observed in the absence of dbcAMP.[31]

3.2 Astrocyte Morphology After LPA Exposure

We then investigated the influence of LPA, a Rho activator, on the morphology of astrocytes from both regions, to examine the involvement of RhoA in MeHg-induced spreading of the astrocyte shapes, since that molecule has been demonstrated to be pivotal in determining astrocyte morphology.[9-11] Since spreading of astrocyte morphology induced by LPA at a concentration of 10 μM occurred more rapidly (Fig. 4) than that induced by MeHg, exposure to LPA was performed 3 h after the medium change in all experiments. In cerebral hemisphere astrocytes, morphology started to spread within 10 min, and the morphology of almost all cells showed a polygonal shape within 30 min after LPA exposure at 10 μM (Fig. 4). In addition, when the cerebral hemisphere astrocytes were added to LPA at a lower concentration (1 μM), morphology started to spread within 20 min, and the morphology of many cells showed a polygonal shape within 30 min (Fig. 4). In contrast, the morphology of many cells remained stellate in cerebellar astrocytes 30 min after the LPA exposure at both concentrations (Fig. 4). Thus, similar to MeHg, LPA easily altered the morphology from stellate to polygonal in cerebral hemisphere astrocytes, whereas it only slightly affected the morphology in cerebellar astrocytes. A similar region-dependent difference has been reported in the spreading of astrocytes induced by not only LPA[23] but also another Rho activator, glutamate.[20]

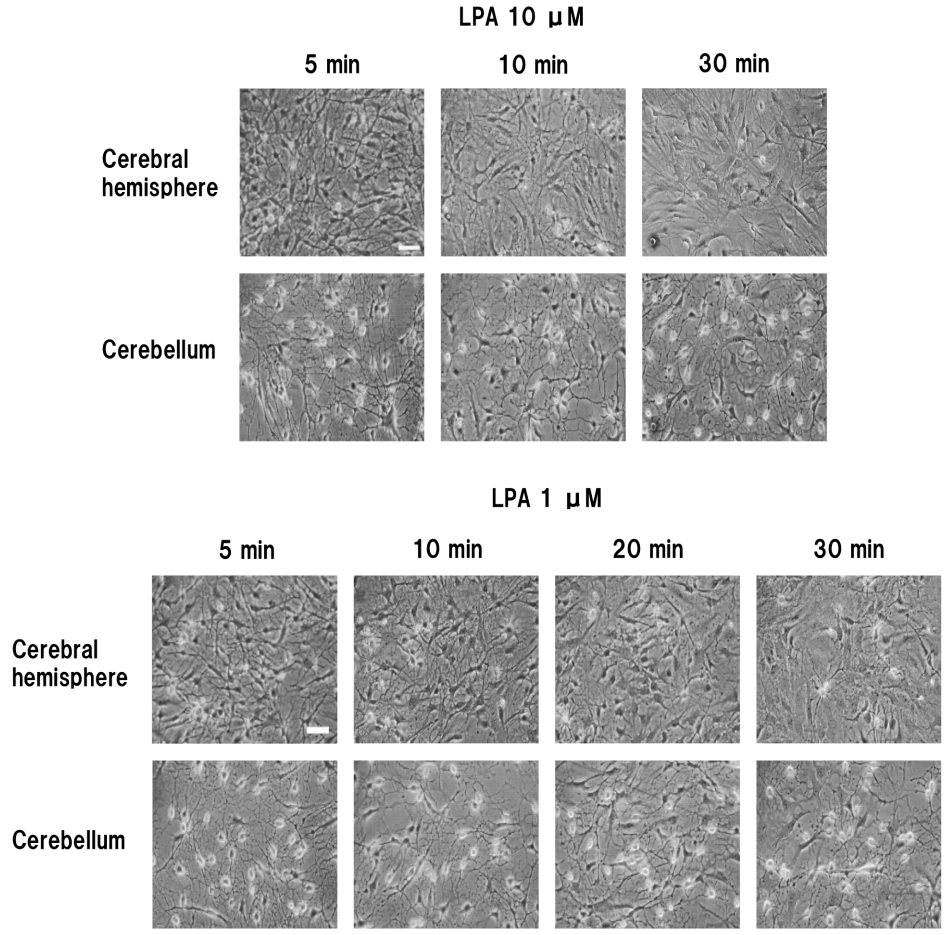

Fig. 4 Time-Dependent Changes in Cell Morphology after Exposure to LPA in Cultured Astrocytes Prepared from Cerebral Hemisphere or Cerebellum

LPA was exposed 1 or 10 μM 3 h after changing the 15% FCS-containing medium to a SFDM containing 0.5 mM dbcAMP. Bar=50 μm.

4. DISCUSSION

In the present study, there were marked region-dependent differences in responses, including morphological changes induced by MeHg in cultured astrocytes. Astrocytes from the cerebral hemisphere and cerebellum similarly showed a flat polygonal shape in the presence of serum, and a process-bearing stellate shape after changing the 15% FCS-containing medium to a SFDM containing dbcAMP, respectively (Figs. 1A, B, 2A). Three hour-exposure to MeHg at a concentration of 3 μM altered the shape of most cerebral hemisphere astrocytes from stellate stimulated by dbcAMP to polygonal, but only a few in cerebellar astrocytes, regardless of the elapsed time from the medium change to its exposure (Figs. 1C, 2B). It has been revealed that changes in astrocyte morphology are related to the organization of actin, and that RhoA, a small GTP-binding protein, is a key molecule for determining changes in morphology and in actin organization in cultured astrocytes. For example, a stellate shape in cultured astrocytes induced by cAMP is changed to a polygonal one by LPA, a Rho activator,[9-11] cAMP-induced stellation is accompanied by a loss of actin stress fibers,[11,13] and LPA-induced spreading is accompanied by actin reorganization.[9-11] In addition, pretreatment of C3 transferase, which inactivates Rho proteins, inhibits LPA-induced spreading and actin reorganization in cultured astrocytes.[9-11] In the present study, when cerebral hemisphere astrocytes were maintained in the SFDM containing 0.5 mM dbcAMP with 3 μM of MeHg for 3 h, transient stellation was followed by changing to a polygonal shape along with the formation of stress fibers (Fig. 1C). Considering the similarity in

effects of LPA[11,23] and MeHg (Figs. 1C, 2B) on astrocyte morphology and actin organization, RhoA might be a key molecule in MeHg-induced shape changes, at least in the cerebral astrocytes. If MeHg-induced spreading of astrocytes occurs through activation of RhoA, LPA should also region-dependently affect the shape. Indeed, we found that LPA, as well as MeHg (Figs. 1C, 2B), change the morphology from a stellate shape to a polygonal one in most cerebral hemisphere astrocytes, but in few cerebellar astrocytes (Fig. 4). A similar result has been reported that glutamate, another Rho activator, region-dependently alters the shape of astrocytes.[20] Therefore, at least in the cerebral hemisphere astrocytes, the MeHg-induced spreading might occur through activation of RhoA. However, further study of the mechanism in MeHg-induced astrocyte morphological changes is necessary, including a probe into the cellular level of whole and active RhoA, to confirm the contribution of this molecule to those changes. In addition to the mechanism in MeHg-induced astrocyte shape change, the fact that Rho activators, LPA [23] (Fig. 4) and glutamate,[20] region-dependently spread astrocyte morphologies, strongly suggests that either Rho activation or downstream regulation of signal transduction might be different between the cerebral hemisphere and cerebellar astrocytes.

It has been demonstrated that MK-801, a non-competitive antagonist of *N*-methyl-D-aspartate (NMDA) receptor, suppressed MeHg-induced neuronal damage, particularly in the cerebral cortex but not in the cerebellum.[35] The protective effect of MK-801 is also reported against MeHg-induced damage in cultures of cortical neurons.[36] These results suggest that the mechanism of MeHg neurotoxicity might differ between the two regions, including the involvement of glutamate. In the present study, most cerebral hemisphere astrocytes altered to a polygonal shape from a stellate shape, whereas few cerebellar astrocytes did so after exposure to MeHg at 3 μM for 3 h (Figs. 1C, 2B). It has been reported that polygonal astrocytes induced by endothelins suppress glutamate uptake,[37] expression levels of its transporters,[37] and the activity of glutamine synthetase[12] compared to stellate astrocytes. Therefore, regulation in extracellular levels of glutamate, including uptake into astrocytes and metabolism into glutamine, might have been disordered in polygonal astrocytes compared to in stellate astrocytes. If so, contributions of glutamate in MeHg intoxication would be greater in cerebrum than in cerebellum, due to the difference in astrocyte morphology after MeHg exposure at 3 μM (Figs. 1C, 2B). Thus, our findings with regard to a region-dependent difference in the spread of astrocytes might lead to an understanding of the varying involvement of glutamate in neuronal cell death induced by MeHg. This hypothesis is consistent with the results in the study[35] using MK-801. Thus, the regional heterogeneity in astrocyte morphological changes might account for the brain region-dependent difference in the protective effect of MK-801 against MeHg toxicity.

In the present study, there were variations in the responsiveness, such as shape changes, in response to mercury compounds, not only between brain regions but also between MeHg and Hg^{2+}, especially at 3 μM, and between the concentrations of MeHg exposed (1 and 3 μM), at least in the cerebral hemisphere astrocytes (Figs. 1C, 2A). MeHg-induced spreading of the shape occurred more easily in cerebral hemisphere astrocytes than in cerebellar astrocytes (Fig. 1C), although the accumulated mercury level after MeHg exposure at 3 μM for 3 h was similar in the astrocytes from both regions (Fig. 3). These results suggest that the region-dependent difference in the spreading effect of MeHg are not caused by the variation in accumulated mercury levels. Therefore, the responsiveness to MeHg, including shape changes, would be markedly different among the brain's regional origins of cultured astrocytes. In contrast to MeHg, Hg^{2+} at that concentration did not influence the morphology in cultured astrocytes from either region (Fig. 2A). Mercury accumulation was markedly lower in Hg^{2+}-treated than in MeHg-treated astrocyte cultures from the respective regions (Fig. 3). In addition, treatment with the lower concentration (1 μM) of MeHg did not affect the shape for at least 3 h. Therefore, neither Hg^{2+} treatment at 3 μM nor MeHg treatment at 1 μM would reach a mercury level high enough to induce a shape change within the first few hours. Another possibility is that the nature of MeHg's effect might be different from that of Hg^{2+}.

In conclusion, although morphology and actin organization are similar in the presence of serum and after dbcAMP stimulation in cerebral hemisphere and in cerebellar astrocytes, there are marked region-dependent differences in the MeHg-induced alterations despite similar mercury accumulations. In addition, LPA-induced spreading of astrocyte morphology is also region-dependently observed, suggesting that MeHg may affect astrocyte morphology through the Rho-dependent signal pathway. It is also suggested that responses to MeHg and to Hg^{2+} are different at the same exposure concentration

(3 μM), especially in cerebral hemisphere astrocytes.

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