METHYLMERCURY-INDUCED MICROTUBULE DEPOLYMERIZATION LEADS TO INHIBITION OF TUBULIN SYNTHESIS

Kyoko MIURA^{1,2}, Yuka KOBAYASHI², Haruka TOYODA³ and Nobumasa IMURA²

¹*Department of Environmental Sciences, Faculty of Economics,
Wako University, 2160, Kanai-cho, Machida-shi, Tokyo, 195-0071, Japan*

²*Department of Public Health and Molecular Toxicology, School of Pharmaceutical Sciences,
Kitasato University, 9-1, Shirokane 5-chome, Minato-ku, Tokyo, 108-8641, Japan*

³*Pharmaceutical and Medical Safety Bureau, Blood and Blood Products Division,
Ministry of Health and Welfare, 1-2-2, Kasumigaseki, Chiyoda-ku, Tokyo 100-8045, Japan*

(Received May 6, 1998; Accepted July 9, 1998)

ABSTRACT —Methylmercury (MeHg) specifically depolymerizes microtubules and inhibits cell proliferation in mouse glioma cells. The effect of microtubule depolymerization by MeHg on tubulin synthesis was studied. Tubulin synthesis analyzed by two-dimensional electrophoresis using [³⁵S]methionine as a tracer was markedly inhibited in mouse glioma cells exposed to 5×10^{-6} M MeHg for 3 hr, which completely depolymerized microtubules. Under this condition, density of the protein bands other than tubulin in autoradiogram remained unchanged on gradient urea-polyacrylamide gels. Furthermore, the decrease in tubulin mRNA level was relevant to that in tubulin synthesis, although actin mRNA levels remained unchanged. In addition, specific transcription rates of β -tubulin genes appeared to be unaffected under the same experimental condition as above. Thus, it is concluded that the disruption of microtubules by MeHg resulted in the inhibition of the synthesis of tubulin itself through autoregulatory repression in post-transcriptional processes as in the case of colchicine treatment.

KEY WORDS: Methylmercury, Growth inhibition, Microtubule depolymerization, Tubulin synthesis, Mouse glioma cells

INTRODUCTION

Methylmercury (MeHg) is known to be a ubiquitous environmental pollutant that affects the nervous system of both exposed humans and experimental animals. While numerous clinical and pathological studies, since the first report by Hunter and Russell (1954), have revealed that MeHg causes neuropathological changes (WHO, 1976, 1990), the mechanisms associated with its toxicity remain obscure. Several studies at cellular levels have shown that MeHg is capable of impairing tubulin polymerization and of depolymerizing microtubules at micromolar concentrations in culture medium (Miura *et al.*, 1978, 1984; Imura *et al.*, 1980; Sager *et al.*, 1983; Sager, 1988; Sager and Syversen, 1984; Wasteneys *et al.*, 1988; Brown *et al.*,

1988; Cadrin *et al.*, 1988). The impairment of microtubules by MeHg resulted in the inhibition of cell division in proliferating cells (Miura *et al.*, 1978) and in the blockade of axoplasmic transport in neuronal cells (Abe *et al.*, 1975). Furthermore, a few reports have demonstrated by *in vivo* experiments that MeHg selectively affects mitotic process in the developing mouse brain (Rodier *et al.*, 1984; Sager *et al.*, 1984). Thus, the sensitivity of microtubules to low concentrations of MeHg has been suggested to account for the MeHg neurotoxicity (Miura and Imura, 1987; Sager and Matheson, 1988).

Microtubules, which are comprised of dimeric subunits of one α - and one β -tubulin polypeptide, participate in diverse cellular functions, including the formation of mitotic spindles, the establishment of axonal

transport and cell motility (Olmsted and Borisy, 1973). In addition to the important functions of microtubules, it has been documented that the synthesis of tubulin is regulated by a process named as an "autoregulatory pathway" (Ben Ze'ev *et al.*, 1979; Cleveland *et al.*, 1981, 1983; Yen *et al.*, 1988), which is closely linked to the pool size of unpolymerized tubulin subunits. The treatment of cells with colchicine, an antimicrotubule drug which induces microtubule depolymerization (Borisy and Taylor, 1967; Shelanski and Taylor, 1967), increased the pool size of depolymerized tubulin subunits (Hiller and Weber, 1978; Spiegelman *et al.*, 1977). The increased unpolymerized tubulin subunits induced a specific repression of new tubulin synthesis (Ben Ze'ev *et al.*, 1979; Cleveland *et al.*, 1981; Pachter *et al.*, 1987). In contrast, vinblastin, which disrupts microtubules to form insoluble paracrystals, showed a mild increase in tubulin synthesis (Ben Ze'ev *et al.*, 1979; Cleveland *et al.*, 1981).

On the other hand, MeHg depolymerizes microtubules by binding to SH groups in the tubulin subunit and increases the pool size of unpolymerized tubulin subunits (Vogel *et al.*, 1985, 1989). Therefore, it is suggested that, like colchicine, MeHg is capable of depressing tubulin synthesis through the increase of unpolymerized tubulin subunits by microtubule disruption.

Since an initial report by Yoshino *et al.* (1966), protein synthesis in nervous tissues has been considered as one of the most sensitive targets for MeHg (Cavanagh and Chen, 1971; Farris and Smith, 1975; Omata *et al.*, 1978; Syverson, 1977, 1982; Verity *et al.*, 1977; Cheung and Verity, 1985). Although evidence in support of the inhibitory effects of MeHg on protein synthesis has accumulated, it has not been clarified which protein species are actually affected by MeHg. It is well known that the content of tubulin in a vertebrate brain is very high, i.e. 10 to 24% of the total protein and up to 42% of the soluble protein in a developing brain (Hiller and Weber, 1978; Fulton and Simpson, 1979). Therefore, it is important to investigate whether the increase in cytoplasmic tubulin subunits caused by MeHg contributes to the depression of tubulin synthesis or not.

In this report, we demonstrate that microtubule disruption by MeHg leads to an inhibition of tubulin synthesis. Furthermore, by determination of tubulin mRNA and tubulin gene transcriptional rates, it is shown that the inhibition of tubulin synthesis by MeHg results from microtubule depolymerization through autoregulatory control of tubulin synthesis.

MATERIALS AND METHODS

Chemicals

Methylmercuric chloride (MeHg, CH_3HgCl) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Labelled [^{35}S]methionine (37 TBq/mmol), [γ - ^{32}P]ATP (37 TBq/mmol), [α - ^{32}P]UTP (30 TBq/mmol), and [α - ^{32}P]dCTP (110 TBq/mmol) were obtained from Amersham International (Bucks, United Kingdom). Fetal bovine serum (FBS, Gibco Oriental, Tokyo, Japan), Eagle's minimum essential medium (EMEM, Nissui, Tokyo, Japan), Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo, Japan), Tris(hydroxymethyl) aminomethane (Tris), ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), trichloroacetic acid (TCA), and Nonidet-P40 (NP-40) were purchased from Wako Pure Chemical Industries Ltd.

Cell culture

Two cell lines, mouse glioma (CDF₁DBT) and neuroblastoma C1300 (N-18 clone) were chosen to investigate the effects of MeHg exposure. Mouse glioma cells were maintained as monolayer cultures in EMEM supplemented with 10% FBS as described previously (Miura *et al.*, 1978). Mouse neuroblastoma cells were cultured in DMEM supplemented with 10% FBS. About 20 hr after seeding on plastic dishes, cells of subconfluent cultures were incubated in fresh medium with or without MeHg (10^{-6} , 5×10^{-6} and 10^{-5}M) at 37°C for 3 and 6 hr. According to our previous studies (Miura *et al.*, 1978, 1984, Imura *et al.*, 1980), MeHg at $5 \times 10^{-6}\text{M}$ was used as the growth-inhibiting concentration, which induced the complete inhibition of cell proliferation through the disruption of microtubules. Colchicine, a microtubule-disrupting reagent, was used at 10^{-5}M as a positive control (Ben Ze'ev *et al.*, 1979).

Tubulin synthesis

The cells (5×10^4 cells/16-mm wells) were preincubated with MeHg (10^{-6} , 5×10^{-6} and 10^{-5}M) or colchicine (10^{-5}M) at 37°C for 3 and 6 hr, then were pulse-labeled with 3.7 MBq/200 μl [^{35}S]methionine for 15 min in a methionine-deficient medium containing 10% dialyzed fetal bovine serum. After washing with phosphate-buffered saline (PBS, pH 7.4), cells were lysed by the addition of a buffer (25 mM Tris-HCl pH 7.4) containing 0.4 M NaCl, 0.1% deoxycholate, 1% NP40 and 0.5% sodium dodecyl sulfate (SDS). Labeled cell extracts were prepared according to the methods described by Cleveland *et al.* (1981).

Incorporation of [^{35}S]methionine into protein was

monitored by measuring TCA-precipitable counts according to the method of Cleveland *et al.* (1981).

Gel electrophoresis

Pulse-labeled proteins from monolayer cultures were analyzed by two-dimensional gel electrophoresis as described by O'Farrell (1975). The pH gradient was 3.5-10. The samples were run on a 10% acrylamide slab gel in the second dimension. For detection of labeled proteins, the gels were exposed to Kodak X-ray film (XAR) at -80°C . Quantitation of radioactivity in individual gel areas was performed by scanning densitometry (CS-9000, Shimadzu Corporation, Kyoto, Japan). On the autoradiogram thus obtained, the spot corresponding to α -tubulin was apparently overlapped by that of 58 K intermediate filament protein as reported by Ben Ze'ev *et al.* (1979). Therefore, the β -tubulin spot was used for quantitative determination of tubulin synthesis.

Cloning of mouse β -tubulin and mouse β -actin cDNAs

cDNAs of mouse β -tubulin and mouse β -actin were cloned from a mouse liver cDNA library (a kind gift from Dr. Kuge of the University of Tokyo) constructed in vector λ gt10. Plaque hybridization was conducted using end-labeled synthetic oligonucleotides (30 mer) containing mouse β 5-tubulin (Wang *et al.* 1986) or β -actin (Tokunaga *et al.* 1986) gene sequences as probes. Nucleotides 813-843 were used to probe for β -tubulin, whereas nucleotides 300-330 and 709-739 were used as probes for β -actin. The inserts were purified and subcloned into a pUC118 plasmid vector at the EcoRI site resulting in the construction of recombinant DNAs pST1301 for β -tubulin and pYA8801 for β -actin. An EcoRI fragment derived from pST1301 or pYA8801, thus obtained, was used as a probe for Northern blot analysis after being uniformly labeled with $[^{32}\text{P}]\text{dCTP}$ by DNA polymerase I according to Maniatis *et al.* (1982). By DNA-sequence analysis it was determined that cDNAs plasmids, pST1301 and pYA8801, carry 43% (nucleotides 718-1468) or 100% (nucleotides 12-1279) of mouse β 5-tubulin and mouse β -actin mRNA sequences, respectively.

RNA preparation and Northern blot analysis

Total RNA was prepared from mouse glioma cells using the guanidium thiocyanate/CsCl methods (Maniatis *et al.* 1982). Equal amounts of RNAs, determined by absorbance at 260 nm, were separated by electrophoresis in 1% agarose gel containing 2.2 M formamide after glyoxylation and were transferred to

nitrocellulose filters. The blots were hybridized at 42°C overnight in a solution consisting of 40% formamide, $3\times\text{SSC}$, 0.1% SDS, $10\times\text{Denhardt's}$ solution and 50 $\mu\text{g/ml}$ of denatured salmon sperm DNA. Hybridization with nick-translated mouse β -tubulin or β -actin cDNA probe was followed by two washings in $0.3\times\text{SSC}$ containing 0.1% SDS at 55°C for 20 min and exposure to Kodak XAR-5 X-ray film at -80°C . Autoradiograms were scanned at 550 nm using a spectrodensitometer (CS-9000, Shimadzu Corporation, Kyoto, Japan). SSC contained 0.15 M NaCl and 0.015 M sodium citrate. Denhardt's solution contained 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin.

Nuclear transcription and RNA isolation

Nuclei were isolated by cell lysis in 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM NaCl, 3 mM MgCl_2 and 0.5 % of NP-40 for 10 min in ice by the method of Groudine *et al.* (1981). Cell lysates were stored in 50 mM Tris-HCl buffer (pH 8.3) containing 40% glycerol, 5 mM MgCl_2 and 0.1 mM EDTA at -80°C . For nuclear transcription studies, isolated nuclei ($1.2\times 10^7/100\mu\text{l}$) were incubated in reaction solution I for 20 min at 30°C . This solution contained 10 mM Tris-HCl (pH 8.0), 5 mM MgCl_2 , 300 mM KCl, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP and $[\gamma\text{-}^{32}\text{P}]\text{UTP}$. Reactions were terminated by the addition of deoxyribonuclease I (DNase I, Takara 980 U/ml) and were further incubated at 30°C for 10 min. The transcribed RNA was extracted using TCA precipitation according to the method of McKnight and Palmiter (1979) as modified by Groudine *et al.* (1981).

Preparation of the slot blots of the β -tubulin and β -actin cDNAs and hybridization

Purified plasmids (7 μg) containing cDNA specific for β -tubulin or β -actin, were immobilized on nitrocellulose as slot blots. The blots were hybridized at 42°C for 48 hr, to 1×10^7 cpm of the transcripts in a solution consisting of 40% formamide, $3\times\text{SSC}$, $10\times\text{Denhardt's}$ solution, 0.1% SDS, 50 mM sodium phosphate, pH 7.0, and 250 $\mu\text{g/ml}$ denatured salmon sperm DNA. Filters were washed and subjected to autoradiography as described above for Northern blot analysis.

Statistical analysis

Student's *t*-test (Gad and Weil, 1982) was used to determine the significance of the difference in protein synthetic activity and mRNA level between non-treated control and MeHg or colchicine treated groups.

RESULTS

We reported previously that MeHg added into the culture medium selectively and completely disrupts microtubules within 1 hr at a growth-inhibiting concentration (5×10^{-6} M) in mouse glioma cells (Miura *et al.*, 1978, 1984; Imura *et al.*, 1980).

To investigate the influence of microtubular disruption on tubulin synthesis, mouse glioma cells previously exposed to 10^{-6} , 5×10^{-6} or 10^{-5} M MeHg for 3 and 6 hr were labeled with [35 S]methionine for 15 min. As a positive control, cells were pretreated with colchicine (10^{-5} M) for 3 hr, then labeled with [35 S]methionine for 15 min, as described by Ben Ze'ev *et al.* (1979). Autoradiograms subsequent to two-dimensional electrophoresis of the [35 S]methionine-labeled cytoplasmic proteins from the control, MeHg (5×10^{-6} M for 3 hr) or colchicine (10^{-5} M for 3 hr) treated cells were shown in Photo. 1. The radioactivity of β -tubulin markedly decreased in MeHg or colchicine-treated cells (Photo. 1B and 1C) compared with that in the control (Photo. 1A). Similar reduction of the radioactivity of β -tubulin on two-dimensional gel electrophoresis as above was observed in neuroblastoma cells exposed to 5×10^{-6} M MeHg for 3 hr (data not shown).

Effect of MeHg on protein synthesis other than β -tubulin was examined by autoradiography after gradient urea(1-4M)-polyacrylamide(4-16%) gel electrophoresis (PAGE). As shown in Photo. 2, density of the protein bands other than tubulin in autoradiogram remained almost unchanged in cells treated with 10^{-6} M and 5×10^{-6} M MeHg for 3 hr (Photo. 2, lanes 2 and 3), while label of β -tubulin protein markedly decreased in cells treated for 3 hr with 5×10^{-6} M MeHg (Photo 2, lane 3) but not with 10^{-6} M. On the other hand, density of almost all protein bands in autoradiogram decreased at a higher concentration of MeHg (10^{-5} M).

For quantitation of the depression in β -tubulin synthesis, the density of the spots corresponding to β -tubulin and actin on autoradiograms of two-dimensional electrophoresis was measured by scanning densitometry. The density of actin spot was taken as the standard, because actin band remained unchanged in 5×10^{-6} M MeHg treated cells (Photo. 2). Fig. 1 shows that β -tubulin synthesis was inhibited by approximately 45% at 5×10^{-6} M MeHg. On the other hand, the inhibition of total protein synthesis monitored by measuring TCA-precipitable counts was less than 10% at 5×10^{-6} M MeHg (Fig. 1). Thus, it was suggested that microtubule disruption by growth-inhibiting concentration of MeHg

Photo 1. Autoradiograms of proteins in mouse glioma cells after two-dimensional gel electrophoresis

Cells were treated with MeHg (5×10^{-6} M) or colchicine (10^{-5} M) for 3 hr at 37°C . The cells were pulse-labeled with [35 S]methionine for 15 min. Proteins were analyzed by two-dimensional gel electrophoresis.

A: control; B: 5×10^{-6} M MeHg; C: 10^{-5} M colchicine. a, α -tubulin plus 58K intermediate filament protein; b, β -tubulin; c, actin.

resulted in a specific inhibition of tubulin biosynthesis in mouse glioma cells as in the case of colchicine treatment.

The effect of microtubule disruption by MeHg on tubulin mRNA level was subsequently examined using mouse glioma cells. Cytoplasmic RNAs from the cells treated with various concentrations of MeHg or 10^{-5} M colchicine for 3 hr were extracted and analyzed by

Northern blot analysis using cloned cDNAs for mouse β -tubulin and β -actin as probes. As shown in Photo 3, two RNA species of different molecular weights hybridized to the β -tubulin probe, as previously reported by Cleveland *et al.* (1981). Radioactivity hybridized to β -tubulin mRNA decreased by MeHg treatment in a concentration-dependent manner. With concentrations higher than 5×10^{-6} M MeHg, a remarkable decrease in the density (lanes 3 and 4) was observed as in the colchicine treated sample (lane 4). However, the actin mRNA level was unaffected by either MeHg or colchicine treatment. By densitometry of the autoradiograms, it is clear that β -tubulin mRNA level declined in response to MeHg or colchicine, however, no significant decline in actin mRNA was observed in 5×10^{-6} M

Photo 2. Gradient urea-polyacrylamide gel electrophoresis of proteins extracted from control and MeHg-treated mouse glioma cells

Cells were incubated with MeHg for 3 hr at 37°C and treated as described in Photo. 1. Proteins were separated on SDS-urea(1-4M)-polyacrylamide(4-16%) gel electrophoresis. M and T: The gel was stained with Coomassie blue. Lane M, standards of molecular weight; Lane T, tubulin protein $20 \mu\text{g}$. Lanes 1-4: The same gel was exposed to X-ray film. Lane 1, control; Lane 2, 10^{-6} M MeHg; Lane 3, 5×10^{-6} M MeHg; Lane 4, 10^{-5} M MeHg; a, α -tubulin; b, β -tubulin; c, actin. Arrow shows β -tubulin. Tubulin proteins shown in lane T were prepared from porcine brain as reported previously (Miura *et al.* 1984).

Fig. 1. Effects of MeHg on β -tubulin synthesis and total protein synthesis in mouse glioma cells

Synthesis of β -tubulin: Cells, which were incubated with MeHg or colchicine for 3 hr at 37°C , were pulse-labeled with [^{35}S]methionine for 15 min. After two-dimensional-electrophoresis of cell extracts, spots of β -tubulin on autoradiograms were analyzed by scanning densitometry. The relative amounts of β -tubulin to those of actin were shown.

CLC: colchicine. Values are mean $\pm$ SD of three determinations. *Significantly different from control (* $p < 0.01$, $n = 3$). Total protein synthesis was determined by measuring radioactivity in the acid-insoluble fraction.

MeHg or colchicine treated cells (Fig. 2).

To analyze the relative rates of transcription of β -tubulin or β -actin RNAs in nuclei derived from glioma cells treated with MeHg (10^{-6} or 5×10^{-6} M for 3 hr), nuclei were isolated from the control and MeHg-treated cells. [32 P]labeled RNAs were extracted from each sample and were hybridized to a filter containing β -tubulin and β -actin cDNAs. Autoradiogram of the resultant filter is shown in Photo 4. No obvious decrease in RNA transcripts in the nuclei of MeHg-treated cells was detected. Densitometric data of the β -tubulin transcripts on the autoradiogram were normalized by comparison with the β -actin signal on the same filter. The results indicated the same relative rates of β -tubulin gene transcription in the 10^{-6} M and the 5×10^{-6} M MeHg-treated cells, as did those in the control cells.

Photo 3. Northern blot analysis of β -tubulin and β -actin genes in mouse glioma cells exposed to MeHg or colchicine.

Total RNAs were isolated from the cells exposed to MeHg or colchicine for 3 hr and analyzed by Northern blot analysis as described in Materials and Methods. T: tubulin; A: actin; 1, control; 2, 10^{-6} M MeHg; 3, 5×10^{-6} M MeHg; 4, 10^{-5} M MeHg; 5, 10^{-5} M colchicine.

Fig. 2. Effects of MeHg on β -tubulin and β -actin mRNA levels in mouse glioma cells.

Levels of β -tubulin and β -actin mRNAs were quantified by scanning densitometry. CLC: colchicine. Values are mean $\pm$ SD of three determinations. *Significantly different from control ($p < 0.01$, $n = 3$).

Photo 4. Effects of MeHg on the transcriptional rates of β -tubulin and β -actin genes in mouse glioma cells.

Cell nuclei were isolated from control and MeHg-treated cells (3 hr at 37°C) and transcriptional rates were analyzed as described in Materials and Methods. c, control; 1, 10^{-6} M MeHg; 2, 5×10^{-6} M MeHg.

DISCUSSION

The results presented here indicate that MeHg at a growth-inhibiting concentration inhibits β -tubulin synthesis in mouse glioma cells. In general, tubulin synthesis is regulated in higher eukaryotes mainly by two processes. One is transcriptional activation (Cleveland, 1987). Another, in most animal cells, is an autoregulatory translation in which the intracellular concentration of tubulin subunits modulates the stability of tubulin messenger RNAs (Ben Ze'ev *et al.*, 1979; Cleveland and Havercroft, 1983; Cleveland and Kirschner, 1982; Cleveland *et al.*, 1981; Cleveland *et al.*, 1983; Cleveland, 1988). From the facts presented above, MeHg seems to affect the autoregulatory pathway of tubulin synthesis in mouse glioma.

By using mouse glioma and neuroblastoma cells, we have conducted a series of experiments in search of the most susceptible target of MeHg in the cells (Miura *et al.*, 1978, 1984; Imura *et al.*, 1980; Nakada and Imura 1980, 1982; Nakada *et al.*, 1980). MeHg added in the medium inhibited the growth of mouse neuroblastoma and glioma cells at concentration ranges from 1 to 5×10^{-6} M. Complete growth inhibition was observed at 5×10^{-6} M (Miura *et al.*, 1978). When mouse glioma cells were incubated with MeHg at growth-inhibiting concentration (5×10^{-6} M), microtubule networks in interphase cells disappeared within 1 hr (Imura *et al.*, 1980; Miura *et al.*, 1984) and mitotic spindles in mitotic cells disappeared completely after 4 hr (Miura *et al.*, 1978). Under the same conditions as above we also observed the accumulation of c-mitotic cells which are arrested at M phase in the cell cycle through microtubule disruption by MeHg (Miura *et al.*, 1978). Furthermore, it was demonstrated that DNA, RNA and protein syntheses remained unchanged under these conditions (Nakada and Imura, 1982; Nakada *et al.*, 1980). Thus, the cellular microtubules seemed to have a structure highly susceptible to MeHg. These data coincided well with the results observed in neuroblastoma, glioma and fibroblast cells by Sager *et al.* (1983) and Sager and Syversen (1984).

In the present experiment, mouse neuroblastoma and glioma cells were pretreated with 5×10^{-6} M MeHg for 3 hr to depolymerize microtubules completely, then tubulin synthetic activity was determined by measuring the incorporation of [35 S]methionine into the protein for 15 min. The effects of MeHg on β -tubulin synthesis observed in mouse neuroblastoma cells were similar to those in mouse glioma cells. In this paper, the data obtained from glioma cells are presented. Photo 1 and

Fig. 1 clearly show that β -tubulin synthesis decreased to 55% of untreated control. Furthermore, since protein synthesis other than β -tubulin remained almost unchanged, the inhibition of β -tubulin synthesis by MeHg appeared to be highly specific.

This selective reduction in β -tubulin synthesis was closely associated with the specific decline in β -tubulin mRNA level (Photo 3, Fig. 2). Furthermore, β -tubulin mRNA level in mouse glioma exposed to MeHg seemed to be regulated at the post-transcriptional process (Photo 4). Colchicine also induced selective decreases both in the level of β -tubulin synthesis and β -tubulin mRNA level by preventing tubulin assembly in mouse glioma cells (Photo 1-3, Fig. 1, 2). These results agreed with those obtained in previous studies using 3T6 cells derived from mouse embryo (Ben Ze'ev *et al.*, 1979; Cleveland *et al.*, 1981), Chinese hamster ovary cells, mouse L cells and N115 mouse neuroblastoma cells (Cleveland *et al.*, 1981) exposed to colchicine. It was demonstrated that the decrease in tubulin mRNA levels caused by colchicine was ascribed to the increased instability of tubulin mRNAs but not to the altered transcription rate of the tubulin gene (Cleveland *et al.*, 1981; Pachter *et al.*, 1987; Gay *et al.*, 1987, 1989; Yen *et al.*, 1988). These results, together with ours as mentioned above, strongly suggest that the inhibition of tubulin synthesis in mouse glioma cells exposed to MeHg is an autoregulatory depression of tubulin synthesis due to an increase in the pool of tubulin subunits resulting from microtubule depolymerization.

Yoshino *et al.* (1966) first reported a 43% decrease in protein synthesis in brain slices from non-symptomatic rats treated with a single dose of MeHg. Since then, several workers have confirmed that MeHg inhibits brain protein syntheses (Cavanagh and Chen, 1971; Farris and Smith, 1975), although an increased protein synthesis in the brain after MeHg exposure has also been reported (Brubaker *et al.*, 1973). Thereafter, numerous studies were carried out (Carmichael *et al.*, 1975; Cheung and Verity, 1985; Omata *et al.*, 1980, 1982; Syversen, 1977, 1982; Sarafian *et al.*, 1984; Cavanagh and Chen, 1971; Nakada *et al.*, 1980). However, the precise mechanisms of the MeHg action on the protein syntheses has not been elucidated. Cheung and Verity (1985) showed that the amino acylation of phenylalanyl tRNA in the brains of infant rats was inhibited by MeHg. On the other hand, Hasegawa *et al.* (1988) reported that three of six aminoacyl-tRNA synthetase species in the rat brain were inhibited on MeHg administration, although only one was activated

and the other two species remained unchanged. Frenkel *et al.* (1985) showed that MeHg possibly affected the transcriptional steps of gene expression in HeLa cells. Recently, Kasama *et al.* (1989) reported that the effect of MeHg on the synthesis of proteins in rat dorsal root ganglia was not uniform for individual protein species at the latent period of MeHg intoxication. They further noted that the tubulin synthesis was reduced by MeHg to a greater extent than the total protein syntheses in rat brain. Thus, as documented above, the mechanism of inhibitory action of MeHg on protein biosyntheses has not yet been fully elucidated and the specific targets of its toxic effect remain unclear.

Our data presented herewith may provide a new mechanism of MeHg cytotoxicity by demonstrating an autoregulatory inhibition of biosynthesis of tubulin, which is a dominant protein in neuronal tissues as stated below, through specific disruption of microtubules at its growth-inhibitory concentration.

Vertebrate brains contain a large amount of microtubules that provide structural support for axonal transport and functions of neuronal cells (Matsumoto and Sakai, 1979; Yoshioka *et al.*, 1978). Hiller and Weber (1978) reported that tubulin content is high in the brain, accounting for 23% and 24% of the total extracted protein in chick and mouse brains, respectively. Liver, by contrast, contains much less tubulin than brain as only 0.6-1% of the total extracted protein. Borisy and Taylor (1967) also showed that the tubulin content of the brain was exceedingly high among seven rabbit tissues (kidneys, liver, muscle, spleen, lung, bone marrow and brain). Therefore, the present experimental results demonstrating a specifically high susceptibility of tubulin synthesis to MeHg may suggest a possible mechanism of neurotoxicity of MeHg together with the data of Rodier *et al.* (1984) which clearly showed a neurotoxic effect of MeHg through microtubule disruption in neonatal mouse brain by *in vivo* experiments.

ACKNOWLEDGMENTS

We thank Professor Robert Louis-Ferdinand of Wayne State University for his valuable advice during the preparation of this manuscript. This work has been supported in part by a grant from the Japanese Environmental Agency.

REFERENCES

- Abe, T., Haga, T. and Kurokawa, M. (1975): Blockage of axoplasmic transport and depolymerization of reassembled microtubules by methylmercury. *Brain Res.*, **86**, 504-508.
- Ben Ze'ev, A., Farmer, S. R. and Penman, S. (1979): Mechanisms of regulating tubulin synthesis in cultured mammalian cells. *Cell*, **17**, 319-325.
- Borisy, G. G. and Taylor, E. W. (1967): The mechanism of action of colchicine. *J. Cell Biol.*, **34**, 525-533.
- Brown, D. L., Reuhl, K. R., Bormann, S. and Little, J. E. (1988): Effects of methylmercury on the microtubule system of mouse lymphocytes. *Toxicol. Appl. Pharmacol.*, **94**, 66-75.
- Brubaker, P. E., Klein, R., Herman, S. P., Lucier, G. W., Alexander, L. T. and Long, M. D. (1973): DNA, RNA and protein synthesis in brain, liver and kidneys of asymptomatic methylmercury-treated rats. *Exp. Mol. Pathol.*, **18**, 263-280.
- Cadrin, M., Wasteneys, G. O., Jones-Villeneuve, E. M. V., Brown, D. L. and Reuhl, K. R. (1988): Effects of methylmercury on retinoic acid-induced neuroectodermal derivatives of embryonal carcinoma cells. *Cell Biol. Toxicol.*, **4**, 61-80.
- Carmichael, N., Cavanagh, J. B. and Rodda, R. A. (1975): Some effects of methylmercury salts on the rabbit nervous system. *Acta Neuropathol. (Berl)*, **32**, 115-125.
- Cavanagh, J. B. and Chen, F. C. K. (1971): The effects of methylmercury-dicyandiamide on the peripheral nerves and spinal cord of rats. *Acta Neuropathol.*, **19**, 208-215.
- Cheung, M. K. and Verity, M. A. (1985): Experimental methyl mercury neurotoxicity: locus of mercurial inhibition of brain protein synthesis *in vivo* and *in vitro*. *J. Neurochem.*, **44**, 1799-1808.
- Cleveland, D. W. (1987): The multitubulin hypothesis revisited: What have we learned? *J. Cell Biol.*, **104**, 381-383.
- Cleveland, D. W. (1988): Autoregulated instability of tubulin mRNAs: A novel eukaryotic regulatory mechanism. *Trends Biochem. Sci.*, **13**, 339-343.
- Cleveland, D. W. and Havercroft J. C. (1983): Is apparent autoregulatory control of tubulin synthesis nontranscriptionally regulated? *J. Cell Biol.*, **97**, 919-924.
- Cleveland, D. W. and Kirschner, M. W. (1982): Autoregulatory microtubule assembly. *Cold Spring Harbor Symp. Quant. Biol.*, **46**, 171-183.
- Cleveland, D. W., Lopata, M. A., Sherline, P. and Kirschner, M. W. (1981): Unpolymerized tubulin modulates the level of tubulin mRNAs. *Cell*, **25**, 537-546.
- Cleveland, D. W., Pittenger, M. F. and Feramisco, J. R.

- (1983): Elevation of tubulin levels by microinjection suppresses new tubulin synthesis. *Nature* (London), **305**, 738-740.
- Farris, F. F. and Smith, J. C. (1975): *In vivo* incorporation of [^{14}C]leucine into brain protein of methylmercury treated rats. *Bull. Environ. Contam. Toxicol.*, **13**, 451-455.
- Frenkel, G. D., Cain, R. and Chao, E. S. E. (1985): Exposure of DNA to methylmercury results in an increase in the rate of its transcription by RNA polymerase II. *Biochem. Biophys. Res. Commun.*, **127**, 849-856.
- Fulton, C. and Simpson, P. A. (1979): Tubulin pools, synthesis and utilization. In *Microtubules* (Roberts, K. and Hyams, J. S. Eds.) pp. 117-174, Academic New York.
- Gad, S. and Weil, C. S. (1982): Statistics for toxicologist. In *Principles and Methods of Toxicology* (Wallance, H. A. Ed.) pp. 273-320, Raven Press, New York.
- Gay, D. A., Yen, T. J., Lau, J. T. Y. and Cleveland, D. W. (1987): Sequences that confer β -tubulin autoregulation through modulated mRNA stability reside within exon 1 of a β -tubulin mRNA. *Cell*, **50**, 671-679.
- Gay, D. A., Sisodia, S. S. and Cleveland, D. W. (1989): Autoregulatory control of β -tubulin mRNA stability is linked to translational elongation. *Proc. Natl. Acad. Sci. (USA)*, **86**, 5763-5767.
- Groudine, M., Perets, M. and Weintraub, H. (1981): Transcriptional regulation of hemoglobin switching in chicken embryos. *Mol. Cell Biol.*, **1**, 281-288.
- Hasegawa, K., Omata, S. and Sugano, H. (1988): *In vivo* and *in vitro* effects of methylmercury on the activities of aminoacyl-tRNA synthetases in rat brain. *Arch. Toxicol.*, **62**, 470-472.
- Hiller, G. and Weber, K. (1978): Radioimmunoassay for tubulin: a quantitative comparison of the tubulin content of different established tissue culture cells and tissues. *Cell*, **14**, 795-804.
- Hunter, D. and Russell, D. S. (1954): Focal cerebral and cerebellar atrophy in a human subject due to organic mercury compounds. *J. Neurosurg. Psychiatry*, **17**, 235-241.
- Imura, N., Miura, K., Inokawa, M. and Nakada, S. (1980): Mechanism of methylmercury cytotoxicity: By biochemical and morphological experiments using cultured cells. *Toxicology*, **17**, 767-771.
- Kasama, H., Itoh, K., Omata, S. and Sugano, H. (1989): Differential effects of methylmercury on the synthesis of protein species in dorsal root ganglia of the rat. *Arch. Toxicol.*, **63**, 226-230.
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982): *Molecular Cloning: a Laboratory Manual* 1, Cold Spring Harbor Lab. Press, N.Y. p619.
- Matsumoto, G. and Sakai, H. (1979): Microtubules inside the plasma membrane of squid giant axons and their possible physiological function. *J. Membrane Biol.*, **50**, 15-22.
- McKnight, G. S. and Palmiter, R. (1979): Transcriptional regulation of the ovalbumin and conalbumin genes by steroid hormones in chick oviduct. *J. Biol. Chem.*, **254**, 9050-9058.
- Miura, K., Inokawa, M. and Imura, N. (1984): Effects of methylmercury and some metal ions on microtubule networks in mouse glioma cells and *in vitro* tubulin polymerization. *Toxicol. Appl. Pharmacol.*, **73**, 218-232.
- Miura, K., Suzuki, K. and Imura, N. (1978): Effects of methylmercury on mitotic mouse glioma cells. *Environ. Res.*, **17**, 453-471.
- Miura, K. and Imura, N. (1987): Mechanism of methylmercury cytotoxicity. *CRC Crit. Rev.*, **18**, 161-188.
- Nakada, S. and Imura, N. (1980): Stimulation of DNA synthesis and pyrimidine deoxyribonucleoside transport systems in mouse glioma and mouse neuroblastoma cells by inorganic mercury. *Toxicol. Appl. Pharmacol.*, **53**, 24-28.
- Nakada, S. and Imura, N. (1982): Uptake of methylmercury and inorganic mercury by mouse glioma and mouse neuroblastoma cells. *Neurotoxicology*, **3**, 249-258.
- Nakada, S., Nomoto, A. and Imura, N. (1980): Effect of methylmercury and inorganic mercury on protein synthesis in mammalian cells. *Ecotoxicol. Environ. Saf.*, **4**, 184-190.
- O'Farrell, P. H. (1975): High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, **250**, 4007-4021.
- Olmsted, J. B. and Borisy, G. G. (1973): Microtubules. *Ann. Rev. Biochem.*, **42**, 507-540.
- Omata, S., Horigome, T., Momose, Y., Kambayashi, M., Mochizuki, M. and Sugano, H. (1980): Effect of methylmercury chloride on the *in vivo* rate of protein synthesis in the brain of the rat: Examination with the injection of a large quantity of [^{14}C]valine. *Toxicol. Appl. Pharmacol.*, **56**, 207-215.
- Omata, S., Momose, Y., Ueki, H. and Sugano, H. (1982): *In vivo* effect of methylmercury on protein synthesis in peripheral nervous tissues of the

- rat. Arch. Toxicol., **49**, 203-214.
- Omata, S., Sakimura, K., Tsubaki, H. and Sugano, H. (1978): *In vivo* effect of methylmercury on protein synthesis in brain and liver of the rat. Toxicol. Appl. Pharmacol., **44**, 367-378.
- Pachter, J. S., Yen, T. J. and Cleveland, D. W. (1987): Autoregulation of tubulin expression is achieved through specific degradation of polysomal tubulin mRNAs. Cell, **51**, 283-292.
- Rodier, P. M., Aschner, M. and Sager, P. R. (1984): Mitotic arrest in the developing CNS after prenatal exposure to methylmercury. Neurobehav. Toxicol. Teratol., **6**, 379-385.
- Sager, P. R. (1988): Selectivity of methylmercury effects on cytoskeleton and mitotic progression in cultured cells. Toxicol. Appl. Pharmacol., **94**, 474-486.
- Sager, P. R., Aschner, M. and Rodier, P. M. (1984): Persistent, differential alteration in developing cerebellar cortex of male and female mice after methylmercury exposure. Dev. Brain Res., **12**, 1-11.
- Sager, P. R. and Matheson, D. W. (1988): Mechanisms of neurotoxicity related to selective disruption of microtubules and intermediate filaments. Toxicology, **49**, 479-492.
- Sager, P. R. and Syversen, T. L. M. (1984): Differential responses to methylmercury exposure and recovery in neuroblastoma and glioma cells and fibroblasts. Exp. Neurol., **85**, 371-382.
- Sager, P. R., Doherty, R. A. and Olmsted, J. B. (1983): Interaction of methylmercury with microtubules in cultured cells and *in vitro*. Exp. Cell Res., **146**, 127-137.
- Sarafian, T. A., Cheung, M. K. and Verity, M. A. (1984): *In vitro* methylmercury inhibition of protein synthesis in neonatal cerebellar perikarya. Neuropathol. Appl. Neurobiol., **10**, 85-100.
- Shelanski, M. L. and Taylor, E. W. (1967): Isolation of protein subunit from microtubules. J. Cell Biol., **34**, 549-554.
- Spiegelman, B. M., Penningroth, S. M. and Kirschner, M. W. (1977): Turnover of tubulin and the N site GTP in Chinese hamster ovary cells. Cell, **12**, 587-600.
- Syversen, T. L. M. (1977): Effects of methylmercury on *in vivo* protein synthesis in isolated cerebral and cerebellar neurons. Neuropathol. Appl. Neurobiol., **3**, 225-236.
- Syversen, T. L. M. (1982): Effects of repeated dosing of methylmercury on *in vivo* protein synthesis in isolated neurons. Acta. Pharmacol. Toxicol., **50**, 391-397.
- Tokunaga, K., Taniguchi, H., Yoda, K., Shimizu, M. and Sakiyama, S. (1986): Nucleotide sequence of a full-length cDNA for mouse cytoskeletal β -actin mRNA. Nucl. Acids Res., **14**, 2829.
- Verity, M. A., Brown, W. J., Cheung, M. and Czer G. (1977): Methylmercury inhibition of synaptosome and brain slice protein synthesis: *In vivo* and *in vitro* studies. J. Neurochem., **29**, 673-679.
- Vogel, D. G., Margolis, R. L. and Motted, N. K. (1985): The effects of methylmercury binding to microtubules. Toxicol. Appl. Pharmacol., **80**, 473-486.
- Vogel, D. G., Margolis, R. L. and Motted, N. K. (1989): Analysis of methylmercury binding sites on tubulin subunits and microtubules. Pharmacol. Toxicol., **64**, 196-201.
- Wang, D., Villaswante, A., Lewis, S. A. and Cowan, N. J. (1986): The mammalian β -tubulin repertoire: Hematopoietic expression of a novel, heterologous β -tubulin isotype. J. Cell Biol., **103**, 1903-1910.
- Wasteneys, G. O., Cadrin, M., Jones-Villeneuve, E. M., Reuhl, K. R., and Brown, D. L. (1988): The effects of methylmercury on the cytoskeleton of murine embryonal carcinoma cells. Toxicol. Cell Biol., **4**, 41-60.
- WHO (1976): Environmental health criteria 1; Mercury. World Health Organization, Geneva, p132.
- WHO (1990): Environmental health criteria 101; Methylmercury. World Health Organization, Geneva, p144.
- Yen, T. J., Machlin, P. S. and Cleveland, D. W. (1988): Autoregulated instability of β -tubulin mRNAs by recognition of the nascent amino terminus of β -tubulin. Nature, **334**, 580-585.
- Yoshino, Y., Mozai, T. and Nakao, K. (1966): Biochemical changes in the brain in rats poisoned with an alkylmercury compounds, with special reference to the inhibition of protein synthesis in brain cortex slices. J. Neurochem., **13**, 1223-1230.
- Yoshioka, T., Pant, H. C., Tasaki, I., Baumgold, J., Matsumoto, G. and Gainer, H. (1978): An approach to the study of intracellular proteins related to the excitability of the squid giant axon. Biochim. Biophys. Acta, **538**, 616-626.