—Minireviews—

Methylmercury Toxicity at Cellular Levels — From Growth Inhibition to Apoptotic Cell Death —

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This review describes our studies on the toxic effects of methylmercury (MeHg) at cellular levels using neuroblastoma, PC12, glioma and HeLa cell lines. MeHg specifically disrupted microtubules and inhibited cell growth by halting the cell cycle at the M phase. Effects on DNA, RNA and protein syntheses were not associated with growth inhibition by MeHg. Microtubule disruption by MeHg led to specific inhibition of β-tubulin synthesis with little or no effect on total protein synthesis. This selective reduction in β-tubulin synthesis was caused by post-transcriptional regulation through increased an tubulin pool resulting from depolymerization of microtubules by MeHg. The cells exposed to MeHg proceeded to apoptotic cell death long after growth inhibition. Since the occurrence of apoptosis was preceded by the G2/M phase arrest after MeHg treatment, it is likely that this arrest is an important event in apoptosis induction by MeHg. This apoptosis was induced *via* a p53-independent pathway in neuronal and nonneuronal cell lines. The study using the MeHg resistant cell line established by us demonstrated that the ability to accumulate MeHg and a low level of intracellular glutathione(GSH) made cells to vulnerable to MeHg. The neuronal cell lines showed a tendency to have a lower GSH level and, consequently, a higher susceptibility to MeHg than nonneuronal cell lines.

Key words —— methylmercury, growth inhibition, microtubule, apoptosis, glutathione

INTRODUCTION

Clinical and pathological studies have revealed that methylmercury (MeHg) causes neurological disturbances and fetal impairments. The ability of MeHg to easily pass through biological membrane seems to be responsible for its accumulation in brain and fetus through blood brain- or placenta-barriers, respectively.[1] Numerous studies on the adverse effects of MeHg at the biochemical level have been published,[1,2] however, little is known about the mechanism of selective damage caused by MeHg in neuronal cells. To determine the most susceptible intracellular target of MeHg and clarify the role of damage in its neurotoxicity, we conducted a series of experiments using cultured mammalian cells derived from neuronal and nonneuronal organs.[3–10]

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Cell Growth Inhibition by MeHg through Microtubule Disruption

The growth inhibitory effects of alkylmercury in cultured mammalian cells were reported to be on the order of concentrations of 3 to 4 $\times$ 10^{-6} M.[2] Cell growth was also inhibited by MeHg at 3—5 $\times$ 10^{-6} M in mouse neuroblastoma cells, PC12 cells and mouse glioma cells.[3,4] Parallel with the growth inhibition of mouse glioma cells, an increase in mitotic cells was observed. The mitotic index increased 2.0- to 2.5-fold after 3 to 6 hr exposure to MeHg and then decreased again within 25 hr.[3] This decrease in the mitotic index was associated with a simultaneous increase in multimicronucleated cells.[3] The incidence of

abnormal mitotic cells caused by alkyl mercury compounds was reported by Ramel (1967)[11] for *Alium cepa* and by Umeda *et al.* (1969)[12] in HeLa cells. It was suggested that organic mercury disrupts the mitotic apparatus, in which microtubules play an important role.

However, there was no direct evidence for the effect of organic mercury compounds on intracellular microtubules. Therefore, cells that remained in the mitotic stage after exposure to 5×10^{-6} M MeHg for 4 hr were observed by electron microscopy.[3] These mitotic cells displayed no microtubules in spite of the presence of centrioles and kinetochores. Furthermore, effects of MeHg on microtubules were found to be specific, because cell organelles other than microtubules were normal in such cells. This was the first time that microtubule disruption by MeHg had been demonstrated directly at cellular levels. From these results, we concluded that MeHg disrupted microtubules and inhibited cell growth by blocking the cell cycle from the M phase to the G1 phase. The formation of multimicronucleated cells was assumed to result from separation of the diffused chromosomes by newly formed nuclear membranes.

Major scientific interest in the toxicity of MeHg at the cellular and subcellular levels has long been focused on its effects on macromolecular biosyntheses. Chao *et al.* (1984)[13] claimed that the effects of mercury compounds on nucleic acid synthesis are closely related to cytotoxic effects. Using HeLa S3 cells Gruenwedel and Cruikshank (1979)[14] reported that DNA, RNA and protein syntheses were inhibited by MeHg in the order of DNA=RNA > protein, followed by reduced cell viability. Therefore, the sensitivity of microtubules to MeHg was compared with macromolecular biosyntheses in mouse glioma cells.[2] DNA, RNA and protein syntheses in mouse neuroblastoma and glioma cells were reduced by 50% at 2×10^{-5} M, 5×10^{-5} and above 2×10^{-5} M, respectively. Inhibition of DNA, RNA and protein syntheses was not caused at concentrations up to 10^{-5} M in glioma cells.[2] Thus, it was confirmed that the decrease in macromolecular biosyntheses was not associated with growth inhibition caused by MeHg at 5×10^{-6} M.

Effects of Mercurials and Other Metallic Compounds on *in Vitro* Tubulin Polymerization and Microtubule Networks

To estimate whether the effect on microtubules is due to characteristic features of MeHg as a metal, the effects of other metallic compounds on *in vitro* tubulin polymerization were examined using porcine tubulin.

Complete depression of tubulin polymerization was caused by 5×10^{-5} M MeHg.[5,6] Other metal ions, Hg^{2+}, Cu^{2+}, Cd^{2+} and Cr^{3+} inhibited tubulin polymerization at 2.5×10^{-5}, 2.5×10^{-4}, 5×10^{-4} and 2×10^{-4} M, respectively.[10] Among the compounds tested, Hg^{2+} showed the strongest tendency to depress *in vitro* tubulin polymerization.

The mechanism underlying the inhibition of polymerization is assumed to be interaction of these metals with tubulin SH groups. The inhibitory effects of Hg^{2+}, Cu^{2+} and Cd^{2+} on tubulin polymerization were closely correlated with the efficiency of these metals in blocking free SH groups of protein, *i.e.*, on the order of $Hg^{2+} > Cu^{2+} > Cd^{2+}$.[6] MeHg also has a high affinity for proteinsulfhydryls and acts much like the SH-reagents such as NEM and PCMPS.[15] The polymerization of tubulin was inhibited by binding of 1.3 moles of MeHg per tubulin dimer.

Next, the effects of these metals on intracellular microtubule networks were estimated by indirect immunofluorescence using anti-tubulin antiserum. Microtubule networks of the cells were examined after exposure to each metal for 30 min to 1 h at each growth inhibiting concentration.[5,6] After incubation for 30 min with MeHg, the density of the fibrous structure of microtubules was thinner than that of the control culture. Complete disappearance of microtubule networks occurred after 1 h of incubation. In contrast, the other metals had no effect on the microtubule networks after 1 h of exposure. From these results it was concluded that MeHg is the only chemical which disrupts microtubules when metals are added to the culture medium at each growth inhibiting concentration.

After publication of our data, Sager and Syversen (1984)[16] noted that the disruption and recovery of cytoplasmic microtubules depended on the cellular mercury level, *i.e.*, mercury concentrations of above 1.2 μg Hg/mg of cellular protein disrupted microtubules within 2 h. Sulfhydryl compounds (glutathione, cysteine, and

dimercaptosuccinic acid) added to the medium with MeHg effectively prevented microtubule disruption and restored microtubule polymerization even when added several hours after MeHg treatment. Their results strongly suggested that MeHg interacts directly with intracellular microtubules.

Methylmercury-induced Microtubule Depolymerization Leads to Inhibition of Tubulin Biosynthesis

Ben Ze'ev *et al.* (1979)[17] demonstrated that the concentration of unpolymerized cytoplasmic tubulin subunits regulates tubulin synthesis, which they referred to as an autoregulatory control system, *i.e.*, the increase of unpolymerized tubulin dimers resulting from microtubule depolymerization by colchicine causes inhibition of tubulin biosynthesis. These results suggested the possibility that MeHg not only disrupts microtubules but also interferes with tubulin synthesis.

To investigate the influence of microtubule disruption by MeHg on tubulin synthesis, [^{35}S]-methionine incorporation into tubulin protein was measured in cells previously exposed to MeHg for 3 h, which had completely depolymerized the microtubules. Two-dimensional gel electrophoresis showed reduction of β-tubulin synthesis by about 50% in glioma and neuroblastoma cells.[7] This reduction in tubulin synthesis was closely associated with a specific decline in β-tubulin mRNA levels. Furthermore, it was demonstrated that the reduced β-tubulin mRNA level was caused by a post-transcriptional change. Thus, it can be concluded that the disruption of microtubules by MeHg resulted in inhibition of the synthesis of tubulin itself through autoregulatory repression in the post-transcriptional processes as in the case of colchicine treatment.

Fernyhough and Ishii (1987)[18] demonstrated that an increase in tubulin mRNA is essential for neurite outgrowth in PC12 cell differentiation. The results suggest involvement of the depression of neuronal differentiation in MeHg-induced neurological abnormalities in prenatal exposure.

Abe *et al.* (1975)[19], on the other hand, observed that MeHg caused cessation of axonal flow in differentiated nerve cells. Based on their report and our above results, it was hypothesized that cessation of axonal transport by depolymerization of axonal microtubules leads to accumulation of tubulin subunits in the cell body and results in the suppression of tubulin synthesis through an autoregulatory control system.

Cell Growth Inhibition by MeHg Results in Apoptotic Cell Death

The cells, whose proliferation was depressed by MeHg, eventually died over time after treatment with MeHg, as reported in neuroblastoma, PC12, and HeLa cell lines.[4] Twenty-four hours after MeHg treatment, the cells became rounder but most retained the ability to exclude trypan blue. Fourty-eight hours after MeHg treatment, the number of shrunken or fragmented cells increased. Fluorescence microscopic observation after staining with Hoechst 33258 clearly showed typical apoptotic changes of these cells with chromatin condensation and nuclear fragmentation in PC12 and HeLa cells exposed to MeHg for 48 h. DNA fragmentation was not observed at 24 h, but was clearly apparent by 48 h in PC12 cells. These results indicated that MeHg at growth-inhibiting concentrations induced apoptosis in both neuronal and nonneuronal cell lines.[4]

Flow cytometric analysis revealed that the induction of apoptosis by MeHg was preceded by the G2/M-phase arrest, *i.e.*, a blockade of the cell cycle in the G2/M-phase first appeared at 8 h and thereafter apoptotic bodies increased from 19 h after MeHg-treatment.[4]

The expression of several proteins reported to be associated with apoptotic cell death was examined. First, it was confirmed that MeHg activated a p53-independent apoptotic pathway because the expression of p53 in cells exposed to MeHg was not enhanced until 48 h. This observation was supported by the evidence that taxol-induced mitotic block triggers p53-independent apoptosis in p53 null cells.[20]

Second, the possibility of apoptosis induction through bcl-2 phosphorylation was examined since Srivastava *et al.* (1998)[21] demonstrated that phosphorylation of bcl-2 by the microtubule-interfering agent colchicine led to activation of caspase 3, a key enzyme in apoptosis. However, in PC12 cells the phosphorylation of bcl-2 was not detected by treatment with either MeHg or colchicine.[4] In contrast, in HeLa cells bcl-2 was phosphorylated by treatment with both MeHg and colchicine.[4]

Thus, it was concluded that G2/M-phase

arrest caused by MeHg through microtubular disruption may play a key role in the induction of apoptosis, as in the case of colchicine. MeHg induces apoptosis through a p53-independent pathway, but the activation pathway following the disruption of microtubules differs in neuronal PC12 and nonneuronal HeLa cells.

Identification of Cellular Factors which Determine the Susceptibility of the Cells to MeHg

MeHg poisoning is characterized by selective damage to neuronal cells. However, few researchers have addressed the question of the special susceptibility of neuronal *versus* other cell types. To identify the factors that determine the susceptibility of cells to MeHg, stable sublines of PC12 cells resistant to MeHg were established by exposure to MeHg.[8,9] The most resistant (PC12/TM) cells exhibited MeHg resistance 8 to 10 times higher than that of parental PC12 cells.

The common characteristics of MeHg-resistant cell lines are the reduced accumulation of MeHg and an increased intracellular GSH level compared with the parent cells. This reduced accumulation resulted from slow uptake (PC12/TM : PC12=0.35 : 1.30 nmol/min) and rapid efflux (PC12/TM : PC12=0.086 : 0.030 nmol/min). An inverse correlation between MeHg resistance and MeHg accumulation was found among 7 sublines of PC12 cells with different sensitivities to MeHg ($p<0.01$, $r=0.91$).[8]

MeHg-resistant PC12/TM cells contained 4 times as much GSH as the parent PC12 cells.[8,9] The intracellular GSH level was correlated with the sensitivity to MeHg in PC12 sublines ($p<0.05$, $r=0.72$).[10] The depletion of GSH in PC12/TM cells to levels similar to those of PC12 cells clearly increased the sensitivity of PC12/TM cells to MeHg.[10]

Furthermore, even in various cell lines derived from different organs and animals, the intracellular GSH level was correlated with the sensitivity to MeHg ($p<0.05$, $r=0.77$).[10] GSH levels in neuronal cell lines (neuroblastoma, PC12 and PC12h), which were relatively sensitive to MeHg, were lower than those in nonneuronal cell lines (glioma, HeLa, L and colon 26).[10] The relatively low GSH level and the high sensitivity to MeHg observed in neuronal cells may explain, at least in part, the selective damage to neuronal cells observed in Minamata disease.

Remarks

Considering the high affinity of MeHg cation to SH groups, it appears reasonable to assume that a large number of cellular functions are affected by MeHg. Therefore, several cell functions were compared in our studies using the same cell lines by means of reactivity with MeHg. The data clearly indicated that MeHg specifically disrupts microtubules in cells. After causing this disruption, MeHg induced G2/M phase arrest, then growth inhibition, and finally apoptotic cell death. Our data were supported by those of Rodier *et al.* (1984)[22] who clearly showed the neurotoxic effect of MeHg through microtubule disruption in neonatal mouse brain by *in vivo* experiments.

The ability of the cells to accumulate MeHg and the level of intracellular GSH were found to be important determinants of cell susceptibility to MeHg. The neuronal cell lines tended to show lower GSH levels than nonneuronal cell lines. It is well known that GSH takes part in scavenging MeHg in the body as MeHg–GSH complex.[23] Thus, microtubules in neuronal cells may have more opportunity to bind with MeHg than they do in nonneuronal cells, and this may result in greater susceptibility to MeHg. Of course, GSH may scavenge free radicals that arise from MeHg-induced intracellular oxidation.[24]

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