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Effects of Methylmercury on Primary Cultured Rat Hepatocytes: Cell Injury and Inhibition of Growth Factor Stimulated DNA Synthesis

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Many more studies deal with the toxicity of methylmercury on nervous tissue than on its toxicity to the liver. Methylmercury accumulates in the liver in higher concentrations than brain and the liver has the primary function of detoxifying methylmercury. According to recent studies, hepatocyte mitochondrial membranes are destroyed by methylmercury (Desnoyers and Chang 1975) and DNA synthesis is inhibited by methylmercury during hepatocyte regeneration (Chen and Mottet 1980). Methylmercury alters the membrane ion permeability of isolate skate hepatocytes (Ballatori et al. 1988), and inhibits the metal-sensitive alcohol dehydrogenase and glutathione reductase of primary cultured rat hepatocytes(Hellström-Lindahl and Oskarsson 1989).

However, little is known about the effect of methylmercury on hepatocyte proliferation in primary cultured rat hepatocytes. We therefore used the primary cultured rat hepatocytes to investigate the effects of methylmercury on cell injury and growth factor stimulate DNA synthesis.

MATERIALS AND METHODS

Hepatocytes were isolated from male Wistar strain rats(200-250g) by the modified two-step collagenase perfusion technique (Seglen,1976). The viability of the isolated hepatocytes was determined by the trypan blue exclusion test. Cells were cultured in William's medium E (Flow Laboratories,Inc. Irvine, Scotland) supplemented with 10% fetal calf serum (Whittaker M.A.Bioproducts, Walkersville,Md.), 10^{-7}M insulin, 10^{-7}M dexamethasone, penicillin(100U/ml), streptomycin(100ug/ml), and fungizone (250ng/ml). Hepatocytes were plated at $1x10^{6}$ viable cells per 35 mm collagen-coated dish(Iwaki glass Co, Japan) and incubated at 37°C in a 5% CO_2 humidified atmosphere. The medium was replaced with the serum-free medium at 4h after cell plating. The medium was changed to fresh serum-free medium and methylmercury chloride was added at 24h after plating. The culture medium was collected at 3h, 6h, 12h and 24h after the addition of methylmercury, in

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order to assay the release of cytoplasmic enzymes, lactate dehydrogenase(LDH) and aspartate aminotransferase(AST) into the medium. At the same time, hepatocytes were harvested for the assay of intracellular glutathione. LDH and AST were determined photometrically using a serum autoanalyzer(Beckman Astra 8). Intracellular glutathione was extracted with 5% trichloroacetic acid by the method of Bannai et al.(1986), and was measured by the enzymatic method of Tietze (1969).

To assay DNA synthesis , $5x10^5$ viable cells were plated in 35mm collagen-coated dishes and were incubated in the same medium as described(serum- and hormone-supplemented medium). The medium was replaced with serum- and hormone-free medium at 4h after the cell plating. After further incubation for 20h, the medium was changed to serum-free medium and human epidermal growth factor (EGF: Earth Chemical Co.,Tokyo,Japan, 10ng/ml) or endothelial mitogen(Biomedical Technologies Ins.,Stoughton,Ma., 100ug/ml) was added. [^{3}H]-Thymidine(1.25uCi/ml, 300mCi/mmol, 1mCi=37MBq ICN Biomedicals Inc. Costa Mesa, Ca.)and methylmercury chloride were added at 12h after the addition of growth factors, and aphidicolin (Wako Chemical Co. Osaka, Japan, 10ug/ml)was added in partial dishes. Hepatocytes were harvested for measuring DNA synthesis at 36h after the addition of growth factors. DNA synthesis was measured by [^{3}H]-Thymidine incorporation into DNA by the method of Nakamura et al.(1983).

RESULTS AND DISCUSSION

Hepatotoxicity of methylmercury was determined by measuring the concentration of cytoplasmic enzymes released from cells into cultured medium in relation to that from the untreated control cells. A time course-analysis of LDH and AST release in the medium after the addition of methylmercury is shown in Fig.1. Fig.2 shows that there is a dose-dependent relationship between methylmercury and the release of LDH and AST after 24h exposure. LDH release from hepatocytes increased at 12h after the addition of $5X10^{-5}$M methylmercury as compared with that of the control. AST release into the medium slightly increased at 24h after the addition of $5x10^{-6}$M methylmercury compared with that of control, and decreased in concentrations ranging from $1X10^{-5}$M to $1x10^{-4}$M methylmercury. AST activity was inhibited by methylmercury at $5x10^{-5}$M. The release of cytoplasmic enzyme from hepatocytes is known to be a sensitive index for hepatic toxicants which cause the changes in membrane permeability and the rupture of cells (Tyson 1987). These results show that LDH release is a more useful indicator of hepatocyte injury by methylmercury than AST release.

The time- and dose-dependent relationship between methylmercury and intracellular glutathione contents in the cultured hepatocytes is shown in Fig.3. Intracellular glutathione contents decreased at 12h after the addition of $5x10^{-5}$M methylmercury and were depleted completely at 24 h. The depletion of intracellular glutathione might be caused by the inhibition of glutathione

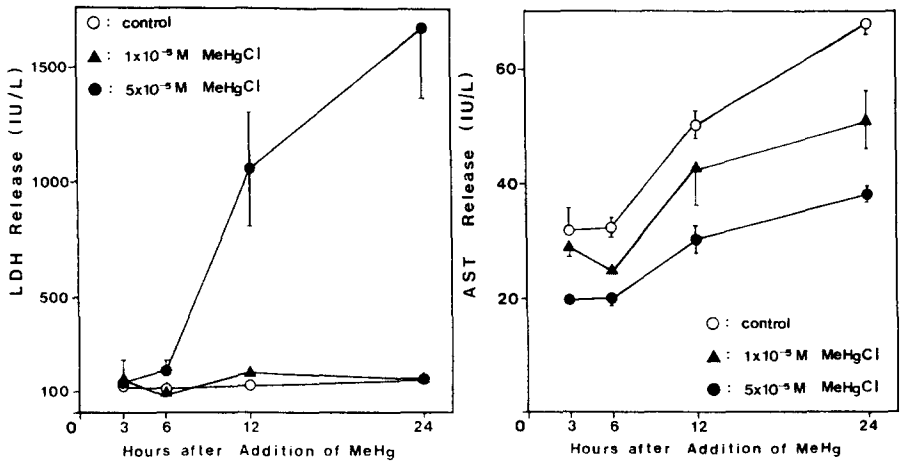

Figure 1. Time-dependent relationships between methylmercury (MeHg) and the release of lactate dehydrogenase(LDH) and aspartate aminotransferase(AST) from primary cultured rat hepatocytes into the culture medium. Each value is the mean±SD of three cultures.

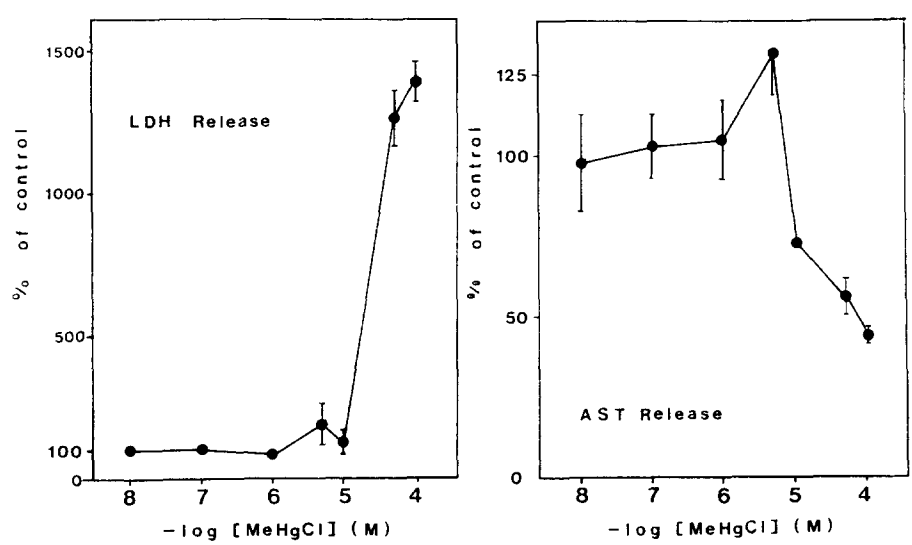

Figure 2. Dose-dependent relationships between methylmercury (MeHg) and the release of lactate dehydrogenase(LDH) and aspartate aminotransferase(AST) from primary cultured rat hepatocytes into the culture medium at 24h after the addition of MeHg. The data are expressed as a percentage of the LDH and AST release value to that in the control cultures. Each value is the mean±SD of three cultures.

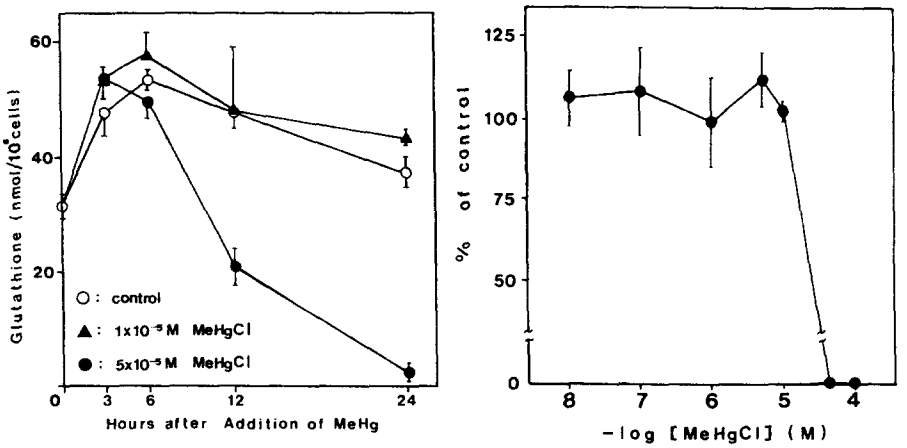

Figure 3. Time- and dose-dependent relationships between methylmercury(MeHg) and the intracellular glutathione levels of primary cultured rat hepatocytes. The data of the dose-dependent relationship are expressed as a percentage of intracellular glutathione levels to that of the control cultures at 24h after the addition of MeHg. Each value is the mean±SD of three cultures.

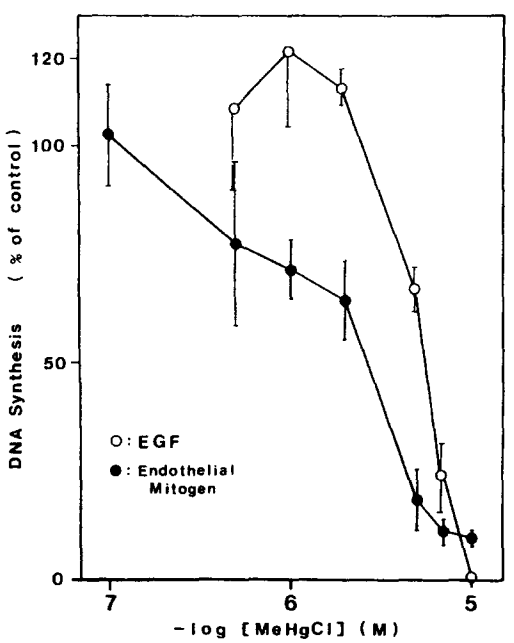

Figure 4. Dose-dependent relationships between methylmercury (MeHg) and DNA synthesis stimulated by EGF and endothelial mitogen in primary cultured rat hepatocytes at 24h after the addition of MeHg. The data are expressed as a percentage of [^{3}H]-Thymidine incorporation into DNA to that in the control cultures. Each value is the mean±SD of three cultures.

synthesis or the leakage of glutathione from the hepatocyte. These results indicate that the hepatotoxicity of methylmercury has a time- and dose-dependent relationship, and that increases of LDH release correlate with depletions of intracellular glutathione. Glutathione plays an important role in the detoxication of methylmercury on biliary secretion from the liver (Alexander and Aaseth 1982). These results suggest that intracellular glutathione may prevent the cytotoxicity of methylmercury on primary cultured rat hepatocytes.

DNA synthesis was assayed by measuring as [^{3}H]-Thymidine incorporation into DNA, and it was stimulated by two kinds of growth factors, EGF and Endothelial mitogen . Fig.4 shows the dose-dependent relationship between methylmercury and DNA synthesis for 24h exposure. EGF stimulated DNA synthesis was inhibited by 67% at $5x10^{-6}$M methylmercury. Endothelial mitogen stimulated DNA synthesis was inhibited by 71% at $1x10^{-6}$M methylmercury. Under the same condition, LDH release from hepatocytes into the medium and the depletion of intracellular glutathione were similar to the untreated control cultures. These results show that hepatocyte DNA synthesis is inhibited at lower concentrations of methylmercury, which is not caused by either LDH release or the depletion of intracellular glutathione. Therefore, it shows that DNA synthesis is the primary target of the cytotoxicity of methylmercury in cultured rat hepatocytes. In addition, these results indicate that methylmercury is a more effective inhibitor of endothelial mitogen stimulated DNA synthesis of primary cultured rat hepatocyte than EGF stimulated DNA synthesis.
Methylmercury inhibited two kinds of growth factors stimulated DNA synthesis at different concentrations. EGF is known to promote the DNA synthesis of primary cultured rat hepatocytes(Richman et al. 1976). Endothelial mitogen, a commercial preparation of crude bovine hypothalamus,contains acidic- and basic-FGF(Klagsbrun and Shing 1985). Both types of FGF also stimulate the proliferation of primary cultured hepatocyte(Kan et al. 1989; Hoffmann and Paul 1990). Hepatic EGF receptor and FGF receptor have been purified and characterized with kinase activity(Cohen et al. 1982; Hou et al. 1991). Methylmercury might cause the inhibition of EGF and FGF binding to each receptor and/or the autophosphorylation of the kinase domain of each receptor. In this study, hepatocyte proliferation was assayed as a DNA synthetic activity. These results indicate that methylmercury inhibits the proliferation of primary cultured rat hepatocytes. This is consistent with the results of the <u>in vivo</u> study that methylmercury inhibits the hepatocyte regeneration of hepatectomy rats (Chen and Mottet 1980).

This study demonstrates that methylmercury has a toxic effect on primary cultured rat hepatocytes, and that the primary effect of methylmercury is to inhibit hepatocyte proliferation assayed by DNA synthesis rather than to cause direct cell injury assayed by LDH release and the depletion of intracellular glutathione.

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