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Identification of Genes Associated with Early and Late Response of Methylmercury in Human Neuroblastoma Cell Line

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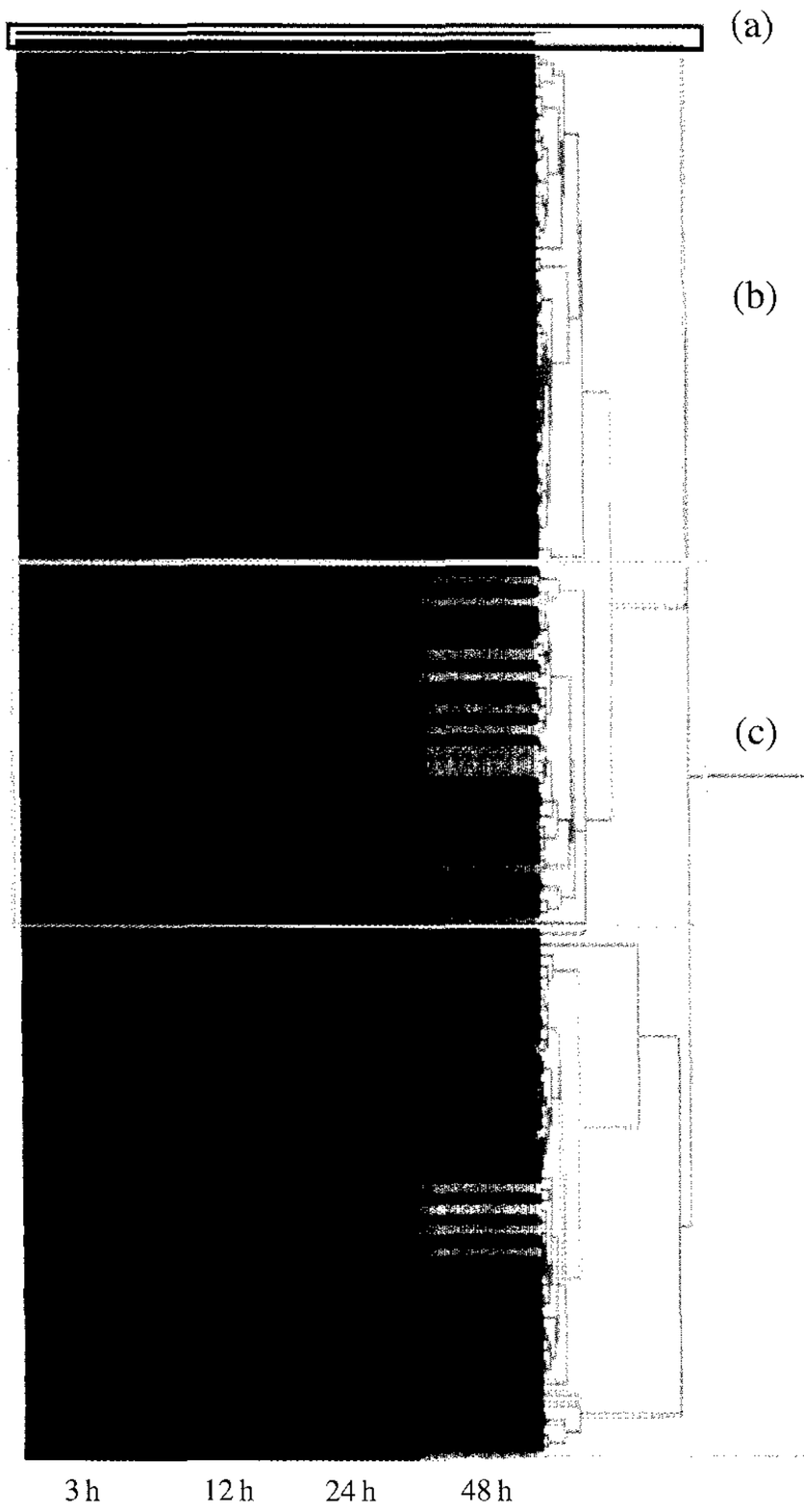
Abstract

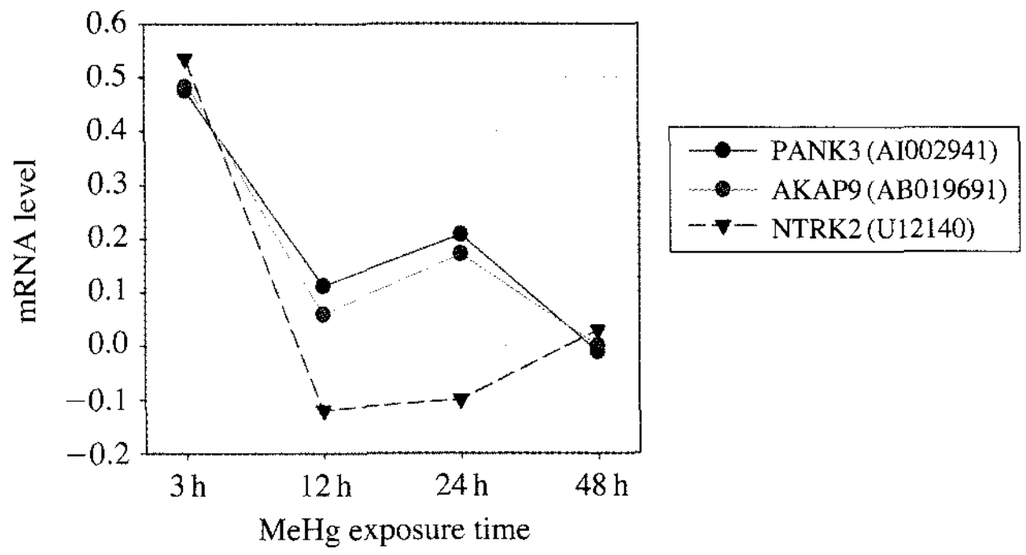
Methylmercury (MeHg) is known to have devastating effects on the mammalian nervous system. In order to characterize the mechanism of MeHg-induced neurotoxicity, we investigated the analysis of transcriptional profiles on human 8k cDNA microarray by treatment of 1.4 μM MeHg at 3, 12, 24 and 48 h in human neuroblastoma SH-SY5Y cell line. Some of the identified genes by MeHg treatment were significant at early time points (3 h), while that of others was at late time points (48 h). The early response genes that may represent those involved directly in the MeHg response included pantothenate kinase 3, a kinase (PRKA) anchor protein (yotiao) 9, neurotrophic tyrosine kinase, receptor, type 2 gene, associated with NMDA receptor activity regulation or perturbations of central nervous system homeostasis. Also, when SH-SY5Y cells were subjected to a longer exposure (48 h), a relative increase was noted in a gene, glutamine-fructose-6-phosphate transaminase 1, reported that overexpression of this gene may lead to the increased resistance to MeHg. To confirm the alteration of these genes in cultured neurons, we then applied real time-RT PCR with SYBR green. Thus, this result suggests that a neurotoxic effect of the MeHg might be ascribed that MeHg alters neuronal receptor regulation or homeostasis of neuronal cells in the early phase. However, in the late phase, it protects cells from neurotoxic effects of MeHg.

Keywords: Methylmercury, Human neuroblastoma cells, Transcriptional profile, Neurotoxic effect, Time responsive genes

Methylmercury (MeHg) is more toxic than other forms and efficiently absorbed into the body and crosses both the blood-brain barrier and the placental barrier[1]. It is known to be a potent neurotoxin and teratogen[2]. Its biological half-life in humans is about 70 days. It has also been shown that mercury is selectively concentrated in the human brain in the medial basal nucleus, amygdala, and hippocampus regions, all of which are involved with memory function, in the granular layer of the cerebellum, and in sensory neurons of the dorsal root ganglia (DRG). Also, the immature central nervous system (CNS) is extremely sensitive to MeHg neurotoxicity and the fetal brain may be affected even if the mother shows no signs of poisoning. Unlike focal damage in adults, the developing brain shows a diffuse and widespread damage. High-dose exposure may result in cerebral palsy, blindness, deafness, and severe mental retardation, due to degeneration and decrease in number of its nerve cells, disappearance of granule cells and reduction of Purkinje cells and white matter astrocytosis[3]. Lower MeHg doses may produce deficits in vision and hearing, delayed walking and speech development, and other subtle developmental problems. The dose-response analysis suggested that an increased risk of fetal brain damage occurs when mercury concentration in maternal hair exceeds 10 ppm (50 nmol/g). The increasing daily fish consumption as a part of a healthy diet may result in chronic low-level dietary intake of MeHg and thus poses a significant toxicological problem, especially to susceptible individuals, such as developing embryos and fetuses.

A number of molecular targets and mechanisms have been proposed to be implicated in its neurotoxic effects. The high affinity of MeHg for thiol groups makes proteins and peptides bearing cysteines susceptible to structural and functional modification by MeHg in all subcellular compartments[4,5]. In recent,





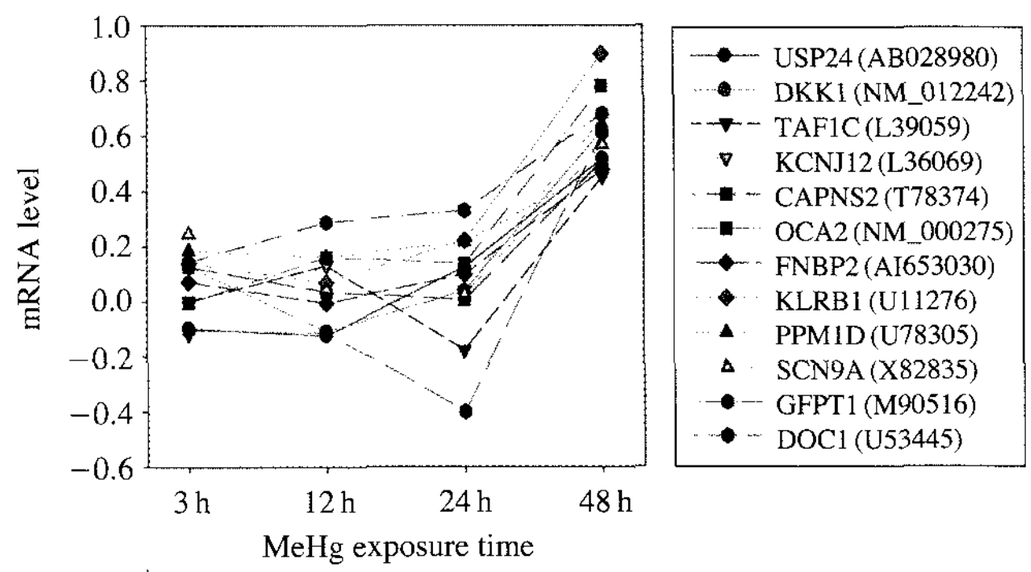


Figure 1. Cluster analysis of MeHg-induced expression profiles of human neuroblastoma SH-SY5Y cells. Cells were treated with 1.4 μM MeHg and then harvested at the indicated times for microarray experiments and the expression data were then analyzed by regression models as described in Materials and Methods. Representative clusters of genes were shown in the clustergram for their temporal pattern of expression. Transition of color for each gene from black to green indicates a gradual decrease in expression with time, and from black to red indicates up-regulation of gene expression. Down-regultation of selenoprotein W at late phase (a), up-regulated genes at early phase (3 h) (b), up-regulated genes at late phase (48 h) (c).

our laboratory elicited that the target protein may be selenoprotein W regulated transcription levels dependent of glutathione depletion in human neuronal cells exposed to MeHg[6]. Also, it is known that MeHg affects the transcription of a number of genes. MeHg induces some genes with protective functions, including those coding for metallothioneins that chelate mercury ions to make it biologically inert, heat shock proteins that play roles in renaturing damaged proteins and oxidative stress related genes[7]. However, these effects of MeHg on gene expression probably represent effects in rodent cells, and there might be a number of unidentified effects on genes relevant to the expression of neurotoxicity.

Therefore, by a comprehensive screening of time dependent altered gene expression with the new and powerful tool of DNA microarray technology, we have been trying to obtain a general picture of the neurotoxic effects caused by MeHg in human neuronal cells. Thus, we investigated the time effects on cell proliferation and the analysis of transcriptional profiles on human 8 k cDNA microarray by treatment of 1.4 μM MeHg at 3, 12, 24 and 48 h in human neuroblastoma SH-SY5Y cell line.

Cytotoxicity of Methylmercury in SH-SY5Y Human Neuronal Cells

Dose dependent cell viability curves were previously reported after from 3 h to 7 days of exposure to MeHg in SH-SY5Y cells, a human neuroblastoma cell line[6]. At 6 h, MeHg exhibited a median inhibition concentration (IC_{50}) of 6.25 μM. After a 48 h exposure,

MeHg showed an IC_{50} of 1.8 μM and IC_{20} of 1.4 μM, which was therefore used for all subsequent experiments.

Gene Expression Altered by Short Term Exposure of Methylmercury

The number of genes induced and repressed by 1.4 μM MeHg for 3 h 1.5 fold or greater were 19 and 6, respectively (Figure 1). The genes are shown in the Table 2. These genes were functionally related to signaling, cell cycle and transport control. Three of these genes specially induced at 3 h but was not changed significantly by 12, 24 and 48 h exposure of MeHg. These composed of PANK3 (*pantothenate kinase 3*: coenzyme A biosynthesis), AKAP9 (*a kinase (PRKA) anchor protein (yoitao) 9*: signal transduction; synaptic transmission) and NTRK2 (*neurotrophic tyrosine kinase, receptor, type 2*: signal transduction; neurogenesis). Especially, we confirmed mRNA gene expression using real time RT-PCR on AKAP9 and NTRK2 (Figure 2).

Table 1. Primer sequences used for real time RT-PCR of early and late response genes by MeHg.

Gene	Primer sequences
AKAP9-F	5′-TGATGGAAAGTGAGTTGGCTGGGA-3′
-R	5′-TGCAGTCCTTCAGTCCCATAGGTAAC-3′
NTRK2-F	5′-GCAGGTGATCCGGTTCCTAA-3′
-R	5′-TGTGTGGCTTGTTTCATTCATATG-3′
USP24-F	5′-TTCTCTGGCAGTGAGGGTTTCTGT-3′
-R	5′-GTCCGTTTCTCTAAGGGTACAGTTCTGC-3′
DKK1-F	5′-TCATCAGACTGTGAATCAGGATTGTG-3′
-R	5′-TTCTGTATCCGGCAAGACAGACCTTC-3′
GFPT1-F	5′-ATGCTGGGTGTTGCATTTCTGGAC-3′
-R	5′-ACACTGCCACTGGATTTAGATACTCC-3′
KLRB1-F	5′-CAGACTCAGGCCCAGAAAGTTCTTCA-3′
-R	5′-TCAGGGCAAATTGATGCCAAGGTG-3′
SeW-F	5′-CGCTTCATGATAGGAAGGACTGA-3′
-R	5′-AACAGCAGCCACGAGAACATC-3′

AKAP9: A kinase (PRKA) anchor protein (yoitao)9, NTRK2: Neurotrophic tyrosine kinase receptor, type 2, USP24: Ubiquitin specific protease 24, DKK1: Dickkopf homolog 1 (Xenopus laevis), GFPT1: Glutamine fructose phosphate transaminase 1, KLRB1: killer cell lectin-like receptor subfamily B, member 1, SeW: selenoprotein W, F: forward primer sequence, R: reverse primer sequence. All primer sequences were determined with established GenBank sequences.

Gene Expression Altered by Long Term Exposure of Methylmercury

The number of genes induced and repressed by 1.4 μM MeHg for 48 h, 1.5 fold or greater were 47 and 10, respectively (Figure 1). The genes are shown in the Table 2. These genes were functionally related to signaling, ion transport, metabolism, regulation of transcription and proliferation control. Nine of these genes specially induced at 48 h but was not changed significantly by 3, 12 and 24 h exposure of MeHg. These composed of USP24 (*ubiquitin specific protease 24*: ubiqitination), DKK1 (*dickkopf homolog 1*: Wnt receptor signaling pathway), TAF1C (*TATA box binding protein (TBP)-associated factor, RNA polymerase 1, C, 110 kDa*: transcription), KCNJ12 (*potassium inwardly-rectifying channel, subfamily J member 12*), CAPNS2 (*calpain small subunit 2*), FBP2 (*forming binding protein 2*: metal ion transport), PPM1D (*protein phosphatase 1D magnesium dependent, delta*

Table 2. List of early and late response genes by 1.4 μM MeHg in SH-SY5Y cell line.

Accesstion No.	Gene name	Gene symbol	Expression ratio	
			3 h	48 h
Early response genes				
AI002941	Pantothenate kinase 3	PANK3	0.4758	−0.0111
AB019691	A kinase (PRKA) anchor protein (yoitao)9	AKAP9	0.4824	−0.0010
U12140	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	0.5354	0.0291
Late response genes				
U67171	Selenoprotein W	SeW	0.1272	−1.1180
AB028980	Ubiquitin specific protease 24	USP24	−0.0976	0.5177
NM_01224	Dickkopf homolog 1 (Xenopus laevis)	DKK1	0.1271	0.6244
L39059	TATA box binding protein (TBP)-associated factor, RNA polymerase I, C, 110 kDa	TAF1C	0.0009	0.4533
L36069	potassium inwardly-rectifying channel, subfamily J, member 12	KCNJ12	−0.1169	0.664
T78374	Calpain small subunit2	CAPNS2	0.1268	0.5104
AI653030	Forming binding protein 2	FNBP2	0.0719	0.4786
U78305	protein phosphatase 1D magnesium-dependent, delta isoform	PPM1D	0.1848	0.4832
X82835	sodium channel, voltage-gated, type IX, alpha	SCN9A	0.2464	0.5685
M90516	Glutamine fructose phosphate transaminase 1	GFPT1	0.1432	0.6791
NM_013431	killer cell lectin-like receptor subfamily B, member 1	KLRB1	0.1401	0.8955

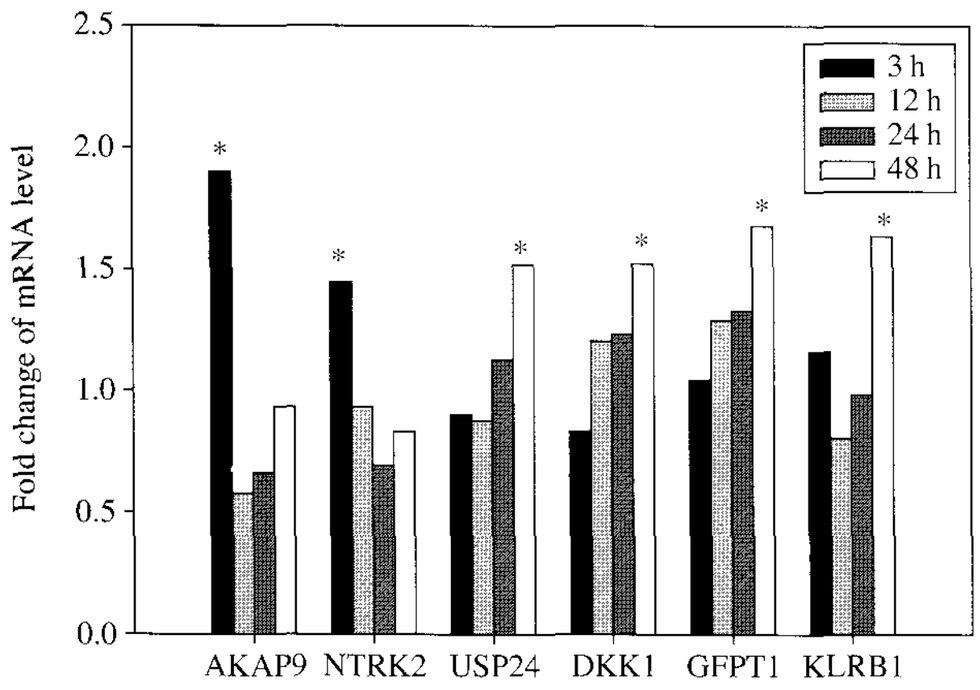


Figure 2. mRNA expression of early and late response genes in cells treated 1.4 μM MeHg examined in each time points. mRNA levels of each genes and GAPDH were determined by real time RT-PCR using SYBR green. Data were expressed as fold change of the mean ratio of each gene mRNA abundance/GAPDH mRNA abundance obtained for each treated cells. Results are shown as means ± SD from three independent experiments. Statistical significance was assessed by a One-way analysis of variance (ANOVA), $*P < 0.05$.

isoform: regulation of cell cycle), SCN9A (*sodium channel, voltage-gated, type IX, alpha*), KLRB1 (*killer cell lectin-like receptor subfamily B, member 1*: signal transduction) and GFP1 (*glutamine-fructose-6-phosphase transaminase 1*). Especially, we confirmed mRNA gene expression using real time RT-PCR on USP24, DKK1, GFP1 and KLRB1 (Figure 2).

Discussion

MeHg is known to have devastating effects on the mammalian nervous system. In order to characterize the mechanism of MeHg-induced neurotoxicity, we investigated the analysis of transcriptional profiles on human 8 k cDNA microarray by treatment of 1.4 μM MeHg at 3, 12, 24 and 48 h in human neuroblastoma SH-SY5Y cell line. The MeHg response of some of the identified genes was significant at early time points (3 h) that of others was at late time points (48 h).

In the report of Wilke *et al.*[7], to help determine which molecular processes were modified by MeHg in the lower concentration range, the transcriptional effect of 1 μM MeHg was then characterized in two distinct neuronal model systems, freshly dissociated rat DRG neurons and PC12 cells. Of the 1,032 genes tested, 189 were associated with greater than a two-fold expression changes (either up or down) in DRG neurons. Fifty-eight of these 189 genes (~32%) could be linked to previously characterized antioxidant defense mechanisms. To ascertain whether the relative distribution of transcriptional changes associated with exposure to MeHg is altered over time, analysis performed in PC12 cells at 6 and 24 h. The more prolonged exposure time was associated with a relative shift toward cell cycling and apoptosis. Of the 1,032 genes studied, 254 demonstrated greater than a two-fold expression change (either up or down) in response to 24 h of 1 μM MeHg. Thirty-eight of these 254 genes (~16%) represented genes assigned to the apoptosis category. At an earlier (6 h) time point, only 17 genes in this category (~9%) were associated with a similar change. This analysis suggested that apoptosis might be occurring in sensory neurons, as well, after 24 h exposure to 1 μM MeHg.

In our study, of the early response genes that may represent those involved directly in the MeHg response, AKAP9 is known to regulate NMDA receptor activity[8,9]. And in the case of NTRK2, perturbations of central nervous system homeostasis have been shown to lead to up regulation of this mRNA.

Also, when SH-SY5Y cells were subjected to a longer exposure (48 h), a relative increase was noted in a gene, glutamine-fructose-6-phosphate transaminase 1 (GFP1). Over-expression of GFP1, the gene encoding GFAT, may lead to the increased resistance to MeHg seen in the study of Miura *et al.*[10]. Thus, there is a possibility that GFAT is an intracellular target of MeHg. DKK1 negatively modulates the canonical Wnt signaling pathway, thus activating the tau-phosphorylating enzyme glycogen synthase kinase-3beta[11]. DKK1 was also expressed by degenerating neurons in the brain from Alzheimer's patients, where it colocalized with neurofibrillary tangles and distrophic neurites. Hwang *et al.*[12] report the hypothesis that MeHg induces the cellular accumulation of a certain protein(s) that causes cell damage and that this protein(s) is degraded after its ubiquitination in proteasomes. USP24 may be affect in this process. Also, since the function of neurons depends on voltage gated ion channels, reduction by MeHg voltage-activated calcium channel currents (VACCCs) is important. By study of Busselberg[13], MeHg reduced potassium channel currents and, at higher concentrations, sodium channel currents. Thus, up regulation of KCNJ12 gene may be associated with change of potassium channel currents.

In addition, MeHg treatment to neuroblastoma cells in the late phase (24 h and 48 h) resulted in a reduction of SeW (selenoprotein W) mRNA level (data not shown). From this result, we previously reported that SeW would be a molecular target of MeHg in SH-SY5Y, a human neuroblastoma cell line and the effects on SeW were further characterized by the mea-

surement of intracellular ROS and GSH induced by MeHg and by addition of Se or GSH[6]. While the precise function of SeW has yet to be elucidated, the results of research have yielded clues[14-16]. This binding with GSH indicated that like other selenoenzymes, SeW may be exerting its activity through a redox mechanism.

Thus, this result suggests that a neurotoxic effect of the MeHg might be ascribed that in the early phase, MeHg alters neuronal receptor regulation or homeostasis of neuronal cells but in the late phase, it protects cells from effects of MeHg. Also SeW as a marker gene of MeHg could be identified in human neuronal cells. Overall, this study may provide information concerning the mechanisms and the possible marker genes on its complicated cellular response to neurotoxicity of MeHg.

Methods

Cell Culture and Treatment

SH-SY5Y cell line was purchased from American Type Culture Collection (CRL-2266) and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The culture medium was 90% culture medium (50% F-12 and 50% MEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) plus penicillin, streptomycin and non-essential amino acid. The medium was renewed every two days. After reaching to 80% confluency, cells were exposed to various concentrations of MeHg (Aldrich, USA) in culture medium for each exposure time. For the cytotoxicity assay, SH-SY5Y cells were seeded at a density of 0.2×10^6 cells/mL and treated with 0.1-10 μM for various times up to 7 days. At each time point, the number of viable cells was determined by MTT assay and cell viability was calculated. The 20% inhibitory concentration (IC_{20}) of a particular agent was defined as that concentration that causes a 20% reduction in the cell number versus the untreated control. The IC_{20} values were directly determined from the semi-logarithmic dose-response curves. All experiments were carried out at least in triplicate.

Microarrays

GenePlorer TwinChip Human-8K was obtained from Digital Genomics (Seoul, Korea). This array contained approximately 8, 170 expressed sequence tags (ESTs/cDNA elements) corresponding to known genes in the GenBank database. All the EST clones have been sequence verified. The array also contained 192 spots of total genomic DNA and 168 housekeeping genes, which serve as reference points for the image analysis software, for normalization and for verifying the homogeneity of the hybridization.

RNA Extraction, Probe Preparation and Labeling, Hybridization and Scanning

RNA was extracted from the SH-SY5Y cells treated to 1.4 μM MeHg for each time using the Trizol reagent (Invitrogen, USA). The quality and integrity of the prepared total RNAs were confirmed with the use of an Agilent 2100 bioanalyzer (Agilent, USA), and by spectrophotometry. Fluorescent labeled cDNA for the microarray analysis was prepared by the reverse transcription of the total RNA in the presence of aminoallyl-deoxyuridine triphosphate (dUTP). This was followed by the coupling of the Cy3 dye for the controls (DMSO) or Cy5 dye for the treated samples (1.4 μM MeHg) at each time points (3, 12, 24 and 48 h) (Amersham-Pharmacia, Sweden). The microarray was hybridized with a mixture of the fluorescent labeled cDNAs from the control and the treated cells of each time point at 58°C for 16 h and then they were washed. After the washing procedure, the microarray was scanned using a ScanArray Lite (Perkin-Elmer Life Sciences, USA). The scanned images were analyzed with GenePix 3.0 software (Axon Instruments, USA) to obtain the gene expression ratios (treated vs. control). Logged gene expression ratios were normalized by a LOWESS regression[17]. The genes were considered differentially expressed when the logarithmic gene expression ratios in four independent hybridizations were more than a 0.5-fold difference in the expression level with 5% of q value cutoff. For the selected genes, the patterns of gene expression were log transformed, centered by median, and subjected to cluster analyses by centered correlation and average linkage as the similarity/distance metric, using the hierarchical cluster algorithm in Cluster and TreeView software suite.

Quantitative RT-PCR Analysis

The mRNA levels for the selenoenzymes in SH-SY5Y, human neuroblastoma cells were analyzed by real time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, USA). The purified total RNAs were reverse-transcribed into cDNAs by using an Omniscript RT kit (Qiagen, USA). The Primer specificity was tested by running a regular PCR for 40 cycles at 95°C for 20 s and 60°C for 1 min, and followed by an agarose gel electrophoresis. The real time RT-PCR was performed by using a SYBR supermix kit (Bio-Rad, USA), and running for 40-45 cycles at 95°C for 20 s and 60°C for 1 min. The PCR efficiency was examined by serially diluting the template cDNA and the melting curve data were collected to

check the PCR specificity. Each cDNA sample was triplicated and the corresponding no-RT mRNA sample was included as a negative control. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was included in every plate to avoid sample variations. The mRNA level of each sample for each gene was normalized to that of the GAPDH mRNA. Relative mRNA level was presented as 2[(Ct/GAPDH-Ct/gene of interest)]). All data shown were the mean ± SD of three separate experiments. For quantitative real time RT- PCR, primers used were as Table 1.

Acknowledgements

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