Effects of Cadmium Chloride on Neurite Outgrowth and Gene Expression in Human Neuroblastoma NB-1 Cells

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Cadmium is a well-studied environmental toxicant. The neurotoxicity of cadmium is a concern, especially in the developing human brain, because it is thought that cadmium exerts long-term and irreversible effects. In the present study cadmium toxicity using human neuroblastoma NB-1 cells was investigated in relation to gene expression and subsequent neurite extension using cDNA macroarray and image analysis techniques. The neurite outgrowth in NB-1 cells was stimulated significantly by the presence of dibutyryl-cyclicAMP (db-cAMP) and by cadmium chloride at sublethal concentrations. Db-cAMP-stimulated neurite outgrowth was associated with a three-fold increased expression of axonal membrane protein growth-associated protein-43 (GAP-43), but cadmium chloride did not affect GAP-43 expression. Db-cAMP also increased dopamine β-hydroxylase gene expression eight-fold and down-regulated muscle/brain cAMP-dependent protein kinase inhibitor gene expression. However, cadmium chloride had no effect on gene expression except for that of metallothionein II (mtII) gene among 1764 genes analyzed. The results demonstrate that the increased neurite outgrowth with cadmium chloride is not associated with the same gene expression profile of that with db-cAMP.

Key words —— human neuroblastoma cell, neurite extension, cadmium chloride, cDNA macroarray

INTRODUCTION

Environmental pollutants have long-term effects on cellular development. Chronic exposure to pollutants like cadmium via food and drinking water is a major human health concern. The transition metal cadmium has been widely used in industry. Toxic doses of transition metals are capable of disturbing the natural oxidation/reduction balance in cells through various mechanisms stemming from their own complex redox reactions with endogenous oxidants and effects on cellular antioxidant systems, which deranges the cellular signaling and gene expression systems.[1)] Ultimately, there is a variety of toxic effects, including apoptosis and carcinogenesis.[2–5)] Cadmium-induced apoptosis occurs through major mitogen-activated protein kinases, which have been shown to regulate apoptosis.[6)]

Different routes of cadmium uptake, such as via airborne particles, smoking, drinking water, and food have been identified.[7)] Cadmium uptake into cells occurs by simple diffusion and cadmium also utilizes the transport pathways for calcium, zinc, and copper.[8)] Cadmium uptake also requires interaction with membrane sulfhydryl groups.[8,9)]

The toxic effects of cadmium in the brain are poorly understood. Although it is reported that cadmium modifies pituitary hormone secretion and amine metabolism in the central nervous system (CNS), the changes in pituitary hormone secretion do not correlate with the modification of CNS metabolism of the neurotransmitters involved in their regulation.[10)] It is difficult for foreign substances to cross the blood-brain barrier, but it is thought that during the brain development, when the blood-brain barrier is not well established, cadmium may enter the brain and exert its toxic effects on brain development.

To investigate the effects of cadmium on the neuronal differentiation, human neuroblastoma NB-

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1 cell is one of the useful tools, because NB-1 cells differentiate in *in vitro* culture system and extend neurites, which is stimulated by the presence of dibutyryl-cyclicAMP (db-cAMP).[11,12] In this report, we studied the neurite outgrowth of NB-1 cells with the gene expression in the presence or absence of cadmium and compared with the positive neurite outgrowth regulator, db-cAMP.

MATERIALS AND METHODS

Materials —— Cadmium chloride and db-cAMP were from Wako Pure Chemicals. [α-^{32}P] dCTP was purchased from Amersham. The human Atlas™ 1.2 and neurobiology cDNA array membranes were obtained from Clontech (CA, U.S.A.). All other tissue culture media, sera, and supplements were from Gibco BRL (MD, U.S.A.).

Cell Culture —— Human neuroblastoma NB-1 cells were cultured in 45% RPMI and 45% Eagle's minimum essential medium containing 10% fetal bovine serum. Cells were subcultured once a week at a split ratio of 1 : 6.[13]

cDNA Array —— Total RNA was prepared with TRIzol (Gibco BRL, MD, U.S.A.) as described in the user's manual. Poly A$^+$ RNA was concentrated with biotin-labeled magnetic beads (Roche Diagnostics). To remove the genomic DNA completely, concentrated poly A$^+$ RNA was digested with DNase I and then phenol-chloroform extracted followed by ethanol precipitation. Poly A$^+$ RNA 1 μg was used for reverse-transcription reaction, which was carried out with Superscript II reverse transcriptase (Gibco BRL, MD, U.S.A.) and [α-^{32}P] dCTP. Reverse-transcribed products were digested with *E. coli* RNase A and double stranded DNA was synthesized with exo-Klenow fragments in the presence of [α-^{32}P] dCTP using the standard protocol. Labeled double-stranded cDNA were purified on a filtration column (Clontech, CA, U.S.A.) and hybridized with the Atlas™ 1.2 and neurobiology membrane, which was prehybridized with salmon sperm DNA for 2–4 hr. After 8–12-hr hybridization at 68°C, the membrane was washed several times with 2 × SSC/0.1% SDS followed by washing two times with 0.1% SSC/0.1% SDS at 68°C. The image was analyzed with BAS 2000 (Fujifilm, Tokyo, Japan), and relative radioactivity was measured with Arraygauge software from Fujifilm (Tokyo, Japan).

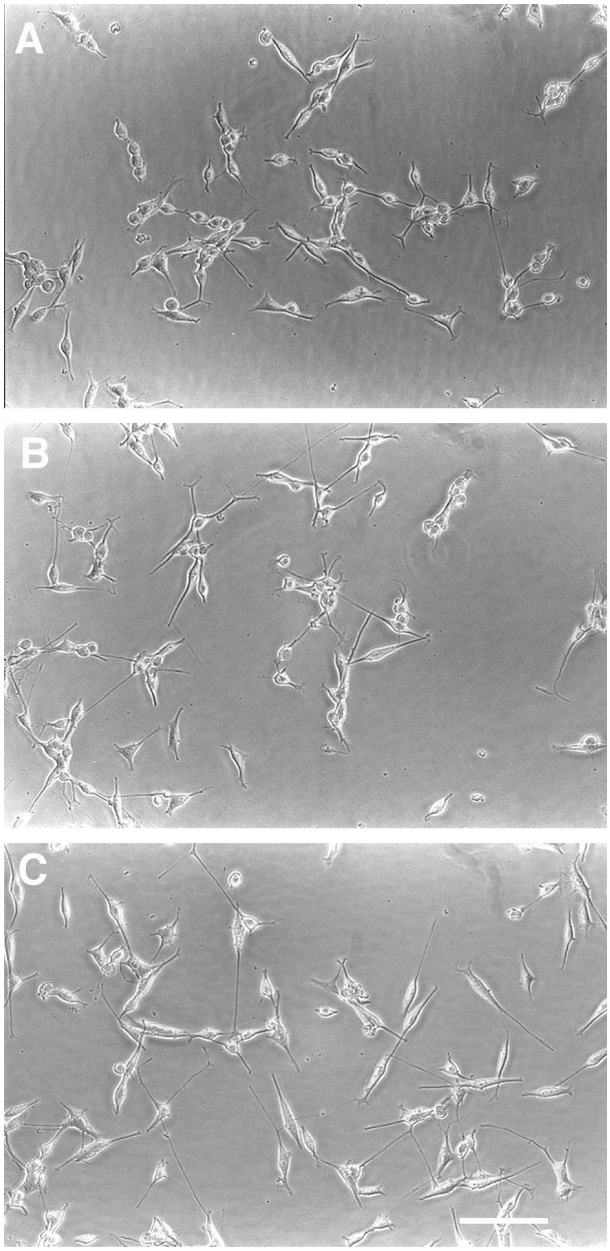

Fig. 1. Phase-Contrast Micrographs of NB-1 Cells

NB-1 cells precultured for 1 day were further cultured in the absence (A) or presence (B) of dibutyryl cAMP 2 mM, or in the presence of cadmium chloride 5 μM (C) for 2 days. Scale bar, 40 μm.

RESULTS AND DISCUSSION

To investigate the effects of cadmium on neuronal differentiation, human neuroblastoma NB-1 cells were cultured in the presence or absence of cadmium chloride and db-cAMP, a positive regulator of neuronal cell differentiation. As shown previously,[13] db-cAMP at 1 mM stimulates the neurite outgrowth of NB-1 cells (Fig. 1B). Cadmium chloride at concentrations as high as 5 μM cadmium chloride had no toxic effect on NB-1 cells viability but

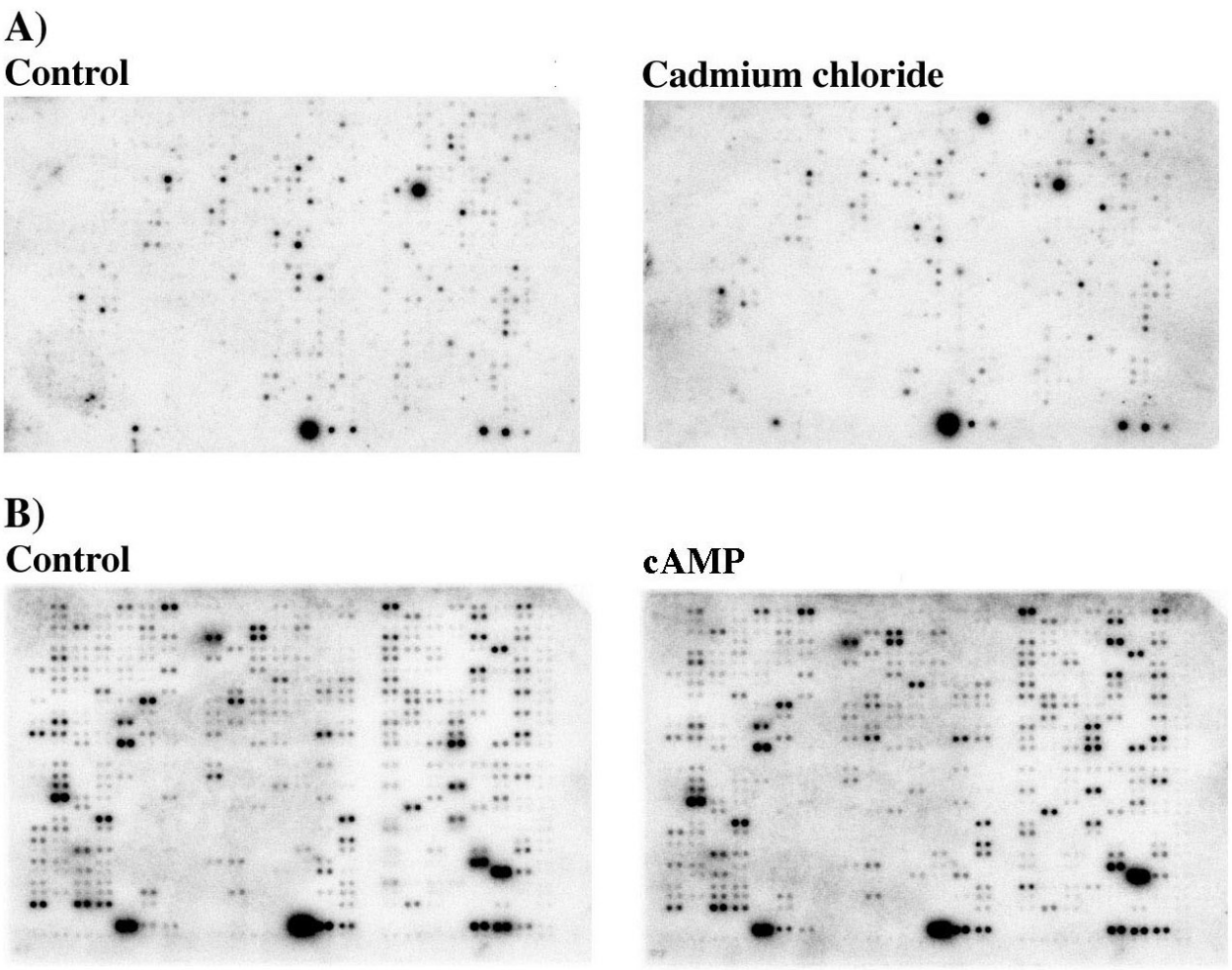

Fig. 2. cDNA Array Analysis of Genes Expressed in NB-1 Cells

NB-1 cells precultured for 1 day were further cultured in the absence or presence of dibutyryl cAMP 2 mM, or in the presence of cadmium chloride 5 μM for 2 days. Poly A$^+$ RNA isolated from the cells was reverse-transcribed to cDNA using gene-specific primers. cDNA array membranes (A: Atlas™ 1.2 and B: human neurobiology membrane) were hybridized with [α-^{32}P] dCTP-labeled double-stranded cDNA. After washing, the membranes were exposed to X-ray films at –80°C overnight.

significantly stimulated neurite outgrowth (Fig. 1C).

Neurite outgrowth is the consequence of related gene expression and their functions. To clarify the involvement of genes in the neurite outgrowth process in NB-1 cells, cDNA macroarray was performed in the presence or absence of cadmium chloride at 5 μM or db-cAMP at 1 mM (Fig. 2). Gene expression was quantified by measuring of relative radioactivity. Cadmium chloride enhanced metallothionein II (mtII) gene expression by about 20-fold (Fig. 3) but did not affect the expression of other genes on the membrane. On the other hand, db-cAMP had no effect on mtII gene expression but it induced the dopamine β-hydroxylase and axonal membrane protein growth-associated protein-43 (GAP-43), and also reduced the muscle/brain cAMP-dependent protein kinase inhibitor, which have already been reported.[13,14] The up-regulation of GAP-43 with db-cAMP explains its involvement in the neurite outgrowth process.[15] However, cadmium chloride did not affect the expression of those genes. These results indicate that the increased neurite outgrowth with cadmium is not associated with the same gene expression profile as that with db-cAMP. While it is not yet known how cadmium exerts its effect, it may perturb the normal neuritogenesis during brain development, which is controlled precisely by internal and external signals.

Gotti *et al*. have reported that long-term cadmium exposure of human neuroblastoma cell line IMR32 did not interfere with neurite outgrowth but increased the expression of cholinergic receptors, α-bungarotoxin binding sites, and muscarinic receptors.[16] However, our observations indicated an in-

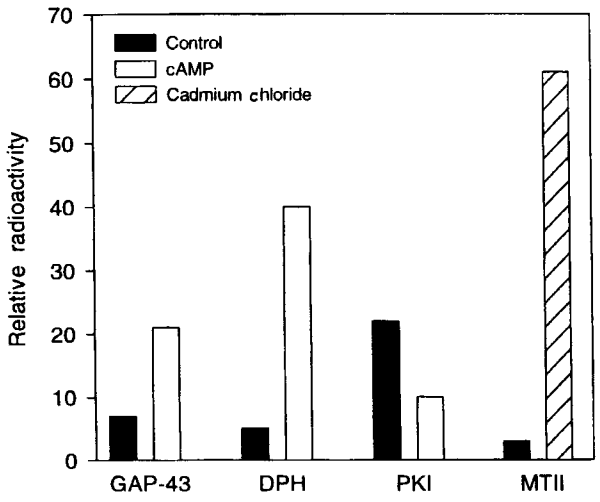

Fig. 3. Altered Gene Expression in NB-1 Cells with Culture with db-cAMP or Cadmium Chloride

Radioactivity of the cDNA array membranes prepared as described in Fig. 2 were measured with BAS 2000 and the relative radioactivity of each spot was analyzed with Arraygauge software. GAP-43, growth associated protein-43; DPH, dopamine β-hydroxylase; PKI, protein kinase inhibitor; mtII, metallothioneine II.

crease in neurite outgrowth in NB-1 cells. This discrepancy may result from the differences in the cell type or in the dose and duration of cadmium exposure.

In this report, only a limited number of genes, which were spotted on the membrane, were analyzed (Fig. 3). To clarify the mechanism of the toxic effects of cadmium in neurons and on human brain development, further investigation is needed in which all expressed transcripts are analyzed. In the future, newly developed techniques such as serial analysis of gene expression (SAGE) will be performed to obtain the complete gene expression profile with cadmium, and the mechanisms of cadmium-induced neurotoxicity will be revealed.

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