Cadmium Causes Increases of N-myc and Multidrug-resistance Gene mRNA in Neuroblastoma Cells

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Abstract : Since cadmium exposure results in neuropathological alterations in central nervous system, we investigated the effects of cadmium on the gene expression of neuroblastoma (GOTO) cells. We observed an increase in mRNA levels of heat-shock protein (hsp) 70, hsp 90, hsp 32 and metallothionein after treatment of GOTO cells with cadmium, although the time courses of the changes of individual mRNA of the heat-shock proteins and metallothionein were somewhat different from each other. An accumulation of N-myc and multidrug-resistance gene (MDR1) mRNA was detected in the presence of cadmium. This is contrary to the previous report, in which an inverse correlation between the expression of MDR1 gene and N-myc oncogene in human neuroblastoma had been described. However, the increase of N-myc and MDR1 mRNA in the present study is not likely due to the loss of regulatory mechanism of these genes by cytotoxic effects of cadmium, because active protective mechanisms such as heat-shock proteins and metallothionein could be induced under these conditions.

Key words : cadmium, metallothionein, heat-shock protein, N-myc, multidrug-resistance gene.

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Introduction

Cadmium is an ubiquitous heavy metal toxicant found throughout the industrial world and accumulating in the environment. Occupational exposure to cadmium may cause kidney, lung and prostate cancers (Sunderman, 1977; Hallenbeck, 1984). The carcinogenic activity of cadmium compounds has been reported by many investigators. Cadmium has been reported to induce chromosomal aberrations (Ochi *et al.*, 1984), transform cultured cells and also to enhance viral transformation (Terracido & Nachtigel, 1986; Casto *et al.*, 1979). On the other hand, cadmium exposure results in neuropathological changes in the central nervous system (Gulati *et al.*, 1986) as well as behavioral changes such as increased hyperactivity and irritability in animals (Rastogi *et al.*, 1977).

The biochemical influence with cadmium is, however, not fully understood at the present time. Recently, Smith *et al.* (1989) have shown that cadmium evoked inositol polyphosphate formation and calcium mobilization in human skin fibroblasts. Cellular Ca^{2+} mobilization may promote cell growth (Rosengurt, 1986) and account in part for the carcinogenicity of the metals (Smith *et al.*, 1989; Miller, 1987). Cadmium induced c-jun

and c-myc mRNA in a concentration dependent manner (Jin & Ringertz, 1990). On the other hand, cadmium induces increased transcriptions of heat-shock protein genes (Misra *et al.*, 1989), metallothionein gene (Karin *et al.*, 1984) and heme oxygenase gene (Alam *et al.*, 1989). Since cadmium promotes lipid peroxidation (Anderson, H. & Anderson, O., 1988; Solé *et al.*, 1990) in the cell membrane and this evidently contributes to the toxicity of cadmium in the central nervous system, we examined the effects of cadmium on the gene expression in neuroblastoma cells.

Material and Method

Cell Culture: GOTO cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo) and grown in RPMI 1640 supplemented with glutamine (2mM), penicillin (50 unit/ml), streptomycin (50 μ g/ml), and 10% fetal calf serum (Gibco Lab., NY) in a humidified atmosphere of 95% air and 5% CO₂. Cells were plated at 5×10^5 cells/ml/cm². After a 24 h subculture, cells were maintained in RPMI 1640 containing 10% FCS and either with or without an addition of CdCl₂ at a final concentration of 10 μ M for a given period.

RNA extraction and Northern Blots: Total cellular RNA was isolated from neuroblastoma cells by using the guanidinium thiocyanate procedure (Chirgwin *et al.*, 1979). Twenty micrograms of total RNA was electrophoresed on 1% agarose (SeaKem, ME) under denaturing conditions (Lehrach *et al.*, 1977) and transferred to nylon filters (Hybond N, Amersham, U. K.). The filters were hybridized with ³²P-labeled nick-translated probes. Hsp 70 [human, BamHI fragment, 0.8kb (Kao & Nevins, 1983)], hsp90 [human, PstI fragment, 1.4kb (Hickey *et al.*, 1986)], hsp32 [rat, EcoRI Fragment, 1.5kb (Kageyama *et al.*, 1988)], N-myc [human, EcoRI-BamHI fragment, 1.0kb (Kohl *et al.*, 1986)], mdr-1 [human, EcoRI fragment, 1.5kb (Roninson *et al.*, 1986)], were used as the probes. Hybridization and washing of filters were carried out as described previously (Murakami *et al.*, 1991b). Quantitation of mRNA was carried out with a Bioimage-Analyzer (BAS 2000, Fujix, Tokyo).

Results

First of all, the induction of metallothionein and several heat-shock genes after treatment of neuroblastoma (GOTO) cells with cadmium was studied. The levels of metallothionein mRNA began to increase at 2 h after treatment and gradually increased until 15 h (Fig. 1). On the contrary, transcriptions of heat-shock proteins, that is, hsp70, hsp90 and hsp32 (heme oxygenase) were stimulated at 2 h and peaked at 6 h and then declined (Fig. 1). The induction of hsp70 was transient, but those of hsp90 and hsp32 were maintained for longer period than that of hsp70. It is unknown whether these different time courses of individual heat-shock protein genes are due to differences of transcriptional activity or half-lives of individual gene products. As far as we know, this is the first demonstration of the

Fig. 1. Time course of the levels of heat-shock protein and metallothionein (MT) mRNA after cadmium treatment. RNA was prepared at the time indicated, electrophoresed on 1% agarose and transferred on nylon filters as described in Material and Methods. The levels of individual heat-shock proteins (hsp70, hsp32, hsp90) and metallothionein (MT) mRNAs were determined after hybridization of these filters with individual ^{32}P -labeled probes (see Materials and Methods).

Fig. 2. Changes of N-myc and multidrug-resistance gene (MDR) mRNA levels after treatment of GOTO cells with cadmium. Northern-blot analysis of N-myc and MDR was carried out as described in the legend to Fig. 1.

induction of hsp90 after cadmium treatment.

Next, we analyzed N-myc gene expression, which is constitutively expressed in GOTO cells, after treatment of these cells with cadmium. Interestingly, N-myc mRNA levels increased in the presence of cadmium and showed a threefold content of control cells at 6 h. After this time point, N-myc mRNA decreased gradually and returned to the control levels at 15 h (Fig. 2).

The effect of cadmium compounds has been studied in many cell culture systems, particularly in relation to development of cadmium-resistant cell populations. The increase of metallothionein synthesis was mostly associated with an amplification of metallothionein genes (Beach & Palmitter, 1981; Gick & McCarty, 1982). Another possible mechanism to develop resistant cells to some compounds is the induction of multi-drug resistance genes, which might be a serious problem of chemotherapy in neuroblastoma. We examined the behavior of MDR1 mRNA content after the addition of cadmium into the culture medium, and observed the peak of MDR1 mRNA at 6 h after cadmium treatment (Fig. 2).

Discussion

Cadmium has been recognized as an environmental pollutant for many years and is found to be toxic to some extent to almost every organ studied. Several studies have shown that cadmium exposure results in neuropathological and neurochemical alterations in the central nervous system (Gabbiani *et al.*, 1967; Gulati *et al.*, 1986). Since divalent ions of transition metals can promote lipid peroxidation and the brain contains high amounts of polyunsaturated fatty acids, the cell membrane of the central nervous system might be a primary target for toxic metals (Gill *et al.*, 1989). In the present study, we examined the effects of cadmium on the gene expression in neuroblastoma GOTO cells as a model system to see the defensive response to toxic metal and to search out any genes affected by this metal.

The results reported in this paper demonstrated that cadmium induced simultaneous accumulation of N-myc, several heat-shock protein genes, and metallothionein mRNAs.

Although the induction of metallothionein and heat-shock proteins after cadmium treatment is useful to protect cells from cytotoxic action of this metal, at least a part of the changes of heat-shock proteins might contribute to other cellular functions (Kingston *et al.*, 1984; Pelham, 1986; Milarski & Morimoto, 1986; Welch, 1986; Ohmori *et al.*, 1990; Murakami *et al.*, 1991a, b). Cadmium has been reported to induce transcription of protooncogene c-myc in rat myoblasts (Jin & Ringertz, 1990). We found the accumulation of N-myc mRNA in neuroblastoma cells after cadmium treatment, although it is unknown how the induction of the myc family participates in the pathogenesis of nervous cells.

Induction of metallothionein was suggested as the mechanism of protection for cadmium toxicity. Furthermore, cadmium-resistant cells have been isolated in many established cell lines. The mechanism of resistance has been attributed to the synthesis of

metallothionein by the cadmium-exposed mutant (Beach & Palmiter, 1981; Gick & McCarty, 1982). On the other hand, Chin *et al.* (1990) have reported that exposure of HTB-46 cells to cadmium chloride led to a 7-to 8-fold increase in MDR1 mRNA. MDR1 gene product, a multidrug transporter, was initially identified as a multidrug efflux pump responsible for cultured cells to natural product toxic drugs (Fojo *et al.*, 1985; Riorden *et al.*, 1985). In the neuroblastoma cells, there was a 3-fold increase of MDR1 mRNA after cadmium treatment (Fig. 2). However, the MDR1 gene product probably does not play a direct role in conferring resistance to heavy metals because some multidrug-resistant cells did not show increased resistance to CdCl₂ (Chin *et al.*, 1990).

The development of resistance to chemotherapy in neuroblastoma is a serious problem. Previously, however, Nakagawara *et al.* (1990) reported an inverse correlation between expression of MDR genes and N-myc oncogene in neuroblastoma-samples obtained from the primary tumors of patients at the surgical operation. An increase at the levels of MDR1 mRNA was also observed after retinoic acid treatment of neuroblastoma cell lines (Bates *et al.*, 1989). These results suggest that the expression of the MDR gene might be associated with less malignant cases of neuroblastoma and also with the differentiated state of neuroblastoma cells having lower levels of N-myc expression. On the contrary, simultaneous increase of both N-myc and MDR1 mRNA was observed in the present study after cadmium treatment. However, the increase of N-myc and MDR1 mRNA is not likely a consequence of merely the loss of the regulatory mechanism of these genes by cytotoxic effects of cadmium, since active induction of mRNA for defensive mechanism such as metallothionein and heat-shock proteins could occur under these conditions. Further study on the relationship between N-myc, heat-shock proteins and lipid peroxidation by cadmium is under way in our laboratory.

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カドミウム処理による神経芽細胞腫培養細胞の N-myc および MDR (多薬剤耐性遺伝子) の mRNA 量の増加

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要 旨: カドミウムは、発癌性を示すばかりでなく、中枢神経系に病変を起こすことも報告されている。神経芽細胞種由来の培養細胞である GOTO 細胞にカドミウム処理をし遺伝子発現への影響をみた。カドミウム投与後、メタロチオネイン, hsp70, hsp90, および hsp32 mRNA の増量がみられ、熱ショック蛋白質の mRNA は、6 時間でピークを示したが、メタロチオネインの mRNA は、さらに 15 時間まで増え続けた。これらの条件下で N-myc と MDR1 (多薬剤耐性遺伝子) の mRNA も増量し約 6 時間でピークがみられた。メタロチオネインや熱ショック蛋白質の活発な誘導パターンからみて N-myc や MDR1 の mRNA の増量は単にカドミウムによる細胞毒性のため、これらの遺伝子の抑制機構が脱落したためとは考えられない。

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