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SHORT COMMUNICATION

Increased Cytotoxicity of Cadmium in Fibroblasts Lacking c-*fos*

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ABSTRACT. Cadmium has been known to induce the expression of the c-*fos* gene in various cell types including fibroblasts. To clarify the biological significance of c-*fos* induction by cadmium, mouse 3T3-like fibroblasts lacking c-*fos* were exposed to cadmium, and the resultant cellular damage was assayed by WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt) conversion, trypan blue exclusion, or lactate dehydrogenase leakage. The c-*fos*-deficient cells (f1 and f10) were affected more severely than the wild-type cells (NIH 3T3 and f20) with respect to both cell growth and cellular damage following exposure to 10 or 20 μM cadmium chloride. These results suggest that c-*fos* may play a protective role against the cytotoxic effects of cadmium at least in these 3T3-like fibroblasts. BIOCHEM PHARMACOL **59**;12:1573–1576, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cadmium; c-*fos*; cell growth; cytotoxicity; fibroblasts; WST-8

Cadmium has a long biological half-life that exceeds 20 years, and has a diversity of toxic effects including nephrotoxicity, carcinogenicity, and teratogenicity [1]. However, the molecular mechanisms responsible for cadmium-induced cellular damage and for recovery have not been fully clarified.

The c-*fos* proto-oncogene, a member of immediate early genes, encodes a DNA-binding protein (c-Fos) that functions as a component of the transcriptional factor AP-1, heterodimerizing with Jun family proteins [2]. The expression of c-*fos* has been shown to modify transcription of target genes such as human collagenase, metallothionein IIA, stromelysin, and interleukin 2, and has been related to cellular proliferation and differentiation [2]. Moreover, induction of the c-*fos* gene is also known to be associated with apoptosis [3]. We have found previously that cadmium induces transcription of the c-*fos* gene in LLC-PK_1 renal epithelial cells [4]. It has also been reported that cadmium causes the accumulation of c-*fos* transcripts in various cell types including fibroblasts [5–7]. However, the biological significance of c-*fos* expression in response to cadmium exposure is not known. In the present study, mouse 3T3-like fibroblasts lacking c-*fos* were exposed to cadmium, and the resultant cellular damage was determined.

MATERIALS AND METHODS

Cell Culture

The cell lines f20 (c-*fos*+/+), f1 (c-*fos*−/−), and f10 (c-*fos*−/−) (provided by Dr. E. F. Wagner, Research Institute of Molecular Pathology) are mouse 3T3-like fibroblasts immortalized from embryos obtained by mating c-*fos*+/− mice, and their characterizations have been described [8, 9]. These 3T3-like and NIH 3T3 fibroblasts (Health Science Research Resources Bank) were grown in DMEM† supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin (GIBCO BRL, Life Technologies, Inc.) in a humidified atmosphere of 5% CO_2, 95% air at 37°.

Cell Growth

A stock solution of $CdCl_2$ (Sigma) was prepared in water and sterilized by filtration. Exponentially growing NIH 3T3, f20, f1, and f10 cells were trypsinized and plated at 5×10^3 cells/well in 96-well culture plates. Two hours after plating, 10 μL of $CdCl_2$ (final concentration of 10 or 20 μM) or water was added to each well. Before (0 day) and after the incubation with or without $CdCl_2$ for 1, 2, 3, or 4 days, cell growth was evaluated with the WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt) assay.

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† *Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; MT, metallothionein; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

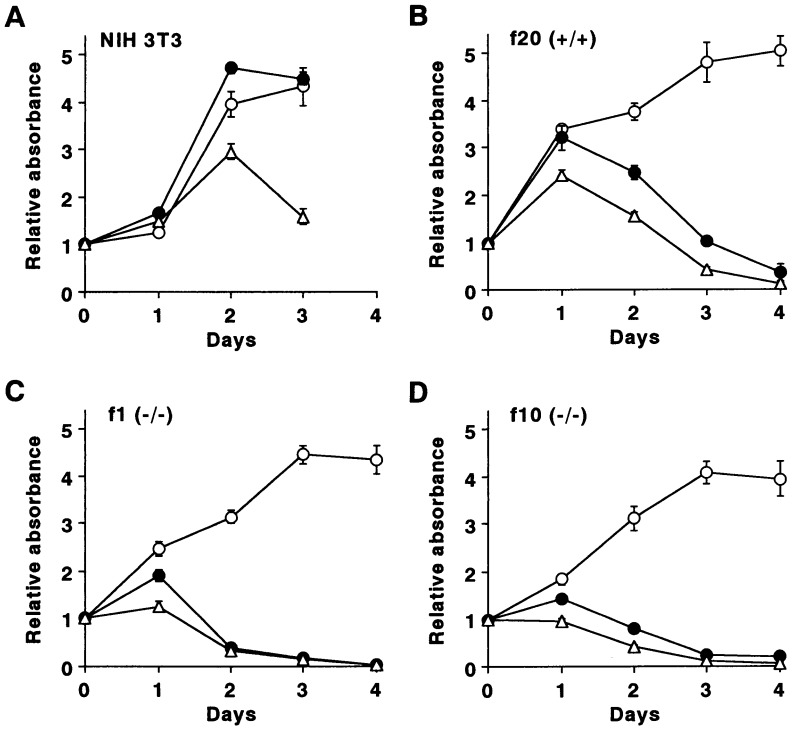

FIG. 1. Cell growth of NIH 3T3 (A), f20 (c-*fos*+/+) (B), f1 (c-*fos*−/−) (C), and f10 cells (c-*fos*−/−) (D). Cells were plated at 5 × 10^3 cells/well in 96-well culture plates at day 0, and were incubated subsequently with water (○), 10 μM $CdCl_2$ (●), or 20 μM $CdCl_2$ (△) for 3 or 4 days. At the indicated times, cell growth was evaluated with the WST-8 assay. Each value (mean ± SD of 8 wells) represents the relative absorbance measured at 450 nm. Points without error bars indicate that the SD was less than the size of the symbol. Another experiment using the MTT assay showed comparable results.

Cytotoxicity

The f20, f1, and f10 cells were cultured for 1 day, and maintained in serum-free DMEM for another day. Each cell line was incubated with serum-free DMEM containing 10 or 20 μM $CdCl_2$ for 18 hr. Untreated control cells were incubated with serum-free DMEM, and cultured the same as the cells exposed to $CdCl_2$. At the end of the incubation, cytotoxicity was assayed by WST-8 conversion, trypan blue exclusion, or LDH leakage.

WST-8 Assay

Both the WST-8 assay and the MTT assay were carried out. In the present study, results obtained with the WST-8 assay are shown mainly because it is more sensitive than the MTT assay, while the principle of the measurement is similar. The WST-8 assay is based on the conversion of the tetrazolium salt WST-8 to highly water soluble formazan by viable cells [10]. Ten microliters of the Cell Count Reagent SF (Nacalai Tesque), which consists of 5 mM WST-8, 0.2 mM 1-methoxy-5-methylphenazinium methosulfate, and 150 mM NaCl was added to each well. After incubation for 1 hr at 37°, the absorbance of each well was measured at 450 nm with a reference wavelength at 655 nm.

Trypan Blue Exclusion Assay

Culture medium was aspirated and reserved. After trypsinization, cells were suspended in DMEM, and the culture medium was returned. The mixture was centrifuged at 800 *g* for 3 min to concentrate the cells. Cellular suspension and 0.4% trypan blue in Hanks' Balanced Salt Solution were mixed (final concentration of 0.07% trypan blue), and the number of viable cells was counted using a hemacytometer in the triplicate samples. The experiment was repeated three times. The percentage of viable cells (cell viability) was calculated as 100 × (unstained cells)/(stained + unstained cells).

LDH Assay

Culture medium was removed and centrifuged at 800 *g* for 10 min to obtain a cell-free supernatant. The activity of LDH in the supernatant was determined using a Cytotoxicity Detection Kit (LDH) (Boehringer Mannheim). The results were expressed as the percentage of the maximum amount of LDH released from cells that had been treated with 1% Triton X-100 (percentage release).

Statistical Analysis

Results are expressed as means ± SD. Statistical significance was determined by one-way ANOVA followed by the Bonferroni multiple comparison test. $P < 0.05$ was considered as statistically significant.

RESULTS

Cell growth was evaluated with the WST-8 assay (Fig. 1). In the absence of $CdCl_2$, both c-*fos*+/+ (NIH 3T3 and f20) and c-*fos*−/− cells (f1 and f10) proliferated well, although some differences in the growth rate among the cell lines were seen. No cell line was affected by treatment with 1 μM $CdCl_2$ for 2 days (data not shown). Incubation with 10 μM $CdCl_2$ did not affect the growth of NIH 3T3 cells (Fig. 1A). However, incubation of NIH 3T3 cells with 20 μM $CdCl_2$ for 2 or 3 days reduced the growth rate. When 3T3-like fibroblasts were incubated with 10 or 20 μM $CdCl_2$ for 1 day, the growth rates of f1 (Fig. 1C) and f10 (Fig. 1D) cells were reduced more than that of f20 cells (Fig. 1B). Thereafter, the reduction of growth rates, probably representing both the lowered cell division and decreased cell viability of each cell line, was observed more markedly with the time of incubation and with the concentration of $CdCl_2$. At days 2 and 3, f1 and f10 cells were affected more severely than f20 cells. At day 4, almost no cells, including f20, survived. Comparable results were obtained with the MTT assay (data not shown).

Cellular damage of 3T3-like fibroblasts exposed to 10 or 20 μM $CdCl_2$ for 18 hr was also determined (Fig. 2). Cell viability assayed by WST-8 conversion (Fig. 2A) and trypan blue exclusion (Fig. 2B) was reduced more in c-*fos*−/− (f1 and f10) than in c-*fos*+/+ (f20) cells. Consistent with these findings, LDH leakage increased more in f1 and f10 cells than in f20 cells (Fig. 2C). A higher concentration of $CdCl_2$ (20 μM) caused more severe cellular damage. When f1 and f10 cells were compared, f10 cells were more sensitive to the incubation with $CdCl_2$.

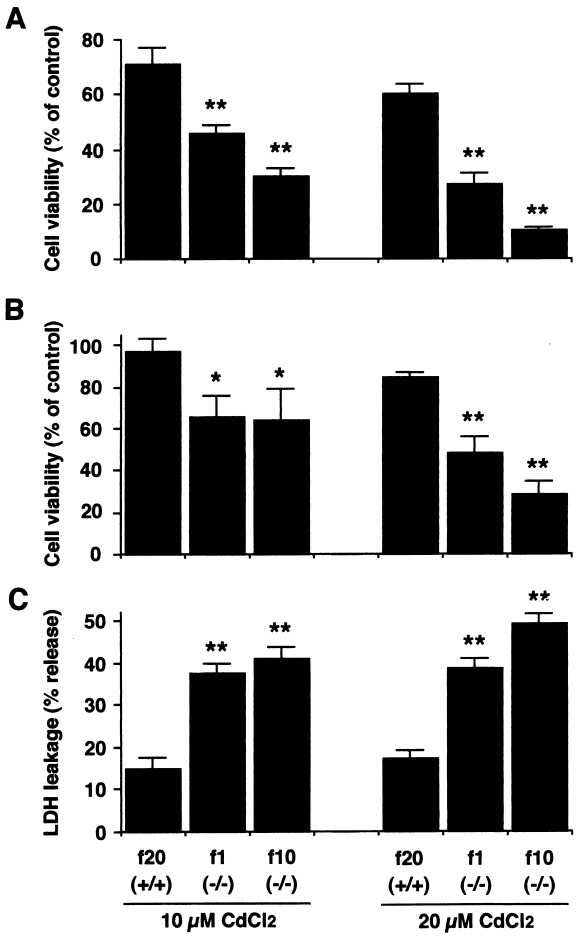

FIG. 2. Cytotoxicity assayed by WST-8 conversion (A), trypan blue exclusion (B), and LDH leakage (C). The f20 (c-*fos*+/+), f1 (c-*fos*−/−), and f10 (c-*fos*−/−) cells were plated at 5×10^3 cells/well in 96-well culture plates (A), 1×10^6 cells/dish in 60-mm culture dishes (B), or 1×10^4 cells/well in 96-well culture plates (C), cultured for 1 day, and maintained in serum-free medium for another day. Then cells were incubated without or with $CdCl_2$ (10 or 20 μM) for 18 hr. Each value of cell viability (A, means ± SD of 8 wells; B, means ± SD of 3 experiments) and LDH leakage (C, means ± SD of 8 wells) represents the percentage of each untreated control culture and the percentage release, respectively. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with f20 cells treated with each concentration of $CdCl_2$. Another set of experiments of WST-8 conversion and LDH leakage showed comparable results.

DISCUSSION

We found that c-*fos*−/− cells (f1 and f10) were affected more severely than c-*fos*+/+ cells (NIH 3T3 and f20) with respect to both cell growth and cellular damage following exposure to 10 or 20 μM $CdCl_2$. These results indicated that the expression of the c-*fos* gene is not the major factor for the development of cellular damage caused by cadmium, but c-*fos* may play a protective role against the cytotoxic effects of cadmium at least in these 3T3-like fibroblasts. The difference in the sensitivity to cadmium exposure between f1 and f10 cells might reflect clonal variation.

MT, a cysteine-rich, metal-binding protein, is induced easily by metals and plays a key role in the detoxification of metals including cadmium [11]. While MT contains the functional AP-1 sites in its promoter [12], no differences were observed in either the kinetics or the magnitude of MT induction between wild-type and c-*fos*-deficient fibroblasts when they were treated with 50 μM $CdCl_2$ [13]. Therefore, c-*fos* does not seem to be essential for MT induction, and the target genes other than MT might be responsible for the increased cytotoxicity of cadmium in c-*fos*-deficient fibroblasts examined in the present study.

Cadmium induces DNA damage by generating reactive oxygen species such as hydrogen peroxide [14]. In previous studies on f1 and f10 cells (c-*fos*−/−), UV radiation has been reported to cause a marked reduction of clonogenic survival and proliferation [9], and increased chromosomal mutations and DNA breakage [15]. In addition, these c-*fos*-deficient fibroblasts were hypersensitive after treat-

ment with agents that cause methylation lesions, bulky adducts, or cross-links in DNA [16]. We have found that another DNA-damaging and carcinogenic metal, hexavalent chromium [14], induces more severe cellular damage in c-*fos*-deficient fibroblasts than in wild-type cells, as cadmium did. The cell viability assayed with WST-8 conversion (means ± SD of 6 wells) in f1, f10, and f20 cells treated with 50 μM Na_2CrO_4 for 18 hr was 46.2 ± 1.0, 47.4 ± 3.4, and 74.8 ± 4.1%, and those treated with 100 μM Na_2CrO_4 for 18 hr was 31.3 ± 1.4, 14.6 ± 2.9, and 56.8 ± 2.7% of each untreated control culture, respectively. Taken together, c-*fos* may be involved in a general cellular protective function, especially against DNA-damaging environmental stresses including heavy metals. Furthermore, the possibility also exists that the expression of the c-*fos* gene contributes to cellular regeneration because the immediate early genes such as c-*fos* are well known to be related to cellular proliferation [2].

On the other hand, the c-Fos protein has been shown to play a causal role in the activation of apoptosis [17]. It has been reported that the administration of cadmium to the experimental animals induces apoptotic cell death in the proximal tubules [18], testis [19], and liver [20]. In cultured $LLC\text{-}PK_1$ renal epithelial cells, we also found that incubation with cadmium results in DNA fragmentation [4]. In contrast, clear findings of apoptosis were not detected in either wild-type or c-*fos*-deficient fibroblasts treated with cadmium (data not shown). These findings suggest that the development of apoptosis by cadmium depends on the cell type, and the possible role of c-*fos* in cadmium-induced apoptosis is still not clear. To reveal the relation between c-*fos* induction and apoptosis, further examinations on susceptible tissues from c-*fos* knockout mice exposed to cadmium are required.

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