(Fig. 4). Our results suggest that the resistance to methylmercury conferred on yeasts by overexpression of these two F-box proteins might be derived from enhanced degradation in proteasomes after enhanced polyubiquitination of a protein or proteins (hereafter collectively designated X-protein) that reinforce the toxicity of methylmercury.

In this study, disruption of the genes for Hrt3 and/or Ylr224w did not affect the sensitivity of yeast cells to methylmercury (data not shown). Thus, it is possible that X-protein might also be recognized and polyubiquitinated by some F-box protein(s) other than Hrt3 and Ylr224w.

Multiple E2s coexist with multiple F-box proteins in yeast cells. Therefore, it is possible that not only F-box proteins but also E2 might be involved in the selection of proteins as substrates for degradation. As described above, we found previously that overexpression of Cdc34, an E2, also rendered yeast cells resistant to methylmercury and, thus, the F-box protein related to this phenomenon might be Hrt3 or Ylr224w. However, when we overexpressed Cdc34 in yeast cells in which the Hrt3 and/or the Ylr224w gene had been deleted, the resistance of all three lines of cells to methylmercury was almost as strong as that of wild-type yeast cells that overexpressed Cdc34 (data not shown). This result suggests that Cdc34 does not confer resistance to methylmercury in cooperation only with SCF complexes that contain Hrt3 or Ylr224w as the F-box protein. However, we have also found that some types of E2 other than Cdc34 confer resistance to methylmercury when overexpressed in yeast cells [4]. Therefore, it is possible that types of E2 other than Cdc34 or multiple types of E2, including Cdc34, might play a role in the polyubiquitination of X-protein by SCF complexes that contain Hrt3 or Ylr224w as the F-box protein.

Their functions of Hrt3 and Ylr224w as F-box proteins were confirmed for the first time in the present study, but no further information is currently available, to our knowledge, about the possible proteins that might be polyubiquitinated after being recognized by these F-box proteins. In the preliminary study, we have already identified some candidates of X-proteins, which play a role in the enhancement of the toxicity of methylmercury and which are degraded by proteasomes after its polyubiquitination, by searching for proteins that can bind to both F-box proteins. The SCF complex, an E3 that includes F-box proteins, is present not only in yeast cells but also in mammalian cells, including human cells, and it has the same function in mammals as in yeast [26,27]. The identification of X-protein should help to clarify the mechanism of damage to the central nervous system that is caused by methylmercury.

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– Toxicogenomics/proteomics Report –

DNA MICROARRAY ANALYSIS OF TRANSCRIPTIONAL RESPONSES OF HUMAN NEUROBLASTOMA IMR-32 CELLS TO METHYLMERCURY

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ABSTRACT — We used DNA microarrays to monitor the transcriptional responses of IMR-32 neuroblastoma cells to methylmercury. Methylmercury increased levels of expression of seven genes, including genes for ETV5 and ID4, and reduced those of two genes.

KEY WORDS: Methylmercury, DNA microarray, IMR-32 cells, ETV5, ID4

INTRODUCTION

Methylmercury is an environmental pollutant that damages the central nervous system, and many cases of methylmercury poisoning have been reported (Castoldi *et al.*, 2003). However, little is known about the molecular mechanism of methylmercury poisoning. We postulated that treatment of neuroblastoma cells with methylmercury would induce various intracellular reactions and, in particular, changes in the levels of expression of certain genes. To examine our hypothesis, we examined levels of transcripts in IMR-32 cells before and after treatment for 6 and 24 hr with methylmercury using DNA microarrays.

MATERIALS AND METHODS

Exposure of cells to methylmercury

IMR-32 human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 10% CO_2 in air. IMR-32 cells (1×10^6 cells) were distributed in six-well plates and cultured in 1-ml aliquots of medium. After incubation for 24 hr, 1 μM methymercury chloride was added to the medium and cells were cultured for 6 hr, during which the cell-survival rate was close to 100%, and for 24 hr, during which fewer than 10% of cells ceased to be viable.

Eextraction of RNA

Control IMR-32 cells and cells that had been treated with methylmercury chloride were lysed with TRIzol (Life Technologies Inc., Rockville, MD, USA) according to the manufacturer's protocol and total RNA was extracted by the standard method and stored at –80°C.

Microarray analysis

Purified RNA (8 μg) was reverse transcribed by Superscript II reverse transcriptase (Life Technologies Inc.) with T7-$(dT)_{24}$, which contains a promoter recognized by T7 RNA polymerase, as primer. Then complementary DNA (cDNA) was synthesized and used as template for transcription *in vitro* of biotinylated complementary RNA (cRNA). Fifteen micrograms of fragmented cRNA were allowed to hybridize to an HG-Focus microarray (Affymetrix, Santa Clara, CA, USA) for 16 hr at 45°C with constant rotation at 60 rpm according to the protocol from Affymetrix. This high-density oligonucleotide-based array is specific for approximately 8,500 human genes selected from the

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Table 1. Genes whose levels of expression changed upon treatment of IMR-32 cells with methylmercury.

Gene name	Accession number	Changes in level of transcript		Function of gene product
		6 hr	24 hr	
[Increased]				
BAIAP2	NM_017450	4.5	2.7	Signal transduction
CDKN1A	NM_000389	3.9	2.2	Signal transduction
DKK1	NM_012242	2.8	2.5	Signal transduction
ETV5	NM_004454	22.6	2.6	Transcription
FZD1	NM_003505	3.8	2.4	Signal transduction
ID4	NM_001546	6.6	2.2	Transcription
NQO1	NM_000903	4.7	2.1	Signal transduction
[Decreased]				
CPO1	NM_173077	0.4	0.5	Tumor progression
MLF1	NM_022443	0.5	0.3	Tumor progression

IMR-32 cells were treated with methylmercury for 6 hr and 24 hr, and then gene expression was analyzed with DNA microarrays. The genes presented in the Table include only those whose levels of expression changed (with an increase of more than two-fold or a decrease of more than 50%) not only when IMR-32 cells were treated with methylmercury for 6 hr but also when they were treated for 24 hr.

Gene Bank database of the National Center for Biotechnology Information (NCBI, USA). A separate microarray was used for the analysis of each individual sample. After hybridization, the microarray was washed and stained on an Affymetrix Fluidics Station and scanned with a confocal argon laser, with emission of 488 nm and detection at 570 nm. Data were analyzed with Genespring software, version 7.0 (Silicon Genetics, San Carlos, CA, USA).

RESULTS AND DISCUSSION

We used DNA microarrays to identify for genes whose levels of expression chaged when IMR-32 cells were treated with methylmercury (1 μM) for 6 hr (cell viability rate, approximately 100%) and 24 hr (cell viability rate, greater than 90%). We found that treatment for 6 hr and for 24 hr more than doubled the levels of expression of seven genes and more than halved those of two genes (Table 1). Among the first set of genes, the gene for CDKN1A has already been identified as a gene whose level of expression increases when cells from rat embryonic limb buds and the midbrain central nervous system are treated with methylmercury (Ou *et al.*, 1999). However, to our knowledge, the remaining eight genes that we identified have not previously been reported to be associated with exposure of cells to methylmercury. Our findings provide useful clues to the nature of the response of neural cells to methylmercury.

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– Letter –

CISPLATIN-INDUCED EXPRESSION OF IRON-RETAINING GENES *FIT2* AND *FIT3* IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT — cDNA microarray analysis indicated that mRNA levels of Fit2p and Fit3p, proteins involved in iron retention within the yeast cell wall, were markedly increased by treatment of *Saccharomyces cerevisiae* with cisplatin. Expression of *FIT2* and *FIT3* is known to be transcriptionally regulated by Aft1p. Northern blotting demonstrated a time- and concentration-dependent increase in the mRNA levels of both proteins following treatment with cisplatin. However, overexpression or disruption of the *FIT2* or *FIT3* genes had little effect on the susceptibility of yeast to cisplatin. Although Fit2p and Fit3p do not appear to be directly involved in protecting against the toxic effects of cisplatin, the present results suggest the existence of an activation system of gene expression in response to cisplatin within yeast cells.

KEY WORDS: Cisplatin, Induction, Gene expression, Fit2, Fit3

INTRODUCTION

Cisplatin is an anticancer agent that has been shown to be effective against various malignant tumors (Einhorn and Williams, 1979). The mechanism of acquired resistance to cisplatin has been widely studied (Siddik, 2003), but few investigations into cellular responses to cisplatin have been reported. We carried out a study of genes whose expression levels were altered by cisplatin in budding yeast (*Saccharomyces cerevisiae*). Since many yeast genes share common functions with those of mammals, including humans, the findings obtained from investigations using yeast are considered useful for elucidating the functions of human proteins.

MATERIALS AND METHODS

Yeast strain and media

The wild-type yeast strain BY4742 (*MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*) and yeast strains with disrupted *FIT2* or *FIT3* (Euroscarf, Frankfurt, Germany) were grown in synthetic dextrose (SD) medium supplemented with amino acids.

Construction of gene expression vectors

FIT2 and *FIT3* were amplified by PCR using yeast genomic DNA as a template with the following primers:

FIT2-F, 5'- TCATAAAGCCATCACAAAACA-3'
and
FIT2-R, 5'-TTTCTGGTTTGAAACCGAGC-3'
for the *FIT2* gene
FIT3-F, 5'-AACTAATAACAATAATCCC-3' and

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FIT3-R, 5'-AAATCGGATATCCCGCATGA-3' for the *FIT3* gene.

The amplified fragments were ligated into the pKT10-GAPDH (*URA3*) expression vector. BY4742 cells were transformed with each plasmid according to the lithium acetate procedure (Naganuma *et al.*, 2000).

Northern blotting

Yeast cells (5×10^6 cells/ml) were precultured in SD medium for 3 hr at 30°C and then cultured in SD medium containing cisplatin. Total RNA was prepared as described elsewhere (Furuchi *et al.*, 2002). The probes for *FIT2*, *FIT3*, and *ACT1* were amplified by PCR from yeast genomic DNA using the following primers:

FIT2-1-F, 5'-ACAGTTATGACTGCCGTCTCG-3' and
FIT2-1-R, 5'-ACACTTGCTCCTTGGAATGCA-3' for the *FIT2* probe
FIT3-1-F, 5'-AACTAATAACAATAATCCC-3' and
FIT3-1-R, 5'-AAATCGGATATCCCGCATGA-3' for the *FIT3* probe
ACT1-1-F, 5'-GGTGATGAAGCTCAATCCAA-3' and
ACT1-1-R, 5'-AGAAGATTGAGCAGCGGTTT-3' for the *ACT1*probe

Northern blotting was performed using the digoxigenin system (Takara Bio Inc., Shiga, Japan), in accordance with the manufacturer's instructions.

Quantitation of cisplatin toxicity in yeast.

Yeast cells were cultured (1×10^4 cells/200 µl) in SD medium containing cisplatin at various concentrations. After 48 hr, the absorbance of the culture at 620 nm was measured to quantify cell growth.

RESULTS AND DISCUSSION

We examined the effect of cisplatin on gene expression in *S. cerevisiae* by cDNA microarray. The results showed that the mRNA levels of Fit2p and Fit3p, proteins involved in the retention of iron within the cell wall (Protchenko *et al.*, 2001), were the most markedly increased (>30 fold) by the cisplatin treatment (data not shown). In contrast to our findings, previous cDNA microarray studies into altered gene expression levels in cisplatin-treated *S. cerevisiae* did not reveal Fit2p or Fit3p to be affected (Birrell *et al.*, 2002). We therefore investigated the effects of cisplatin on Fit2p and Fit3p by Northern blotting and showed that the mRNA levels of both proteins increased after cisplatin treatment in a dose-dependent manner (Fig. 1A). After 1 hr incubation with cisplatin, mRNA levels

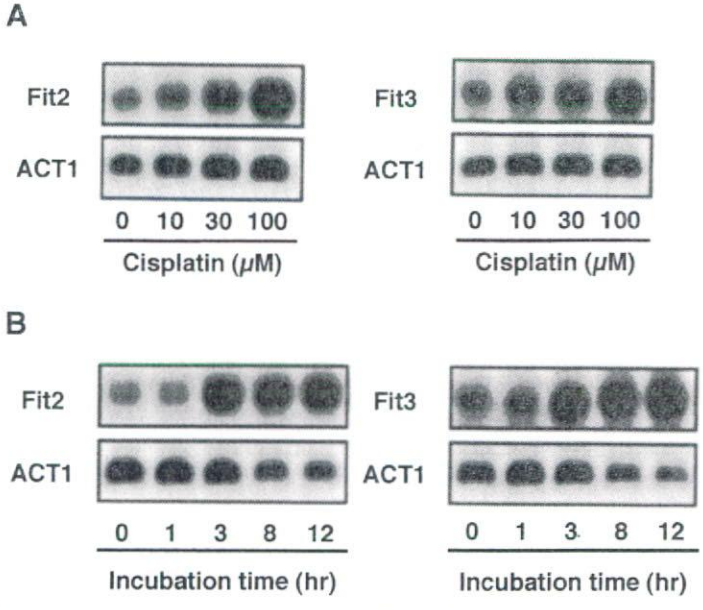

Fig. 1. Effect of cisplatin on Fit2p and Fit3p mRNA levels in yeast cells. *S. cerevisiae* was cultured with various concentrations of cisplatin for 3 hr (A), or with 100 µM cisplatin for various incubation times (B). Each lane contains 10 µg total RNA.

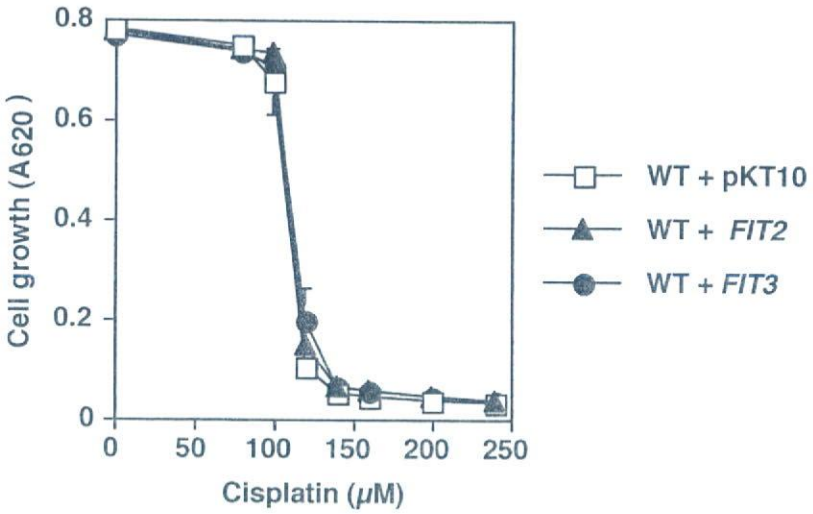

Fig. 2. Effect of *FIT2* or *FIT3* overexpression on sensitivity of yeast cells to cisplatin.
Yeast strains (BY4742) carrying pKT10 (open square), pKT10-*FIT2* (closed triangle) or pKT10-*FIT3* (closed circle) were grown in SD (-Ura) medium containing cisplatin. After 48 hr incubation, the absorbance at 620 nm was measured spectrophotometrically. Each point and bar represents the mean value and standard deviation (S.D.), respectively, of the results from three cultures. The absence of a bar indicates that the S.D. falls within the symbol.

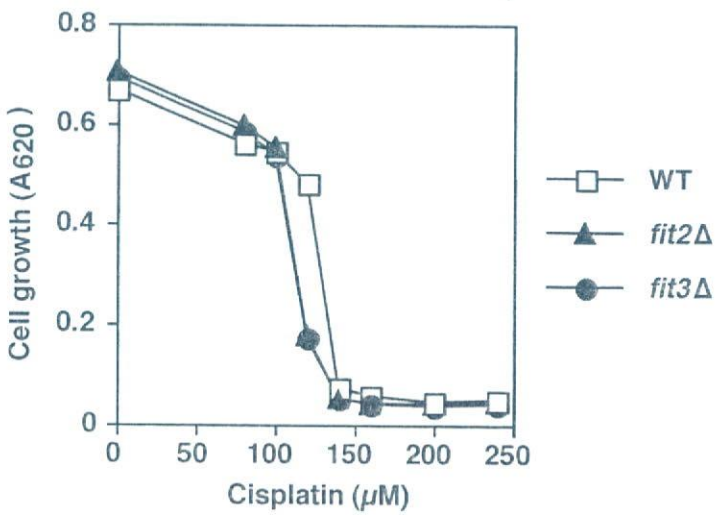

Fig. 3. Effect of *FIT2* or *FIT3* disruption on the sensitivity of yeast cells to cisplatin.
Yeast cells (BY4742 (open square), *fit2Δ* (closed triangle) or *fit3Δ* (closed circle)) were grown in SD medium containing cisplatin. After 48 hr incubation, the absorbance at 620 nm was measured spectrophotometrically. Each point and bar represents the mean value and S.D., respectively, of the results from three cultures. The absence of a bar indicates that the S.D. falls within the space occupied by a symbol.

remained low, but increased markedly after 3 hr incubation and remained high thereafter (Fig. 1B).

FIT2 and *FIT3* have been identified as genes that are transcriptionally activated by the transcription factor Aft1p during iron deprivation (Protchenko *et al.*, 2001). Aft1p is usually located in the cytoplasm, but insufficient cellular iron uptake causes it to enter the nucleus, where it enhances the expression of genes involved in the maintenance of iron homeostasis (Yamaguchi-Iwai *et al.*, 1995; Yamaguchi-Iwai *et al.*, 2002). It is therefore possible that cisplatin might increase Fit2p and Fit3p mRNA levels by activating Aft1p.

The marked increase in Fit2p and Fit3p expression suggests that the proteins might have a defensive function against the toxic effects of cisplatin. We therefore investigated the cisplatin susceptibility of yeasts overexpressing or lacking the *FIT2* or *FIT3* genes. As shown in Figs. 2 and 3, the susceptibilities of these yeasts to cisplatin were almost comparable with that of the wild-type strain, indicating that Fit2p and Fit3p are not directly involved in protecting against the toxic effects of cisplatin. However, the fact that cisplatin markedly increases Fit2p and Fit3p mRNA levels strongly suggests the existence of an activation mechanism of gene expression in response to cisplatin within the cell. A detailed further investigation of the effects of cisplatin on the activation of Aft1p is expected to elucidate the cell response mechanism to cisplatin.

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CHARACTERIZATION OF AN IMMORTALIZED HEPATIC STELLATE CELL LINE ESTABLISHED FROM METALLOTHIONEIN-NULL MICE

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ABSTRACT — Hepatic stellate (HS) cells were isolated from the livers of metallothionein (MT)-null and control mice and used to establish IMS/MT(-) and IMS/N cell lines, respectively, using SV40 virus transformation. Cellular morphology, incorporation of vitamin A and expression of α-SMA, desmin and SV40 T-antigen were used to confirm that both cell lines were immortal HS cells. The growth rates of both cell lines were similar and there was little difference between cell line sensitivity to zinc. MT-null IMS/MT(-) cells were more sensitive to cadmium and mercury, although both cell lines accumulated almost equal amounts of cadmium during a 24-hr culture period. As HS cells play an important role in hepatic fibrosis and are activated by heavy metals such as cadmium or reactive oxygen, the MT-null HS cell line derived in this study should be a useful experimental model for examination of the role of MT in HS cell activation.

KEY WORDS: Metallothionein-null cells, Hepatic stellate cells, Cadmium, Immortalization

INTRODUCTION

Metallothionein (MT) is a unique protein consisting of 30% cysteine but lacking the usual cysteine S-S bonds (Webb, 1979; Lazo and Bahnson, 1989; Kägi, 1991). Various physiological effects of MT have been reported such as detoxification of heavy metals, scavenging of active oxygen and maintenance of zinc (Zn) homeostasis (Bremner, 1987; Lazo and Bahnson, 1989; Kägi, 1993; Sato and Bremner, 1993). Its role in detoxification has been particularly well studied and was originally examined using MT-inducing agents.

More recently, MT-transgenic cell lines and mice have been produced and used to demonstrate that MT provides protection against the toxicity of heavy metals such as cadmium (Cd) and mercury (Hg) (Kaina *et al.*, 1990; Dalton *et al.*, 1996) and the oxidative stress from radiation or reactive oxygen species (Lohrer and Robson, 1989; Kang *et al.*, 1997; Pitt *et al.*, 1997). Moreover, transgenic mice deficient in MT-I and -II, major molecular species of MT, have elucidated the basal level functions of MT (Michalska and Choo, 1993; Masters *et al.*, 1994). Model experimental systems using MT-null cell lines are of use in examining the precise physiological functions of MT. To this end, we have established and characterized an immortalized hepatic stellate (HS) cell line from the livers of MT-null mice.

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MATERIALS AND METHODS

Materials

All reagents were purchased from Wako Pure Chemical unless otherwise stated.

Isolation and immortalization of HS cells

HS cells isolated from MT-null mice (Masters *et al.*, 1994) and normal 129/Sv mice were immortalized by infection of SV40 virus. Cells were maintained in Dulbecco's modified minimum essential medium (DMEM, Nissui Pharmaceutical) supplemented with 10% fetal calf serum and 60 mg/ml kanamycin and cultured at 37°C in a humidified atmosphere containing 5% CO_2. These cells were designated IMS/MT(-) (immortalized mouse stellate cells from MT-null mice) and IMS/N (immortalized mouse stellate cells from normal mice), respectively (Miura and Naganuma, 2000).

Reverse transcription-PCR of SV40 large T-antigen

Total RNA from IMS/N and IMS/MT(-) cells was extracted and reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen), AmpliTaq Gold (Applied Biosystems), oligo (dT) primers (Invitrogen) and a specific primer pair for the SV40 large T-antigen: forward, 5'-TAA CAC TGC CAT CCA AAT AAT CC-3'; reverse, 5'-TGT GTT GTT ATT GCT TGG GAT AT-3'. The reaction included an initial incubation at 95°C for 10 min followed by 30 cycles of 96°C for 30 sec, 65°C for 15 sec and 72°C for 60 sec. The PCR product was 667 bp.

Vitamin A accumulation

IMS/N and IMS/MT(-) cells (1×10^4 cells) were seeded in 8-well glass chambers (No 4118; Falcon) and incubated in cell medium containing 25 mM retinol acetate (Sigma-Aldrich) dissolved in dimethyl sulfoxide for three days (Matsuura *et al.*, 1989). After washing the cells with phosphate buffered saline (PBS), vitamin A autofluorescence in the cytoplasm was observed under a fluorescence microscope using a broad-range wavelength (330 to 385 nm) excitation filter (U-MWU; Olympus).

Cell growth rate and cell survival rate

IMS/N and IMS/MT(-) cells were seeded at 1×10^5 cells per well in 6-well plates and cultured for 24, 48, 72 or 96 hr. Cells were harvested by trypsinization and the cell number was counted by a hemocytometer. The survival rate against various types of external stress was estimated using AlamarBlue reagent (Becton Dickinson) as previously described (Miura *et al.*, 1999). Briefly, IMS/N and IMS/MT(-) cells (1×10^4 cells/well) were seeded in 96-well plates, and different concentrations of stimulants were applied 24 hr later. Cell survival rates were estimated after a further 24 hr.

Western blotting analysis

IMS/N and IMS/MT(-) cells were harvested, collected and sonicated in PBS for 15 s on ice. The samples were mixed with an equal volume of 2 × sample buffer (100 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 12% β-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue) and boiled for 5 min followed by centrifugation at 12,000 × *g* for 10 min to remove insoluble cell debris. Cellular proteins (approximately 45 mg) were resolved on a 7.5% SDS-polyacrylamide gel, and electroblotted onto Immobilon polyvinylidene fluoride membrane (Millipore). Membranes were incubated overnight at room temperature with a rabbit polyclonal anti-desmin antibody (1:100 dilution; PC-10570, Cosmo Bio), followed by a 1 hr incubation with an alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG secondary antibody (1:3,000 dilution; Santa Cruz Biotechnology). Proteins were visualized by chemiluminescence using the ECL kit (Amersham) with α-smooth muscle actin (SMA) expression determined in a similar manner using the monoclonal anti-α-SMA antibody (1:100 dilution; A2547, Sigma-Aldrich) and the AP-conjugated goat anti-mouse IgG secondary antibody (1:3,000 dilution; Santa Cruz Biotechnology).

Accumulation and distribution of Cd in cells after $CdCl_2$ treatment

IMS/N and IMS/MT(-) cells (3×10^5 cells) were seeded in 6-well plates in triplicate and incubated overnight. Medium was changed to fresh DMEM containing 30 mM $CdCl_2$ and incubated for 8 hr or 24 hr. Cells were scraped, collected and sonicated in PBS. Cd concentrations in the sonicated sample were measured by atomic absorption spectrometry. Cellular total protein levels were determined by the Bradford reagent (Sigma-Aldrich).

To investigate Cd distribution, 2×10^6 cells were seeded in 100 mm dishes (four dishes per cell line). Cells were incubated with $CdCl_2$ (30 mM) for 24 hr, harvested and all cells collected into one tube. The cell concentration was adjusted by addition of PBS. Cells (4×10^6 cells in 400 ml PBS) were sonicated for 1 min on ice followed by centrifugation at 105,000 *g* for 1 hr

at 4°C. Supernatants were collected and filtered (0.45 mm pore size). The distribution profile of Cd was measured by high-performance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICP-MS). A 100 ml aliquot of supernatant was loaded on the TSK GEL SW3000 column (Tosoh Corporation) and eluted with 50 mM Tris-HCl (pH 8.4) at a flow rate of 0.8 ml/min (HP1100; Yokokawa Analytical Systems). The eluate was introduced directly into the ICP-MS instrument (HP4500; Yokokawa Analytical Systems). Cd concentrations were determined at mass numbers of 111 and 114 m/z.

RESULTS

Characterization of immortalized HS cells

IMS/MT(-) and IMS/N cell lines were grown for more than 30 passages under standard culture conditions supplemented with 10% FCS. Expression of SV40 large T-antigen, an index of cell immortalization, was identified in both cell lines by RT-PCR (Fig. 1a). Both cell lines assumed a similar morphology with elongated asteroidal multicellular nodules, which are characteristic of HS cells (Fig. 1b). Growth rates of both cells were similar, with doubling times of approximately 27 hr, as calculated from their growth curves (Fig. 1c). Both cell lines also lifted off the base of the dish in sheets at high confluency.

The contractile proteins desmin and α- SMA can be used as markers for HS cells as they are specifically expressed in this cell type (Yokoi *et al.*, 1984; Rockey *et al.*, 1992; Shimizu *et al.*, 1999). We therefore investigated the expression of these proteins using western blotting and identified 42 kDa and 50 kDa bands corresponding to the molecular weights of α-SMA and desmin, respectively, in both cell lines (Fig. 2).

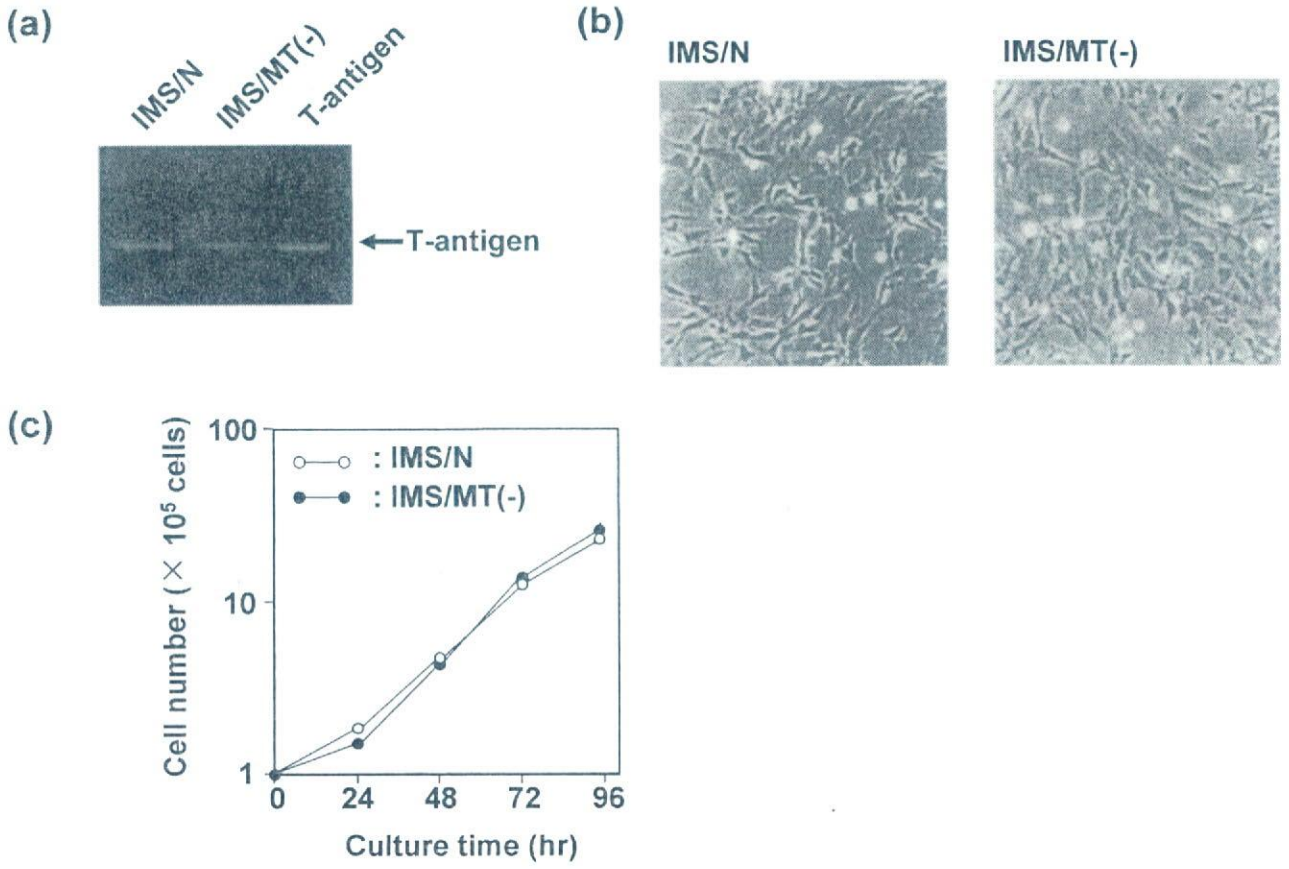

Fig. 1. Biochemical features of IMS cells. (a) SV40 T-antigen mRNA expression determined by RT-PCR. (b) Morphological characteristics of IMS/N and IMS/MT(-) cells shown by phase contrast microscopy (magnification × 100). (c) Cell proliferation curve of IMS/N (open circles) and IMS/MT(-) (closed circle) cells.

HS cells play important roles in vitamin A metabolism and are known to store vitamin A in ester form intracellularly (Hendriks *et al.*, 1985; Matsuura *et al.*, 1989). To investigate the incorporation of vitamin A into both established cell lines, we cultured them for three days with 25 μM all-trans retinol acetate. Fluorescence microscopy revealed clear droplet-like blue-green vitamin A autofluorescence in the cytoplasm of both cell lines, confirming their incorporation and accumulation of vitamin A (Fig. 3). Little morphological differences were observed between the cell lines after culture with retinol. These results suggest that IMS/MT(-) and IMS/N cells are immortalized HS cell lines and therefore suitable cell models for the examination of MT deficiency.

Sensitivity to environmental stress

Cell survival rates after 24-hr culture with different concentrations of $CdCl_2$ were determined to compare Cd sensitivity. MT-null IMS/MT(-) cells were shown to be more sensitive to Cd than normal IMS/N cells (Fig. 4-a). In addition, the cell lines demonstrated morphological differences after 24-hr culture with 30 μM Cd: IMS/N cells were elongated, while IMS/

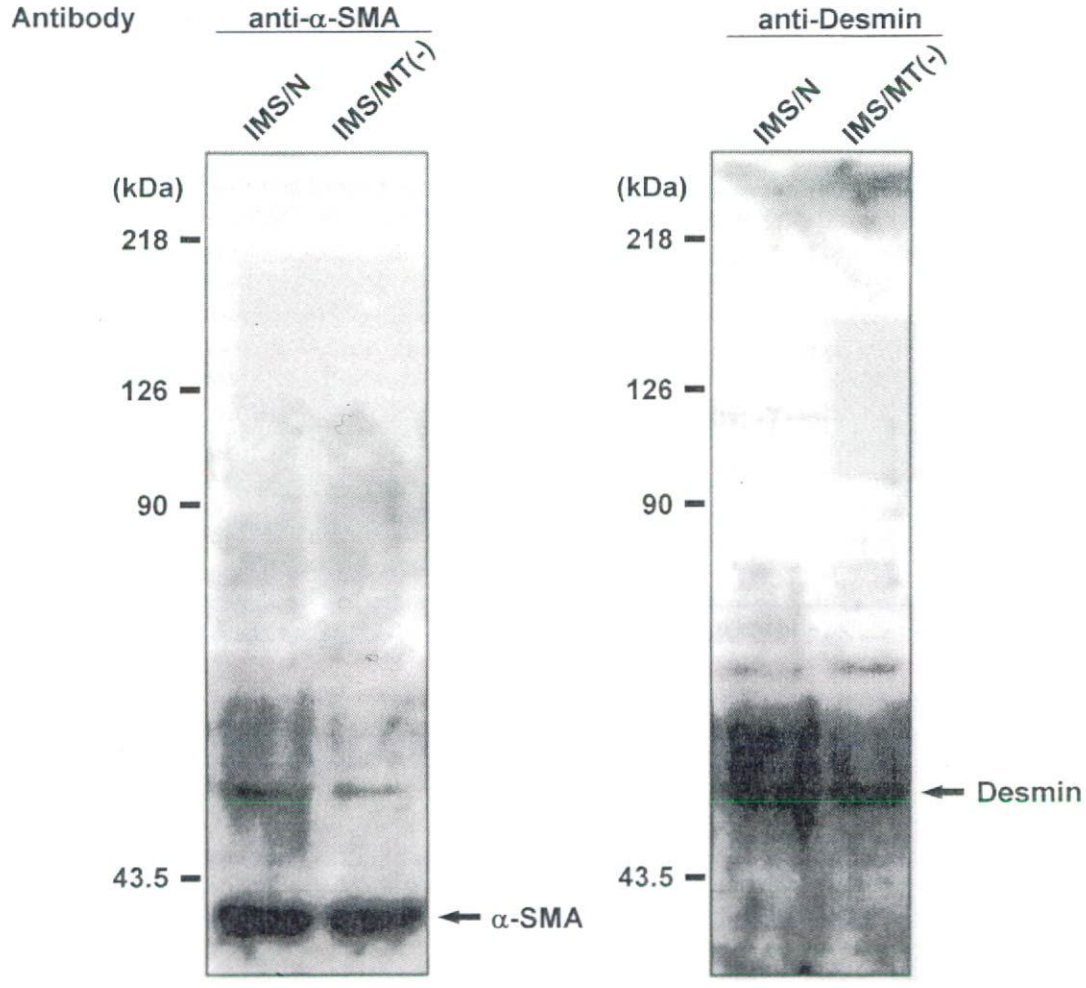

Fig. 2. Expression of α-SMA and desmin proteins as HS cell markers. Cellular proteins (approximately 45 mg) from IMS/N and IMS/MT(-) cells were resolved on 7.5% SDS-PAGE. Western blotting analysis was performed using an anti-α-SMA antibody or an anti-desmin antibody.

MT(-) cells were rounder in appearance and floated off the dish (Fig. 4b). IMS/MT(-) cells also showed a higher sensitivity to $HgCl_2$ than IMS/N cells, although their sensitivity to $ZnCl_2$ and retinol acetate was comparable (Fig. 4c, 4d, 4e). Little difference was observed in cell line sensitivity to paraquat and tert-butylhydroperoxide (TBH), both of which generate reactive oxygen and hydrogen peroxide (data not shown).

Intracellular Cd accumulation

Because of the distinct differences observed in Cd sensitivity between the two cell lines, we determined the intracellular Cd accumulation 8 hr and 24 hr after addition of $CdCl_2$. Cd accumulation increased with time in both cell types without significant difference in rate (Fig. 5a). The distribution of Cd as a soluble fraction was then examined after 24 hr culture with CdC_2 using HPLC/ICP-MS. This analysis showed that 41% Cd existed as a MT fraction in IMS/N cells (Fig. 5b, left), while almost all Cd existed as a high-molecular-weight fraction in MT-null IMS/MT(-) cells. In MT-null cells Cd appeared to be conjugated with high-molecular-weight protein(s), but not low-molecular-weight peptides or proteins (Fig. 5b).

DISCUSSION

Immortalized fibroblast cell lines from MT-null mice have been previously established from the kidney (Butcher *et al.*, 2004), lung (Jiang *et al.*, 2002) and fetus (Kondo *et al.*, 1999; Himeno, 2002; Mahboobi *et al.*, 2003). By contrast, the IMS/MT(-) and IMS/N cells established in this study are HS cells from the liver. HS cells are also known as Ito cells, fat-storing cells, perisinusoidal cells and lipocytes, and are non-parenchymal cells of mesenchymal origin, located in the hepatic Disse space. They are involved in the storage and metabolism of vitamin A and accumulate 80-90% of intrahepatic vitamin A as fat droplets (Hendriks *et al.*, 1985; Blomhoff and Wake, 1991).

HS cells play a major characteristic role in hepatic fibrosis, where they are activated to transform

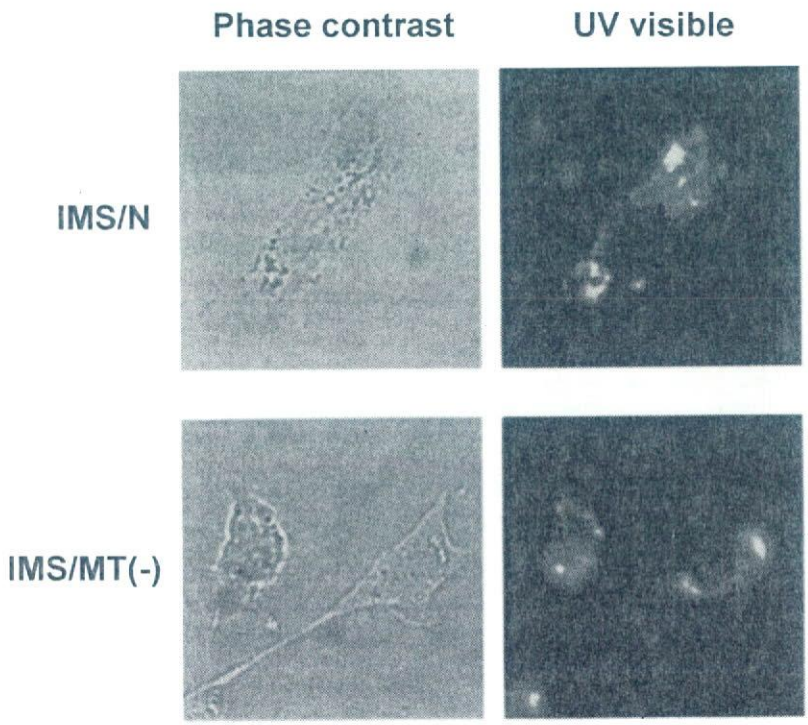

Fig. 3. Accumulation of vitamin A in IMS cells. IMS/N and IMS/MT(-) cells were incubated with 25 mM retinol acetate for three days and visualizedunder UV light (right) and phase contrast (left) (magnification × 400).

into myofibroblast-like cells, generating contractile proteins such as α-SMA and extracellular matrices such as collagen (mainly type I) or fibronectin (de Leeuw *et al.*, 1984; Rockey *et al.*, 1992). Previously-known factors that activate HS cells include the active oxygen generated by chronic alcohol drinking and viral infection (Schuppan *et al.*, 2003), while experimental HS cell activation by carbon tetrachloride has also been reported (Greenwel *et al.*, 1991). Del Carmen *et al.* (2002) reported that Cd induces expression of collagen type I in HS cells of rat liver. Increases in hepatic collagen contents by metals such as Hg, Pb, Mo, Cu and Cr have also been reported (Rana and Prakash, 1986). Moreover, HS cells help regulate sinusoidal bloodflow; in this way the flow rate of portal blood into the sinusoid is controlled by endothelin-1 contraction and nitric oxide relaxation (Rockey, 2001).

In these varied ways, the physiological activity of HS cells is controlled by many factors. Recent reports also revealed a relationship between increased intrahepatic MT and suppression of hepatic fibrosis (Cheng *et al.*, 2002, 2004), suggesting that increasing or decreasing the amount of MT protein could lead to HS cell activation. The immortal HS cell lines established in this study demonstrated varied sensitivity to heavy metals such as Cd and Hg. A more precise investigation into the effects of xenobiotics and oxidative stress on the function of both cell lines, rather than on their cytotoxicity, might elucidate the role of MT in hepatic disorders.

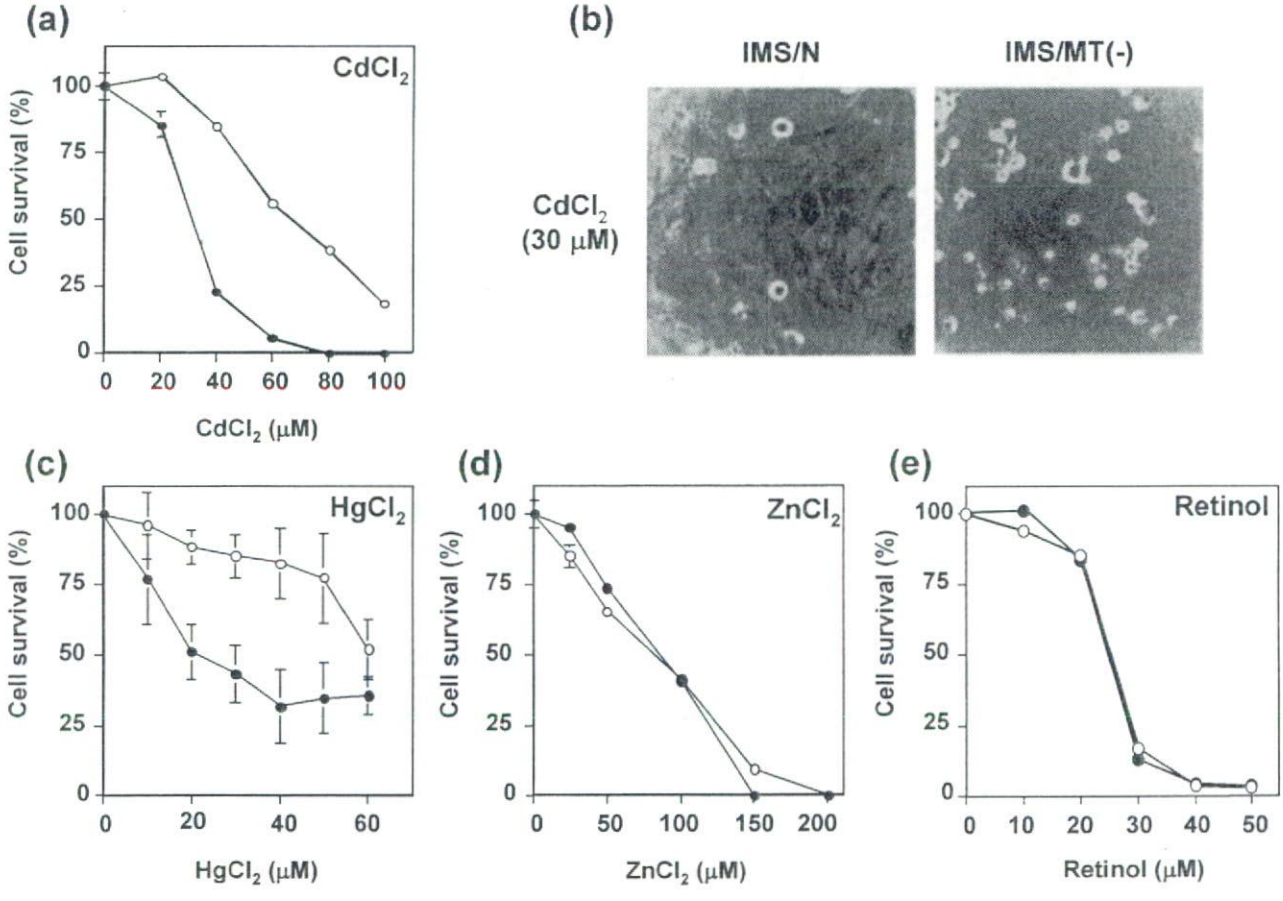

Fig. 4. Sensitivities of IMS cells to heavy metals and retinol. (a) IMS/N and IMS/MT(-) cells (1×10^4 cells/well) were incubated with the indicated concentrations of $CdCl_2$. Cell viability was estimated by addition of AlamarBlue. (b) Morphological changes of IMS/N and IMS/MT(-) cells after incubation with 30 mM $CdCl_2$ were observed by phase contrast microscopy (magnification × 100). Cell survival rates of other heavy metals: $HgCl_2$ (c), $ZnCl_2$ (d), and retinol acetate (e) were also estimated. IMS/N and IMS/MT(-) cells are represented by open and closed circles, respectively.

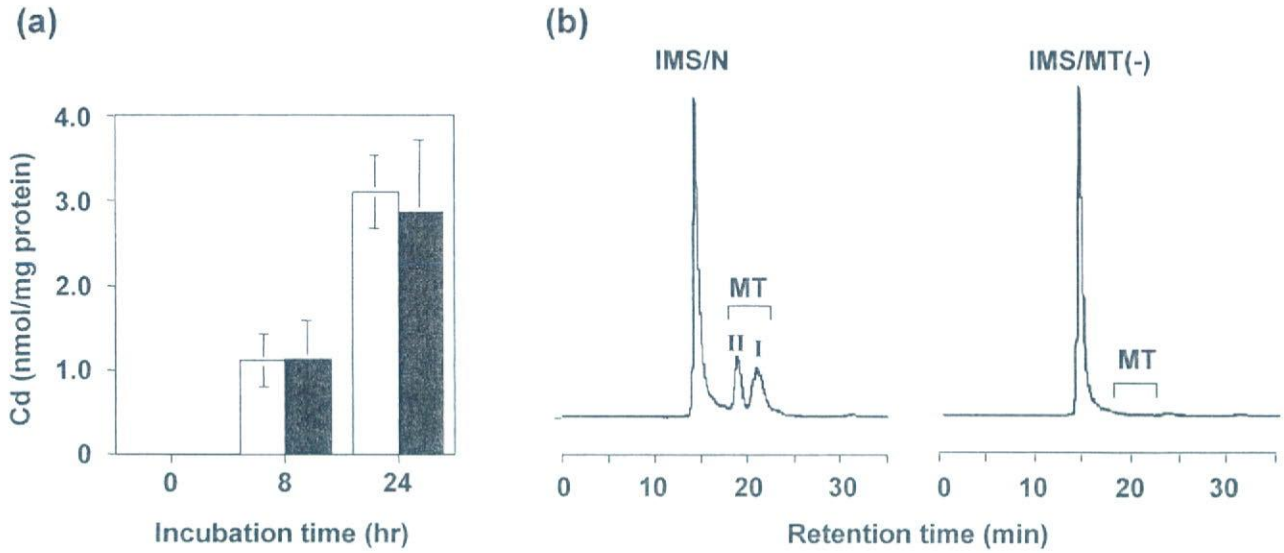

Fig. 5. Accumulation and distribution of Cd in IMS cells. (a) IMS/N and IMS/MT(-) cells (n=3) were incubated in medium containing 30 mM $CdCl_2$ for 8 hr or 24 hr and intracellular Cd concentrations determined by atomic absorption spectrometry. (b) IMS/N and IMS/MT(-) cells were incubated with 30 mM $CdCl_2$ and the Cd distribution profile was analyzed by HPLC/ICP-MS.

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ORIGINAL INVESTIGATION

Potential effect on cellular response to cadmium of a single-nucleotide A → G polymorphism in the promoter of the human gene for metallothionein IIA

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Abstract Most people generally ingest cadmium in their food. Cadmium that has accumulated in tissues induces the synthesis of metallothioneins (MTs) which are metal-binding proteins that bind tightly to cadmium to inhibit its renal toxicity. Individuals whose ability to induce the synthesis of MTs is low seem likely to be particularly susceptible to the toxic effects of cadmium. In this study, we analyzed the polymorphism of the promoter region of the gene for MT-IIA, the major species of MT in humans, in 119 adult Japanese subjects. We found that about 18% of the subjects had an A → G single-nucleotide polymorphism in the core region of the promoter near the TATA box. A reporter-gene assay using HEK293 cells showed that replacement of A by G at position −5 reduced the efficiency of the cadmium-induced transcription of the gene for MT-IIA. This single-nucleotide polymorphism inhibited the binding of nuclear proteins to the core promoter region of the gene for MT-IIA. When the promoter region upstream of the TATA box was replaced by a sequence that contained three dioxin-responsive elements, the reporter-gene assay demonstrated that the A → G single-nucleotide polymorphism resulted in a marked reduction in the rate of dioxin-induced transcription. These results suggest that the A → G single-nucleotide polymorphism reduces the efficiency of those aspects of the transcription of the gene for MT-IIA that are controlled by general transcription factors.

Introduction

Metallothioneins (MTs) are proteins of low molecular weight whose synthesis is induced by heavy metals, such as cadmium (Cd), zinc (Zn) and mercury (Hg), and which bind tightly to these heavy metals to suppress

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their toxicity (Kägi 1991; Webb 1979). Treatment of mice with inducers of the expression of MT, such as Cd and Zn, to promote the synthesis of MT markedly reduces the toxicity of subsequently administered Cd (Webb 1979). Moreover, mice with defective genes for MT are ten times more sensitive than normal mice to Cd toxicity (Liu et al. 2000). Many researchers have reported similar data and it seems clear that MTs are the main determinants of the sensitivity of mammals and cultured mammalian cells to Cd.

Humans generally ingest Cd in their food, and people who ingest excessive amounts of Cd have a high incidence of renal tubular dysfunction (Friberg and Vahter 1983; Saito et al. 1977). The half-life of Cd in humans is estimated to range from 10 to 30 years (Webb 1979) and Cd that has accumulated in the body is excreted in very small amounts. Thus, the amount of Cd in the human body increases with age (Yoshida et al. 1998). Furthermore, since Cd induces the synthesis of MT, the concentration of MT in tissues also increases with age (Yoshida et al. 1998). Much of the Cd that accumulates in tissues is bound to MT with resultant suppression of the heavy metal's toxicity. It has been postulated that the accumulation of Cd in the kidney exceeding the rate of synthesis of MT induces renal tubular dysfunction.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has estimated that the critical concentration of renal cortical Cd, namely, the concentration that causes renal tubular dysfunction in 10% of individuals exposed to Cd, is 200 μg/g tissue (WHO 1993). The mean renal cortical concentration of Cd in the general Japanese population of 41–60 years of age has been reported to be 68.9 μg/g tissue (Yoshida et al. 1998), being one-third of the estimated critical concentration. However, in MT-deficient mice, renal dysfunction can be detected even at renal concentrations of Cd below 10 μg/g tissue (Liu et al. 2000). These findings suggest that individuals with abnormalities in the synthesis of MT might be prone to renal dysfunction due to exposure to Cd. In this study, we analyzed the polymorphism of the promoter of the gene for MT-IIA, the main species of MT in humans, and found that approximately 18% of the subjects examined had an A → G single-nucleotide polymorphism near the TATA box, which reduced the level of expression of the gene for MT-IIA.

Materials and methods

Cells

Human embryonic kidney 293 (HEK293) cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% fetal bovine serum and 60 μg/ml kanamycin.

Isolation of DNA

DNA was obtained from samples of blood from 119 unrelated Japanese individuals (mean age 58 ± 11 years, range 34–79 years; 28.6% men) and was stored at −20°C prior to analysis. Written informed consent was obtained from all individuals, as required by the research protocol that had been approved by the Medical Ethics Committee of Tohoku University.

Amplification of the promoter of the gene for MT-IIA

A 222-bp fragment of the promoter of the gene for MT-IIA was amplified from 200 ng of high-molecular-weight DNA by PCR. This fragment extended from a site that was 202 bases upstream of the site of initiation of transcription (including MRE-a, -b, -c, -d and -e) to the 5′-end of exon 1 (20 bases downstream of the site for initiation of transcription; see Fig. 1). Amplification by PCR was performed in a buffered solution that contained 10 μM each primer (forward, 5′-GGG CCG CCT TCA GGG AAC TG-3′; and reverse, 5′-GGA CTT GGA GGA GGC GTG GT-3′), 200 μM dNTP mix, 10% dimethylsulfoxide and 0.025 units of AmpliTaq Gold (PE Applied Biosystems, Foster, CA) in a total volume of 100 μl. Samples were heated at 95°C for 10 min and then subjected to 30 cycles of incubation at 96°C for 30 s, at 61°C for 30 s and at 72°C for 30 s. The products of PCR were purified with a High Pure PCR Product Purification Kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to manufacturer's instructions. The samples were stored at −20°C prior to analysis.

Analysis of single-strand conformation of polymorphism (SSCP)

Eight microliters of a solution of the product of PCR were mixed with 15 μl of F-dye solution (95% formamide, 10 mM EDTA, pH 8.0, 0.05% xylene cyanole and 0.05% bromophenol blue), denatured by boiling for 5 min, and snap-chilled in iced water for 5 min. Samples were then loaded onto a 10% polyacrylamide gel that had been prepared in 1× TBE and subjected to electrophoresis at 180 V for approximately 90 min in 0.5× TBE. DNA was silver-stained with PlusOne DNA Silver-Staining Kit (Amersham Pharmacia Biotech, Buckinghamshire, England) according to manufacturer's instructions.

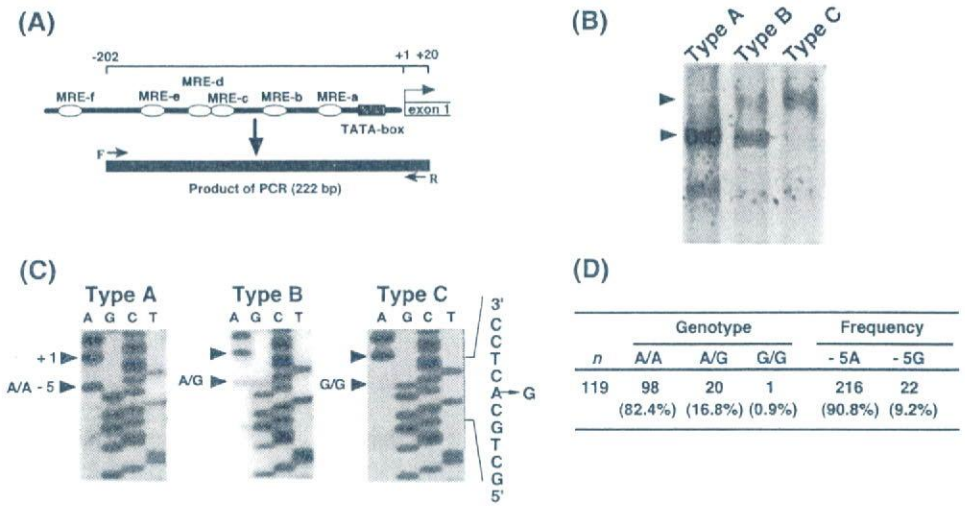

Fig. 1 Identification of polymorphism in the promoter region of the gene for MT-IIA. **a** The region of the gene for MT-IIA that was amplified for SSCP analysis. *F* and *R* indicate positions of primers. **b** Representative results of SSCP analysis. The patterns of bands after electrophoresis were of three types, as indicated. **c** Nucleotide sequencing of the DNA isolated from each band indicated in **b**. Re-amplified samples of the DNA were subjected to direct sequencing. **d** Genotypes and allele frequencies of the core promoter region of the gene for MT-IIA

Analysis of restriction fragment length polymorphism (RFLP) by PCR

The core promoter region of the gene for MT-IIA was amplified by PCR with the primers; forward, 5′-CGC CTG GAG CCG CAA GTG AC-3′; and reverse, 5′-TGG GCA TCC CCA GCC TCT TA-3′. Amplified fragments of 185 bp were digested with *Bsg*I (New England Biolabs, Hertfordshire, UK), fractionated on a 2% agarose gel, and visualized by staining with ethidium bromide. The lengths of fragments obtained by digestion of each 185-bp fragment by *Bsg*I were 144 bp and 41 bp for the A/A type (see below), 185 and 144 bp for the A/G type, and 185 bp for the G/G type.

Direct sequencing

The bands of products of PCR were excised from a silver-stained dried gel (see above) and transferred to microtubes. After addition of 50 μl of molecular-grade water, tubes were incubated at 37°C for 10 min and then at 85°C for 15 min to elute the DNA from each slice of gel. To amplify these samples of DNA, we performed PCR as described above, using 1 μl of the solution of eluted DNA as template. We purified the products of PCR and subjected an aliquot (350 fmol) to sequencing reactions using a Themo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech) and the ^{32}P-labeled forward or reverse primer (see above) as the sequencing primer. Samples were mixed with loading buffer, heat-denatured, and resolved on a 6% sequencing polyacrylamide gel at 3,000 V in 0.6× TBE buffer. The gel was dried on 3 MM paper (Whatman, Middlesex, UK) and bands were visualized by autoradiography.

Construction of plasmids

A bacterial expression vector, pcDNA3.1/*lac*Z (Invitrogen, Carlsbad, CA), in which expression of the gene for β-galactosidase is driven by the CMV promoter, was used to construct a reporter plasmid. The vector was first double-digested with *Nru*I and *Nhe*I to eliminate the CMV promoter, blunted and ligated to yield a promoter-less vector, namely, pcDNA3.1a/*lac*Z. Then the A/A type or the G/G type promoter region of the gene for MT-IIA including the 5′-end of exon 1 (positions −202 to +20; Fig. 1) was amplified by PCR using the following primers: common 5′ primer, 5′-GGG CCG CCT TCA GGG AAC TG-3′; A/A-type 3′ primer, 5′-GGA CTT GGA GGA GGC GTG GTG GAG TGC AGC GCG-3′; and G/G-type 3′ primer, 5′-GGA CTT GGA GGA GGC GTG GTG GAG CGC AGC GCG-3′. The two products of PCR were blunted and cloned into the *Eco*RV-digested pcDNA3.1a/*lac*Z vector. The resultant plasmids were designated simply −5A (A/A type) and −5G (G/G type), and each was sequenced with the Thermo Sequenase Cycle-Sequencing Kit to confirm the sequence of the inserted promoter.

Assay of reporter-gene expression

To assess the transcriptional activities of the A/A- and G/G-type promoters, we plated HEK293 cells (3.2×10^4/well) in a 24-well plate and cultured them overnight. We then transfected cells with the A/A-type or G/G-type plasmid (0.13 μg) and a reference plasmid (pRSV/Luc; 0.13 μg) using the lipofection reagent FuGene™6 (0.2 μl; Roche Diagnostics, Tokyo, Japan). After a 12-h incubation, cells were washed once with DMEM and incubated with fresh DMEM that contained non-lethal concentration of $ZnCl_2$ (100, 150, 200 or 250 μM) or $CdCl_2$ (0.125, 0.25, 0.5 or 1.0 μM) for a further 24 h. Cells were then washed with cold PBS (−) and combined with 100 μl of Reporter Lysis Buffer (Promega, Madison, WI) and lysed by freezing (−80°C for 30 min) and thawing (25°C for 20 min). To determine the activity of β-galactosidase as measure of the transcriptional activity of each MT-IIA promoter, we combined aliquots of lysed cells (45 μl) with an equal volume of a 0.2% solution of *o*-nitrophenyl-β-D-galactopyranoside, as substrate, and incubated the mixture at 37°C for 2–6 h. Each reaction was terminated by addition of 100 μl of 1 M Na_2CO_3 and then absorbance were measured at 405 nm with a microplate reader. For measurements of luciferase activity, the same samples as described above (45 μl) were combined with 10 μl of luciferin (Reconstituted Substrate; Promega) and the mixture was shaken on a vortex mixer for 10 s. Then luminescence was estimated with a luminescent plate reader.

Preparation of nuclear extract

HEK293 cells were plated at 1.2×10^6 cells per well of a 6-well plate with 2 ml of medium per well and incubated at 37°C for 24 h. Cells were then incubated with $ZnCl_2$ (final concentrations, 100 and 250 μM) for another 4 h. Nuclear extracts were prepared as described by Muller et al. (Muller et al. 1989) with slight modifications. In brief, cells were harvested with ice-cold PBS (−) and collected by centrifugation (2,000×*g*, 1 min, 4°C). The cell pellet was resuspended in 350 μl of buffer A (10 mM HEPES–KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.25% (v/v) NP−40, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin A) and mixed vigorously for 10 s. The suspension was then centrifuged as described above to pellet the nuclei. Nuclei were resuspended in 35 μl of buffer C (50 mM HEPES–KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 20% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin A) and mixed with gentle shaking for 30 min at 4°C. Then the suspension was centrifuged at 20,000 × *g* for 15 min at 4°C. The supernatant was collected and frozen in aliquots at −80°C. Concentrations of protein were determined by Bradford's protein assay method with a kit from Bio-Rad (Hercules, CA) and bovine serum albumin as the standard.

Electrophoretic mobility shift assay (EMSA)

Synthesized oligonucleotide probes are shown in Fig. 3a. The double-stranded probes were end-labeled with [γ−^{32}P] ATP by T4 polynucleotide kinase for 30 min at 37°C, and then they were purified on Micro-Spin G-50 columns (Amersham Pharmacia). Each reaction mixture (23 μl) contained 12 mM HEPES–KOH (pH 7.6), 50 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$, 12% (v/v) glycerol, 0.6 mM DTT, 100 μM $ZnSO_4$, 3 μg poly(dI–dC)-(dI–dC), and 10 μg of nuclear extract. The binding reaction was started by addition of a [γ-^{32}P]-labeled probe (10,000 cpm/μl; 2 μl), and reaction mixtures were incubated for 20 min at room temperature. Then they were loaded on a 4% non-denaturing polyacrylamide gel that contained 1× TGE (25 mM Tris–HCl (pH 8.5), 190 mM glycine, 0.5 mM EDTA) and 100 μM $ZnSO_4$, and fractionated by electrophoresis at 120 V for 100 min at room temperature in a running buffer that consisted on 1× TGE plus 100 μM $ZnSO_4$. The gel was dried and autoradiographed on Scientific Imaging Film (Kodak).

Results and discussion

We subjected DNA that had been isolated from the leukocytes of 119 Japanese subjects to SSCP analysis in an attempt to identify polymorphisms in the upstream region of the gene for MT-IIA (from position −202 to position +20; Fig. 1a). We classified the resulting electrophoretic patterns into three types (Fig. 1b). Direct sequencing of several products of PCR of each type revealed the presence of an A → G single-nucleotide polymorphism at the fifth nucleotide (−5) from the site of initiation of transcription (+1; Fig. 1C). This single-nucleotide polymorphism was located in the core promoter between the TATA box and the site of initiation of transcription (Fig. 2). Since the A → G substitution abolished the *Bsg*I restriction site, we were able to easily determine the frequency of the A/A genotype. We examined the corresponding region of nucleotide sequence in all samples from individuals with genotypes other than A/A to identify the nucleotide at position −5. We found that the A/A, A/G, and G/G

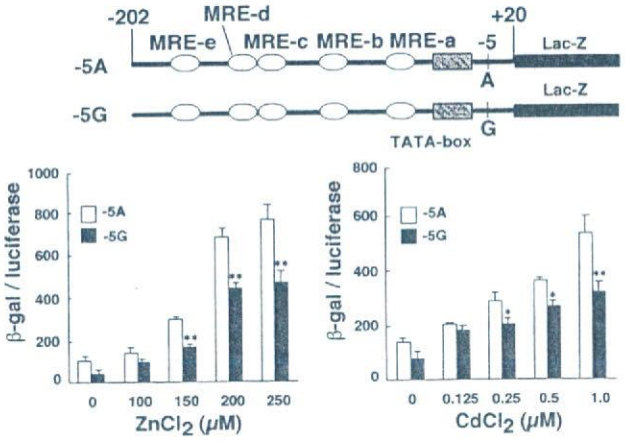

Fig. 2 Effects of the single-nucleotide A → G polymorphism on the promoter activity of the gene for MT-IIA. HEK293 cells were transfected with a plasmid with the A/A type of promoter (−5A) or the G/G type of promoter (−5G). After a 12-h incubation, cells were treated with $ZnCl_2$ or $CdCl_2$ and incubated for a further 24 h. The activities of β-galactosidase (an index of the activity of the promoter derived from the gene for MT-IIA) and of luciferase (an index of the efficiency of transcription) were measured. Results are mean ± SD ($n = 5$). A schematic representation of the relevant regions of the reporter plasmids is shown above the histograms. *, **Significantly different from the −5A type (*$P < 0.001$, **$P < 0.0001$, Student's t test)

genotypes accounted for 98 (82%), 20 (17%), and 1 (0.9%), respectively, of the 119 samples (Fig. 1d).

To examine the effects of the single-nucleotide polymorphism in the core promoter on the induction of transcription of the gene for MT-IIA by Zn and by Cd, we performed reporter-gene assays using HEK293 cells. We found that, although the transcription from both the −5A type and the −5G type promoter increased in a dose-dependent manner upon treatment of cells with Zn and with Cd, transcription from the −5G type of promoter was less efficient than that from the −5A type at all concentrations of metal ions tested (Fig. 2). The almost same results are also observed in HepG2 cells (data not shown). These findings suggested that the A → G single-nucleotide polymorphism in the core region of the promoter reduced the efficiency of the induction of transcription of the gene for MT-IIA by heavy metals. The extent of inhibition of the induction of the transcription of the gene for MT-IIA due to the single-nucleotide polymorphism was not very large. However, since people ingest Cd on their food and very little of the Cd that accumulates in their bodies is excreted, the ratio of the concentration of MT to the concentration of tissue Cd (MT/Cd) might become significantly lower in individuals with the G/G variant, who have a limited ability to induce the synthesis of MT, and somewhat lower in those with the A/G variant than it is in those with the A/A type during a human lifetime of more than 70 years. In short, the possibility cannot be excluded that the A → G single-nucleotide polymorphic variant renders an individual more prone to Cd toxicity than does the A/A type.

The main transcription factor involved in the regulation of expression of the gene for MT-IIA is metal transcription factor-1 (MTF-1) (Kita et al. 2001; Otsuka et al. 1994; Westin and Schaffner 1988). This factor is activated by Zn and by Cd and it binds to the consensus sequence (TGCRCNC) of the metal-responsive element (MRE) within the promoter of the gene for MT-IIA gene, thereby promoting transcription of this gene (Carter et al. 1984; Karin et al. 1984; Stuart et al. 1985). There are six MREs (MRE-a through MRA-f) upstream of the TATA box of the promoter of the gene for MT-IIA, and MTF-1 binds most strongly to MRA-a (Koizumi et al. 1999). A sequence identical to that of MRE-a has been found in the region between the site of initiation of transcription and the TATA box (Stuart et al. 1985). The single-nucleotide polymorphism that we found in the present study is an A → G substitution located in the center of the consensus sequence TGCACTC (from position −2 to −8; Fig. 3a). Therefore, we examined the binding of MTF-1 to this MRE-like sequence in the core promoter region. We radio labeled an oligonucleotide that corresponded to the MRE-a-containing region (from position −61 to −36) and used it as a probe (^{32}P-MRE-a oligo). We mixed the probe with a nuclear extract from Zn-treated cells and subjected the mixture to an electrophoretic mobility shift assay (EMSA). Our results confirmed previous reports that Zn dose-dependently increased the binding of MTF-1 to the ^{32}P-MRE-a probe (Fig. 3b, lanes 1–3). Treatment of the nuclear extract with a 250-fold molar excess of unlabeled MRE-a probe prior to be addition of the labeled probe almost completely abolished this binding (Fig. 3, lane 4). However, formation of the MTF-1/MRE-a complex was barely inhibited by prior treatment of the nuclear extract with an unlabeled oligonucleotide (−5A oligo or −5G oligo) that corresponded to the region (from −15 to +11) that included the MRE-like sequence with a single-nucleotide polymorphism (Fig. 3b, lanes 5–8). These findings suggested that MTF-1 might not bind to the MRE-like sequence in the core promoter region and is, thus, uninvolved in the difference in transcriptional activity that was due to the single-nucleotide substitution. However, since a number of basic transcription factors bind to the polymorphic core promoter region, we postulated that the single-nucleotide substitution might inhibit such binding. Therefore, we allowed the ^{32}P-labeled −5A and