

CHARACTERIZATION OF AN IMMORTALIZED HEPATIC STELLATE
CELL LINE ESTABLISHED FROM METALLOTHIONEIN-NULL MICENobuhiko MIURA^{1,2}, Yoshitaka KANAYAMA², Wakako NAGAI², Tatsuya HASEGAWA³,
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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, Immobilization

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% CO₂. 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 $2 \times$ 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 $12,000 \times g$ 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 CdCl₂ 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 CdCl₂ 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 CdCl₂ (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

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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 condi-

tions 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).

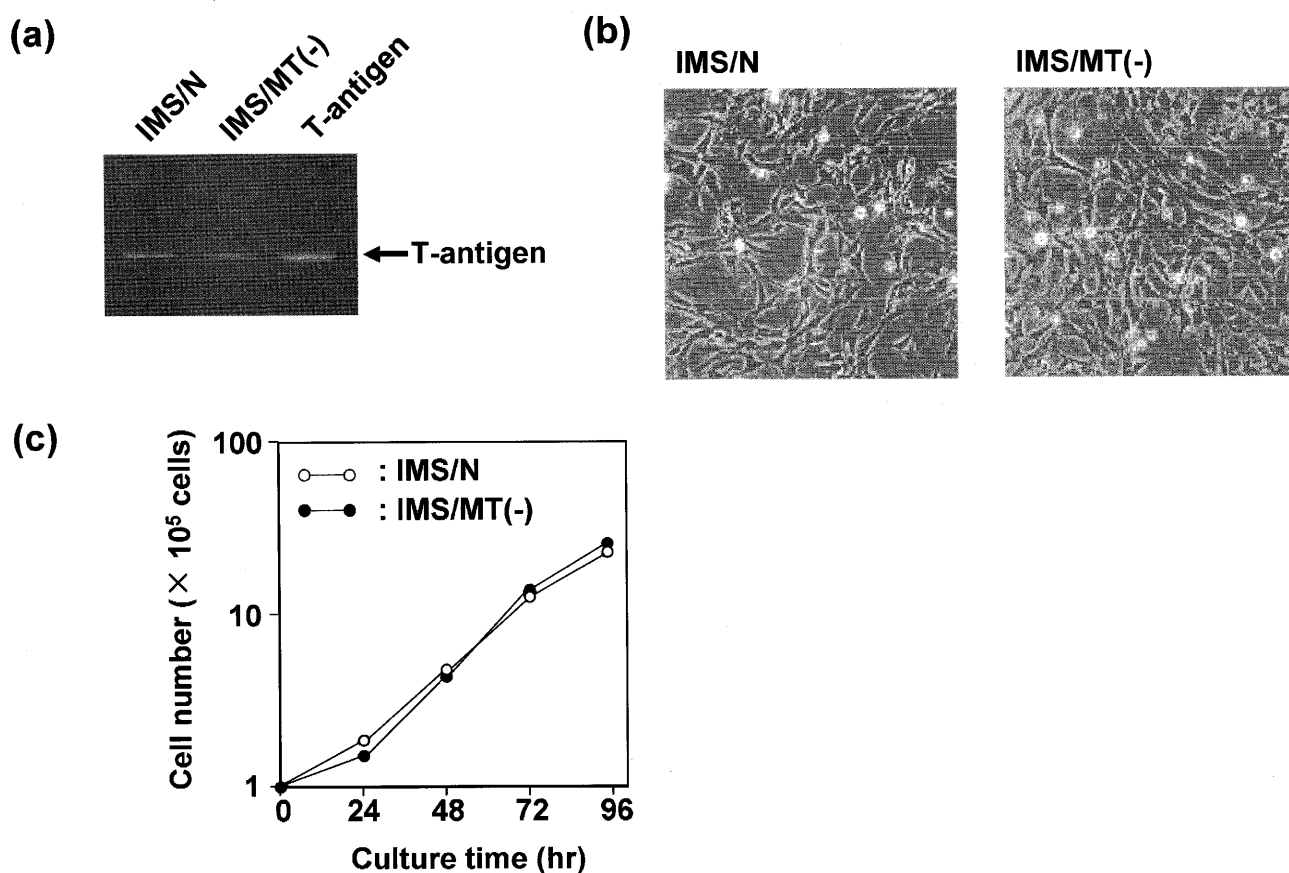


Fig. 1. Biochemical features of IMS cells. (a) SV40 T-antigen mRNA expression determined by RT-PCR. (b) Morphological characteristics of IMS/N and IMS/MT(-) cells shown by phase contrast microscopy (magnification $\times 100$). (c) Cell proliferation curve of IMS/N (open circles) and IMS/MT(-) (closed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

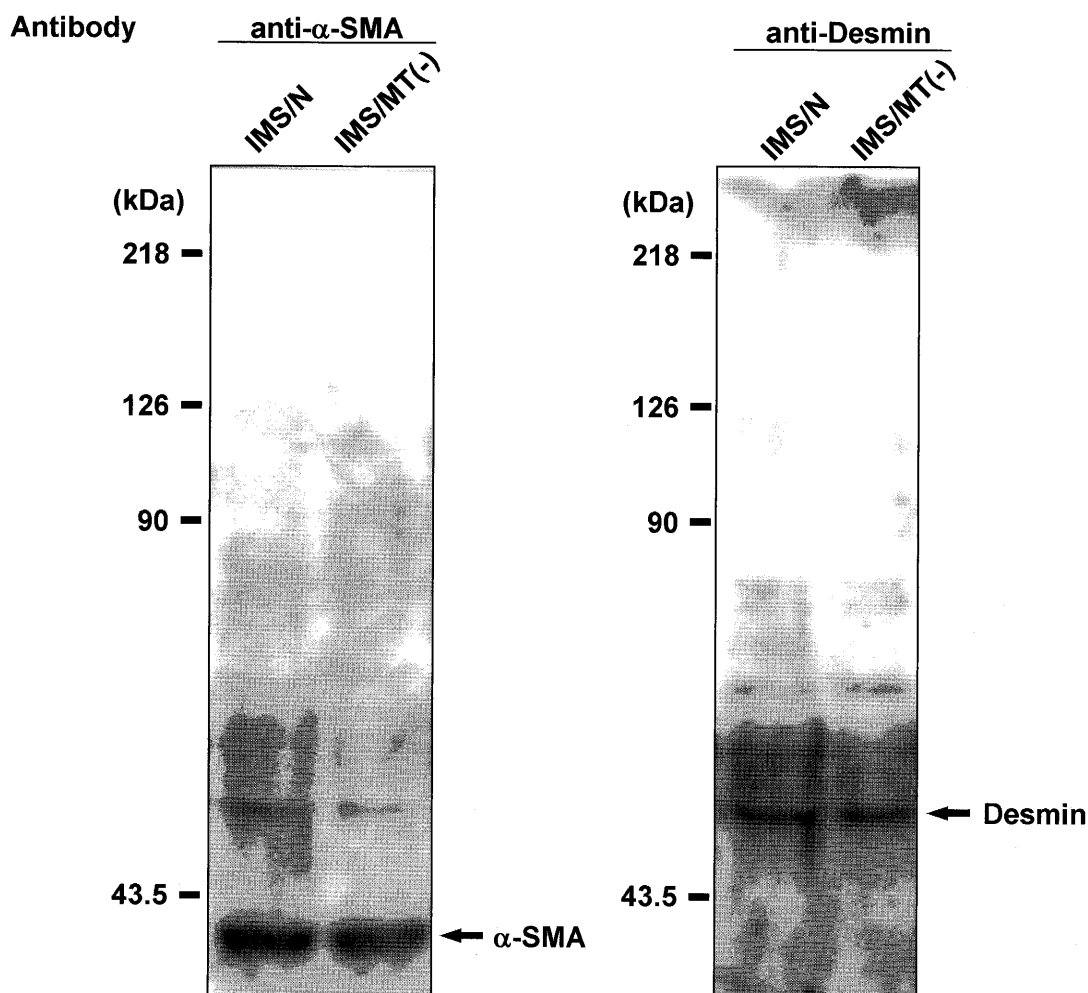


Fig. 2. Expression of α -SMA and desmin proteins as HS cell markers. Cellular proteins (approximately 45 mg) from IMS/N and IMS/MT(-) cells were resolved on 7.5% SDS-PAGE. Western blotting analysis was performed using an anti- α -SMA antibody or an anti-desmin antibody.

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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 CdCl_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

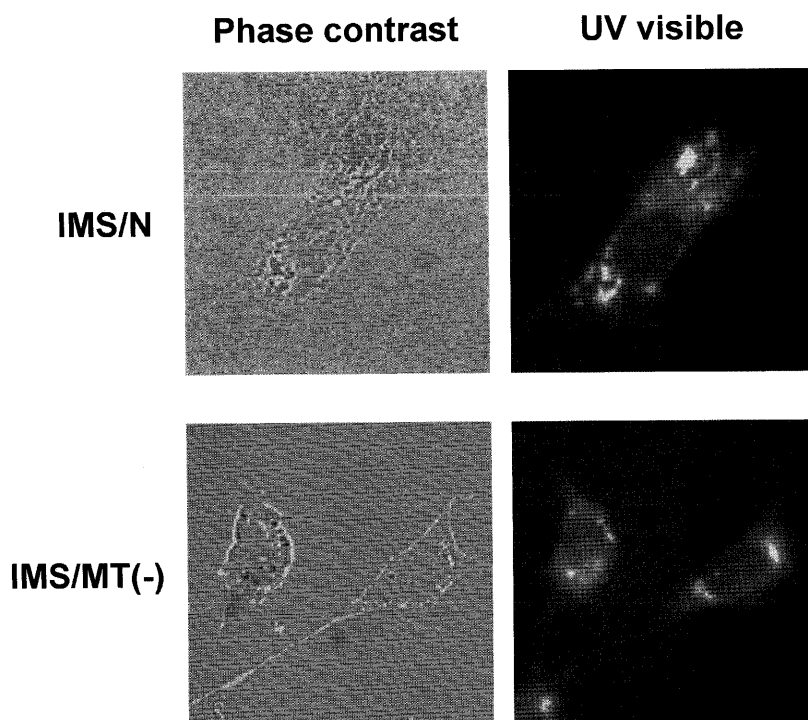


Fig. 3. Accumulation of vitamin A in IMS cells. IMS/N and IMS/MT(-) cells were incubated with 25 μM retinol acetate for three days and visualized under UV light (right) and phase contrast (left) (magnification $\times 400$).

into myfibroblast-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.

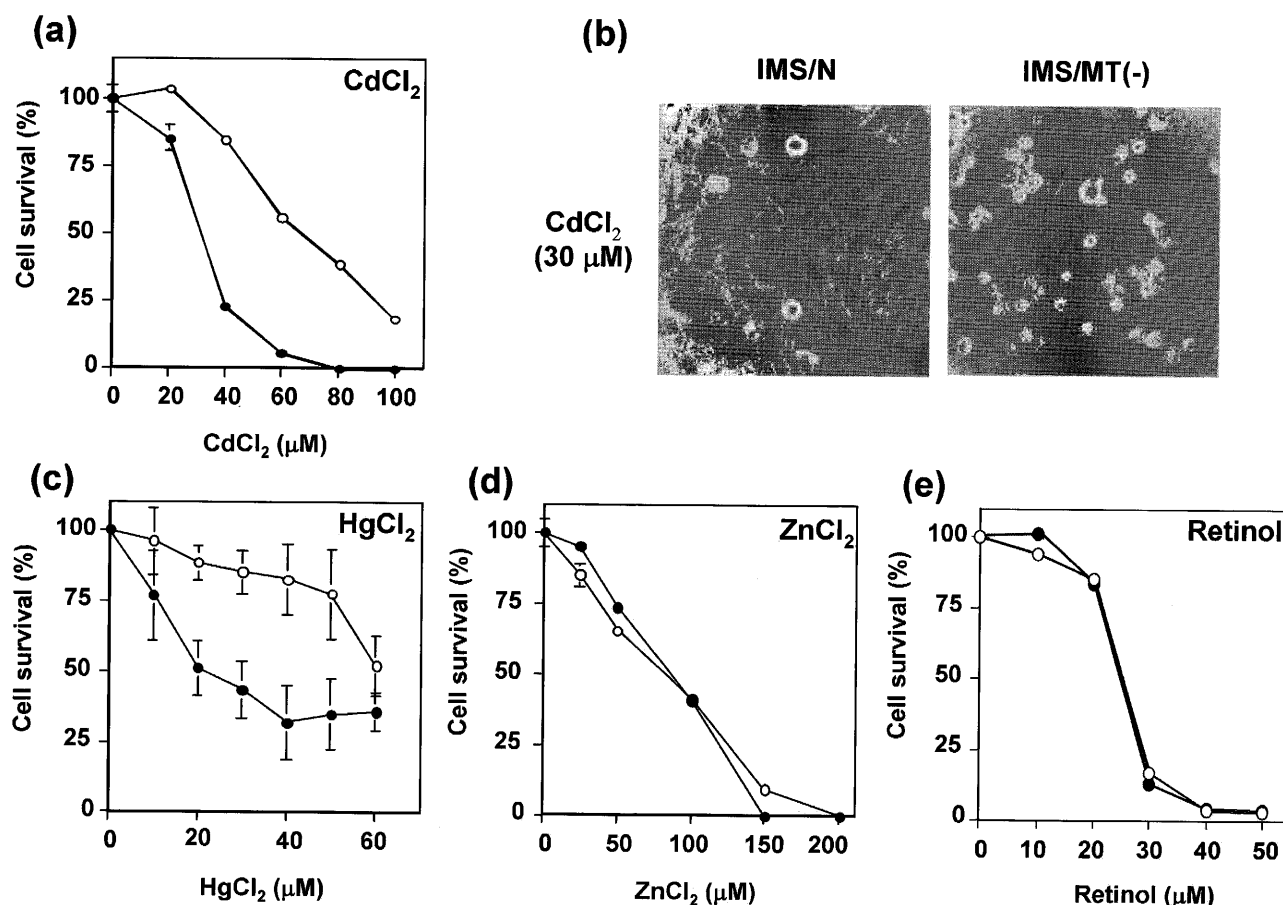


Fig. 4. Sensitivities of IMS cells to heavy metals and retinol. (a) IMS/N and IMS/MT(-) cells (1×10^4 cells/well) were incubated with the indicated concentrations of CdCl₂. Cell viability was estimated by addition of AlamarBlue. (b) Morphological changes of IMS/N and IMS/MT(-) cells after incubation with 30 mM CdCl₂ were observed by phase contrast microscopy (magnification $\times 100$). Cell survival rates of other heavy metals: HgCl₂ (c), ZnCl₂ (d), and retinol acetate (e) were also estimated. IMS/N and IMS/MT(-) cells are represented by open and closed circles, respectively.

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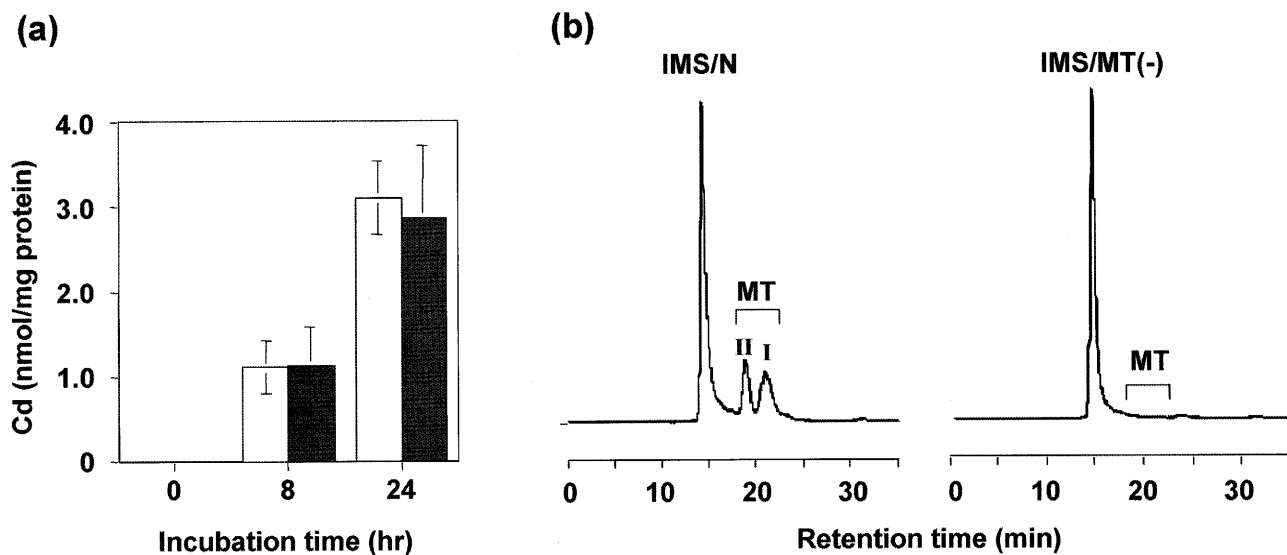


Fig. 5. Accumulation and distribution of Cd in IMS cells. (a) IMS/N and IMS/MT(-) cells (n=3) were incubated in medium containing 30 mM CdCl₂ for 8 hr or 24 hr and intracellular Cd concentrations determined by atomic absorption spectrometry. (b) IMS/N and IMS/MT(-) cells were incubated with 30 mM CdCl₂ and the Cd distribution profile was analyzed by HPLC/ICP-MS.

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