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Cadmium Inhibits *All-Trans*-Retinoic Acid-Induced Increase of Nitroblue Tetrazolium Reduction Activity and Induces Metallothionein 1G Expression in Human Acute Myelocytic Leukemia HL-60 Cells

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Cadmium is an environmental pollutant. Metallothioneins are cysteine-rich, low-molecular-weight proteins that are induced by cadmium, which they chelate for detoxification. In humans, the functional metallothionein isoforms are MT1A, 1B, 1E, 1F, 1G, 1H, 1M, 1X, 2A, 3, and 4. It has been reported that overexpression of MT1G inhibits *all-trans*-retinoic acid (ATRA)–induced hematopoietic differentiation. Here, we found that cadmium inhibited ATRA-induced nitroblue tetrazolium reduction activity, a marker of hematopoietic differentiation, in human acute myelocytic leukemia HL-60 cells. Reverse transcription – quantitative polymerase chain reaction analysis of HL-60 cells revealed the expression of the metallothionein isoforms MT1G, MT1X, and MT2A and showed that MT1G expression increased significantly after cadmium treatment. ATRA treatment significantly attenuated this cadmium-induced increase in MT1G expression; however, MT1G expression remained significantly higher than that in untreated cells. No significant changes in MT1X and MT2A expression were observed. We also found that the cadmium-induced increase in MT1G expression was independent of CpG demethylation. ATRA is a standard chemotherapy treatment for acute promyelocytic leukemia; our findings suggest that cadmium may inhibit the effect of this chemotherapy.

Key words cadmium, hematopoietic differentiation, metallothionein

INTRODUCTION

Cadmium is a naturally occurring metal but also an environmental pollutant.[1)] Cadmium is toxic to humans, with the major routes of exposure being tobacco smoking and the consumption of rice that has taken up cadmium from the soil. Because of cadmium's high renal toxicity, the provisional tolerable weekly intake has been set at 7 μg/kg-body weight/week by the Joint FAO/WHO Expert Committee on Food Additives on the basis of the urinary α_1-macroglobulin content, a biomarker of renal tubular dysfunction.[2)] Cadmium is toxic not only to renal tubular cells but also to other cells, such as hematopoietic cells.[3)] Therefore, cadmium influences immunity, possibly via the modification of immune cell numbers. Indeed, Zhang *et al.* have reported that cadmium increases myeloid cell numbers and decreases lymphocyte numbers in mice.[3)]

Metallothioneins (MTs) are cysteine-rich, low-molecular-weight proteins that are induced by cadmium via the activation of metal regulatory transcription factor 1 (MTF1).[4)] MTs are capable of chelating cadmium for detoxification.[5)] MT1 and MT2 (which contain 61 amino acids each) are the major MT isoforms found in all tissue types.[6)] Two other isoforms, MT3 (62 amino acids) and MT4 (68 amino acids), are also expressed in a limited range of tissues. In humans, the functional MT1 isoforms are MT1A, 1B, 1E, 1F, 1G, 1H, 1M, and 1X. Several lines of research have revealed MT isoform–specific functions.[7)]

In human leukemia cell lines (K562, NB4, and THP-1 cells), transcription factor PU.1, a myeloid master regulator, downregulates the expression of MT isoforms 1A and 1G in response to hematopoietic differentiation, probably because of CpG methylation via PU.1 recruitment to the promoter region.[8–10)] Indeed, it has been reported that PU.1 binds to the MT1A promoter region at base pairs –602 to –299 from the transcription start point in K562 cells and base pairs –887 to –602 in THP-1 cells, and to the MT1G promoter region at base pairs –596 to –297 in K562 cells, although the precise binding sites are yet to be elucidated. Overexpression of MT1G inhibits all-*trans* retinoic acid (ATRA)–induced hematopoietic differentiation.[9)] ATRA is used in the standard chemotherapy in acute promyelocytic leukemia.[11)] Cadmium might have an inhibitory potential for the chemotherapy.

Here, we examined the effect of cadmium on MT expression and cell differentiation in the human acute myelocytic leukemia HL-60 cell line. We found that cadmium increased MT expression in a CpG demethylation–independent manner and that the induced MTs inhibited ATRA-induced HL-60 cell differentiation.

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MATERIALS AND METHODS

Cell Culture HL-60 cells were purchased from RIKEN BioResource Center Cell Bank (Tsukuba, Japan) and cultured in RPMI1640 (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaillé, France) and a mixture of Antibiotic-Antimycotic solution (Nacalai Tesque, Inc.) at 37°C under a 5% CO_2 atmosphere. The cells were seeded at a density of 1.68×10^5 or 8.4×10^4 cells/mL every 2 or 3 d. Before treatment with ATRA (Sigma-Aldrich, St. Louis, MO, USA), cells were seeded at a density of 8.4×10^4 cells/mL. Several studies have shown that the average blood cadmium concentration in humans is around 20 nM, with the maximum around 80 nM[12–15]; therefore, here HL-60 cells were treated with a cadmium concentration of 50 nM.

Nitroblue Tetrazolium Reduction Activity Assay To evaluate the differentiation of HL60 cells, nitroblue tetrazolium (NBT) reduction activity was measured.[16] Cells were collected and resuspended in fresh RPMI1640 at a density of 2.0×10^5 cells/0.5 mL. Then, an equal amount of phosphate-buffered saline containing 0.2% NBT and 20% fetal bovine serum was added to the cell suspension and the cells were incubated with 12-*O*-tetradecanoylphorbol-13-acetate (final concentration, 2 μM; Tocris Bioscience, Bristol, UK) at 37°C for 30 min. After the incubation, blue formazan deposited inside the cells was dissolved by 500 μL dimethyl sulfoxide and the absorbance at a wavelength of 550 nm was measured.

Evaluation of MT Isoform Expression For quantitative reverse transcription PCR (RT-qPCR), RNA was isolated from cells by using Isogen RNA extraction reagent (Nippon Gene Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. Extracted total RNA was converted to the complementary DNA by using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a random primer. For qPCR, we used TB Green *Premix Ex Taq* (Tli RNaseH Plus) (Takara Bio Inc., Kusatsu, Japan) as the qPCR reagent and an Eco Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). Gene-specific primers for the MT isoforms are shown in Table 1. The primer set for MT1M was purchased as predesigned PrimeTime qPCR Primer Assays (Integrated DNA Technologies, Inc., Coralville, IA, USA). The others were designed previously.[17] To calculate the copy number of each cDNA in the total RNA, cDNA cloned vectors, which were quantified by Qubit dsDNA high-sensitivity assay (Thermo Fisher Scientific, Inc.), were used as DNA standards. The lower limit of detection was around 500 copies/10 ng RNA.

For comparison of our RT-qPCR results with previously published findings, HL-60 cell gene expression data were obtained from the Reference Expression Dataset (RefEx).[18] The URL of the HL-60 sample annotations for CAGE human PRJDB3010 (FANTOM5) is http://fantom.gsc.riken.jp/5/sstar/FF:10829-111D1.

Bisulfite Sequencing Assay Purification of genomic DNA, bisulfite conversion, PCR amplification, cloning into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and sequencing and visualization for CpG methylation status analysis by using QUMA (QUantification tool for Methylation Analysis[19]) were performed as described previously.[20] The sequences of the sense and antisense primers for PCR amplification are shown in Table 1. These primers were designed by using Methyl Primer Express software (v1.0; Applied Biosystems, Foster City, CA, USA).

Table 1. Primers Used for qPCR and for Bisulfite Sequencing Assay

	Gene		Sequence
qPCR	MT1A	F	5'-GCTCGAAATGGACCCCAAC-3'
		R	5'-TAAATGGGTCAGGGTTGTATGG-3'
	MT1B	F	5'-CTCCTGCCCTGACTTCTCA-3'
		R	5'-CTCTGATGAGCCTTTGCAGAC-3'
	MT1E	F	5'-GCTTGTTCGTCTCACTGGTG-3'
		R	5'-CAGGTTGTGCAGGTTGTTCTA-3'
	MT1F	F	5'-CGCTTCTCTCTTGGAAAGTCC-3'
		R	5'-CTGTTTACATCTGGGAGAAAGGTTG-3'
	MT1G	F	5'-CTAGTCTCGCCTCGGGTTG-3'
		R	5'-GCAGCTGCACTTCTCCGAT-3'
	MT1H	F	5'-GCTCCTGCAAGTGCAAAAAG-3'
		R	5'-AGGAATGTAGCAAATGAGTCG-3'
	MT1M	F	5'-AGCAGTCGCTCCATTTATCG-3'
		R	5'-GCTCTTCTTGCAGGAGGTG-3'
	MT1X	F	5'-TTGATCGGGAACTCCTGCTT-3'
		R	5'-CAGCAGCTGCACTTGTCTG-3'
	MT2A	F	5'-CTAGCCGCCTCTTCAGCTC-3'
		R	5'-AAGTCGCGTTCTTTACATCTGG-3'
	MT3	F	5'-TCCTGCAAGTGCGAGGGATG-3'
		R	5'-GGACACCAGCCACACTTCA-3'
	MT4	F	5'-GCAAATGCACAACCTGCAAC-3'
		R	5'-AGACACTTCCCAGGTTTCTC-3'
BSP	MT1A	F	5'-GGATTAGGGTGGAAGGTAATT-3'
		R	5'-ATCCCAAACAAAAAATTAAAAAAC-3'
	MT1G	F	5'-GGTAATTTTAGGGAAGTTGGG-3'
		R	5'-TTTAACATCCCAAAACACAAAA-3'
	MT1X	F	5'-ATGTAAAGGAGGATTTGGGTAA-3'
		R	5'-CTTACCTACCCAATTTCCCTAA-3'
	MT2A	F	5'-AGGGAGGGGAGTTGTGTATA-3'
		R	5'-ACTAAACATCCCCAACCTCTTA-3'

F: forward primer, R: reverse primer, BSP: primers for bisulfite sequencing assay; underlining indicates T and A nucleotides that are C and G nucleotides, respectively, in the original genomic sequence.

Statistical Analysis Data were analyzed with Tukey's test in PASW Statistics 18 software (IBM, Armonk, NY, USA). Differences between groups were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

To clarify the effects of cadmium on ATRA-induced HL-60 cell differentiation, we measured the induction of NBT reduction activity. HL-60 cells were cultured for 14 d with or without 50 nM cadmium and then treated with ATRA for 4 d at the indicated concentrations. As shown in Fig. 1A, there was no effect of cadmium (50 nM for 14 d) on NBT reduction activity in ATRA-untreated (undifferentiated) HL-60 cells. NBT reduction activity was significantly increased about 3-fold after ATRA treatment (125–500 nM) compared with that in untreated cells (Fig. 1B). However, in HL-60 cells exposed to cadmium, NBT reduction activity was significantly increased only about 2-fold after ATRA treatment (250 or 500 nM) compared with that in ATRA-untreated cells. This suggests that cadmium inhibited cell differentiation in the promyelocytic leukemia HL-60 cells; it is consistent with the findings of Zhang *et al.*, who reported that cadmium increases myeloid cell numbers but decreases lymphocyte numbers in mice.[3]

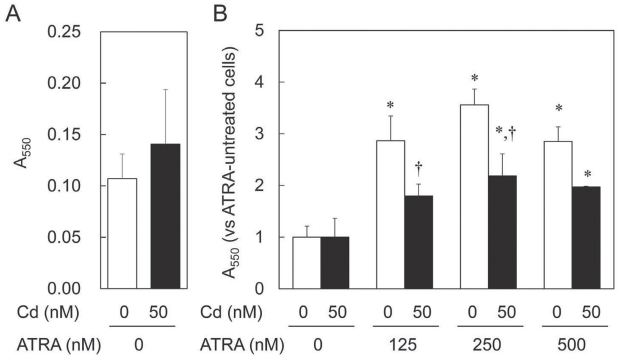

Fig. 1. Cadmium Inhibits ATRA-Induced NBT Reduction Activity in HL-60 Cells

(A) Cells were cultured for 18 d with or without 50 nM cadmium and then NBT reduction activity was measured as the change in absorbance at a wavelength of 550 nm. (B) Cells were cultured with or without 50 nM cadmium. On the 14th day, ATRA was treated at the indicated concentrations. Four-day after ATRA treatment, NBT reduction activity was measured. Data are presented as means ± SD of three independent experiments. *$p < 0.05$, significantly different from the ATRA-untreated group; †$p < 0.05$, significantly different from the cadmium-untreated group.

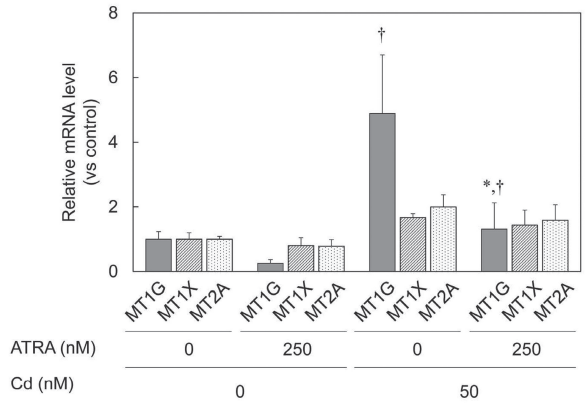

Fig. 2. Effect of Cadmium on Metallothionein Expression in ATRA-Treated HL-60 Cells

HL-60 cells were treated as described in the caption to Figure 1. MT1G, MT1X, and MT2A mRNA was measured 1-day after ATRA treatment by RT-qPCR with values normalized to total RNA content. Data are presented as means ± SD of three independent experiments. *$p < 0.05$, significantly different from the ATRA-untreated group; †$p < 0.05$, significantly different from the cadmium-untreated group.

Table 2. Metallothionein Isoforms Expressed in HL-60 Cells

Isoform	CAGE value (relative log expression)	RT-qPCR ($\times 10^5$ copies/10 ng RNA)
MT1A	0	n.d.
MT1B	0	n.d.
MT1E	no data	n.d.
MT1F	0.66	n.d.
MT1G	2.82	9.8 ± 2.3
MT1H	0	n.d.
MT1M	0	n.d.
MT1X	4.76	78.5 ± 15.5
MT2A	0.15	69.9 ± 6.1
MT3	0	n.d.
MT4	0	n.d.

n.d.: not detected; CAGE values were obtained from the RefEx database.

MTs are negative regulators of hematopoietic differentiation.[9] Therefore, we measured the expression of MTs in HL-60 cells by means of RT-qPCR analysis (Table 2). MT1G, MT1X, and MT2A mRNA were detected. Although a CAGE value for MT1F (0.66) was obtained from RefEx, we did not detect MT1F mRNA in our analysis. In addition, the expression level differed between CAGE and RT-qPCR. These inconsistencies possibly occurred because of the different measurement methods used—CAGE value was from cap analysis gene expression technology, whereas RT-qPCR was performed as an intercalator-based real-time PCR.

Figure 2 shows the relative expression of the three detected MTs in HL-60 cells treated with cadmium or ATRA or both. Expression of MT1G was significantly increased 4.9-fold after cadmium treatment compared with that in untreated cells. Expression of MT1G tended to decrease with ATRA treatment; however, the expression in 50 nM cadmium/250 nM ATRA cells remained higher than that in cadmium- and ATRA-untreated cells. No significant changes in MT1X and MT2A expression were observed.

The inhibitory effects of MT isoforms on hematopoietic differentiation have been examined by Hirako *et al*., who reported that MT1G inhibited hematopoietic differentiation.[9] This suggests that induction of MT1G might be the reason why we found that cadmium treatment inhibited differentiation in HL-60 cells. MT1G expression profile can be viewed in RefEx. Highly expressed tissues are liver, kidney, pancreas and thyroid grand. The amino acid sequences of the MT1/2 isoforms are highly conserved; in MT1G, 58 of 61 amino acids are conserved. The 13th amino acid (serine) is unique, and the 9th and 11th amino acids (alanine and valine, respectively) do not appear often in other MT1/2 isoforms. Therefore, it is possible that the hematopoietic differentiation inhibitory activity of MT1G is mediated by these unique or rare amino acids. To confirm the involvement of MT1G on the inhibitory activity, experiments at the protein level are essential. Our results suggest that MT1X and MT2A do not have such an inhibitory function, but whether other MT isoforms do remains unknown. Further experiments are needed to clarify the functions of the other MT isoforms.

Cadmium is also an epimutagen.[21] Previously, we showed that long-term cadmium exposure resulted in CpG demethylation of the MT1 promoter in mouse lymphosarcoma P1798 cells.[20] To clarify the involvement of CpG methylation status in MT isoform expression, we performed bisulfite sequencing for MT1A, 1G, 1X, and 2A. HL-60 cells were cultured for 14 d with or without 50 nM cadmium. After the culture, genomic DNA was extracted and bisulfite sequencing was performed. MT1G, 1X, and 2A, but not MT1A (which has a PU.1 binding site[8,10]), were detected by RT-qPCR. We then verified the CpG methylation status and number of putative MTF1 binding sites, as predicted by JASPAR,[22] of the MT isoforms in cells with and without exposure to 50 nM cadmium (Fig. 3). The promoter of the undetected MT isoform, MT1A, showed an extremely high CpG methylation ratio. In contrast, the promoters of the three detected isoforms all showed low CpG methylation ratios. No marked change of the CpG methylation ratio was observed among the MT isoforms in cells with and without exposure to cadmium, suggesting that the observed cadmium-induced induction of MTs occurred in a CpG-demethylation–independent manner.

In summary, cadmium exposure inhibited ATRA-induced HL60 differentiation, possibly via an increase in MT1G expression in a CpG-demethylation–independent manner.

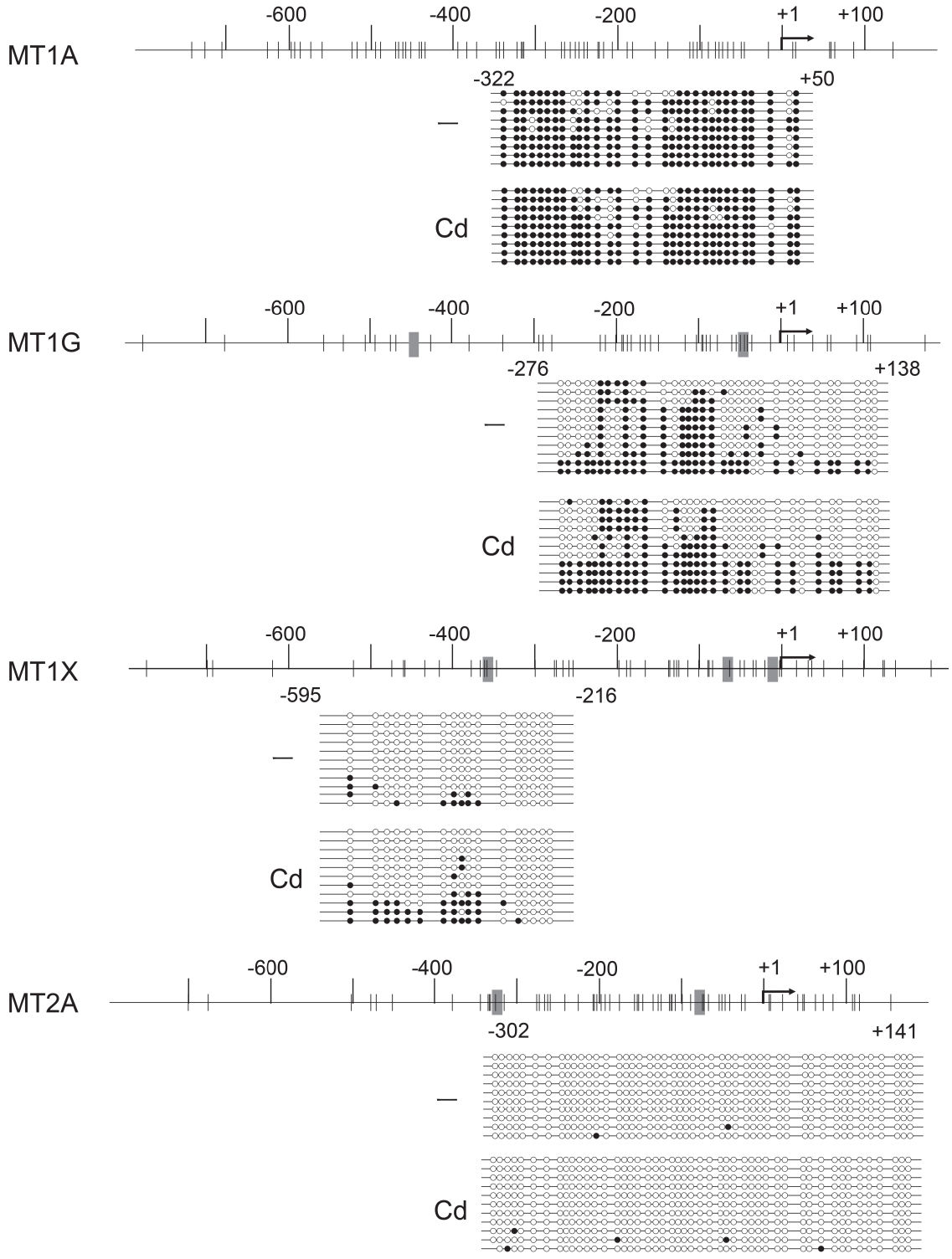

Fig. 3. Effect of Cadmium on CpG Methylation Status of MT1A, 1G, 1X, and 2A in HL-60 Cells

HL-60 cells were cultured for 14 d with or without 50 nM cadmium. After the culture, genomic DNA was extracted and bisulfite sequencing was performed. In the schematic, CpG dinucleotides are represented by | . Open circles represent unmethylated cytosine; closed circles represent methylated cytosine. Gray boxes represent putative MTF1-binding sites, as discovered via a search of the JASPAR database of transcription factor profiles[22] (http://jaspar.genereg.net; matrix ID:MA0863.1; relative profile score threshold: 80%).

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Conflict of interest The authors declare no conflict of interest.

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