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Metallothionein 1 Isoform Gene Expression Induced by Cadmium in Human Peripheral Blood Lymphocytes[1]

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Objective To study the gene expression of metallothionein 1 (MT-1) isoforms in human peripheral blood lymphocytes (HPBLs). **Methods** The expression of mRNA representing the seven active MT-1 genes was determined in HPBLs by quantitative RT-PCR before and after exposure to cadmium. **Results** Basal expressions of MT-1X, and MT-1A in HPBLs were similar to expression of housekeeping gene. In contrast, the basal gene expressions of MT-1H, 1F, 1E, and 1G were a little transcripts in human HPBLs. No signal was detected for MT-1B. There was a sex difference ($P<0.05$). in basal gene expression of MT-1E. The levels of gene expression of MT-1A, 1E, 1F, 1G, 1H, and 1X increased, but the level of MT-1B did not increase after exposure to cadmium. **Conclusions** Gene expressions of MT-1G, MT-1H, MT-1F, and MT-1X in HPBLs can be used as a potential biomarker of cadmium exposure.

Key words: Metallothionein 1; Gene; Human peripheral blood lymphocytes; Cadmium

INTRODUCTION

The metallothioneins (MTs) are a group of cysteine-rich, inducible, intracellular stress proteins with a low molecular weight (M_r=6000-7000), best known for their high affinity to heavy metals. The MTs are widely recognized and accepted as a major tool in the cellullar armamentarium for protection against and recovery from environmental insult. They are believed to play an important role in the homeostasis of essential metals such as Zn and Cu during growth and development as well as in the detoxification of heavy metals such as Cd, rendering the MTs important mediators/attenuators of heavy metal-induced toxicity[1-4].

In humans, four MT isoforms, MT-1, MT-2, MT-3, and MT-4, have been found so far but these also have subtypes with 17 MT genes identified in humans, of which 10 are known to be functional, clustered on chromosome 16q13[5]. The human MT-1 gene family is composed of seven active genes (MT-1A, 1B, 1E, 1F, 1G, 1H, and 1X) and six pseudogenes (MT-1C, 1D, 1I, 1J, 1K, and 1L). The human MT-1 isoform genes have been shown to exhibit unique expression profiles with examples of inducer-specific, tissue-specific, and development-specific regulation[6]. Different cells express different MT isoforms with varying levels of expression perhaps as a result of the different function of each isoform. To define the regulation of expression of the MT-1 gene isoforms in HPBLs exposed to cadmium, the expression of mRNA representing the seven active human MT-1 genes was determined in cultured HPBLs before and after exposure to cadmium in this study.

MATERIALS AND METHODS

Subjects

Twelve healthy volunteers (6 males and 6 females), aged 25-34 yr were enrolled, in the study. All subjects were nonsmokers and considered to be healthy without history of illness or chronic medication.

Blood Cadmium Determination

Twenty μL of venous whole blood was diluted with 0.1% HNO_3. Then blood cadmium was analyzed by atomic absorption spectrometry[7].

Culture, Cadmium Treatment, and Viability Determinations of HPBLs

HPBLs were isolated using density-gradient

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centrifugation[8]. A total of 10 mL of venous whole blood was collected in an acid-washed, heparin-containing vacutainer. Whole blood was mixed 1:1 with phosphate-buffered saline (PBS) and laid onto Ficoll-Paque. Lymphocytes were collected at the interface after centrifugation at 400 g for 30 min. Then lymphocytes were washed twice with PBS and once using RPMI 1640. Isolated HPBLs were then divided in 24-well plates. The cells were incubated at 37℃, in humidified atmosphere with 5% CO_2. After culturing for 1 h, $CdCl_2$ was added to the medium in the appropriate concentrations of 10 μmol/L, 20 μmol/L, 40 μmol/L, 80 μmol/L. The viability of HPBLs was assayed by the leakage of lactate dehydrogenase (LDH)[9]. After exposure to 4 h, 12 h, 24 h, the lymphocytes were collected by centrifugation at 400 g for 5 min.

RNA Isolation

Total RNA was isolated according to the protocol supplied with TRI Reagent (Intrivitrogen Life Technologies). TRI Reagent (1 mL) was added to cells, homogenized. chloroform (200 μL) was added, and the sample was mixed thoroughly. After phase separation at room temperature for 15 min, the sample was centrifuged at 12 000×g for 15 min at 4℃. RNA was precipitated from the aqueous phase with 0.5 mL isopropanol. Following centrifugation at 12000×g for 10 min at 4℃, liquid was decanted, leaving the RNA pellets. The pellets were washed once with 0.5 mL 75% ethanol and air-dried at room temperature. RNA was resuspended in diethylpyrocarbonate-treated distilled water. The concentration and purity of RNA samples were determined using UV spectrophotometer.

RT-PCR

Total RNA (3 μg) was reverse transcribed using the AMV first strand cDNA synthesis kit (BBI). The primers developed for analysis of each of the active MT genes have been previously described[10] (Table 1).

TABLE 1

Primer Sequence and Product

Gene	GenBank GI	Primer Sequence		Product (bp)
		Upper	Lower	
GAPDH	GI：0226183	5′CGGAGTCAACGGATTTGGTCGTAT3′	5′AGTCTTCTCCATGGTGGTGAAGAC3′	307
MT-1A	GI:31342255	5′CTCGAAATGGACCCCACT3′	5′ATATCTTCGAGCAGGGCTGTC3′	219
MT-1B	GI:27414494	5′GCTTGTCTTGGCTCCACA3′	5′AGCAAACCGGTCAGGTAGTTA3′	287
MT-1E	GI:31581520	5′CTCATTGCCCGTGTCATTC3′	5′AGAACCCAGACCCAGAGGAT3′	159
MT-1F	GI:28866946	5′AGTCTCTCCTCGGCTTGC3′	5′ACATCTGGGAGAAAGGTTGTC3′	232
MT-1G	GI:10835229	5′CTTCTCGCTTGGGAACTCTA3′	5′AGGGGTCAAGATTGTAGCAAA3′	309
MT-1H	GI:10835084	5′CCTCTTCTCTTCTCGCTTGG3′	5′GCAAATGAGTCGGAGTTGTAG3′	315
MT-1X	GI:31543213	5′TCTCCTTGCCTCGAAATGGAC3′	5′GGGCACACTTGGCACAGC3′	151

The reverse transcribed product was then used for PCR amplification with a DNA thermocycler (Perkin-Elmer-Cetus 9600) programmed to cycle at 94℃ for 5-min initial step, then at 94℃ for 30s and at 55℃ for 1 min, with a final elongation step at 72℃ for 10 min. The resulting PCR product was electrophoresed on 2% agarose gel, along with appropriate size markers. The levels of transcripts from the MT-1 genes as well as the GAPDH gene, as a reference, were determined by RT-PCR. To obtain quantitative results, the intensity of specific mRNA bands was quantified by densitometric analysis. Relative MT-1 isoform mRNA abundance was defined as

$$\frac{\text{MT-1mRNAdensity}}{\text{GAPDHmRNAdensity}}$$

at the same sample of RNA.

Statistical Analysis

The data were analyzed with the SPSS 11.0 statistical program. To analyze the data statistically, we performed a multivariate and factor analysis. We then used LSD to determine which means achieved significant differences between the treated-group and the control group. $P<0.05$ was considered statistically significant.

RESULTS

Blood Cadmium

In the 12 subjects, the mean concentration of blood cadmium was 0.0064±0.0025 μmol/L; it was similar between the genders (0.0061±0.0016 μmol/L for males and 0.0068±0.0032 μmol/L for females, $P>0.05$).

LDH Activity

Very little or no detectable extracellular LDH activity was released from the control group and the treated group (Table 2).

TABLE 2

LDH Activity in HPBLs Exposed to Cadmium (U, $\bar{x} \pm s$, n=12)

$CdCl_2$ (μmol/L)	4 h	12 h	24 h
0	105.54±34.18	122.25±33.89	126.65±36.30
10	102.56±37.44	123.74±43.30	139.78±41.99
20	124.12±33.40	116.21±33.79	139.33±43.86
40	100.03±23.03	121.58±32.34	131.80±41.65
80	119.79±35.47	115.62±59.18	134.48±32.16

Basal Expression of Human MT Isoform 1 mRNA in PBLs

Basal mRNA expressions of MT-1X and 1A in HPBLs were similar to expression of the housekeeping gene glyceraldehydes 3-phosphate dehydrogennase (Table 3). In contrast, mRNAs representing the basal expressions of 1E, 1G, and 1H were very low in HPBLs. No signal was observed for the MT-1B. Basal expression of MT-1E showed sex difference ($P<0.05$).

TABLE 3

Basic Expression Levels of MT-1 Isoform Genes in HPBLs

Isoform	Male	Female	Total
MT-1A	0.7±0.18	0.8±0.42	0.75±0.32
MT-1E	0.71±0.19	0.58±0.19*	0.64±0.2
MT-1F	0.74±0.16	0.73±0.19	0.73±0.18
MT-1G	0.67±0.19	0.57±0.16	0.62±0.18
MT-1H	0.7±0.33	0.77±0.24	0.74±0.28
MT-1X	0.78±0.25	0.79±0.19	0.79±0.22

Note. $^*P<0.05$ vs. males.

Cadmium-induced MT-1 mRNA in HPBLs

The expressions of MT-1X, MT-1E, MT-1F, MT-1A, MT-1G, and MT-1H mRNA were up-regulated after exposure to cadmium in HPBLs (Fig. 1), excluding MT-1B. The maximal induction of MT-1

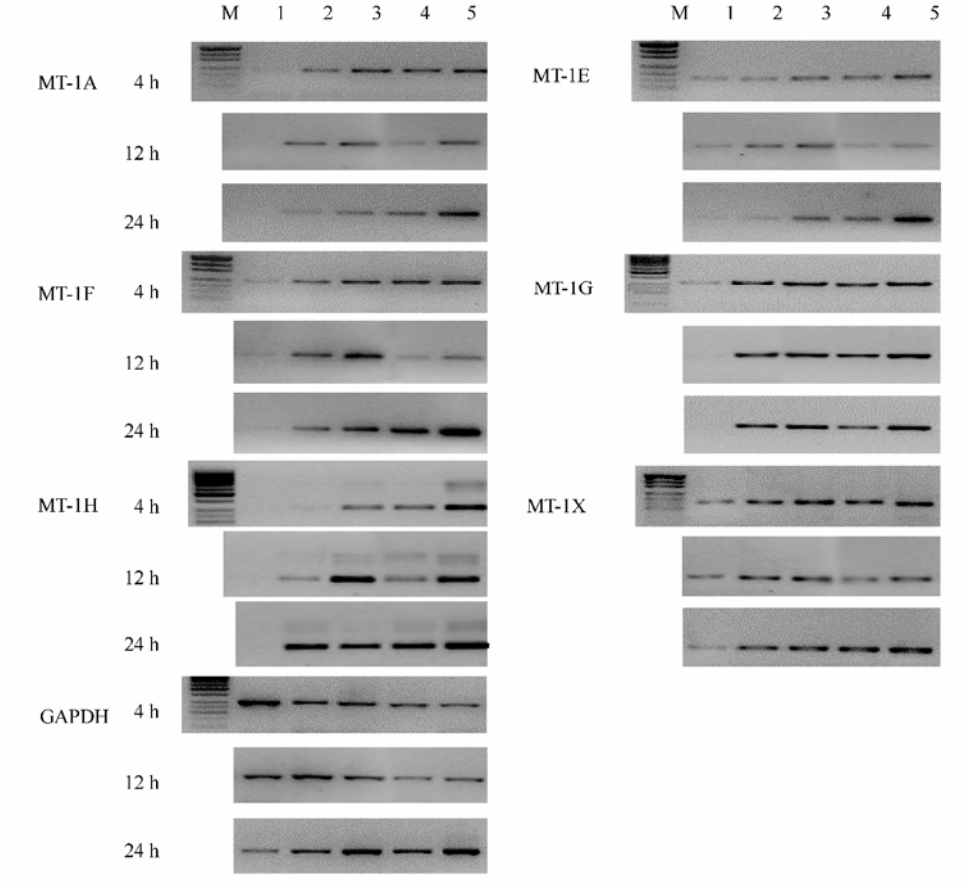

FIG. 1. Expression of MT-1A,E,F,G,H,X mRNA in HPBLs exposed to cadmium.
M：marker; 1：control; 2：10 μmol/L Cd; 3：20 μmol/L Cd; 4：40 μmol/L Cd; 5：80 μmol/L Cd.

isoforms was found in human HPBLs after exposure to cadmium for 4 h (Table 4). The maximal induction ratio of MT-1G mRNA and MT-1X mRNA was 1.72 and 1.32, respectively.

TABLE 4

Expression of MT-1 A, E, F, G, H, X mRNA in HPBLs After Exposure to Cadmium for 4 h

Isoform	Basal Expression	Maximum Expression	Maximum Ratio
MT-1A	0.77±0.32	0.98±0.30	1.35±0.34
MT-1E	0.64±0.18	0.85±0.24	1.41±0.49
MT-1F	0.69±0.23	1.01±0.24	1.58±0.57
MT-1G	0.62±0.15	0.99±0.21	1.72±0.69
MT-1H	0.79±0.31	1.19±0.26	1.69±0.67
MT-1X	0.78±0.23	0.99±0.32	1.32±0.36

We further used mRNA expression to compare the housekeeping gene GAPDH with the MT-1 isoform mRNA abundance (Fig. 2). Level of MT-1 isoform mRNA was significantly up-regulated in cadmium-treated peripheral blood lymphocytes in different concentrations and at different time points ($P<0.05$). Each of MT-1 isoform genes was unique in its dose- and time-dependent response. For example, MT-1A gene responded slowly, reaching a maximum at 40 μmol/L Cd^{2+}. But MT-1G gene responded more rapidly, reaching a maximum at 10 μmol/L Cd^{2+}. We also examined the time-dependent changes of gene expression after exposure of cells to Cd^{2+}. When induced by Cd^{2+}, MT-1G gene reached a maximum at 20 μmol/L Cd^{2+} after 4 h, and at 10 μmol/L Cd^{2+} after 24 h.

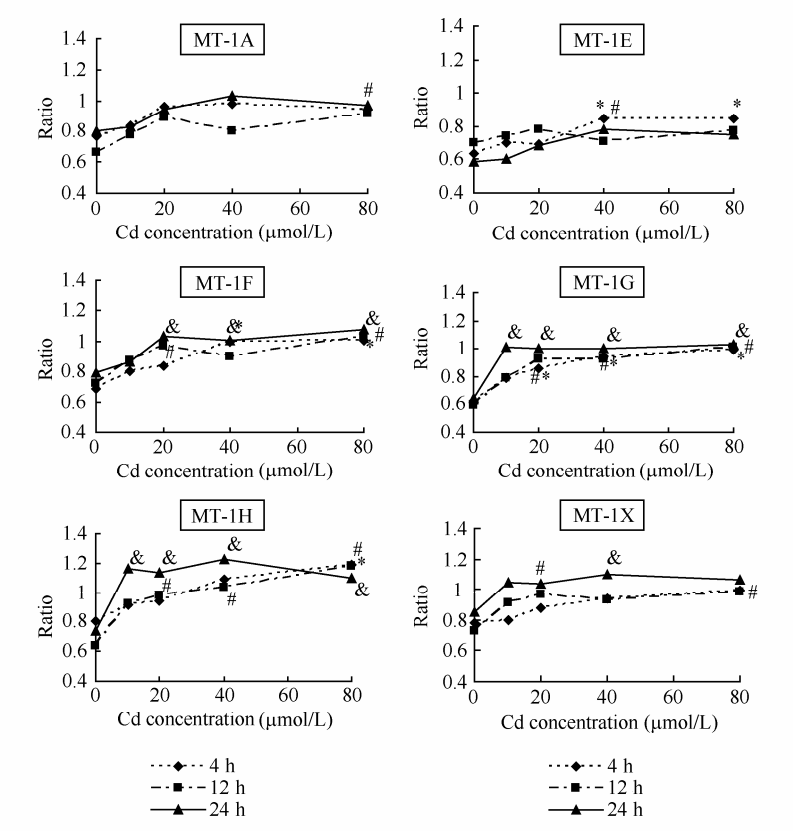

FIG. 2. Expression of MT-1A, E, F, G, H, X mRNA in HPBLs exposed to cadmium.
*$P<0.05$ vs. control at 4 h; #$P<0.05$ vs. control at 12 h; & $P<0.05$ vs. control at 24 h.

Factor Analysis of MT-1 Isoforms

The MT-1 isoforms were significantly correlated between themselves (Table 5). Kaiser-Meyer-Olkin measure was 0.814 and P value was less than 0.001 by Bartlett's test of sphericity. Factor analysis was performed for the variables and principal factors were extracted. Factors were retained only with the eigenvalue greater than 1 by the criterion. Two factors were retained. They accounted for 70 percent of the variance. In the factor analysis for MT-1 isoform variables, associations were found between

MT-1A, F, G, H, and X, and in MT-1E (Table 6). Table 7 shows the relationship between factor 1 as a "new" variable and exposure to cadmium. There was a better dose-effect relationship between factor 1 and exposure to cadmium, but not between factor 2 and exposure to cadmium.

TABLE 5

Correlation Matrix of MT-1 Isoforms

	MT-1A	MT-1E	MT-1F	MT-1G	MT-1H
MT-1E	0.357				
MT-1F	0.404	0.443			
MT-1G	0.452	0.299	0.619		
MT-1H	0.456	0.111	0.378	0.497	
MT-1X	0.523	0.219	0.537	0.627	0.559

TABLE 6

Factor Analysis for MT-1 Isoforms

	Component	
	1	2
MT-1A	0.727	0.016
MT-1E	0.500	0.791
MT-1F	0.779	0.248
MT-1G	0.821	-0.073
MT-1H	0.698	-0.472
MT-1X	0.818	-0.257

TABLE 7

Relationship Between Factor 1 and Exposure to Cadmium

$CdCl_2$ (μmol/L)	Factor 1		
	4 h	12 h	24 h
0	-0.877	-1.259	-0.583
10	-0.588	-0.356*	0.344*
20	-0.246	0.254*	0.575*
40	0.339*	-0.005*	0.806*
80	0.465*	0.604*	0.530*

Note. $^*P<0.05$ vs control.

DISCUSSION

In contrast to the single MT-1 gene of mice, the human MT-1 gene family is composed of seven active genes and six pseudogenes. The coding regions of the genes for the various MT isoforms are highly conserved and do not contain sufficient sequence diversity to allow the generation of specific RT-PCR primers. This study used specific RT-PCR primers from the divergent 5' and 3' untranslated regions of each MT isoform[10]. The expression of mRNA representing the seven active human MT-1 genes was determined in cultured human PBLs after exposure to cadmium. Analysis of the MT isoform-specific mRNA demonstrated that MT-1A, 1E, 1F, 1G, 1H, and 1X genes were expressed in HPBLs under basal conditions (background BCd 0.0064±0.0025 μmol/L), but the MT-1B genes did not. Treatment with cadmium resulted in a significant increase in the expressions of the MT-1A, 1E, 1F, 1G, MT-1H, and 1X genes, but no expression of the MT-1B gene was demonstrated. The expression profiles are consistent with the observation in zinc-treated HPBLs[11]. But each of MT-1 isoform genes is unique in its dose- and time-dependent response. One possibility is that among the human MT-1 gene family promoters, marked sequence conservation exists. However, the mRNA accumulation level in each species was found to be quite unique. This differential regulation of MT-1 promoters is primarily due to single base alterations in their TATA motifs[12]. Another possibility is that a high level of expression of the endogenous hMT-1B gene exhibiting tissue specificity of expression could be detected only in human hepatoma and renal carcinoma cell lines[13]. Previous studies suggested that the MT 1F gene shows cell-type-specific expression and is differentially regulated by metals and glucocorticoids[6]. Induction of the human MT family therefore does not represent an equivalent elevation in the level of expression of individual genes, but is the sum of the differential responses of active members. The differential response is due to functional differences of the respective promoter/regulatory regions of the genes as shown by gene-fusion experiments. The hMT-2A promoter is responsive to cadmium, zinc, and glucocorticoids, while the MT-1A promoter mediates response only to cadmium[14].

MT is a unique low-molecular-weight protein that plays an important role in metal metabolism. HPBLs provide candidate cells to analyze MT expression *in vivo*, since the effect of cadmium on MT expression in blood cells has been studied thoroughly. The results in the present study show that the expression of MT-1 isoform genes is significantly induced by cadmium, but no cytotoxicity was observed. This finding in turn suggests that MT expression levels in HPBLs can be used as a potential early exposure biomarker of cadmium. The analysis of induction ratio of expression of MT-1 after exposure to cadmium for 4 h revealved that though MT-1G, 1H, and 1F have a lower basal expression, they all have a higher induction ratio. MT-1X has a higher basal expression with a lower individual

difference. It is suggested that gene expression of MT-1G, 1H, 1F, and MT-1X in human HPBLs can be used as a potential exposure biomarker. Factor analysis is often preferred as a method for data reduction. There is a high correlation between MT-1A, F, G, H, and X, which can be integrated as a representative variable, thus facilitating the analysis. Considering the complication of regulation of MT expression, further study is needed to demonstrate the feasibility of using MT genes as biomarkers.

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