Metallothionein I Isoform mRNA Expression in Peripheral Lymphocytes as a Biomarker for Occupational Cadmium Exposure

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It is reported that metallothionein (MT) mRNA expression in human peripheral blood lymphocytes (HPBLs) could be used as exposure biomarkers in occupationally cadmium-exposed workers. Several MT isoforms have been identified in humans. The relationship between MT isoforms and cadmium toxicity has not been fully elucidated in occupational settings. In this study, the MT-IA, IE, IF, IX mRNA expressions in HPBLs were tested by RT-PCR technique, and the relationship between MT isoforms mRNA expression and cadmium-induced renal dysfunction was evaluated in cadmium-exposed workers. The MT-IE, IF, IX mRNA levels were found to increase with increasing blood cadmium (BCd) levels and MT-IA mRNA levels increased with increased urinary cadmium (UCd) levels. The MT-IE, IF, IX mRNA levels were significantly correlated with the BCd levels ($P < 0.05$), and MT-IA mRNA levels were significantly correlated with the UCd levels. This confirmed that MT-I isoforms mRNA expression in HPBLs is a biomarker of cadmium exposure and internal dose. The MT-IA mRNA levels were significantly correlated with renal dysfunction biomarkers, such as urinary β_2-microglobulin (Uβ_2-MG) ($r = 0.294$, $P < 0.01$) and urinary albumin (UALB) ($r = 0.305$, $P < 0.01$). The latter finding indicates that MT-IA mRNA expression in HPBLs may be used as a biomarker for renal dysfunction in occupational cadmium exposure. Exp Biol Med 234:666–672, 2009

Key words: cadmium; metallothionein; mRNA; isoforms; biomarker

Introduction

Cadmium (Cd) is considered as a harmful pollutant worldwide (1). Long-term low level exposure to cadmium is presently the focus in toxicological research on cadmium. Accurate human risk assessment requires sensitive methods to evaluate dose-response relationships, especially following low level exposures. To prevent the adverse health effects of cadmium, it is important to develop sensitive and specific biomarkers. The metallothioneins (MTs) are a family of Cd-binding proteins with low-molecular-mass, rich in cysteine, inducible and with high affinity for divalent metals, e.g., zinc and cadmium (2). Presently four major forms, MT I-IV and a number of MT-I isoforms, have been identified, of which MT-I and MT-II genes are ubiquitously expressed in most tissues. MT-III is mainly expressed in brain and MT-IV is found in stratified squamous epithelia (3–5). Among the various physiological functions of MT, the role of MT in the detoxification of toxic metals is most well established (6, 7). MTs have been demonstrated to participate in the detoxification of toxic metals such as cadmium (8), in zinc homeostasis (9) and in protection against oxidative stress (10). Because MT plays an important role in the metabolism of toxic metals, especially cadmium, the possibility of using MT expression levels in tissues as a biomarker of metal exposure has been suggested. In previous studies, it was found that MT protein in urine can be used as a sensitive biomarker of renal tubular dysfunction in a cadmium-exposed population (11, 12). The expression of MT mRNA in peripheral blood lymphocytes has been shown that it can be used as a biomarker of susceptibility to cadmium-induced renal dysfunction (13–15).

Humans have a complex MT gene family including MT isoforms, and to date 10 functional genes and 7 nonfunctional MT ones have been identified. All the functional genes in humans are located on chromosome 16 (3, 16, 17). The human MT-I isoforms mRNA exhibit unique expression profiles such as inducer-specific, tissue-specific, and developmental-specific regulation (18). In our previous study, MT-IA, IB, IE, IF, IG, IH and IX mRNA expressions

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in response to cadmium were tested by specific RT-PCR primers in vitro (19). It was shown that induction of each of the MT-I isoforms gene transcription was unique with dose- and time-dependent response in cadmium-treated human peripheral blood lymphocytes (HPBLs) (19). However, MT-I isoforms mRNA expressions have never been explored in a human population exposed to cadmium. The relationship between MT-I isoforms mRNA expression and cadmium-induced renal dysfunction is still unclear. In the present study, MT-IA, IE, IF and IX isoforms mRNA expression was measured in HPBLs of workers occupationally exposed to cadmium in China. The relationships between the MT-IA, IE, IF, and IX isoforms mRNA expression, cadmium exposure, and cadmium-induced renal dysfunction were evaluated.

Materials and Methods

Study Population. The exposed population consisted of 91 workers (male, 75; female, 16) who were from a cadmium smelter located in Hunan Province, central China. Workers having more than one year occupational cadmium exposure were chosen as exposure group and the average working span is 10.3 ± 8.8 years. Twenty-six health care officers (male, 21; female, 5) in the factory hospital were chosen to be the control group. Personal data of each subject were collected, including socio-economic conditions, life style factors, such as smoking and drinking habits, medicine-taking history, and toxic metals exposure history.

The study was carried out after permission of the local government and approved by the ethics committee of Fudan University, China. Informed consent was obtained from each participant.

Collection and Analysis of Biological Samples. A total of 3 ml of venous whole blood was collected in an acid-washed, heparin-containing Vacutainer after the skin had been cleaned. A 1 ml sample was taken for blood cadmium (BCd) analyses and stored at –70°C until analysis (20). Lymphocytes used for MT mRNA expression measurement were isolated from the remaining 2 ml of whole blood by centrifugation by Ficoll-Paque gradient (15).

The treatment of urine samples was as described previously (21). Spot urine samples were collected in the morning in metal-free polyethylene bottles and stored at –20°C until analysis. Each urine sample was distributed into three parts immediately after it was collected. Urinary cadmium (UCd) was assayed on an acidified sample. Urinary β_2-microglobulin (Uβ_2-MG) was measured on a spot-urine sample which had been alkalinized in order to prevent degradation. Cadmium in urine and blood was analyzed by graphite furnace atomic absorption spectrometry (GF-AAS). The standard addition method was used as previously described (22). Urinary albumin (UALB) and Uβ_2-MG were used as indicators of renal dysfunction. The levels of Uβ_2-MG and UALB were measured by ELISA method. The levels of creatinine (Cr) were measured using the Jaffe reaction method. All urinary variables were standardized by the levels of creatinine in urine.

Measurement of Levels of MT-I Isoforms mRNA. HPBLs were isolated from blood samples collected from subjects by density gradient centrifugation of heparinized blood. 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 counted. Total RNA was isolated according to the protocol supplied with TRIzol (Invitrogen Life Technologies). The concentration and purity of RNA samples were determined using UV spectrophotometer.

Total RNA (3 μg) was reversely transcribed using the AMV first strand cDNA Synthesis Kit (BBI, Toronto, Canada). MT-IA, IB, IE, IF, IG, IH and IX isoforms mRNA levels were attempted to be tested in this study. However, MT-IB does not express in lymphocytes (19), while MT-IG and 1H isoforms mRNA expressions were too low to get further quantitative results. The primers developed for analysis of each of the active MT-I genes have been described previously (19) and presented in Table 1. The reverse transcribed product was then used for PCR amplification with a DNA thermocycler (Perkin-Elmer-Cetus 9600) programmed to cycle at 94°C for 5 min initial step, then 94°C for 30 s and 55°C for 1 min, with a final elongation step of 72°C for 10 min. The resulting PCR product was electrophoresed on 2% agarose gel, along with Lambda DNA/PstI Marker (MBI). The levels of transcripts from the MT-I isoforms gene 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-I isoforms mRNA abundance was normalized with the GAPDH (23), and expressed as relative units.

Statistical Analysis. The data were analyzed with the SPSS 11.0 statistic program. Distributions of the biological measurements were normalized through logarithmic transformation. The data were expressed as geometric means and 95% confidence intervals for geometric means. The logarithm data are transformed to geometric means. To analyze the statistical significance, we used ANOVA and ANCOVA to determine the differences between two and more than two groups. The Pearson and Spearman correlation regression analysis were also performed. Equations were selected from the stepwise regression analysis if all regression coefficients were significant. Statistical significance was set at P values < 0.05 throughout this study.

Results

Cadmium Exposure and Renal Dysfunction in the Study Population. Table 2 showed the main characteristics and results of exposure and effect measure-

Table 1. Primer Sequence and Product

Gene	GenBank GI	Primer sequence		Product (bp)	Cycles
		Upper	Lower		
GAPDH	GI:0226183	5′-CGGAGTCAACGGATTTGGTCGTAT-3′	5′-AGTCTTCTCCATGGTGGTGAAGAC-3′	307	26
MT-I A	GI:31342255	5′-CTCGAAATGGACCCCACT-3′	5′-ATATCTTCGAGCAGGGCTGTC-3′	219	30
MT-I E	GI:31581520	5′-CTCATTGCCCGTGTCATTC-3′	5′-AGAACCCAGACCCAGAGGAT-3′	159	32
MT-I F	GI:28866946	5′-AGTCTCTCCTCGGCTTGC-3′	5′-ACATCTGGGAGAAAGGTTGTC-3′	232	28
MT-I X	GI:31543213	5′-TCTCCTTGCCTCGAAATGGAC-3′	5′-GGGCACACTTGGCACAGC-3′	151	30

ments in both the control and the exposed groups. Some of the basic characteristics—such as age, gender and drinking habits—were comparable between the two groups (Table 2). While the percentage of smokers in the exposed group was significantly higher than that of the control group ($P = 0.016$). Because the cadmium in cigarettes may contribute to cadmium exposure, an analysis of covariance (ANCOVA) was further performed using smoking index (defined as the number of cigarettes smoked per day divided by 20 and multiplied by smoking years) as a covariate (Table 2). In the present study, we used UCd and BCd as indicators of cadmium exposure and body burden. The levels of UCd and BCd in the exposed group were statistically significantly higher than those in the control group ($P < 0.05$, ANCOVA). In the present study, $U\beta_2$-MG and UALB were used as the biomarkers of cadmium-induced renal tubular and glomerular damage. Table 2 showed that the excretion of $U\beta_2$-MG, and UALB in the exposed group was statistically higher than that in the control group ($P < 0.05$, ANCOVA).

Table 3 displayed the levels of the urinary indicators of renal dysfunction at different UCd levels. When UCd > 5 μg/g Cr, the levels of $U\beta_2$-MG and UALB increased significantly compared with 2–5 μg/g Cr and < 2 μg/g Cr UCd groups. There were significant correlations between UCd and $U\beta_2$-MG, UALB, and the Spearman correlation coefficients were 0.33 and 0.24 ($P < 0.05$), respectively.

MT-I Isoforms mRNA Expressions, Cadmium Exposure, and Renal Dysfunction. The expression levels of MT-IA, IE, IF and IX mRNA were significantly higher in the exposed groups ($P < 0.01$, ANCOVA, Table 4) compared to the control group. To further elucidate the relationship between expression of MT-I isoforms mRNA and the cadmium exposure dose, based on BCd levels (0–5, 5–10, $>$ 10 μg/l) and UCd levels (0–2, 2–5, $>$ 5 μg/g Cr), the subjects were divided into 3 groups (Table 4). The gender and age distributions among the groups were not significantly different, but the percentage of smokers is higher in the highest BCd group ($>$ 10 μg/l group) (data not shown). When smoking index is controlled as a covariate, the MT-IA, IE, IF and IX mRNA levels were significantly different between BCd groups ($P < 0.05$, ANCOVA). MT-IA, IE, IF and IX mRNA levels increased with the increase of BCd levels, and the expressions were statistically higher in the $>$ 10 μg/l BCd group than those groups $<$ 5 μg/l BCd group ($P < 0.05$, ANCOVA). There were no significant differences between the three categories of UCd shown in groups in the gender, age, and smoking habit distributions. MT-IA mRNA levels increased significantly with the increase of UCd levels ($P < 0.01$, ANOVA). MT-IE and IX mRNA levels in the 2–5 μg/g Cr UCd group were significantly higher than that in the < 2 μg/g Cr UCd group ($P < 0.05$, ANOVA). A similar relationship was observed between MT-IF mRNA levels and UCd, although, the differences were not statistically significant ($P > 0.05$, ANOVA).

Table 2. Characteristics of Study Population[a]

	Control group	Exposure group	*P* value
Number	26	91	
Gender (male/female)[c]	21/5	75/16	0.931
Age (years)[b]	40.15 ± 11.55	38.25 ± 7.12	0.304
Number of smokers (%)[c]	8 (30.7)	53 (58.2)	0.016
Number of drinkers (%)[c]	12 (46.2)	28 (30.8)	0.205
BCd (μg/l)[d]	2.88 (1.90–4.35)	6.74 (5.44–8.20)	0.0001
UCd (μg/g Cr)[d]	1.64 (1.15–2.32)	2.77 (2.23–3.43)	0.044
Uβ_2-MG (μg/g Cr)[d]	50.06 (39.44–63.54)	79.02 (65.89–94.77)	0.017
UALB (mg/g Cr)[d]	5.03 (3.92–6.46)	6.87 (5.92–7.95)	0.043

[a] Values in brackets indicate a 95% confidence interval for geometric means.
[b] Mean ± standard deviation, ANOVA.
[c] Chi-squared test.
[d] ANCOVA, adjusted for smoking index.

Table 3. Indicator of Renal Dysfunction at Different Urinary Cadmium Levels[a]

UCd levels (μg/g Cr)	*n*	Uβ_2-MG (μg/g Cr)	UALB (mg/g Cr)
< 2	43	53.77 (43.15–66.99)	6.03 (4.93–7.38)
2–5	57	67.66 (55.36–82.70)	5.98 (4.90–7.24)
> 5	17	160.40* (104.58–246.01)	9.03* (6.40–12.75)

[a] Values in brackets indicate a 95% confidence interval for geometric means.
* Compared with the < 2 μg/g Cr group, $P < 0.05$, ANOVA.

Table 4. MT-I Isoform IA, IE, IF, IX mRNA Expressions in HPBLs at Different Levels of Cadmium Exposure (Relative Units)

Group	*n*	MT-IA	MT-IE	MT-IF	MT-IX
Control	26	0.20 ± 0.20	0.20 ± 0.20	0.69 ± 0.27	0.98 ± 0.23
Exposed	91	0.39 ± 0.25**	0.39 ± 0.25**	0.91 ± 0.34**	1.17 ± 0.32**
BCd levels					
< 5 μg/l	56	0.31 ± 0.24	0.40 ± 0.22	0.74 ± 0.28	1.05 ± 0.28
5–10 μg/l	30	0.27 ± 0.17	0.42 ± 0.20	0.97 ± 0.33†	1.15 ± 0.24
> 10 μg/l	31	0.47 ± 0.26††	0.55 ± 0.25†	1.00 ± 0.36†	1.24 ± 0.37†
UCd levels					
< 2 μg/g Cr	43	0.31 ± 0.27	0.39 ± 0.20	0.80 ± 0.31	1.04 ± 0.26
2–5 μg/g Cr	57	0.33 ± 0.20	0.50 ± 0.25‡	0.92 ± 0.34	1.18 ± 0.34‡
> 5 μg/g Cr	17	0.51 ± 0.27‡‡	0.46 ± 0.23	0.88 ± 0.38	1.17 ± 0.32

** Compared with the control group, $P < 0.01$, ANCOVA, adjusted for smoking index.
† Compared with the < 5 μg/l BCd levels group, $P < 0.05$; †† $P < 0.01$, ANCOVA, adjusted for smoking index.
‡ Compared with the < 2 μg/g Cr UCd levels group, $P < 0.05$; ‡‡ $P < 0.01$, ANOVA.

Spearman correlation analysis was performed between MT-IA, IE, IF and IX mRNA levels and the cadmium dose indicators (Table 5). It was shown that MT-IA, IE, IF and IX mRNA expression levels were significantly correlated with BCd ($P < 0.05$), and MT-IA and IX mRNA expression levels were significantly correlated with UCd ($P < 0.05$) (Table 5). The relationship between MT-IA, IE, IF and IX mRNA levels and the occurrence of cadmium-induced renal dysfunction were also explored by Spearman correlation (Table 5). It was shown that MT-IA mRNA level was significantly correlated with Uβ_2-MG, and UALB ($P < 0.05$), MT-IE and IX mRNA levels were significantly correlated with Uβ_2-MG ($P < 0.05$).

The quantitative relationships between MT-I isoforms

Table 5. Spearman Correlation Analysis Between MT Isoforms mRNA Expressions in HPBLs and BCd, UCd and Indices of Renal Dysfunction

	MT-IA		MT-IE		MT-IF		MT-IX	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
BCd	0.251	0.009	0.233	0.016	0.262	0.005	0.354	0.000
UCd	0.235	0.015	0.185	0.058	0.152	0.111	0.271	0.004
Uβ_2-MG	0.294	0.002	0.284	0.003	0.027	0.777	0.246	0.009
UALB	0.305	0.002	0.020	0.844	0.163	0.099	0.149	0.129

(MT-IA, IE, IF, IX) mRNA expression levels and cadmium dose indices (BCd and UCd) were examined by Stepwise regression analysis in all subjects. Age, smoking index, logarithms of the concentration of BCd (logBCd) and logarithms of the concentration of UCd (logUCd) were used as the independent variables; MT-IA, IE, IF and IX mRNA levels were used as the dependent variables, respectively. LogUCd showed significant effects on MT-IA mRNA levels. LogBCd showed significant effects on MT-IE, IF and IX mRNA levels respectively, whereas other variables didn't show such a significant effect on MT-I isoforms mRNA expression. The stepwise regression analysis ($P_{enter} = 0.05$, $P_{exit} = 0.10$) yielded equations as follows: MT-IA = 0.296 + 0.147 × logUCd, MT-IE = 0.343 + 0.151 × logBCd, MT-IF = 0.743 + 0.181 × logBCd, MT-IX = 0.933 + 0.234 × logBCd. The quantitative relationships between cadmium-induced renal dysfunction indicators and MT-IA, IE, IF and IX mRNA levels were examined by Stepwise regression analysis, using Uβ_2-MG and UALB as the dependent variable, respectively; using age, smoking index, MT-IA, IE, IF and IX mRNA levels and logBCd and logUCd as the independent variables in all the subjects. MT-IA mRNA levels showed significant effects on both Uβ_2-MG and UALB. The stepwise regression analysis ($P_{enter} = 0.05$, $P_{exit} = 0.10$) yielded equations as follows: logUβ_2-MG = 1.229 + 0.428 × MT-IA + 0.012 × (age), logALB = 0.631 + 0.482 × MT-IA.

Discussion

Cadmium has a long biological half-life (about 10–30 years) in humans (1, 24) and is mainly stored in the liver and kidneys. The kidney has been recognized as the critical organ in long-term exposure to cadmium (25). Animal studies showed that the synthesis of MT in the kidney is induced by cadmium administration (26). It is not practical to measure the synthesized MT in the kidney. However, the PBLs provide an ideal indicator medium. They are easy to collect and most of the cadmium in blood is present in blood cells (1). There have been some applications of MT gene expression in HPBLs for detecting cadmium exposure (13, 27–29). In our previous studies, the MT mRNA levels in HPBL were measured in occupationally and environmentally cadmium-exposed populations, and it is shown that MT mRNA level could be used as a biomarker of cadmium exposure (14, 15). However, both studies only concentrated on the MT-II expression, and the MT-I including different MT-I isoforms expressions have never before been studied in a cadmium-exposed population.

In contrast to the single MT-I gene of the rat and mouse, the human MT-I gene family consists of seven active genes and six pseudogenes (16). The MT isoforms may have unique functions. Thus far, the functional isoforms of MT have been reported to be involved in different functions. The MT-IIA isoform is associated with cell proliferation (22); MT-IA isoforms are reported to protect against apoptosis and oxidative stress (30). It has also been pointed that MT-IF and MT-IIA isoforms may be involved in histological differentiation (31, 32). Our previous in vitro study showed that the induction of each MT-I isoforms mRNA presented a unique dose- and time-dependence response in cadmium-treated HPBL (19). In the present study, the relationship between cadmium exposure and MT-I isoforms mRNA expression in HPBLs was examined in an occupationally exposed population. MT-IE, IF, IX mRNA levels were found to increase with the increase of BCd levels and MT-IA mRNA levels increased with the elevation of UCd levels. MT-IE, IF, IX mRNA levels were significantly correlated with the logBCd levels, and MT-IA mRNA levels and the logUCd levels. MT-I isoforms mRNA in HPBLs increase dramatically with internal cadmium exposure biomarkers which indicated that MT-I isoforms mRNA could be used as biomarkers of cadmium exposure and internal dose.

In this study, workers in the smelter have a relatively high level of ongoing cadmium exposure and the UCd and BCd of the exposed workers were statistically significantly higher than that of the control population. The exposure was high enough to cause a slight renal dysfunction (Table 2). When urinary cadmium exceeded 5 μg/g Cr, the levels and the prevalence of Uβ_2-MG and UALB increased dramatically. These results on cadmium exposure and renal dysfunction are in accordance with the results of previous studies (22, 33). In Stepwise regression analysis, an interesting result was found that logUCd was significantly correlated with MT-IA mRNA level and that MT-IA mRNA level showed significant correlation with logUβ_2-MG and logUALB, whereas logBCd was significantly correlated with MT-IE, IF and IX mRNA levels. BCd is mainly related to recent exposure when there is an ongoing exposure (34) and urinary cadmium mainly reflects past exposure, body

burden and renal accumulation. A possible explanation for this situation is that MT-IA mRNA level may reflect past exposure and MT-IE, IF and IX mRNA levels may reflect ongoing exposure. $U\beta_2$-MG is a well established early and sensitive biomarker of renal dysfunction caused by cadmium (1). Albumin with high molecular weight and an increased level of albumin in urine may reflect glomerular dysfunction. Since albumin is easy to quantify in urine, increased urinary albumin is widely used as an index of glomerular damage (35). In the present study, MT-IA mRNA expression level shows a significant effect not only on low molecular biomarker $U\beta_2$-MG, but also on high molecular biomarker UALB. It suggested that MT-IA mRNA expression could be used as a potential biomarker of cadmium-induced renal dysfunction. The MT-I isoforms mRNA difference might be explained by functional differences of the respective promoter/regulatory regions of the genes (36, 37). MT-IA mRNA was detected only at concentrations of $CdCl_2$ producing cell cytotoxicity or at the time point immediately preceding cell death due to Cd^{2+} exposure, which suggests that the MT-IA protein could have potential for development as a biomarker for toxic metal exposure and/or toxicity (38, 39). However, further studies on larger population groups are warranted to develop MT-I isoforms mRNA as an ideal biomarker of cadmium risk assessment.

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