Human Metallothionein Gene Expression Determined by Quantitative Reverse Transcription-Polymerase Chain Reaction as a Biomarker of Cadmium Exposure[1]

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Abstract

Expression of the metallothionein (*MT*) gene in frozen human lymphocytes has been developed as a new molecular biomarker of heavy metal exposure. Workers at a Polish battery factory with high exposures to cadmium were monitored for airborne exposure and blood cadmium levels. A novel quantitative reverse transcription-PCR (RT-PCR) technique, making use of a homologous internal standard, was used to assess the level of MT-specific mRNA in frozen stored aliquots of blood samples taken from exposed and control workers. Results from this assay showed a statistically significant 2.5-fold increase in MT mRNA in exposed compared to control workers. The RT-PCR results also showed significant correlation with airborne cadmium, as registered on personal monitors and with blood cadmium levels. The results suggest that gene induction measured by quantitative RT-PCR is a promising approach for application as a biomarker of biologically effective dose in small samples of frozen tissues or cells.

Introduction

Our approach to the development of new molecular biomarkers is based on the well-known phenomenon of the inducibility by specific toxic agents of genes that code for detoxifying proteins. For example, our studies using expression of the *CYP1A1* gene in human lymphocytes (1) and placenta (2) have confirmed the potential utility of using the induction of this gene as a biological monitor for human exposure to environmental polycyclic aromatic hydrocarbons. MTs[3] are low-molecular weight proteins that form complexes with toxic metals and the synthesis of which is induced by exposure to heavy metals such as cadmium, nickel, mercury, or zinc (3). MT proteins have been used as markers of human exposure to toxic metals such as cadmium (4). In a population of Canadians, a correlation was found between cadmium or zinc concentration and MT levels in kidney and liver tissue (using autopsy materials; Ref. 5). In similar studies using human tissues from a metal-contaminated region of Poland, high cadmium and zinc levels were associated with increased MT protein level (6, 7). In addition to cadmium accumulation in liver and kidney, there is a measurable amount of cadmium found in whole blood after chronic exposure that is associated principally with nucleated blood cells (8). *In vitro* studies have shown that PBLs are capable of induction of *MT* gene expression in response to cadmium treatment (9–11).

We wanted to determine whether industrial exposure to cadmium could be reliably monitored by assessment of the degree of expression of the *MT* gene at the level of mRNA. We have hypothesized that the direct detection of inducible gene transcription should provide a sensitive, specific, and reproducible approach to the development of biomarkers of biologically effective dose. We describe here our results using a novel quantitative RT-PCR assay on stored whole blood and compare these with experiments using Northern blot analysis on mitogen-stimulated lymphocytes in culture. The most critical aspect of the use of RT-PCR to measure specific mRNAs in a quantitative fashion is to design an appropriate external standard that can be used for accurate quantitation. Many laboratories have published strategies for use of standards in quantitative PCR for a number of genes (12, 13). Our approach has been to use as an internal standard homologous gene sequences from rodents, which share exact sequence homology to the human gene in the primer regions, but which differ by a unique restriction site within the amplified product. This is similar to certain competitive RT-PCR assays described previously (14, 15), but differs in some important respects. First, it is unnecessary to use synthetic standards, and second, titration of each sample is avoided by the use of a positive control for quantitative normalization. This approach allows for simultaneous amplification of the human target and the rodent external standard using one set of primers and, by normalization with respect to the constant control sample, leads to accurate comparative assessment of the degree of expression of the human gene in multiple samples from different individuals.

Materials and Methods

Study Population

Study subjects were all male employees of the Centra Battery Factory in Poznan, Poland. Exposed workers were chosen from those involved in the production and assembly of nickel-cadmium batteries, as well as from the chemistry and maintenance departments. Controls were chosen from administration personnel at the factory. Each subject was given a brief questionnaire to collect demographic data, height, weight, smoking status,

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[3] The abbreviations used are: MT, metallothionein; PBL, peripheral blood lymphocyte; RT-PCR, reverse transcription-PCR; HIS, homologous internal standard; PHA, phytohemagglutinin.

alcohol consumption, use of pharmaceutical drugs and other potential exposures to metals (such as dietary supplements), age, and working history. Blood was drawn into vacutainer tubes, frozen and stored until RNA isolation or treated immediately for isolation and culture of viable lymphocytes.

Sample Collection

PBLs were isolated from 50 ml whole venous blood donations and cultured in the presence of 2% PHA (Life Technologies, Inc.) as described previously (1). Total cellular RNA was isolated using a rapid guanidinium-phenol extraction method.

Northern Analysis

For Northern blot analysis, 10 μg RNA were denatured by glyoxalation, electrophoresed through 1% agarose gels, blotted onto nylon membrane filters by capillary action, and fixed via UV irradiation (Stratlinker-Stratagene) and baking at 80°C. Filters were then hybridized overnight at 65°C in the presence of dextran sulfate with the nick-translated, ^{32}P-labeled cDNA pr41es probe for human MT-II using a modification of the procedure of Wahl *et al.* (16). Filters were then washed in 0.5× SSC-0.1% SDS at 65°C to remove the non-specifically bound cDNA probe, and mRNA was subsequently visualized by film autoradiography using XAR-5 film (Kodak) with Cronex Lightning Plus intensifying screens (DuPont). The autoradiograms were subjected to scanning laser densitometry using a LKB Ultroscan laser densitometer to quantitate results. Gene expression values for MT were normalized using actin as a control.

Quantitative RT-PCR

Titration for Optimization of the HIS RT-PCR Assay. Various amounts (from 10 to 150 ng) of RNA from the HEPG2 human liver cell line were mixed with a constant amount (35 ng) of Chinese hamster ovary RNA. After RT-PCR and HgiA1 digestion as described below, bands corresponding to HEPG2 and hamster amplicons were cut out and counted in a liquid scintillation counter, and ratios of band intensities were determined.

Application to Human Samples. Total RNA from whole blood (1 μg) was mixed with 35 ng of Chinese hamster ovary RNA, and cDNA synthesis was initiated by addition of 1 μg of the antisense primer 5′ TCAGGCGCAGCAGCTGCACTT 3′. The reaction mixture, also containing 400 μg of all four dNTPs, 10 units AMV reverse transcriptase (United States Biochemical Corp.), and the RNase inhibitor RNAsin (United States Biochemical Corp.), was incubated for 2 h at 42°C. The sense primer 5′ TGCAAATGCAAAGAGTGCAAA 3′ was end labeled by reaction with T4 polynucleotide kinase and [^{32}P]ATP and purified over a Sephadex G-50 spin column. The PCR was initiated by adding 500 ng of the above end-labeled sense primer, 400 μg of dNTPs, and 2 units of Amplitaq (Perkin-Elmer Cetus) to the cDNA product. The PCR conditions were: denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. After 30 cycles, there was a final extension at 72°C for 10 min. Because the human and hamster genes share exact homology in the primer sequences, both genes are simultaneously amplified with equivalent efficiency. The PCR product was digested with the restriction enzyme HgiA1 at 60°C for 2 h. Both human and hamster PCR products are 130 bp, but only the hamster sequence contains a HgiA1 site, allowing for cleavage of the product in half. Digested product was electrophoresed in a 7% urea acrylamide gel. Radioactive bands at 130 (human) and 75 (hamster) bp were cut out and counted by liquid scintillation. Every experiment also included RNA from the cell line HEPG2 as a normalization standard. Quantitation of human MT message was calculated by dividing the ratio of the human to hamster band for each sample by the ratio for the HEPG2 standard.

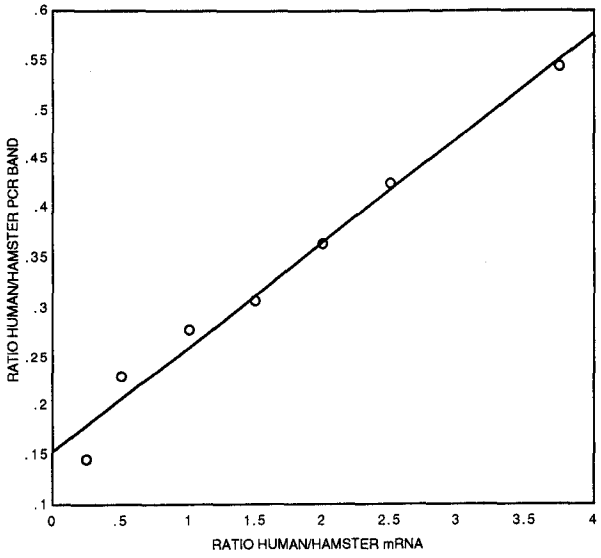

Fig. 1. Titration of human/hamster PCR product band intensities as a function of the ratio of human/hamster RNA added to the tube. A fixed amount of hamster RNA (35 ng) was added to each tube, along with varying amounts of human RNA to give the ratios shown on the abscissa. *Ordinate*, values by scintillation counting of the human MT RT-PCR product at 130 bp compared to the hamster band at 75 bp ($r = 0.99$; $P < 0.0001$).

Chemical Analysis

Ambient exposure was monitored for each subject by lapel-mounted 37-mm cellulose ester membrane filter cassette monitors. Air flow rate through the filter was 2 L/min, and monitoring was continuous for a full working shift. After exposure, filters were wet ashed and analyzed for metal content by atomic absorption spectrometry. Blood cadmium was determined in a graphite furnace atomic absorption after deproteinization by nitric acid.

Statistical Analysis

Student's *t* test was used to compare mean values. Data were log transformed to obtain normal distribution. Pearson's correlation coefficients were calculated. Multiple logistic regression was used to assess the effect of possible confounders on the association between exposure to cadmium at work and levels of *MT* gene expression.

Results

The HIS method for quantitative RT-PCR is illustrated in Fig. 1. Relative intensities of the human band at 130 bp to the hamster band at 75 bp were found to increase linearly with a slope less than 1.0 (R = 0.99) with increasing ratios of HEPG2 human to hamster RNA added to the tube. Experiments using RNA isolated from human blood gave similar results, except that the level of MT expression was considerably less than in the cell line. For analysis of unknown human samples, we

Table 1 Markers of exposure to cadmium and other metals in battery plant workers

Marker (method, units)	Control, mean ± SE (*n*)	Exposed, mean ± SE (*n*)	Exposed: control ratio	*P*[a]
Cd (monitor, μg/m³)	8.3 ± 6.5 (8)	3081 ± 780 (8)	371	0.006
Ni (monitor, μg/m³)	4.4 ± 3.0 (8)	776 ± 294 (10)	176	0.028
Pb (monitor, μg/m³)	2.8 ± 1.3 (8)	11.4 ± 3.4 (10)	4.1	0.036
Cd (blood, μg/L)	2.94 ± 0.97 (8)	35.7 ± 6.2 (10)	12.1	0.0005
mRNA (RT-PCR)	0.90 ± 0.21 (8)	2.29 ± 0.46 (10)	2.5	0.017

[a] *P* value calculated from unpaired *t* test.

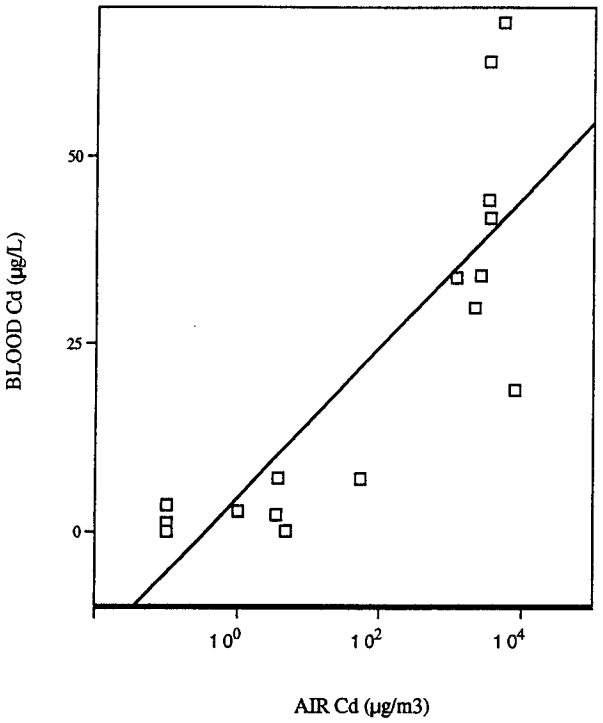

Fig. 2. Correlation between airborne cadmium (μg/m³) from personal monitoring and blood cadmium (μg/L; $R = 0.83$; $P = 0.0001$).

therefore chose to add 1 μg human RNA to a constant 35 ng hamster RNA. The ratio of specific mRNAs was in the same range for the experimental and titration experiments.

Workers at the battery factory were exposed to levels of cadmium, nickel, and lead as shown in Table 1, which presents results of the analysis of personal monitors worn during a complete work shift. Cadmium values for two of the monitors were not available because of technical problems. Blood levels of cadmium, determined on the same individuals, showed a good correlation with ambient exposure levels ($r = 0.83$; $P = 0.0001$; Fig. 2). The values of MT mRNA as determined by the quantitative HIS RT-PCR procedure showed a significant 2.5-fold increase in exposed *versus* unexposed workers (Fig. 3 and Table 1). No effect of smoking, alcohol, age, or other demographic characteristics was seen after logistic regression analysis. The mRNA values of MT were significantly correlated with ambient exposures levels (Fig. 4) of cadmium ($r = 0.61$; $P = 0.012$), but not of nickel (data not shown). There was a somewhat weaker correlation ($r = 0.43$, $P = 0.07$) between MT mRNA levels and blood cadmium (Fig. 5), and with ambient

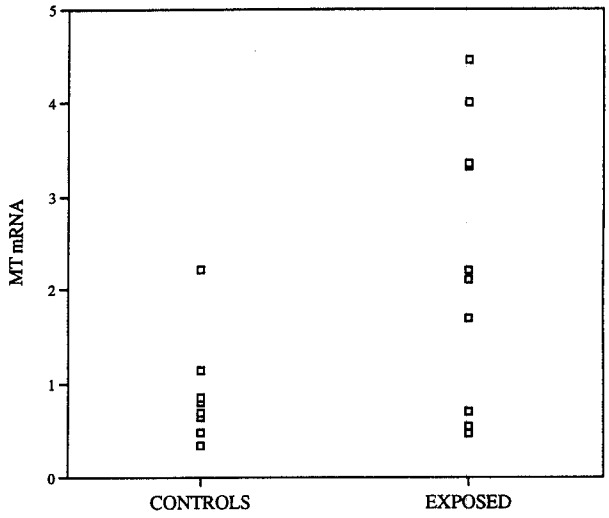

Fig. 3. □, values for MT mRNA for the individual control and exposed workers (see Table 1). Data were calculated from the ratio of the radioactivity in human/hamster PCR bands relative to the ratio for the HEPG2 control.

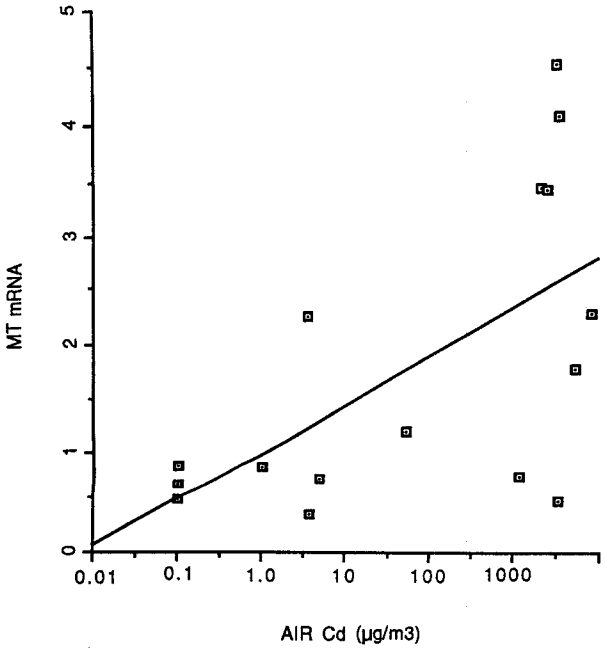

Fig. 4. Correlation between MT mRNA (in relative units) and airborne cadmium exposure as measured by personal monitoring ($r = 0.61$; $P = 0.012$).

lead exposure (data not shown). The difference between exposed and control workers for ambient cadmium was over 300-fold, for blood cadmium was 12-fold, and for mRNA was 2.5-fold. No differences were seen in mRNA levels between workers exposed at different sites or work descriptions in the factory.

In a separate experiment, control and exposed workers at the same plant were asked to donate 50 ml of blood, from which lymphocytes were extracted within 4 h after sampling and cultured in the presence of the mitogen PHA as described in "Materials and Methods." The levels of MT mRNA from these

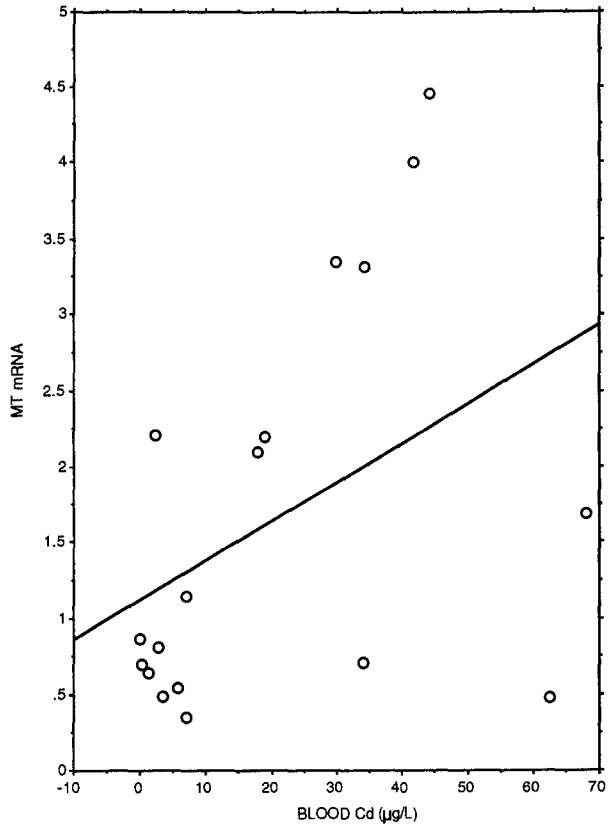

Fig. 5. Correlation between MT mRNA and blood cadmium (r = 0.43; P = 0.07)

samples as determined by Northern blot analysis showed no differences between exposed and control workers (Table 2).

Discussion

When PBLs are exposed *in vitro* to metals such as cadmium, they respond quite rapidly with a major induction of the *MT* gene. This response, like that for most genes, requires that the cells are stimulated to grow in culture by a mitogen such as PHA. Our previous work with the *CYP1A1* gene (1, 2) has led us to the conclusion that mitogen-stimulated PBLs may not be useful for detection of *in vivo* human exposure by assays based on gene expression. The requirement for *in vitro* mitogenic stimulation may mask any effect of previous *in vivo* exposure. In the present study, our attempt to apply Northern blot analysis to detect changes in MT mRNA in mitogen-stimulated PBLs failed to detect any differences between control and cadmium-exposed workers. Similar negative results using Northern analysis on mitogen-stimulated PBLs were obtained for individuals exposed to ZnO (unpublished data). However, by using a new quantitative RT-PCR based assay, we were able to demonstrate that MT mRNA in small samples of frozen blood cells is a useful and reliable biomarker of metal exposure. Besides the lack of any requirement for *in vitro* culture and mitogen stimulation of viable lymphocytes, other advantages of this method include the fact that very small quantities of blood (or other cellular material) are required, and that stored, frozen, and nonviable cells may be used. In the present study, blood samples used had been stored at −70°C for a period of approximately 1 year.

Table 2 *MT* gene induction by Northern analysis in mitogen-stimulated lymphocytes from battery factory workers

Exposure category	Mean ambient Cd exposure ($\mu g/m^3$)	Maximum Cd exposure ($\mu g/m^3$)	*MT* mRNA mean ± SE (*n*)
Controls	7 ± 5	54	1.7 ± 0.46 (9)
Assembly (low)	655 ± 413	3386	1.9 ± 0.25 (10)
Panel production (medium)	598 ± 153	3562	2.1 ± 0.31 (10)
Chemistry (high)	1358 ± 395	7834	1.7 ± 0.21 (11)

Quantitative PCR requires careful use of an internal standard to avoid artifacts resulting from the amplification process. Investigators have proposed a number of approaches, including the use of genes on the same chromosome, use of synthetic sequences, etc. We have developed a method for the quantitation of human DNA or mRNA called the HIS procedure.[4] In this method, one single set of primers amplifies both the human and the homologous rodent gene, thus avoiding any potential problems arising from primer differences. The PCR products differ in that the HIS rodent (in this case, hamster) amplicon has a restriction site near the middle of the amplicon fragment. By addition of a fixed amount of standard rodent RNA to all the human samples (including a control), followed by RT-PCR and digestion with the appropriate enzyme, it is possible, after comparison of the ratios of human to hamster bands, to achieve a quantitative comparison of the target gene RNA levels between human samples. Competitive RT-PCR using synthetic sequences with differences in amplicon length or restriction sites have been described (14, 15). In this approach, each sample is titrated with various amounts of competitor standard RNA, and the dilution at which equivalent amplification of target and standard occurs is informative of the initial target RNA concentration. This method is accurate but difficult to apply as a biomarker because of the necessity to titrate each of many individual samples. Our assay avoids the titration step by the use of a single control (human HEPG2 liver cell line RNA) run with all samples. Ratios of target (human) to internal standard (hamster) amplicon levels for each test sample are compared with the ratio of the control for each experiment to give a quantitative comparison of target RNA concentrations within and between experiments. Other potential problems with the use of synthetic standards include difficulty in preparation of a new standard for each gene to be analyzed and possible errors arising from differences between samples and standard with respect to size and sequence context, etc. Besides this work on the *MT* gene, we have used this approach to quantitate human c-*myc* gene amplification in small samples of archived tissue blocks and to measure *CYP1A1* gene expression in human cells.

Our results from this field study at a Polish battery factory have some interesting implications for the role of biomarkers of exposure. The workers were clearly exposed to quite high levels of cadmium, as well as other metals and presumably other pollutants. Specificity is a critical issue in all biomarker development, and many assays suffer from lack of specificity for single or even classes of compounds. In this context, the results on *MT* gene expression shown here must be interpreted carefully with respect to the utility of this type of assay as a

[4] In preparation.

biomarker for a specific agent such as cadmium. The *MT* gene is inducible by a number of xenobiotics and endogenous factors, including stress. Although some such agents were controlled for here, it remains possible that one or more confounders may be contributing to (or masking) the effect of cadmium exposure on MT mRNA induction. In addition, given the complexities of cadmium toxicokinetics and storage within the body, more research will be required to determine whether detectable increases in blood (or other tissue) MT mRNA reflect recent or historical exposures.

Our data show an interesting trend as we examine markers of exposure of increasing biological relevance. Whereas ambient air concentrations were over 300-fold higher in the exposed compared to the control workers, blood levels were only 12-fold higher. The level of MT mRNA, which represents a still deeper level of biologically effective dose, was further reduced to 2.5-fold above control. The value of a molecular biomarker of biologically effective dose (as compared to ambient air monitoring) or blood levels has been discussed in the literature, and has been the rationale for the use of markers such as DNA adducts (17). However, not all environmental or even carcinogenic toxicants form DNA adducts, and such assays are not always as sensitive as would be hoped (18). It is therefore important to develop and validate new biomarkers of effective dose and new strategies for their use in the field. The approach described here should be applicable to the development of other markers based on gene induction (such as *CYP1A1* by hydrocarbon exposure). Finally, a larger study of *MT* gene induction, including people exposed to less extreme conditions, would clarify the limits of the assay for detection of exposure to metals.

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