

Lymphocyte Metallothionein-mRNA as a Sensitive Biomarker of Cadmium Exposure

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Received September 11, 2000 and accepted December 6, 2000

Abstract: Human peripheral blood lymphocytes (PBLs) produce metallothioneins (MTs) in response to a variety of heavy metal ions. MTs could therefore be a candidate for a marker that represents the biological effect of heavy metals. Since it is practically difficult to measure MT protein levels in PBLs, we examined if MT-mRNA could serve as a biomarker of heavy metal exposure. It is difficult to obtain RNA from PBLs without degradation, but we found that intact RNA can be prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method which minimizes nuclease digestion of RNA during purification steps. By Northern blot analysis of RNA isolated by this method from PBLs cultured with or without CdSO₄, we demonstrated that MT-mRNA is induced by 0.1 to 0.5 μM CdSO₄ in a dose-dependent manner. The blood cadmium levels of exposed humans have been reported to be up to 0.5 μM. This suggests that our assay is able to detect quantitative changes in the PBL MT-mRNA level resulting from *in vivo* cadmium exposure. Thus, PBL MT-mRNA could be used as a sensitive biomarker reflecting exposure to cadmium, and probably to several other MT-inducing heavy metals.

Key words: Biomarker, Cadmium, Metallothionein, mRNA, Peripheral blood lymphocyte

Metallothioneins (MTs) are low-molecular-weight metal-binding proteins that are assumed to be involved in detoxification and homeostasis of heavy metals¹. We have reported that cultured human peripheral blood lymphocytes (PBLs) produce MTs in response to several heavy metals^{2–4}. In these studies, induction of MTs was evident after exposure of PBLs to 0.5 μM CdSO₄. This concentration of cadmium is comparable to the reported blood cadmium levels of humans who suffered from occupational and environmental cadmium exposure^{5–7}. Therefore it was predicted that detectable amounts of MT proteins might be induced in PBLs of cadmium-exposed humans, and that MTs may be a unique biomarker that responds to blood cadmium levels. However, our methods for the detection of MTs used in the *in vitro* studies are not appropriate for the measurement of PBL MTs from cadmium-exposed humans. One of these methods,

fluorographic detection of ³⁵S-cysteine-labeled MTs³) is sensitive enough and has a very low background level, but is impractical because of the difficulties of isotopic labeling of PBLs *in vivo*. Another method, modified silver staining⁸) can successfully detect MTs synthesized in response to 0.5 μM CdSO₄⁴), but high backgrounds are a serious problem for quantification. Since heavy metal-induced MT synthesis is mainly regulated at the transcription level⁹), MT-mRNA could be an alternative biomarker of cadmium exposure. However, accurate and reproducible determination of PBL mRNA levels has been very difficult, because it was hard to obtain intact RNA from human PBLs without degradation. Fortunately, a new technique for isolating intact RNA has recently been developed¹⁰). This method involves cell lysis and RNA extraction in the presence of guanidinium thiocyanate and phenol, which can dramatically minimize enzymatic degradation of RNA during the purification processes. In the present work, we tried to determine accurate

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PBL MT-mRNA levels before and after cadmium exposure with this new technique, in order to examine whether MT-mRNA is a suitable candidate as a biomarker of cadmium exposure.

Experimental procedures used in this study were as follows. In all experiments, peripheral blood mononuclear cells and lymphocytes were cultured in Growth Medium (RPMI 1640 medium supplemented with 10% fetal calf serum, 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 units/ml penicillin) at 37°C under a 5% CO₂ atmosphere; RPMI 1640 medium was obtained from Nissui Seiyaku Co. Ltd., Japan, and other cell culture reagents were from GIBCO-BRL, USA. Mononuclear cells were isolated from 25 ml of heparinized blood taken from a healthy adult male Korean by centrifugation through a Ficoll-Paque (Pharmacia) step gradient. The cells were incubated in 35-mm plastic dishes (Falcon) with Growth Medium for 1 h to remove adherent monocytes. The purified lymphocytes (1×10^7 cells) were incubated for 6 h in Growth Medium with or without CdSO₄ (Nakarai Tesque Inc., Japan). Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) method¹⁰⁾ using Trisol reagent (GIBCO-BRL). The preparation of digoxigenin (DIG)-labeled single-stranded RNA probes was described previously¹¹⁾; the hMT-IIA and β -actin probes detect human metallothionein-IIA and human β -actin mRNAs, respectively. Northern blot analysis with DIG-probes was described previously¹¹⁾. The mRNA bands detected on X-ray film were quantified by an imaging analyzer model TIAS 2300S (ACI Japan).

Total cellular RNA was isolated by the AGPC method from human PBLs incubated with CdSO₄ in a concentration range of 0 to 5 μM . The RNA preparations were electrophoresed on 1% agarose gel and stained with ethidium bromide (Fig. 1). The discrete 28S and 18S ribosomal RNA bands were observed in all the preparations tested, indicating that RNAs, as an entire population, escaped damage caused by ribonucleases. These RNA preparations were subjected to Northern blot analysis with a DIG-labeled hMT-IIA probe, as well as a DIG- β -actin probe (as a reference). In this assay, a discrete specific RNA band was observed; the β -actin probe detected a main band 2.2 kilobases long as shown in Fig. 2a. This result indicates that very little enzymatic degradation of specific mRNA occurred. A similar result is shown in Fig. 3a, where the hMT-IIA probe clearly detected a 450 base-long hMT-IIA mRNA. The β -actin mRNA level was not altered by exposure to 0.1 or 0.5 μM CdSO₄, and the results of quantitative densitometric analysis are shown in Fig. 3b. In contrast, the hMT-IIA mRNA level completely

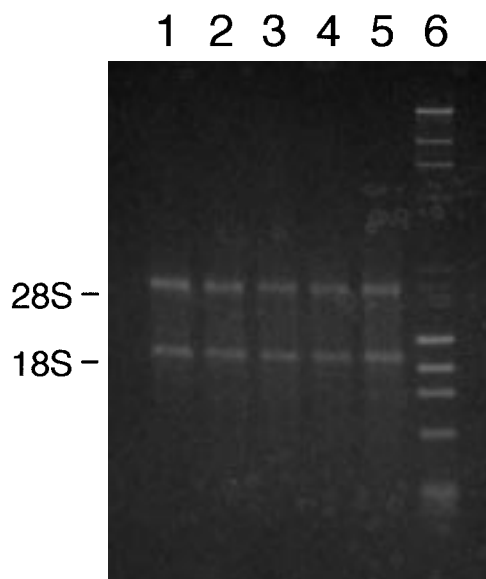


Fig. 1. Quality of human PBL RNA isolated by the AGPC method.

Total RNAs was prepared from human PBLs cultured with 0, 0.01, 0.1, 1 or 5 μM of CdSO₄ (lanes 1 to 5, respectively) for 6 h. The RNAs (0.1 $\mu\text{g}/\text{lane}$) were electrophoresed together with DNA markers (lane 6; DRigest III, Pharmacia) in 1% agarose gel and were visualized by ethidium bromide staining.

depended on the concentration of cadmium. In the absence of cadmium the hMT-IIA mRNA level was quite low, but incubation of PBLs with increasing concentrations of cadmium dramatically increased the hMT-IIA mRNA level in a dose-dependent manner. It should be noted that this assay shows a quite low background, making an accurate analysis possible. Reproducibility of these results were confirmed by repeated experiments.

The experiments described above provided the following conclusions. (1) Intact RNA can be isolated from PBL samples by the AGPC method. (2) Our assay is sensitive enough to determine the hMT-IIA mRNA level in PBLs of cadmium-exposed humans. (3) PBLs synthesize MT-mRNA in response to 0.1 to 0.5 μM CdSO₄ in a dose-dependent manner.

Blood cadmium levels observed for itai-itai disease patients⁶⁾, a worker in a Swedish cadmium-nickel battery factory⁵⁾ and some cadmium-exposed workers in a battery factory in the United States⁷⁾ were within the range in which we can detect quantitative changes in the hMT-IIA mRNA level in cultured PBLs (0.1 to 0.5 μM). Thus the PBL MT-mRNA can be expected to serve as a novel biomarker that reflects cadmium exposure. It may be used as a marker

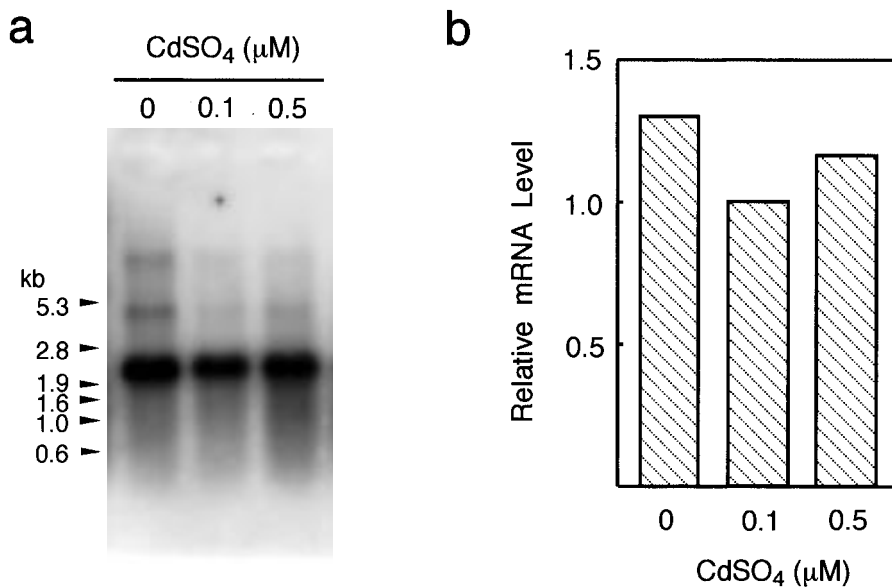


Fig. 2. Effect of cadmium exposure on β -actin mRNA levels in human PBLs.

Total RNA was isolated by the AGPC method from the purified human PBLs cultured with 0, 0.1, or 0.5 μ M CdSO₄ for 6 h. The RNAs (0.16 μ g each/lane) were subjected to Northern blot analysis using DIG- β -actin probe (100 ng/ml). a, X-ray film image. The positions of co-electrophoresed DIG-labeled RNA markers (Roche) are indicated on the left. b, Densitometric analysis. Density relative to the 0.1 μ M CdSO₄-exposed sample (taken as 1.0) is shown.

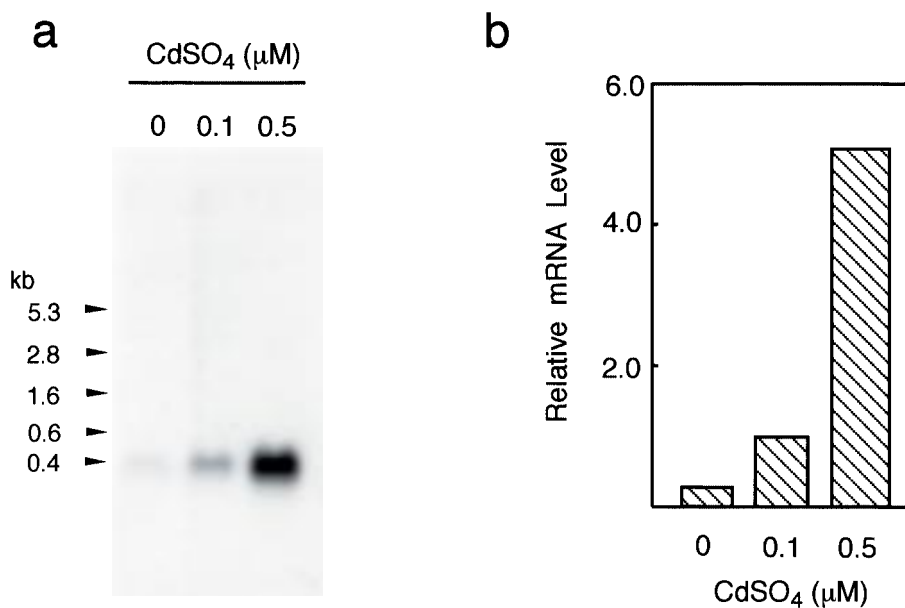


Fig. 3. Effect of cadmium exposure on hMT-IIA mRNA levels in human PBLs.

Total RNA was isolated by the AGPC method from the purified human PBLs cultured with 0, 0.1, or 0.5 μ M CdSO₄ for 6 h. The RNAs (4.2 μ g each/lane) were subjected to Northern blot analysis using DIG-hMT-IIA probe (1,000 ng/ml). a, X-ray film image. The positions of co-electrophoresed DIG-labeled RNA markers (Roche) are indicated on the left. b, Densitometric analysis. Density relative to the 0.1 μ M CdSO₄-exposed sample (taken as 1.0) is shown.

with properties distinct from the blood cadmium level currently being used as a marker of cadmium exposure. Since MTs are thought to be involved in the protection system against heavy metals¹⁾, MT-mRNA might serve as a biomarker that reflects resistance to heavy metals. Its kinetics of response may possibly be different from that of the blood cadmium level, and these two markers could be used for different monitoring purposes. However, to assess these points further studies will be needed, including detailed analyse of time- and dose-dependent changes of the mRNA level and studies with blood samples from humans or experimental animals exposed to cadmium *in vivo*.

The AGPC method enables not only the preparation of RNA without degradation, but also the processing of many samples simultaneously, because it is much simpler and more convenient than older methods. Furthermore, this method would be more practical as a routine assay by introducing the PCR technique, which is quite effective in raising the sensitivity of detection and reducing the amounts of starting materials (i. e. blood samples). The assay with an mRNA biomarker could be widely used for the biological monitoring of various environmental hazardous factors. The mRNA markers, different from other markers such as proteins, have the advantage that an identical procedure can be used for estimating the biological effects of any toxicant, only if appropriate probes are prepared. The PBL mRNA markers are therefore expected to be quite useful as practical biomarkers in the future.

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