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Metallothionein RNA levels in HL-60 cells

Effect of cadmium, differentiation, and protein kinase C activation

Tomas Hanke*, Michael Tyers and Calvin B. Harley

Department of Biochemistry and **Department of Pathology, McMaster University, 1200 Main St. W., Hamilton, Ontario L8N 3Z5, Canada*

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Metallothionein (MT) gene transcription is regulated in a developmental and tissue-specific manner by metal ions and other agents. We examined MT expression in the human promyelocytic leukemia cell line HL-60 during differentiation along macrophage and neutrophil lineages. All HL-60 phenotypes showed similar basal levels of MT RNA with significant induction following exposure to Cd^{2+} but not activators of PKC. MT RNA did not correlate with growth state or with known levels of PKC activity, thus our data do not support a role for MT in HL-60 differentiation or for PKC in MT expression.

Differentiation; Metallothionein; RNA; Cadmium; Protein kinase C; (HL-60 cell)

1. INTRODUCTION

Metallothioneins (MTs) are small (6 kDa), conserved proteins which bind IB and IIB metal ions (Cu^+, Ag^+, Au^+, Zn^{2+}, Cd^{2+}, and Hg^{2+}) [1,2]. A role for MTs in metal metabolism and/or detoxification is suggested by their extreme affinity for and their induction by these ions, and a correlation between levels of MT expression and resistance to metal poisoning. However, other functions for MT, such as scavenging free hydroxyl radicals [3] and a role in stress response and control of cell growth and differentiation [4], have been postulated based on in vitro or circumstantial evidence.

MT gene expression is predominantly regulated at the level of transcription [5–9]. Though the mechanism of regulation is poorly understood, rapid induction occurs upon exposure to metals [5] and, in mammalian cells, to various circulating factors such as glucocorticoid hormones [6], α-interferon [7], and interleukin-1 and other mediators of the inflammatory response [8,9]. It has been suggested that a common pathway for MT induction involves protein kinase C (PKC) activation since MT levels increase in serum-starved cells after treatment with growth factors and TPA, agents which function in part through PKC activation [10,11]. However, data showing that multiple *cis*- and *trans*-acting factors involved in the TPA response act in a gene- and tissue-specific manner are accumulating [12].

Since changes in PKC activity have been well characterized during differentiation of HL-60 human promyelocytic leukemia cells [13], it was of interest to examine the effect of cadmium and activators of PKC on MT RNA levels. The active vitamin D_3 metabolite, calcitriol (1,25-di(OH) vitamin D_3) [14] and TPA (alone [15] or synergistically with calcium ionophore [16]) induce HL-60 cells to acquire macrophage-like characteristics, while retinoic acid (RA) induces maturation to neutrophil-like cells [17–19]. We found that MT is induced by Cd^{2+} in all phenotypic states of

Correspondence address: C.B. Harley, Department of Biochemistry, McMaster University, 1200 Main St. W., Hamilton, Ontario L8N 3Z5, Canada

Abbreviations: MT, metallothionein; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Ra, retinoic acid; PKC, protein kinase C (EC 2.7.1.-)

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HL-60 cells, that dose- and time-responses are affected slightly by differentiation, but that PKC activation appears neither necessary nor sufficient for MT induction in HL-60 cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

HL-60 cells (American Type Culture Collection) were grown as described previously [16]. To induce differentiation, cultures were treated with 20 nM TPA or 1 nM TPA plus 400 nM A23187 for 2 days, or 100 nM calcitriol or 1 μM RA for 5 days, and monitored by standard procedures including morphology, cell cycle exit, adherence, and biochemical markers [16,20]. Stock solutions of TPA (1000 × in ethanol), RA (1000 × in ethanol), and calcitriol (1000 × in dimethylsulfoxide) were stored at −20°C. $CdCl_2$ was added from a sterile stock solution (1000 × in water) to final concentrations as indicated.

2.2. *Total cellular RNA preparation*

Total cellular RNA was prepared by a modified guanidinium isothiocyanate method and quantified with a Sequoia-Turner model 450 fluorometer [21,22]. Possible DNA contamination was determined by fluorometer measurements after digestion with pancreatic RNase (10 μg/ml, 5 min, room temperature).

2.3. *RNA dot blot analysis*

RNA was spotted (0.5 μg per dot) in quadruplicate onto nitrocellulose and processed as described [21,23] with one of two end-labelled synthetic oligonucleotides complementary to highly conserved regions of human MT mRNA (bases 79–96 and 670–689 [24]). These probes were highly specific for MT RNA (fig.1), but one probe (MTAP) also reacted significantly with human genomic DNA (not shown). Therefore RNA preparations containing more than 5% DNA contamination were repurified or discarded. MT RNA was detected by overnight hybridization at 48°C and washing 4 times (5 min each) with 2 × SSC at 48°C before autoradiography. Autoradiograms were quantified by densitometric scanning after exposure for various lengths of time to obtain signals within the linear range of the film. MT signals were normalized to total poly(A)$^+$ RNA by using an oligo$(dT)_{18}$ probe on both identical and replicate filters [21]. The mean (± SD or ± SE, as indicated) of the quadruplicate averages from independent experiments is reported. Northern analyses (not shown) gave similar results, but were less reproducible than dot blots.

3. RESULTS AND DISCUSSION

3.1. *Effect of differentiation on basal levels of MT RNA*

In the absence of metal ion induction, HL-60 cells express low, variable levels of MT RNA comparable to peripheral blood leukocytes and other cell types (fig.2, table 1, and unpublished data). There was no significant change in basal MT RNA levels upon terminal differentiation during the time frame of cell-cycle exit (6 h for differentiation with TPA [15], 1 day for differentiation with calcitriol [14], and 3–4 days with RA [17]). Thus, changes in MT RNA levels are not an early event in terminal differentiation of HL-60 cells, which argues against a central role for MT in control of cell proliferation [4,10]. However, long-term culture of cells differentiated with TPA showed a significant decrease in basal MT RNA levels (table 1). This may not be an MT-specific event since these cells have reduced viability (not shown).

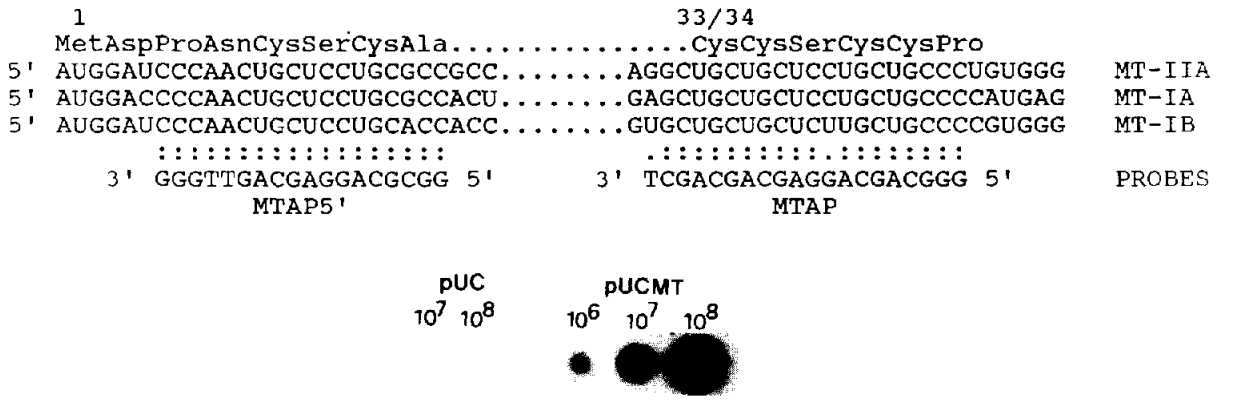

Fig.1. MT oligonucleotide probes. Oligonucleotide (MTAP5', MTAP) complementary to two regions of human MT mRNAs (top) were synthesized and used for hybridization on RNA dot blots as described in section 2. Specificity of the probes is shown by hybridization of MTAP to a MT-cDNA plasmid (pUCMT) but not the parent plasmid (pUC). The number of linear plasmid molecules applied to the nitrocellulose filter is indicated above the dots.

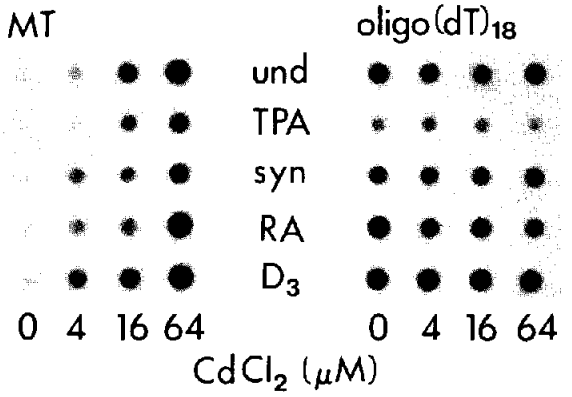

Fig.2. Dot blot analysis of MT RNA. Total cellular RNA from Cd^{2+}-induced HL-60 cells was spotted onto nitrocellulose filters and probed with a synthetic oligonucleotide complementary to MT mRNA (MT) or with oligo$(dT)_{18}$, as described in section 2. Cells were either undifferentiated (und) or differentiated along the macrophage lineage with 20 nM TPA for 2 d (TPA), 1 nM TPA plus 400 nM A23187 for 2 d (syn) or 100 nM calcitriol (D_3) for 5 d, or along the neutrophil lineage with 1 μM retinoic acid (RA) for 5 d prior to incubation with Cd^{2+}. A representative autoradiogram of a 6 h Cd^{2+} dose response is shown.

3.2. *Effect of differentiation on cadmium induction of MT RNA*

Because basal MT RNA levels did not vary significantly with differentiation and were relatively low and difficult to quantify, induction with Cd^{2+} was determined relative to the maximum induction observed in a given experiment. Incubation of both differentiated and undifferentiated HL-60 cells for 6 h with increasing concentrations of Cd^{2+} led to a significant elevation of MT RNA (fig.3, left panel) which reached 20- to 45-fold basal levels in both undifferentiated and differentiated cells after exposure to 64 μM Cd^{2+}. In other experiments, MT RNA levels were substantially reduced after treatment with 240 μM Cd^{2+} (not shown) probably due to acute cytotoxic effects of high Cd^{2+} concentrations. Both macrophage- and neutrophil-like differentiated cells were less sensitive at 6 h to lower concentrations of Cd^{2+} than undifferentiated cells. It is not known whether this difference reflects altered Cd^{2+} uptake or MT response, or if it correlates with resistance to Cd^{2+}.

To investigate the time course of MT RNA induction in HL-60 cells, we chose a relatively low concentration of Cd^{2+} since levels which give a peak response after acute exposure eventually affect differentiation and viability (unpublished). Differentiation altered the time course of MT RNA induction at 4 μM Cd^{2+} (fig.3, right panel): while control cells maintained or increased their induced MT RNA level, differentiated cells had reduced levels after 12–24 h of continuous cadmium treatment. In summary, MT RNA was clearly induced with Cd^{2+} in all HL-60 phenotypes, but the dose- and time-responses were dependent on the differentiated state. MT protein levels were measured in a subset of these studies and found to correspond to MT RNA levels (not shown).

Table 1

Basal MT RNA expression

Differentiated states	Relative basal level
Macrophage-like cells	
TPA 2 d	0.87 ± 0.38 (n = 8)
TPA 4 d	0.26 ± 0.06* (n = 2)
Calcitriol 5 d	0.68 ± 0.17 (n = 2)
TPA + A23187 2 d	0.68 ± 0.28 (n = 2)
Neutrophil-like cells	
RA 5 d	0.80 ± 0.28 (n = 7)
RA 7 d	1.06 (n = 1)

Uninduced levels of MT RNA levels were determined in HL-60 cells differentiated as indicated. Values are mean ± SD of replicate determinations relative to uninduced cells. Levels significantly different ($p < 0.05$) from undifferentiated cells are marked with an asterisk

3.3. *PKC activation is neither necessary nor sufficient for MT induction in HL-60 cells*

TPA-induced macrophage differentiation of HL-60 cells is thought to result in part from prolonged PKC activation [25]. However, continual exposure of HL-60 cells to TPA severely downregulates PKC activity by 6–12 h [19], presumably through proteolysis [26]. Similar rapid loss of specific phorbol ester binding, PKC activity and immunoreactivity has been observed after mitogenic stimulation of 3T3 fibroblasts with TPA [27,28]. In contrast, PKC activity increases 2–4-fold in HL-60 cells after differentiation with RA or calcitriol [19]. Thus, if PKC activity was involved in MT induction, a decreased response to Cd^{2+} would be expected in TPA-differentiated HL-60 cells, while RA- or calcitriol-differentiated cells might have an augmented response. The dose response after 6 h exposure to Cd^{2+} (fig.3) shows

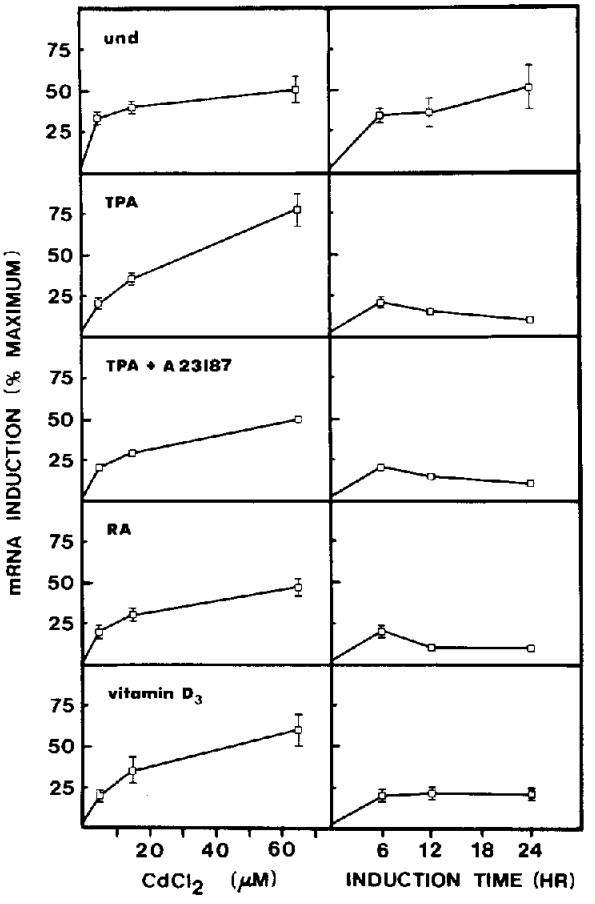

Fig.3. Dose and time response of Cd^{2+} induction of MT RNA. Undifferentiated or differentiated HL-60 cell cultures (as described in fig.2) were incubated with indicated concentrations of $CdCl_2$ for 6 h (left panel) or with 4 μM $CdCl_2$ for indicated periods of time (right panel). In each experiment, MT RNA levels, normalized to the abundance of poly(A)$^+$ species in the total cellular RNA preparation, were expressed as a percentage of maximum induction observed in that experiment. Shown are the means (± SE) for 2 experiments. Where not indicated, standard errors lie within the data symbols.

Table 2

Effect of TPA on MT RNA expression

(a) Undifferentiated cells

Time (h)	TPA (nM)							
	0.5	2	4	8	16	64	100	200
1	1.0	0.9	0.8	0.7	0.9	0.8	–	–
6	1.0	–	–	0.8	0.6	0.5	0.5	0.6
16	0.8	0.9	0.9	0.8	0.9	0.8	–	–
48	0.9	1.0	1.0	1.4	0.9	0.9	–	–

(b) Retinoic acid differentiated cells treated with 20 nM TPA

Time (h)	0	0.08	0.33	1	4	6	24	48
Relative RNA	1	1.5	1.6	1.4	1.1	1.4	1.3	1.2

Levels of MT RNA were determined in undifferentiated (a) and retinoic acid differentiated HL-60 cells (1 μM RA, 5 d) after treatment with TPA as indicated. Values are means relative to untreated cells from two experiments

that neither of these predictions is substantiated. Similarly, the time course of MT induction by Cd^{2+} peaked at 6 h and decreased thereafter independent of the mode of differentiation (fig.3). Thus, PKC activity does not correlate with, or appear necessary for, Cd^{2+} induction of MT RNA.

To test whether PKC activation alone is sufficient for MT induction in HL-60 cells, as suggested for serum-starved primary human fibroblasts, HeLa and HepG2 cell lines [10], both undifferentiated and retinoic acid-differentiated cells were exposed without Cd^{2+} treatment to concentrations of TPA known to activate PKC [25] (table 2). As shown, even very high concentrations of TPA failed to significantly increase MT RNA levels. We observed a similar, negligible effect of TPA on HepG2 cells grown under normal culture conditions (1.3-fold induction after 8 h exposure with 170 nM TPA). It is noteworthy that in fibroblasts, HeLa and HepG2 cells, Imbra and Karin [10] reported MT induction with TPA and other mitogens after serum starvation for 40 h. MT induction following mitogenic stimulation of serum-starved cells may involve mechanisms other than, or in addition to PKC activation. It is also possible that MT induction by PKC activators is tissue-specific, much like the glucocorticoid response [2,6].

3.4. *Role of metallothionein in control of oxidative damage*

Phorbol esters induce the superoxide burst of HL-60 cells differentiated along the neutrophil or macrophage lineage with RA or calcitriol, respectively [14,17]. In vivo, the superoxide burst results in intracellular release of active oxygen species normally involved in destruction of phagocytosed microorganisms. Since the action of free radicals appears nonspecific, it has been proposed that proteins such as superoxide dismutase and metallo-

thionein provide self-protection by neutralizing superoxide anions and free hydroxyl radicals, respectively [3]. Our results do not support a role of MT in the specialized function of macrophages and neutrophils since neither of these lineages of HL-60 cells expressed elevated MT RNA, nor was MT RNA induced by TPA. Similar results have been observed in studies of human peripheral blood polymorphonuclear cells ([29] and Harley et al., in preparation). These observations do not exclude a role for the low, basal levels of MT in scavenging free radicals.

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