Cell- and inducer-specific accretion of human isometallothioneins

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The synthesis of metallothionein (MT) was investigated in three different human epithelial cell lines, each derived from one of the embryonic germ layers. The accretion of different isoforms of the protein was monitored using a sensitive neutral-pH h.p.l.c. method. Induction of MT synthesis by zinc ions and dexamethasone revealed differences between the three cell lines, both

with respect to the number and the amounts of the different isoMTs formed. Dose-response experiments showed that an increase in dexamethasone concentration enhances MT accretion asymptotically to a limit, whereas within the concentration range explored zinc produces an exponential augmentation.

INTRODUCTION

Metallothioneins (MTs) are small (M_r approx. 6500) ubiquitous proteins characterized by an extremely high cysteine and metal content. Their synthesis is readily induced by a variety of metal ions, glucocorticoid hormones, cytokines and many electrophilic agents. MT is thought to play a role in the control of cellular metabolism of essential elements such as zinc and copper and its involvement in detoxification mechanisms against toxic heavy metal ions such as cadmium is also discussed (for review see Kägi and Kojima, 1987; and references therein).

As shown for most other mammals, human MTs occur in two electrophoretically distinct fractions designated as MT-1 and MT-2 (Bühler and Kägi, 1974). Whereas the MT-2 fraction of human liver is nearly homogeneous the MT-1 fraction is polymorphous, containing at least five isoforms which differ in their amino-acid composition and are resolvable by h.p.l.c. (Hunziker and Kägi, 1985).

Human MT is encoded by a multigene family located on chromosome 16 (West et al., 1990). It contains 14 closely linked genes including non-functional pseudogenes. The promoter regions 5'-upstream of the MT genes contain several differentially activatable cis-regulatory elements responsible for the regulation of the expression of each gene (Lee et al., 1987). The lack of one or more of these distinct regulatory elements in the 5'-region of the different isoMT genes was suggested to be responsible for their unequal expression in particular tissues upon exposure to different MT-inducing agents (Richards et al., 1984; Schmidt and Hamer, 1986; Jahroudi et al., 1990). Differences in the expression of the various human MT genes have been noticed in different cell lines (Heguy et al., 1986; Jahroudi et al., 1990) and have been attributed tentatively to the origin of the cell lines from the three different embryonic germ layers (Schmidt and Hamer, 1986). All of these results were obtained by mRNA probing and no information on the accretion of human MT isoproteins has yet become available.

In this report we demonstrate that human MT isoproteins can be analysed in minute amounts using h.p.l.c. The method was applied to assess the accretion of these proteins in three different human cell lines exposed to zinc ions and dexamethasone. Besides documenting the cell- and inducer-specific synthesis of MT isoproteins the results disclosed that the induction of at least one isoform is triggered by an as yet unknown mechanism.

EXPERIMENTAL

Materials and chemicals

Cell culture dishes (100-mm diam.) were obtained from Corning. Dulbecco's modified Eagle's medium (DMEM) and amphotericin B (Fungizone) were purchased from Gibco, glutamine and gentamicin from Serva, and fetal-calf serum (FCS) from Amimed. Dexamethasone 21-phosphate (Dex) and Tris (Trizma base) were obtained from Sigma and [35 S]cysteine (20–150 mCi/mmol) from Amersham. All other chemicals including ZnCl_2 were of analytical grade and obtained from either Merck or Fluka.

Cell culture

All cell lines were obtained from Flow Laboratories. Confluent cultures were split routinely 1:5 and cells were grown for 3–4 days before starting the experiments. All cell lines were grown in DMEM supplemented with 2 mM glutamine, 2.5 $\mu\text{g}/\text{ml}$ Fungizone, 50 $\mu\text{g}/\text{ml}$ gentamicin and 10% (v/v) FCS. Induction of MT synthesis was achieved by addition of ZnCl_2 or Dex to the medium of subconfluent cultures for 16 h. Cell viability was measured in a haemocytometer by Trypan Blue exclusion.

Isotopic labelling

Before the addition of ZnCl_2 or Dex the culture medium was replaced by medium of the same composition but lacking cysteine. For metabolic labelling of MT 10 μCi of [35 S]cysteine per dish was added together with the MT-inducing agents.

Sample preparation

Cells were harvested and resuspended in 2 ml of 25 mM Tris/HCl, pH 7.5. After sonication (four times for 15 s intervals) the homogenate was incubated at 80 °C for 4 min, cooled on ice, and centrifuged at 100 000 g at 4 °C for 90 min. The resulting supernatant was analysed directly by h.p.l.c.

H.p.l.c.

An h.p.l.c. system Model 215 (LKB/Pharmacia) was used. Samples were loaded on to an Aquapore RP-300 butyl column [Brownlee, 4.6 mm (i.d.) $\times$ 220 mm, 7 μm particle size] through a 2 ml sample loop. Chromatography was performed at a flow rate

Abbreviations used: Dex, dexamethasone 21-phosphate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; MT, metallothionein; GRE, glucocorticoid-responsive element; MRE, metal-responsive element.

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of 0.6 ml/min using a linear gradient formed between buffer A (25 mM Tris/HCl, pH 7.5) and buffer B [same as buffer A but containing 60% (v/v) acetonitrile]. The gradient used was as follows: 0% buffer B for 5 min; 0–33% buffer B within 81 min. The effluent was monitored photometrically at 220 nm. For radioactivity measurements fractions were collected every minute, mixed with 5 ml of scintillant mix (LKB/Pharmacia, OptiPhase HiSafe II) and counted in a Beckman liquid scintillation counter Model LS 1701.

Sequence analysis

N-terminal sequences of MT-2 and MT-1e were determined as described (Hunziker, 1991).

RESULTS

The synthesis of different MT isoforms in response to zinc ions and Dex was monitored in the cytosol of three different human cell lines by reversed-phase h.p.l.c. under neutral-pH conditions. The h.p.l.c. elution profiles (Figure 1) indicated resolution of at least eight protein fractions with retention times characteristic of human isoMTs (Hunziker and Kägi, 1985). As deduced from the integrated peak-areas (Figure 1a) 1200–1900 pmol of total MT was recovered from 6×10^6 – 7×10^6 cells. Very similar h.p.l.c.

Figure 1 Reversed-phase h.p.l.c. profiles of cytosols of Chang liver (left), HeLa-S3 (middle) and NCTC 2544 (right) cell lines

Semi-confluent cells were treated with 200 μ M ZnCl₂ and samples for analysis (approx. 6×10^6 cells) were prepared as described in the Experimental section. Chromatography was performed at a flow rate of 0.6 ml/min on an Aquapore RP-300 butyl column (4.6 mm (i.d.) $\times$ 220 mm) using a gradient formed between buffer A (25 mM Tris/HCl, pH 7.5) and buffer B [same as buffer A containing 60% (v/v) acetonitrile]. The gradient used was as follows: 0% buffer B for 5 min; 0–33% buffer B within 81 min. (a) Detection by photometry at 220 nm. Fractions 1 and 2 correspond to the isoforms MT-2 and MT-1e respectively; fractions 3–8 belong to MT-1 subforms not identified as yet with particular isoforms. The MT-2 and MT-1e fractions contained approx. 750 pmol and 300 pmol of protein respectively. (b) Detection by radioactivity measurement ([³⁵S]cysteine). Fractions were collected every minute and analysed by liquid scintillation counting. The relative radioactivity was calculated as c.p.m. of each fraction per total c.p.m. recovered.

patterns were obtained by monitoring the absorbance at 220 nm (Figure 1a) and by measuring the radioactivity of protein-incorporated [³⁵S]cysteine (Figure 1b). SDS/PAGE of the carboxymethylated, [³⁵S]cysteine-labelled protein fractions followed by autoradiography (results not shown) yielded in all eight fractions, the fast-migrating band typical of mammalian MTs (Andersen and Weser, 1979; Sone et al., 1987). N-terminal sequence analysis of the most abundant protein fractions unambiguously identified fraction 1 as human MT-2 (Karin and Richards, 1982) and fraction 2 as human MT-1e (Schmidt et al., 1985). The appearance of a double-peak in these fractions is due to differences in the post-translational modification of these proteins (M. Cavigelli, J. H. R. Kägi and P. E. Hunziker, unpublished work).

Cell-specific isoMT synthesis in response to zinc ions

When semi-confluent cultures of the three cell lines were grown in the presence of 200 μ M ZnCl₂ for 16 h more than 90% of the cells were viable as measured by Trypan Blue exclusion, and no morphological changes were observed. Cytosol of zinc-treated HeLa-S3 and NCTC 2544 cells showed the same elution profiles with respect to the number of MT isoforms (Figure 1a, middle and right respectively). In both cell lines, MT-2 (fraction 1) and MT-1e (fraction 2) were the most abundant species. There were appreciable differences, however, in the relative abundance of the six minor isoforms, as reflected by the relative increase of fractions 3 and 4 and the relative decrease of MT-2 (fraction 1) in NCTC 2544 cells (Table 1, left). In Chang liver cells, MT-2 and MT-1e were also the most abundant isoforms (Figure 1a, left). Of the minor isoMTs four were present in about the same amounts as in HeLa-S3 cells, while two isoforms (fractions 3 and 4) were missing (Figure 1b, left).

Cell-specific isoMT synthesis in response to Dex

Upon addition of 10 μ M Dex to the culture medium the same relative amounts of MT-2 and MT-1e were recovered, both in Chang liver and in HeLa-S3 cells (Table 1, right). In NCTC 2544 cells treated with Dex no MT was detected.

Inducer specificity

When Chang liver and HeLa-S3 cells were cultured in the presence of 200 μ M zinc ions approx. 60% and 20% of the total amount of MT synthesized were found in the MT-2 and MT-1e fractions respectively (Table 1, left). The remaining 20% were distributed among the isoMTs recovered in fractions 3–8. In cells cultured in the presence of 10 μ M Dex, 75% of total MT were found in the MT-2 fraction and 25% in the MT-1e fraction. No significant accretion of the isoMT fractions 3–8 was observed (Table 1, right).

Dose-response curves of sub-confluent Chang liver cells treated either with zinc ions or Dex are shown in Figure 2. In Dex-treated cells the relative distribution of the MT isoforms (Table 1, right) remained unchanged and was independent of Dex dosage. The increase of total MT synthesis relative to that in untreated sub-confluent cells was 11-fold at 10^{-8} M Dex and reached a constant maximum level of 18-fold at concentrations above 10^{-7} M. In contrast, the induction of MT by zinc ions with concentrations ranging from 100 μ M to 200 μ M showed a 10- to 65-fold increase in total MT synthesis (Figure 2). Again the ratios of the different isoforms remained the same at all zinc concentrations (Table 1, left).

As documented in Table 2 comparable accretion of the

Table 1 Relative ratios of MT isoforms in Chang liver, HeLa-S3 and NCTC 2544 cells cultured in the presence of ZnCl₂ or Dex

The numbers given are relative values calculated with reference to the total amount of MT recovered from h.p.l.c. The amount of MT in the effluent was determined photometrically by measuring the absorbance at 220 nm. The fraction numbers correspond to the numbering of isoMTs in Figure 1.

Fraction	Amount of MT isoforms present (%)					
	ZnCl ₂ -treated (200 μ M)			Dex-treated (10 μ M)		
	Chang liver cells	HeLa-S3 cells	NCTC 2544 cells	Chang liver cells	HeLa-S3 cells	NCTC 2544 cells
1	65	52	35	75	75	0
2	21	22	18	25	25	0
3	0	4	16	0	0	0
4	0	5	15	0	0	0
5	3	4	4	0	0	0
6	3	5	3	0	0	0
7	3	2	4	0	0	0
8	5	3	6	0	0	0

Figure 2 Relative MT accretion in Chang liver cells grown in the presence of increasing concentrations of ZnCl₂ or Dex

Semi-confluent cells were cultured in the presence of either zinc ions or Dex as indicated. Cells were harvested after 16 h and cytosols were analysed as described in Figure 1. The amount of MT was determined by summing up the MT peak areas monitored photometrically at 220 nm. All numbers are calculated relative to the amount of MT recovered from untreated cells.

Table 2 MT accretion in Chang liver cells cultured in the presence of ZnCl₂ and/or Dex

All values are relative to the amount of MT-2 recovered from cells grown in the presence of 100 μ M ZnCl₂. The amount of MT in the h.p.l.c. effluent was determined photometrically by measuring the absorbance at 220 nm.

Treatment	MT-2 (Peak 1)	MT-1e (Peak 2)	Minor MT-1 isoforms (Peaks 3–8)
Zinc (100 μ M)	1	0.3	0.2
Dex (100 μ M)	0.9	0.2	0
Zinc (100 μ M) + Dex (100 μ M)	4.3	1.2	0.4

principal MT isoforms was achieved in the presence of either 100 μ M zinc ions or 100 μ M Dex. When both inducers, zinc and Dex were added simultaneously, cellular MT contents increased by approx. 4-fold compared with the addition of either reagent alone.

DISCUSSION

Because of its high sensitivity reversed-phase h.p.l.c. carried out at neutral pH allows the detection of the minute amounts of MT isoproteins endogenously formed in human cell lines. The recognition of some of the fractions eluting with retention-times between 35 min and 55 min as isoMTs was based on the correspondence of the photometric profiles (Figure 1a) with the profiles obtained by monitoring the [³⁵S]cysteine content of the h.p.l.c. effluent of metabolically labelled samples (Figure 1b). After S-carboxymethylation each of these fractions showed, on SDS/PAGE, the characteristic fast-migrating band known to be typical of MT (Andersen and Weser, 1979; Sone et al., 1987). The identity of the most abundant fractions, 1 and 2, with human MT-2 and human MT-1e respectively was established by partial protein-sequence analysis. Since fractions 3–8 contained only small amounts of MT no attempts were made in this study to specify these isoforms further.

Judging from the size of the profiles recorded by photometry at 220 nm (Figure 1a) the method was sensitive enough to follow the emergence of the various isoMTs in induction experiments in as little as 1×10^6 cells. After increasing the sensitivity of the detector to 0.04 full-scale deflection at 220 nm, neutral pH h.p.l.c. measurement of basal-level amounts of MT (3–4 pmol/ 10^6 cells) was accomplished with about 1×10^7 cells.

Upon stimulation with 200 μ M zinc ions the cells of each of the three cell lines derived from different embryonic germ layers responded with substantial accretion of total MT. The h.p.l.c. elution profiles showed that the summed amount of MT-2 and MT-1e was quite comparable in all three cell lines (Figure 1a). In NCTC 2544 cells derived from ectoderm there was, however, an appreciable greater abundance of all minor isoforms, and in particular of those eluting as fractions 3 and 4. In the mesodermic HeLa-S3 cells these two isoforms occurred only in small quantities and they were completely absent in the endodermic Chang liver cells.

Quite disparate results were obtained when the three cell lines

were treated with 10 μ M Dex. Both in Chang liver and HeLa-S3 cells only MT-2 and MT-1e were expressed (Table 1). No MT at all was detectable in NCTC 2544 cells. The reason for this lack of MT synthesis is not known. It could be explained either by incomplete functioning of glucocorticoid receptors, or by the absence of glucocorticoid-responsive elements (GREs) at the 5'-end of the human MT-2a gene in this particular cell line. The results are in agreement with the earlier proposal that cells from different embryonic germ layers respond with transcription of different MT genes when stimulated with diverse inducing agents (Schmidt and Hamer, 1986).

The total amount of MT in Chang liver cells exposed to 10^{-7} M Dex was 18-fold higher than that in untreated control cells (Figure 2). The dose-response curve indicates that a maximum amount of MT is approached at this concentration as even a 100-fold increase of the Dex concentration did not lead to a substantially higher accretion of MT. By contrast, when zinc was used as the inducing agent the amount of protein formed increased exponentially with increasing zinc concentration in the medium. A 65-fold increase in MT content was attained upon exposure to 200 μ M zinc (Figure 2). Above this concentration considerable cell attrition was setting in, as expected from the half-lethal dose of approx. 220 μ M zinc (M. Cavigelli, P. E. Hunziker and J. H. R. Kägi, unpublished work).

As previously observed (Iijima et al., 1990) the response of MT synthesis to the simultaneous exposure to zinc and Dex was more than additive (Table 2). The MT content achieved when both inducing agents were combined was in the range attained with Chang liver cells cultured in the presence of 150–200 μ M zinc. At least for MT-2 this result could be explained by a cooperative action of metal-responsive elements (MREs) and of GREs, both

present in the 5' region of the human MT-2a gene (Lee et al., 1987). In contrast, in the MT-1e gene, which contains MREs in its promoter region (Schmidt et al., 1985), no GREs have been found so far. Nonetheless, in our experiments MT-1e was induced both in Chang liver and in HeLa-S3 cells by exposure to Dex. This unexpected response of the MT-1e gene could indicate the presence of an as yet unknown overriding regulator of at least this particular MT gene.

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