Induction of metallothionein synthesis by cadmium and zinc in cultured rabbit kidney cells (RK-13)

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The effects of increasing concentrations of Zn(II) and Cd(II) on the expression of the four isometallothioneins (isoMTs), namely MT-1a, MT-2a, MT-2d and MT-2e, in rabbit kidney cells (RK-13) and the development of cellular tolerance to these metal ions were studied. The results showed that, whereas in parental cells MT concentration was low and composed nearly exclusively of MT-2a and MT-1a, all four isoMTs increased massively in abundance when the cells were exposed to toxic concentrations of Zn(II) or Cd(II), the relative increase being largest in the two minor isoforms MT-2d and MT-2e. While the response of the four isoMTs to the challenge by Zn(II) or Cd(II) was qualitatively comparable, there were differences in sensitivity and delay time, Cd(II) being the more efficient inducer and much faster in eliciting the onset of isoMT synthesis. An even larger production of isoMTs resulted when RK-13 cells were cultured in the

presence of a series of metal concentrations yielding sub-lines of increased metal tolerance. In this instance too, there were marked differences in the response to Cd(II) and Zn(II). Thus, in cells of sub-lines selected for tolerance to moderate concentrations of Cd(II) the kinetic analysis of isoMT accretion gave indications of a saturable induction process while no such evidence was forthcoming for Zn(II). In sub-line cells selected for tolerance to the highest concentrations of Cd(II) or Zn(II) isoMT formation was increased by another order of magnitude, reaching for some isoforms a 100- to 1000-fold augmentation over the amounts measured in cells of the unexposed parental cells. A potentiation of this magnitude goes beyond the range of ordinary regulation of gene expression. It is to be viewed instead as an enlargement of the capacity of isoMT synthesis acquired by a variety of mechanisms in the surviving cells.

INTRODUCTION

Metallothioneins (MTs) are cysteine-rich, metal-binding proteins believed to be involved in metal homeostasis and in the protection of cells and tissues from the cytotoxic effects of soft heavy-metal ions such as Zn(II), Cd(II), Cu(I) and Hg(II) [1]. In many species MTs are polymorphous proteins occurring in several isoforms (isoMTs) encoded by different genes. Thus, the human genome is known to contain 14 MT genes, all of them located on chromosome 16 [2]. More recent studies also gave evidence for the existence of at least six distinct isoMTs in rabbit tissues (P. E. Hunziker, P. Kaur, M. Wan and A. Känzig, unpublished work).

MT gene promoters are structurally complex, and consist of multiple cis-acting DNA sequences required for basal, as well as for inducible, gene transcription [3]. Although the fine structure of the promoter regions of the various MT genes is quite disparate all MT gene promoters contain cis-acting DNA sequences termed metal-responsive elements (MREs), which are thought to represent binding sites for trans-acting metal transcription factors modulating the level of transcription of the MT genes in response to the environmental metal concentration [4,5]. The fine structure of the rabbit MT-1 gene promoter showed that it contained 11 MREs in the 5'-untranslated upstream sequence of this gene [6].

Since MT is inducible by many agents and conditions, such as metal ions, hormones, cytokines and various forms of physical and chemical stress [7], and since these effects are known to be mediated through different promoters and enhancers, transcrip-

tion factors and signalling pathways [8], it is an attractive hypothesis that the various isoforms can be elicited differentially by the appropriate stimuli and may in fact play somewhat different cellular roles. Earlier studies have indeed shown that the expression of certain isoMT genes can differ in their response to inducing factors [9,10] and that it is also cell- and tissue-specific [7]. Thus, while dexamethasone was found to be a good inducer of MT synthesis in murine liver [11] it had no significant effects in kidney [12], a finding also reported for the RK-13 cell-line derived from rabbit kidney [13].

We have demonstrated that RK-13 cells express four distinct isoMTs previously identified in rabbit liver and kidney and we have established a chromatographic method for their separation, characterization and accurate quantification [14]. In the present study we used this method to monitor, on the protein level, the expression of the four rabbit isoMT genes in response to the exposure of parental RK-13 cells and of sub-line cells resistant to different concentrations of Cd(II) and Zn(II).

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM), gentamicin and amphotericin B were obtained from GIBCO (Paisley, Scotland, U.K.). Fetal-calf serum (FCS) and 0.5% trypsin/0.2% EDTA without Ca(II) and Mg(II) were purchased from Amimed (Muttentz, Switzerland). Glutamine was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; LC₅₀, half lethal concentration; MRE, metal-responsive element; MT, metallothionein; RK-13, rabbit kidney cell line; RCd60, RK-13 sub-line cells resistant to 60 μ M Cd²⁺ in the culture medium. The number indicates the Cd²⁺ concentration to which the cells were adapted; RZn100, RK-13 sub-line cells resistant to 100 μ M Zn²⁺ in the culture medium. The number indicates the Zn²⁺ concentration to which the cells were adapted; TFA, trifluoroacetic acid.

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grade and purchased either from Merck (Darmstadt, Germany) or from Fluka (Buchs, Switzerland).

Cell culture

RK-13 cells were purchased from Flow Laboratories (Rickmansworth, Herts., U.K.). The cells cultured in 100-mm-diam. tissue-culture dishes (Corning, New York, NY, U.S.A.) at 37 °C, with 10% CO₂ in DMEM supplemented with 10% (v/v) FCS, 2.5 mg/l amphotericin B, 50 mg/l gentamicin and 292 mg/l glutamine. Metal stock solutions (CdCl₂ or ZnCl₂, in 5 mM HCl) at an appropriate concentration were added to the cultures 24 h after the cells were seeded. They were grown in the presence of a constant metal concentration for the time periods indicated. Cells from 2–3 culture dishes were used for the quantification of isoMTs. Viable cells were determined by Trypan Blue exclusion and counted in a haemocytometer.

Cd- or Zn-resistant sub-line cells were obtained by a stepwise increase of the concentration of metal ions in the culture medium. Cells surviving after each increment were cultured at a constant metal concentration until they resumed a near-normal growth rate. Subsequently, a portion of these cells was exposed to the next increased metal concentration. The sub-line cells adapted to the various metal concentrations were kept frozen in liquid nitrogen until further use in these studies.

Determination of LC₅₀ values for Zn(II) and Cd(II)

In a 24-well culture plate about 5000 trypsinized RK-13 cells suspended in 0.5 ml of medium were seeded per well. After 24 h appropriate volumes of a 1 mM metal stock solution were added to yield the final metal concentrations. The cells were harvested and counted 24 h after metal administration. For each metal concentration cells from four wells were used to obtain a mean value. The half lethal concentration (LC₅₀) was determined by interpolation from graphs of the logarithm of the number of living cells versus the metal concentration in the medium.

Isolation and quantification of isoMTs

The isolation and quantification were carried out according to the method of Wan et al. [14]. Cells (8×10^7) of the parental cell-line and 2×10^7 – 3×10^7 cells of the various metal-ion-adapted substrains were harvested by trypsinization and homogenized by freezing and thawing in liquid nitrogen. The cytosol fraction of lysed cells was collected by ultracentrifugation (100 000 *g*, 4 °C, 90 min) and loaded on to a Sephadex G-75 column [0.9 cm (i.d.) $\times$ 85 cm] equilibrated in 10 mM Tris/HCl, pH 7.5 and eluted at a flow rate of 9 ml/h with the same buffer. Depending on the sub-line used zinc and cadmium contents were monitored by atomic absorption spectrophotometry. MT-containing fractions were pooled, diluted with an equal volume of water and loaded on to a TSK DEAE-5PW h.p.l.c. column equilibrated with 5 mM Tris/HCl, pH 7.5. The sample was eluted at a flow rate of 0.8 ml/min using a linear gradient from 0–15% buffer B (5 mM Tris/HCl, pH 7.5 containing 1 M NaCl) within 40 min. The two MT fractions were analysed by reversed-phase h.p.l.c. at a flow rate of 0.6 ml/min using a gradient formed between solvent A [0.1% (v/v) trifluoroacetic acid (TFA)] and solvent B [same as A, but containing 60% (v/v) acetonitrile] as follows: 0% solvent B for 5 min, 0–25% solvent B within 10 min, 25–50% solvent B within 50 min. IsoMTs were quantified by

peak-area integration using a computerized data system. All results given here are the means of at least two experiments.

RESULTS

Toxicity of Cd(II) and Zn(II) and MT accretion in parental RK-13 cells

The LC₅₀ values of Cd(II) and Zn(II) for parental RK-13 cells were about 22 μ M and 160 μ M respectively. Table 1 documents the cell attrition caused by metal concentrations higher than the LC₅₀ values. While reaffirming the greater toxicity of Cd(II) these results indicate that Zn(II) manifests its toxicity over a narrower concentration range once its LC₅₀ value is exceeded. Table 2 shows that under these conditions MT synthesis was strongly enhanced. Thus, in the presence of 50 μ M Cd(II) the total amount of MT per cell increased 7.4-fold within 24 h compared with the control, and with 100 μ M Cd(II) an 11.1-fold increase within 12 h was observed. Exposure to 140 μ M Zn(II) resulted in a 3.6-fold augmentation within 24 h. Quantification of the individual isoforms, as performed by reversed-phase h.p.l.c. (Figure 1), shows that the amounts of all isoMTs were enhanced by metal exposure although to a widely different extent. On a relative scale the two minor isoforms MT-2d and MT-2e underwent the strongest increases, to be followed by MT-1a. MT-2a remained the most abundant species but with the upsurge of the minor isoforms its relative contribution to total MT decreased (Table 2).

Table 1 Effects of exposure of confluent parental RK-13 cells to high concentrations of Cd(II) and Zn(II)

Time (h)	Number of viable cells per dish (units of 10^6 cells)				
	Control	50 μ M Cd	100 μ M Cd	150 μ M Zn	200 μ M Zn
4	10.3	9.0	9.2	7.0	5.3
8	12.6	7.2	6.1	8.6	2.8
12	10.9	7.4	5.9	5.6	1.1
24	11.0	4.5	2.3	6.3	0

Table 2 Time- and dose-dependence of isoMT accretion in confluent parental RK-13 cells exposed to Cd(II) or Zn(II)

Abbreviation: n.d., not determined.

Metal concn. in medium	Duration of exposure (h)	MT content (pmol/ 10^6 cells)				
		Total MT	MT-1a	MT-2d	MT-2e	MT-2a
Control	4–24	49.6	11.2	1.2	0.9	36.2
50 μ M Cd	4	94.6	18.8	n.d.	n.d.	75.8
	8	177.7	35.7	18.9	16.0	107.1
	12	259.7	68.3	36.5	24.7	130.2
	24	367.7	100.6	55.8	33.5	177.8
100 μ M Cd	4	109.2	21.4	n.d.	n.d.	87.8
	8	301.8	57.7	49.2	37.5	157.4
	12	549.1	137.8	102.8	36.2	272.3
	24	0	0	0	0	0
150 μ M Zn	24	177.5	37.3	16.3	22.9	101.0

* As compared with control cells no change was observed during the first 12 h.

Figure 1 Representative reversed-phase h.p.l.c. separation of the four MT isoforms of RK-13 cells

Chromatography was performed on an Aquapore RP-300 column at a flow rate of 0.6 ml/min using a gradient formed between solvent A [0.1% (v/v) TFA] and solvent B [same as A, but containing 60% (v/v) acetonitrile]. The gradient used was as follows: 0% solvent B for 5 min, 0–25% solvent B within 10 min, 25–50% solvent B within 50 min. The effluent was monitored at 220 nm and MT fractions 1–4 were quantified by peak-area integration. Upper trace: DEAE fraction 1; lower trace: DEAE fraction 2. Fraction numbers 1–4 correspond to MT-1a, MT-2d, MT-2e and MT-2a respectively [14].

IsoMT accretion in metal-tolerant RK-13 sub-line cells

To assess the relationship between metal-tolerance and MT accretion the four isoforms were quantified in confluent cells of RK-13 sub-lines selected for resistance to different concentrations of Cd(II) or Zn(II) (see the Materials and methods section). As exemplified in Figure 2 these cells retained the epithelial character of the parental cells but differed characteristically in size. Thus, cells tolerant to the highest possible concentration of Cd(II) were about twice as large, and cells tolerant to the highest concentration of Zn(II) were about half as large as the parental RK-13 cells. As the comparative measurements of isoMT accretion (Figure 3) were made in cells cultivated to confluence these size differences account for the opposite changes in cell number encountered in the sub-lines selected for increasing resistance to Cd(II) or Zn(II) (Figure 3, top panels).

The left-hand panels of Figure 3 display the variations in cellular isoMT content in the different sub-lines ranging in resistance to concentrations of Cd(II) between 1 μ M and 150 μ M. Sub-lines tolerant to concentrations higher than 150 μ M could not be obtained. The total MT contents increased with the elevated metal resistance of the cells. However, there were marked differences in dose-response between cell-lines adapted to different concentrations of the metal. In cells tolerant to concentrations between 1 μ M and 20 μ M Cd(II) there was an approx. 10-fold rise in total MT content, approaching a plateau at a cellular concentration of about 500 pmol of MT/ 10^6 cells. As shown in the inset of the central panel all four isoforms contributed to this augmentation, with the minor forms MT-2d

Figure 2 Difference in cell size of cultured parental cells (a), cells from Cd-resistant RK-13 sub-line (RCd150; b) and cells from Zn-resistant RK-13 sub-line (RZn350; c)

The cultures were photographed 24 h after seeding. The cell numbers counted per confluent dish (96 h in culture) were 11×10^6 cells/dish in parental RK-13 cells, 3.4×10^6 cells/dish for the RCd150 sub-line culture and 17.5×10^6 cells/dish for the RZn350 sub-line culture.

and MT-2e undergoing the largest relative increases in abundance. In cells tolerant to up to approx. 40 μ M Cd(II) the same ratio of isoforms was maintained (bottom panel). In cells of the sub-lines resistant to 60 μ M Cd(II) and above there was a continuous increase of MT, leading in the cells adapted to 150 μ M Cd(II) to a 200-fold greater MT content than that found in parental cells (Table 2). Interestingly, in the cells with the highest tolerance to Cd(II) there was an absolute and relative decrease of isoform MT-2d.

Figure 3 MT accretion in different RK-13-derived Cd-resistant (left-hand panels) or Zn-resistant (right-hand panels) sub-line cells

Each point refers to cells of a distinct sub-line selected for tolerance to the metal concentration indicated and grown to confluence (96 h in culture). The changes in cell number per dish (top panels) are thought to arise from the metal-induced changes in cell size (see Figure 2). The total MT content (top panels) is the sum of the contents of the four isoMTs (centre panels). The inset in the left-hand centre panel displays the isoMT accretion in the cells tolerant to low concentrations of Cd(II). The bottom panels give the relative distribution of the isoMTs.

Figure 4 Time courses of growth and total MT (top panels) and of isoMT accretion (bottom panels) in growing parental RK-13 cells following administration of 20 μM Cd(II) (left) and 100 μM Zn(II) (right) after 24 h of culture

The right-hand panels of Figure 3 shows that cells tolerant to increasing concentrations of Zn(II) showed an analogous accretion of MT. In accordance with the lower toxicity of Zn(II)

(see above) the response of the Zn-resistant cells in producing MT was displaced to a metal concentration greater than that observed in the studies with Cd(II), and there were also quan-

Figure 5 Time courses of MT accretion in proliferating cultures of Cd-resistant RK-13 sub-line cells (RCd60, left; RCd150, right) following re-exposure to 60 μM or 150 μM Cd(II) respectively, after 24 h of culture

Upper panels: total MT accretion and growth curves. Centre panels: isoMT accretion. Lower panels: ratios of isoMTs. Note the different scales of the vertical axis of the left- and right-hand panels.

titative differences in dose-response. Thus, the total MT content in sub-lines tolerant to lower concentrations of Zn(II), i.e. up to 100 μM , increased monotonically without giving evidence for a saturable process. In cells of the sub-lines resistant to 150 μM Zn(II) and above there was again a continuous increase, leading in cells tolerant to 300 μM Zn(II) to an approx. 120-fold augmentation over that measured in parental RK-13 cells. The somewhat lower MT content in cells resistant to 350 μM Zn(II) was probably due to the delayed accretion of MT in cells of this sub-line (see Figure 6). Differences to the Cd-tolerant cells were also noticed in the development of the relative isoMT distribution (Figure 3, bottom panels). While with increasing tolerance to Zn the minor isoforms MT-2d and MT-2e underwent strong absolute and relative increases no constancy in isoform ratio was attained. In fact, in cells of sub-lines tolerant to concentrations of 200 μM Zn(II) and more MT-2d vanished progressively and became almost undetectable in RZn350 cells.

Time courses of isoMT accretion in growing cells exposed to Cd(II) or Zn(II)

Metal-specific differences were also manifested in the temporal response to the metal challenge in growing cells. Thus, upon addition of 20 μM Cd(II) to proliferating parental RK-13 cells the cellular content of all four isoMTs increased rapidly, reaching a maximum within 24 h (Figure 4, left-hand panels). Another increase occurred after 72 h of culturing, possibly reflecting the onset of cellular selection for tolerance. In contrast with Cd(II) exposure, the response to Zn(II) was more delayed. Thus, the same cells growing in the presence of 100 μM Zn(II) reached a

maximum MT content at about 72 h after metal addition (Figure 4, right-hand panels).

Comparable disparities in the response to metal exposure were observed in growing cells of metal-resistant RK-13 sub-lines. The time course of isoMT accretion and of cellular growth observed with cells selected for resistance to 60 μM and to 150 μM Cd(II) are compared in Figure 5. The cells of both sub-lines showed elevated initial levels of MT and responded upon renewed exposure to their tolerated concentrations of Cd(II) with an increase in cellular MT content to the levels measured previously in confluent cultures (Figure 3). The two sub-lines differed, however, in the speed of adjustment. While RCd60 cells responded within 24 h with a growth-independent augmentation of MT (Figure 5, left-hand panels), there was a growth-correlated rise of cellular MT in the RCd150 cells. In both Cd-resistant cell-lines the relative distribution of the four isoforms remained unchanged throughout the observed growth period. The actual ratios of the isoforms were, however, quite different in the RCd60 and the RCd150 cells. The percentage of MT-2a in the RCd150 cells was higher than that in the RCd60 cells and the relative amounts of MT-1a and of MT-2d were lower than in the RCd60 cells.

The time course of isoMT accretion and the growth curves of RK-13 sub-line cultures selected for tolerance to 200 μM and 350 μM Zn(II) are compared in Figure 6. Again as with Cd(II) the cells adapted to the lower metal concentration (RZn200) responded to the tolerated 200 μM Zn(II) within 24 h with a growth-independent increase of cellular MT to the concentration range observed previously in confluent cultures (Figure 3). In the RZn350 sub-line cells substantial MT accretion occurred only

Figure 6 Time courses of MT accretion in proliferating cultures of Zn-resistant RK-13 sub-line cells (RZn200, left; RZn350, right) following re-exposure to 200 μM or 350 μM Zn(II) respectively, after 24 h of culture

Upper panels: total MT accretion and growth curves. Centre panels: isoMT accretion. Lower panels: ratios of isoMTs. Note the different scales of the vertical axis of the left- and right-hand panels.

after 96 h and after termination of the growth period. The results also show that at both concentrations of Zn(II) the onset of logarithmic growth is strongly delayed. In contrast with the stable isoform profiles in the Cd-resistant cells both the RZn200 and the RZn350 cells showed changing ratios of the four isoforms during the observed growth period. Another difference to the Cd-resistant cells was the much greater relative abundance of the principal isoform MT-2a.

DISCUSSION

The present results confirm our previous findings that RK-13 cells respond to the exposure to elevated concentrations of Cd(II) and Zn(II) with an increased accretion of all four isoMTs [14]. However, the results also demonstrate that there are marked differences in the time course of the response, in its dose dependence and in the morphological accompaniments of the adaption of the cells to high concentrations of metal ions. Thus, while the cells adapted to 150 μM Cd(II) cover a surface about twice as large as the parental RK-13 cells (Figure 2b), this relationship is opposite in the cells adapted to 350 μM Zn(II) (Figure 2c). While the causes of these morphological changes remain to be identified it is obvious that the respective volume differences must be considered when estimating the actual concentration of MT in the Cd- or Zn-exposed cells. We have measured the total MT concentration in RK-13 cells adapted to 150 μM Cd(II) to be about 10700 pmol/10⁶ cells, while the concentration of total MT in cells adapted to 350 μM Zn(II) is only 5300 pmol/10⁶ cells. However, accepting a more than 4-fold difference in volume of the cells of these two metal-adapted cell-lines given in Figure 2, the amounts of MT indicate that the

molar MT concentration in the Zn-adapted cells might be actually higher than that of the Cd-adapted cells.

Cd(II) is more toxic to RK-13 cells ($\text{LC}_{50} = 22 \mu\text{M}$) than Zn(II) ($\text{LC}_{50} = 160 \mu\text{M}$). It is also much faster and more efficient than Zn(II) in eliciting the formation of MT (Table 2). Thus, while the MT concentration increased within a few hours of exposure to Cd(II) a similar response to comparable toxic concentrations of Zn(II) required as much as 24 h. This result can be compared with the observation in pig renal proximal tubule cells where MT induction by Cd(II) was ten times as efficient as by Zn(II) [15].

The strong variation in the isoMT profiles upon exposure to increased concentrations of Cd(II) and Zn(II) afford evidence for a differential regulation of isoMT accretion in RK-13 cells (Figure 3). Comparable variation in the quantitative and qualitative response to different metals has also been observed in human cell-lines. Thus, as monitored by Northern-blot hybridization, exposure of HepG2 cells to Cu(II), Cd(II) or Zn(II) resulted in widely different patterns of expression of the human MT-2, MT-1f and MT-1g genes [16].

The response of all four active MT genes to the metal challenge is an argument for their organization in a gene cluster, as demonstrated previously for human MT genes [2]. The observed dose-dependent variations in the relative abundance of the various isoforms could arise either from changes in their ability to be activated by the sets of available transcription factors, or from some metal-induced changes in the access of the transcriptional machinery to the different genes in the chromosomal environment. That such metal-dose-dependent changes in regulatory potential contribute to the increased production of isoMTs is supported by our recent finding that the

exposure of RK-13 cells to subtoxic concentrations of Cd(II) and Zn(II) leads to a 2–3-fold increase in the supply of metal transcription factors required for the activation of MT transcription (M. Wan, R. Heuchel, F. Radtke, P. E. Hunziker and J. H. R. Kägi, unpublished work). The differences in the absolute and relative abundance of the various isoforms in cells adapted to the highest concentration of Cd(II) and Zn(II) are probably related to changes in the organization of the MT genes brought about by the selective gene amplification which we recently demonstrated in metal-tolerant RK-13 cells (M. Wan, R. Heuchel, F. Radtke, P. E. Hunziker and J. H. R. Kägi, unpublished work).

The different dose–response of MT formation in the cells adapted to lower concentrations of Cd(II) and Zn(II) could indicate some differences in the mechanism of induction of MT synthesis by these two metals (Figure 3). The plateauing of the accumulation of MT with increasing Cd(II) concentration probably reflects a saturation phenomenon. Since the rabbit MT-1a gene carries 11 potential MREs in its 5'-upstream sequence [6] it is conceivable that the putative transcription factors saturable by Cd(II) and interacting with the MREs are in limited supply. The progressively increasing accretion of MT with increasing Zn(II) concentration could result from contributions of additional MT-induction pathways not open to Cd(II).

Differences between the effects of Cd(II) and Zn(II) are also seen in the preservation of the isoMT profiles with time. Thus, while in cell-lines adapted to a given concentration of Cd(II) the isoMT profiles remained constant throughout the culturing time (Figure 5) there is a temporal evolution in the profiles of the Zn-adapted cells and there is also a striking dissociation of MT accretion and cell proliferation not seen in the Cd-adapted sub-

line cells. It is thus clear that isoMT accretion in response to Cd(II) or Zn(II) and to different concentrations of these metals must be viewed as the result of a variety of different adaptive mechanisms.

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