Isolation of mouse isometallothioneins

A comparison of isometallothioneins in growing cells and post-mitotic cells

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Mouse metallothioneins (MTs) were separated into three isoforms by an anion-exchange h.p.l.c. column; conventionally isolated MT-1 and MT-2 showed a single peak (MT-1-1) and two peaks (MT-2-1 and MT-2-2), respectively. In growing cells, developing hepatocytes and growing tumour cells, MT-1/MT-2 ratios were less than 0.6, irrespective of the type of MT inducer, whereas adult liver post-mitotic cells had a ratio of more than 1.0. A large amount of the MT-2-2 subfraction was found in dexamethasone-treated FM3A cells; 90 % of MTs was MT-2-2, suggesting that glucocorticoid hormone mainly induces MT-2-2 in tumour cells.

INTRODUCTION

Metallothioneins (MTs) are low-molecular-mass proteins containing high amounts of cysteine. They bind Zn^{2+} , Cu^{2+} , Cd^{2+} and Hg^{2+} through cysteine thiolate bridges in polypeptides, and have been postulated to be involved in homoeostatic mechanisms such as detoxification of heavy metals and regulation of essential-metal metabolism (Cherian & Goyer, 1978). MTs have been found in all eukaryotic cells, in which they are induced by heavy metals such as Zn^{2+} (Failla & Cousins, 1978), Cu^{2+} (Weiner & Cousins, 1980) and Cd^{2+} (Failla *et al.*, 1979) or by glucocorticoid hormones (Karin *et al.*, 1980, 1981; Karin & Herschman, 1980, 1981). In mammals there are two major isoforms of MT, generally designated MT-1 and MT-2. They differ in certain amino acids, but are alike in conserving the number and location of 20 cysteine residues within the molecule (Nordberg & Kojima, 1979). These conventional isoMTs, MT-1 and MT-2, have been separated into several isoforms by using reverse-phase h.p.l.c. (Klauser *et al.*, 1983).

It has been reported that the mouse MT-1 gene is transcriptionally regulated by Cd, but not by glucocorticoid (Mayo & Palmiter, 1982). In human, MTs are encoded by a multi-gene family containing at least 14 closely related genes and pseudogenes (Karin *et al.*, 1984; Beau *et al.*, 1985), but only half of these genes appear to be expressed at the protein level (Hunziker & Kagi, 1985). Two of these genes, hMT-I_A and hMT-II_A, encode functional proteins which each show a different induction response to heavy metal and glucocorticoid (Richards *et al.*, 1984). This differential response is due to functional differences in the respective promoter/regulatory regions of the genes; the hMT-I_A promoter responds only to Cd^{2+} , but the hMT-II_A promoter is responsive to Cd^{2+} , Zn^{2+} and glucocorticoid.

During development, MT contents are high in fetal and neonatal rodent liver and decrease to basal values in adult liver (Bell, 1980; Ouellette, 1982; Anderson *et al.*, 1983). During pregnancy, glucocorticoid concentrations are elevated in both the maternal and fetal circulation at

the time when MT mRNA concentrations rise (Barlow *et al.*, 1974; Dalle *et al.*, 1978). Quaife *et al.* (1986) have proposed the hypothesis that glucocorticoid acts at the level of transcription to stimulate MT synthesis and that the sequestration of Cu and Zn by MT is a consequence of the induction rather than the cause. A similar situation occurs in regenerating adult liver, since after partial hepatectomy there is an increase in liver Zn and a concomitant synthesis of MT (Ohtake & Koga, 1979). In regenerating liver, the rate of MT-2 synthesis is twice that of MT-1; however, in the liver of Zn-injected animals, both iso-MTs are synthesized in equal amounts (Cain & Griffiths, 1984). In a previous study, we have demonstrated that in a tumour-cell line, mouse ascites sarcoma S180A, MT-2 was accumulated to a much greater extent than in host liver, the target organ (Kobayashi & Sayato-Suzuki, 1988). Although the exact functions of MT in cell metabolism are unknown, these results suggest that there is a close association between cell growth and MT synthesis.

As we were interested in the relationship between cell growth and MT synthesis, we carried out an analysis of isoMTs in neonatal-mouse liver, and in Zn-, Cd- or glucocorticoid-treated mouse tumour cells, using an anion-exchange h.p.l.c. column and the results were compared with those for metal-treated adult liver. In the present paper, we describe the findings obtained, which suggest that MT-2 has a relationship with glucocorticoid in growing cells.

MATERIALS AND METHODS

Animals

Eight-week-old ddY mice purchased from Sankyo Labo Service Co., Tokyo, Japan, were housed under standardized conditions with a period of 12 h of fluorescent light per day (08:00–20:00 h), room temperature of 23 °C and a relative humidity of 50–60 %. Individual 8-week-old females weighing 20–25 g were each placed with a single 8-week-old male mouse for 2

days. A vaginal smear was taken and, if sperm was detected, the female was used for the experiment. Pregnant females had access to standard food and water *ad libitum*, and gestation was allowed to proceed normally. The newborn mice at 2 days of age were killed by decapitation and livers were quickly removed and stored at -70°C . Zinc (10 mg/kg body wt.) or cadmium (1 mg/kg body wt.) as a solution of ZnSO_4 or CdCl_2 in 0.9% NaCl was administered to 8-week-old mice as a single subcutaneous injection, and the mice were then killed by decapitation 24 h after metal administration. The livers were removed and stored at -70°C .

Cell cultures

Mouse mammary carcinoma FM3A cells, at a concentration of 1×10^5 cells/ml in ES medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with

2.5% (v/v) fetal-calf serum, were incubated at 33°C in a humidified atmosphere of CO_2/air (1:19). In the present study, Zn ($5 \mu\text{g}/\text{ml}$), Cd ($1 \mu\text{g}/\text{ml}$) or $1 \mu\text{M}$ -dexamethasone was added to the cells at a concentration of 1×10^6 cells/ml (exponential-phase growth) and incubation was carried out for 24 h. The treated cells were washed four times with Hanks' balanced salt solution to remove non-specifically bound metals from the cell surface. The cell suspension was kept frozen at -70°C until used.

Sarcoma S180A cells at a concentration of 1×10^7 cells/ml suspended in 0.9% NaCl containing 10 units of penicillin G/ml and $100 \mu\text{g}$ of streptomycin/ml were injected intraperitoneally into ddY female mice. After 3 days (exponential-phase growth), the tumour-bearing mice were injected intraperitoneally with $5 \mu\text{g}$ of Zn/g body wt. and then killed 24 h after the start of metal administration. The tumour cells were removed

Fig. 1. H.p.l.c. profiles of the isolated MT-1 and MT-2 (a) and the MT fraction from gel filtration (b) of a sample from liver of a Zn-treated adult mouse

(a) For this, 10-week-old ddY female mice were given an intraperitoneal dose of 10 mg of Zn/kg body wt. and killed after 24 h. Two adult liver isoMTs, MT-1 and MT-2, were isolated by gel filtration on a Sephadex G-75 column and anion-exchange chromatography with a DEAE-Sephadex A25 column. A $10 \mu\text{l}$ sample of MT-1 (containing $0.35 \mu\text{g}$ of Zn) or MT-2 (containing $0.46 \mu\text{g}$ of Zn) was applied to an anion-exchange h.p.l.c. column (Asahipak ES-502N, $13 \pm 0.5 \mu\text{m}$ particle size; $7.6 \text{ mm} \times 100 \text{ mm}$). (b) The MT fraction ($V_e/V_0 = 2$) was obtained by Sephadex G-75 gel filtration of cytosol from livers of Zn-treated adult mice. A $10 \mu\text{l}$ sample (containing $0.61 \mu\text{g}$ of Zn) of the MT fraction was applied to an anion-exchange h.p.l.c. column. Chromatography was performed at $0.5 \text{ ml}/\text{min}$, with a gradient of 4–52 mM-potassium phosphate buffer, pH 7.5. The peaks revealed by A_{220} were collected with a fraction collector by switching from the u.v. monitor, and Zn (□) and Cu (■) concentrations in the peaks were determined by atomic-absorption spectrometry.

Fig. 2. H.p.l.c. elution profiles of MT fractions by gel filtration of cytosol from Cd-treated adult mouse liver (a) and Zn-treated ascites-sarcoma S180A cells (b)

(a) A 10 μg sample from Cd-treated liver (containing 0.125 μg of Cd) was applied to the anion-exchange h.p.l.c. column. Sarcoma-S180A cells at a concentration of 1×10^7 cells/ml were injected intraperitoneally into ddY mice. After 3 days (exponential-phase growth), tumour-bearing mice were injected with 5 μg of Zn/g body wt. and killed 24 h after the start of metal administration. (b) A 10 μg sample of the MT fraction of tumour cells from Zn-treated sarcoma-S180A-bearing mice (containing 0.191 μg of Zn) was applied to the anion-exchange h.p.l.c. column. Further treatment is described in Fig. 1 legend.

from mice and washed with Hanks' balanced salt solution at 4 °C, and frozen at -70 °C until used.

Purification of metallothioneins

The newborn-mouse liver, metal-treated adult-mouse liver and metal-treated carcinoma cells were homogenized with 5 vol. of 0.01 M-Tris/HCl buffer containing 0.01 M-NaHSO₃ (pH 7.5), 2 mM-dithiothreitol, 2 μg of antipain/ml and 1 μM -pepstatin in a Potter-type homogenizer at 4 °C and centrifuged at 170000 g for 60 min. Antipain and pepstatin were added to protect the MT from hydrolysis by various proteinases. Dithiothreitol (1 mM) and CdCl₂ (10 μM) were added to the homogenates obtained from FM3A cells treated with dexamethasone, to protect the MT from oxidation. The cytosols were applied to a Sephadex G-75 column (1.8 cm $\times$ 45 cm) equilibrated with 0.01 M-Tris/HCl buffer (pH 7.5) containing 0.01 M-NaHSO₃ and 2 mM-dithiothreitol and

eluted with the same buffer at a flow rate of 10 ml/h. The metals in each fraction were measured by atomic-absorption spectrometry. The MT fraction eluted from the column ($V_e/V_0 = 1.8$ –2.0) was pooled and concentrated with Dextran T-40 in cellulose dialysis tubes (M_r cut-off 1000; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) at 4 °C. The concentrated MT fraction was used for isoMTs analysis by h.p.l.c. on an anion-exchange column.

Mouse liver Zn-MT-1 and Zn-MT-2 were isolated as described by Huang *et al.* (1977, 1981) and used as standard samples for isoMTs analysis.

Analysis of isoMTs by h.p.l.c.

The h.p.l.c. instrument consisted of a chromatograph (Shimadzu HPLC CL-6A Gradient System; Shimadzu Co., Kyoto, Japan) and an anion-exchange chromatography column (Asahipak ES-502N, $13 \pm 0.5 \mu\text{m}$

particle size; 7.6 mm $\times$ 100 mm column; Asahi Chemical Industries Co., Kawasaki, Japan). A 10 μ l portion of the concentrated MT fraction obtained from gel filtration, containing 0.1–1.0 μ g of metal, was applied to the column and eluted with 4 mM-potassium phosphate buffer, pH 7.5, at a flow rate of 0.5 ml/min. Subsequently, the sample was eluted with the same buffer for 10 min and then with a linear gradient of 4–52 mM potassium phosphate buffer, pH 7.5, for 30 min at 20 $^{\circ}$ C, and the A_{220} was determined with a Shimadzu SPD-6A u.v. detector. The peak fractions detected by the A_{220} were collected with a Frac-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) by switching them from the u.v. monitor, and heavy-metal concentrations were determined by atomic-absorption spectrometry.

RESULTS

Analysis of conventionally isolated adult mouse liver Zn-MT-1 and Zn-MT-2 was performed with an anion-exchange h.p.l.c. column (Fig. 1a). The MT-1 and MT-2 subfractions showed a single peak and then two

Fig. 3. H.p.l.c. elution profile of MT fraction obtained by gel filtration of cytosol from neonatal-mice livers

A 10 μ g sample from neonatal-mouse liver (containing 0.79 μ g of Zn and 0.69 μ g of Cu) was applied to the anion-exchange h.p.l.c. column. Further treatment is described in Fig. 1 legend.

peaks, designated MT-1-1, MT-2-1 and MT-2-2, which were eluted at retention times of 13–14 min (4 mM-potassium phosphate buffer), 26–27 min (7–8 mM) and 29–30 min (11–12 mM) respectively (Fig. 1a). The recoveries of the applied amounts of Zn were: MT-1-1, 80%; MT-2-1, 10%; MT-2-2, 62%. The Cu contents in these subfractions were less than 10% of total metals (Zn plus Cu). The recovery of total metals, Zn plus Cu, was approx. 70–80%. The ratios of the total A_{220} /total metal content for the individual peaks all showed the same values. These results suggested that mouse MTs were separated into three isoforms, two major and one minor. The elution profile (Fig. 1b) of the MT-containing Sephadex G-75 fraction of the cytosolic preparation of Zn-treated adult liver showed behaviour identical with that of the isolated MT-1 and MT-2 on anion-exchange h.p.l.c. The Zn recoveries for the individual peaks, MT-1-1, MT-2-1 and MT-2-2, were 36%, 5% and 33% respectively. The amounts of Cu associated with the individual subfractions were 15–17% of those of Zn. The ratios of the total A_{220} /total metal content of individual fractions showed similar values. These results indicated that, by application of the MT fraction from gel filtration on an anion-exchange h.p.l.c. column, mouse isoMTs could be separated and analysed.

Fig. 2(a) demonstrates the elution profile of the Cd-containing MT fraction obtained from gel filtration of the Cd-treated adult mouse liver; the two large peaks and one minor peak were found at the same retention times as those in the Zn-containing MT fraction. The recoveries of total metals, Cd plus Zn, for MT-1-1, MT-2-1 and MT-2-2 were 43%, 3% and 38% respectively. The amounts of Zn in individual subfractions were approx. 40% of those of Cd. The ratio of the total A_{220} /total metal contents for the individual peaks all showed the same values. These results suggest that MT obtained from Cd-treated adult liver also is separated into three isoMTs, as with Zn treatment.

The MT fractions obtained by gel filtration from mouse ascites-sarcoma S180A cells showed a different elution profile from that of adult liver on h.p.l.c. (Fig. 3); Zn recoveries for MT-1-1, MT-2-1 and MT-2-2 were 13%, 26% and 29% respectively. The Cu contents in these subfractions were 15–17% of total metals. The ratios of the total A_{220} /total metal contents were the same for all individual subfractions. These results indicated that the MT-2-1 subfraction in sarcoma S180A cells was not a minor one, the amounts of Zn bound to MT-2-1 and MT-2-2 being equal to each other.

Fig. 3 demonstrates the elution profile of the Zn- and Cu-containing MT fraction obtained from gel filtration of the cytosol prepared from livers from neonatal mice (day 2 after birth). Two large peaks at retention times of 13–14 min and 29–30 min in the profile were identical with those of adult mouse liver MT-1-1 and MT-2-2, but the minor peak, MT-2-1, could not be detected. The recoveries of Zn and Cu in MT-1-1 or MT-2-2 subfractions, 20% and 11% or 36% and 19% respectively, and the ratios of the total A_{220} /total metal contents for individual subfractions indicated similar values. A Cu-associated peak at a retention time of 11–12 min was observed; 13% of Zn and 14% of Cu in the MT gel fraction were recovered in the peak. The ratio of the total A_{220} /total metal content for this fraction was 3 times those of MT-1-1 and MT-2-2; moreover, the peak had high A_{280} . The peak was not detected in the

Fig. 4. H.p.l.c. elution profiles of MT fractions obtained by gel filtration of cytosols from (a) Zn- or (b) Cd-treated FM3A cells

(a) For this, 5 μg of Zn/ml was administered to FM3A cells at a concentration of 1×10^6 cells/ml, and incubation was conducted for 24 h. A 10 μg sample of the MT fraction from cytosol of Zn-treated FM3A cells (containing 0.224 μg of Zn) was applied to the anion-exchange h.p.l.c. column. (b) For this, 1 μg of Cd/ml was administered to FM3A cells at a concentration of 1×10^6 cells/ml, and incubation was conducted for 24 h. A 10 μl sample of the MT fraction from the cytosol of Cd-treated FM3A cells (containing 0.115 μg of Cd) was applied to the h.p.l.c. column. Further treatment is described in Fig. 1 legend.

isoMTs of metal-treated adult liver on the same column, but it was not clear whether these metal-containing peaks were mouse isoMT or not.

As shown in Figs. 4(a) and 4(b), isoMTs in the Cd- and Zn-treated cultured mouse FM3A cells were separated into three peaks with the same retention times as those in Cd- and Zn-treated adult livers; Cd or Zn recoveries of MT-1-1, MT-2-1 and MT-2-2 were 28 %, 4 % and 38 %, or 14 %, 12 % and 32 % respectively. The ratios of the total A_{220} /total metal contents for each isoMT were similar to those for adult liver. These results indicated that the elution profile from cultured FM3A cells showed a pattern typical of mouse isoMTs, being similar to that from adult liver.

When FM3A cells were treated with dexamethasone, harvested and then homogenized with Cd-containing buffer, the h.p.l.c. elution pattern showed a single Cd-associated peak at a retention time of 29–30 min, corresponding to MT-2-2; Cd recovery was 62 % (Fig. 5). The amounts of Cd associated with the MT-1-1 and MT-2-1 subfractions were slight. These results strongly

suggest that the major mouse isoMT induced by glucocorticoid hormone is the MT-2-2 subfraction.

Fig. 6 shows MT-1 (MT-1-1)/MT-2 (MT-2-1 + MT-2-2) or MT-2-1/MT-2-2 ratios in the metal-treated adult liver, neonatal liver, metal-treated mouse ascites-tumour cells and metal- or dexamethasone-treated cultured mouse tumour cells. The ratios were expressed as percentages of the total isoMTs, which were calculated from the total amounts of metals associated with individual isoMTs. In the Zn- or Cd-treated adult liver, the ratio of MT-1/MT-2 was more than 1.0, but the others had a ratio of less than 0.6. These results indicate that in developing hepatocytes and growing tumour cells, MT-1/MT-2 ratios were less than 0.6 irrespective of the type of MT inducer, but metal-treated adult liver had a ratio of more than 1.0. In dexamethasone-treated FM3A cells, the amount of MT-1 was only 5 % of the total isoMTs, suggesting that the major isoMT induced by glucocorticoid in mouse tumour cells is MT-2-2. In cultured mouse tumour cells, the amounts of MT-1 accumulated in Cd-treated cells were high in comparison

Fig. 5. H.p.l.c. elution profile of the MT fraction obtained by gel filtration of cytosol from dexamethasone-treated FM3A cells

The FM3A cells, at 1×10^6 cells/ml, were treated with $1 \mu\text{M}$ -dexamethasone for 24 h. To homogenates of the cells were added 1 mM-dithiothreitol and $10 \mu\text{M}$ - CdCl_2 , and incubation was performed for 10 min at 37°C . A $10 \mu\text{g}$ sample of the MT fraction from the cytosol of dexamethasone-treated FM3A cells (containing $0.044 \mu\text{g}$ of Cd) was applied to the h.p.l.c. column. Further treatment is described in Fig. 1 legend.

with those in Zn-treated cells, being 40 % for Cd and 25 % for Zn. The MT-2-1/MT-2-2 ratios in neonatal liver, Cd- or dexamethasone-treated FM3A cells were less than 0.1, but in Zn-treated cultured tumour cells and ascites-sarcoma S180A cells the ratios were 0.6 and 1.0 respectively.

DISCUSSION

Metallothioneins in Zn-treated adult mouse liver were separated into three isoforms on an anion-exchange h.p.l.c. column; the conventionally isolated MT-1 and MT-2 were separated as a single peak (MT-1-1) and as two peaks (MT-2-1 and MT-2-2). Suzuki *et al.* (1985) have demonstrated that minor peaks other than the two major isoMT peaks (MT-1 and MT-2) were present in Zn-treated adult rat liver by line switching from gel-filtration to ion-exchange columns by h.p.l.c.-atomic-absorption spectrophotometry. Klauser *et al.* (1983) also demonstrated that rat MTs could be separated into three

isoforms by reverse-phase h.p.l.c.; yields from the reverse-phase support were typically 60–70 % for the isoMTs. In the present study, the average metal recovery for isoMTs was 70 %, although only a small amount of metal was applied, indicating that application of the metal-containing fraction from Sephadex G-75 to an anion-exchange h.p.l.c. column is an available approach for analysis of isoMTs.

In the present study, the ratio of MT-1/MT-2 in the livers of neonatal mice was 0.5, indicating that the major isoMT in neonatal liver is MT-2 (MT-2-2). On the other hand, it was suggested that in mouse FM3A cells the major isoMT induced by the glucocorticoid hormone dexamethasone was MT-2-2 (Fig. 5). Quaife *et al.* (1986) have demonstrated that, during development, the MT mRNA content is regulated by large increases in corticosterone (the predominant mouse glucocorticoid hormone). They proposed a hypothesis that glucocorticoid acts at the level of transcription to stimulate MT synthesis, and that the sequestration of Cu or Zn by MT is a consequence of the induction, rather than the cause. Thus, in the developing liver, the gene expression of MT-2-2 might primarily be stimulated by glucocorticoid hormone, and Zn or Cu, which increase as a result of MT synthesis, might bind to the isoMTs.

In the present experiments, the ratios of the two isoMTs (MT-1/MT-2) accumulated in the livers of neonatal mice and in ascites sarcoma S180A cells from Zn-treated tumour-bearing mice and cultured Zn-, Cd- and dexamethasone-treated FM3A cells were less than 0.6, irrespective of the type of MT inducer. However, in adult liver, MTs induced by Cd or Zn had an MT-1/MT-2 ratio of more than 1.0. These results indicated that in growing cells the rate of synthesis of MT-2 is much greater than that of MT-1, but in post-mitotic cells the rates for both isoMTs were equal. In the regenerating liver, the MT-1/MT-2 ratio was 0.5, suggesting the possibility that the synthesis of MT-2 is related to cell growth (Cain & Griffith, 1984). Similar findings were obtained by Ujjani *et al.* (1986), who examined Ehrlich-tumour-bearing mouse liver for the presence of Zn-MT. Interestingly, it was found that, in mice with larger tumours, the liver primarily contained Zn-MT-2. Thus the synthesis of MT-2 in host liver may be induced by tumour cells, which act as a stress agent. On the basis of these observations, we propose that, with regard to the physiological roles of MT-1 and MT-2, MT-1 acts as a protection system against heavy metals, such as Cd or extremely high concentrations of Zn, and that MT-2 synthesis is related to cell growth or various forms of stress.

In Zn-treated ascites sarcoma S180A cells, the MT-2-1 peak was not minor, being three times more abundant than that in the host liver (Kobayashi & Sayato-Suzuki, 1988). The amounts of the MT-2-1 subfraction in Cd-treated or dexamethasone-treated FM3A cells and neonatal liver were very small or non-detectable, but in Zn-treated FM3A cells and ascites-sarcoma S180A cells the MT-2-1 isoform showed a high concentration. The function of the MT-2-1 isoform is not clear, but its synthesis may be related to Zn metabolism and cancerous stress.

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Fig. 6. Relative ratios of two or three isoMTs isolated by anion-exchange h.p.l.c.

Ratios of isoMTs were expressed as percentages of the total metal contents of MT-1-1 (stippled column) and MT-2-1 plus MT-2-2 (white column). The black bars marked 1 and 2 refer to the ratios for MT-2-1 and MT-2-2 respectively.

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