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Zinc and copper accumulation and isometallothionein induction in mouse ascites sarcoma S180A cells

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To investigate Zn and Cu accumulation and isometallothionein (iso-MT) induction in ascites-sarcoma S180A cells, 5 μg of Zn^{2+} or Cu^{2+}/g body weight was administered to tumour-bearing mice intraperitoneally. In the tumour cells the Zn or Cu concentration increased more than in the host liver, which is the target organ for those metals; the maximum Zn or Cu level was about 2–3 times that in the host liver. The amounts of Zn-MT or Cu-MT accumulated in the tumour cells and host liver were proportional to such dose accumulation levels in the each cytosol; the maximum level of Zn-MT or Cu-MT was 4 or 2 times higher than in the host liver. MT accumulated in the tumour cells showed two subfractions (MT-1 and MT-2); the ratio of Zn (or Cu) bound to MT-1 to that bound to MT-2 in the host liver and tumour cells was 1.0 (or 1.0) and 0.7 (or 0.25) respectively, suggesting that the induction level of MT-2 in the tumour cells is more than that of MT-1. The h.p.l.c. profiles (using an anion-exchange column) of the isolated MT-1 and MT-2 subfractions from Zn-treated normal-mouse liver showed a single peak (MT-1-1) and two peaks (MT-2-1 and MT-2-2) respectively; mouse MTs were separated into three isoforms. In the ascites cells, the MT fraction obtained by a gel filtration was also separated into three isoforms; however, the amount of MT-2-1 isoform was 3 times that in the Zn-treated normal-mouse liver.

INTRODUCTION

A number of studies have reported disturbed Zn and Cu homoeostasis in human cancer patients (Tessmer *et al.*, 1972*a*; Thorling & Thorling, 1976; Shah-Reddy *et al.*, 1980; Fisher *et al.*, 1981; Choen *et al.*, 1984; Margalioth *et al.*, 1985; Miatto *et al.*, 1985). Frequently an increase in serum Cu occurs in conjunction with a decrease in serum Zn (Inutsuka & Araki, 1978; Fisher *et al.*, 1981). Increased Cu levels have also been observed in leukaemic lymphocytes (Carpentieri *et al.*, 1986) and malignant human tissues such as carcinomas of the breast, urinary bladder and stomach (Tessmer *et al.*, 1972*b*; Hrgovcic *et al.*, 1973), but the Zn concentration in malignant tissues is not significantly different from that in corresponding normal tissues (Margalioth *et al.*, 1983). Lefkowitch *et al.* (1983) have found an increased Cu level and a Cu-binding metallothionein (MT)-like protein in human liver carcinoma.

Ujjani *et al.* (1986) demonstrated Zn redistribution between plasma and liver show in mice injected with Ehrlich ascites-tumour cells. Within 24 h of injection, plasma Zn levels decrease and Zn appears in newly synthesized liver MT and this response is dependent upon the number of tumour cells injected into host mice. They reported that the tumour cells act as a stress agent which induces liver MT synthesis. Karin *et al.* (1981) have suggested that stress induction of MT can be explained by stress-induced elevation of circulating glucocorticoids. Thus various human cancers may cause Zn redistribution from serum to malignant tissues by glucocorticoids, consequently a decreased level of Zn in serum and an increased Zn level in malignant tissues are found in human cancer patients. We have demonstrated in previous studies that dexamethasone, a synthetic glucocorticoid, inhibits, rather than stimulates, Cu transport into a cultured human liver cell line (Kobayashi *et al.*, 1985*a*). Thus it is difficult to explain high Cu concentration in serum and tissues of human cancer patients as being attributable only to metal redistribution through stress.

We are interested in the relationship between Cu and Zn metabolism and the biological role of MT in growing cells. In the present study, we investigated Zn and Cu accumulation, and MT induction, in growing, compared with post-mitotic, cells; mouse ascitic sarcoma S180A cells and host mouse liver were used. Furthermore, iso-MTs induced in the tumour cells were examined by h.p.l.c. on an anion-exchange column.

MATERIALS AND METHODS

Mice bearing ascitic sarcoma S180A cells

Female ddY mice were purchased from Sankyo Labo Service Co. (Tokyo, Japan); they were 7 weeks old and weighed about 25 g at tumour transplantation. They were housed under standardized conditions (12 h of fluorescent light/day (08:00–20:00 h), 23 °C room temperature and 45–55 % relative humidity. Tumour-bearing mice and control mice (no tumour) respectively were divided into two groups of 54 animals and injected i.p. (intraperitoneally) with 5 μg of Cu^{2+} (as $CuSO_4$) or Zn^{2+} (as $ZnSO_4$)/g body wt. and fed a standard laboratory diet *ad libitum*. Six mice of each group were killed at 0, 1, 3, 6, 12, 24 and 48 h after start of metal injection. Sarcoma S180A cells at a concentration of 1×10^7/ml suspended in 0.9 % NaCl containing 10 units of penicillin G and 100 μg of stretomycin/ml were injected i.p. into mice for 3 days (exponential growth phase) before an injection of heavy metals. The livers and tumour cells were removed from mice and washed with Hanks balanced solution at 4 °C. The cells were washed four times with 10 mM-Tris/HCl buffer, pH 8.6, containing 10 mM-$NaHSO_3$ buffer, pH 8.6, 1 mM-dithiothreitol, antipain (2 μg/ml) and 1 mM-pepstatin and stored at −80 °C until use.

Determination of metals in liver and cells

The livers (6 g wet wt.) and tumour cells (5×10^8 cells) from six tumour-bearing and normal mice were homogenized in 5 vol. of 0.01 M-$NaHSO_3$, pH 8.6, 2 mM-dithiothreitol, antipain (2 μg/ml) and 1 μM-pepstatin in a Potter-type homogenizer at 4 °C, and then centrifuged at 170000 *g* for 60 min. The precipitates were re-extracted by the above methods. A 3 ml portion of the cytosol obtained from six animals was digested with HNO_3, and metals in the cytosols were assayed by atomic-absorption spectrometry. The amounts of metals in the cytosol were expressed as nmol/mg of cytosolic protein. The protein concentration in the total cytosols was measured by the dye-binding method of Bradford (1976), using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Purification of MTs

A 3 ml portion of cytosol obtained from cells and livers by the above methods was applied to a Sephadex G-75 (1.8 cm × 45 cm) column, equilibrated with 0.01 M-Tris/HCl buffer, pH 8.6, containing 0.01 M-$NaHSO_3$ and 2 mM-dithiothreitol. The cytosol was eluted with the same buffer at a flow rate of 10 ml/h, and fractions (2 ml each) were collected. The metal contents of each fraction were measured by atomic-absorption spectrometry. Metal absorption indicated three peaks; most of the metals in the cytosol were distributed in the second peak ($V_e/V_o = 2.0$). In a previous study, we showed that the second peak was the MT fraction (Kobayashi *et al.*, 1985*b*). The amounts of MT-binding metals were expressed as the total amounts of Zn plus Cu, and Zn or Cu, in the MT fraction obtained by Sephadex G-75 column chromatography per mg of cytosol protein. The MT fraction was pooled and concentrated with dextran T-40 by using cellulose dialysis tubes (M_r cut-off 1000; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) at 4 °C. The concentrated samples were applied to a DEAE-Sephadex A25 column (0.95 cm × 20 cm) equilibrated with 0.01 M-Tris/HCl buffer, pH 7.5 at 4 °C. After the sample was applied to the column, 0.01 M-Tris/HCl buffer was used as the eluent for 30 fractions at a rate of 15 ml/h. Then a linear gradient of NaCl (0–200 mM) in 0.01 M-Tris/HCl buffer, pH 7.5, was used as the eluent. The metal-binding fractions were re-chromatographed, dialysed against 10 mM-NH_4HCO_3, freeze-dried and stored at −80 °C until analysed.

Polyacrylamide-gel electrophoresis

The purities of the two major metal-binding subfractions from ion-exchange chromatography were determined by 7.5%-(w/v)-polyacrylamide-gel electrophoresis. Electrophoresis was performed as described by Davis (1964) in 1 mm-thick slab gels. The gels were fixed for 30 min in 34.5% (v/v) sulphosalicylic acid/11.5% (v/v) trichloroacetic acid, washed in 5% (v/v) acetic acid, and stained with silver.

Amino acid analysis

The purified subfractions from ion-exchange chromatography were oxidized with performic acid, hydrolysed with 6 M-HCl at 110 °C for 24 h, and analysed with a Hitachi amino acid analyser model KLA-5 (Hitachi Co., Tokyo, Japan).

Isolation of MT isoforms

MT isoforms were analysed by h.p.l.c. with the use of a Shimadzu (Kyoto, Japan) LC-6A system equipped with an anion-exchange chromatography column (Asahipak ES-502N; 13±0.5 μm particle size; 7.6 mm × 100 mm; Asahi Chemical Industry Co., Kawasaki, Japan) equilibrated with 4 mM-phosphate buffer, pH 7.5 at 20°C. After the sample had been applied to the column, 4 mM-phosphate buffer was used as the eluent for 10 min at a rate of 0.5 ml/min. Then the sample was chromatographed with a linear gradient of 4–52 mM-phosphate buffer, pH 7.5, at a flow rate of 0.5 ml/min for 30 min at 20 °C. The A_{220} was determined with a u.v. spectrometer (Shimadzu SPD-6A; Shimadzu Co., Kyoto, Japan). The Zn or Cu levels in the A_{220} peaks were measured by atomic-absorption spectrometry.

RESULTS

Accumulation of Zn and Cu in mouse ascites sarcoma S180A cells, host liver and control liver

The sarcoma S180A cells at a concentration of 1×10^7/ml were transferred i.p. into mice and maintained for 3 days (exponential growth phase). Administration of 5 μg of Zn^{2+} or Cu^{2+} or Cu^{2+}/g body body weight i.p. to tumour-bearing mice did not affect the growth curves for sarcoma S180A cells growing in mouse (results not shown). More than 75% of the Zn and 85% of the Cu in the homogenate from ascites cells or livers were distributed in the cytosol fraction (results not shown). After metal administration, the Zn or Cu level in the cytosol of tumour cells rapidly increased during 12 h or 9 h after injection respectively, and then decreased (Figs. 1*a* and 1*b*). The maximum Zn or Cu concentrations accumulated in tumour cells were 2–3 times those in the host liver and control liver respectively, indicating that tumour cells accumulate higher Zn and Cu concentrations than does the liver, which is a target organ for those heavy metals.

On the other hand, in the host liver, the Zn concentration rapidly decreased during 3 h and then increased. The maximum Zn level in the host liver was lower than the initial level, despite Zn administration, suggesting that Zn is transported from the host liver into tumour cells. However, Cu levels accumulated in the host liver increased rapidly to reach a maximum level at 12 h after the administration. Zinc administration did not affect the endogenous Cu level in the tumour cells, host liver and control liver, but the endogenous Zn in them was increased by Cu administration.

Time courses of metals bound to MT accumulated in ascites sarcoma S180A cells and host liver

Zn and Cu distribution in the cytosols of cells and livers from metal-treated and non-treated tumour-bearing mice were analysed by gel filtration on a Sephadex G-75 column. In non-treated tumour-bearing mice, 30 and 36% of the Zn in the cytosols of cells and host liver respectively were associated with the MT fraction ($V_e/V_o = 2$), but there was no Cu associated with the MT fraction. After Zn administration, the amounts of Zn associated with the MT fraction from gel filtration in the cytosol of cells, host liver or normal liver was proportional to the Zn level accumulated in each cytosol; 60% of Zn in each cytosol was associated with MT

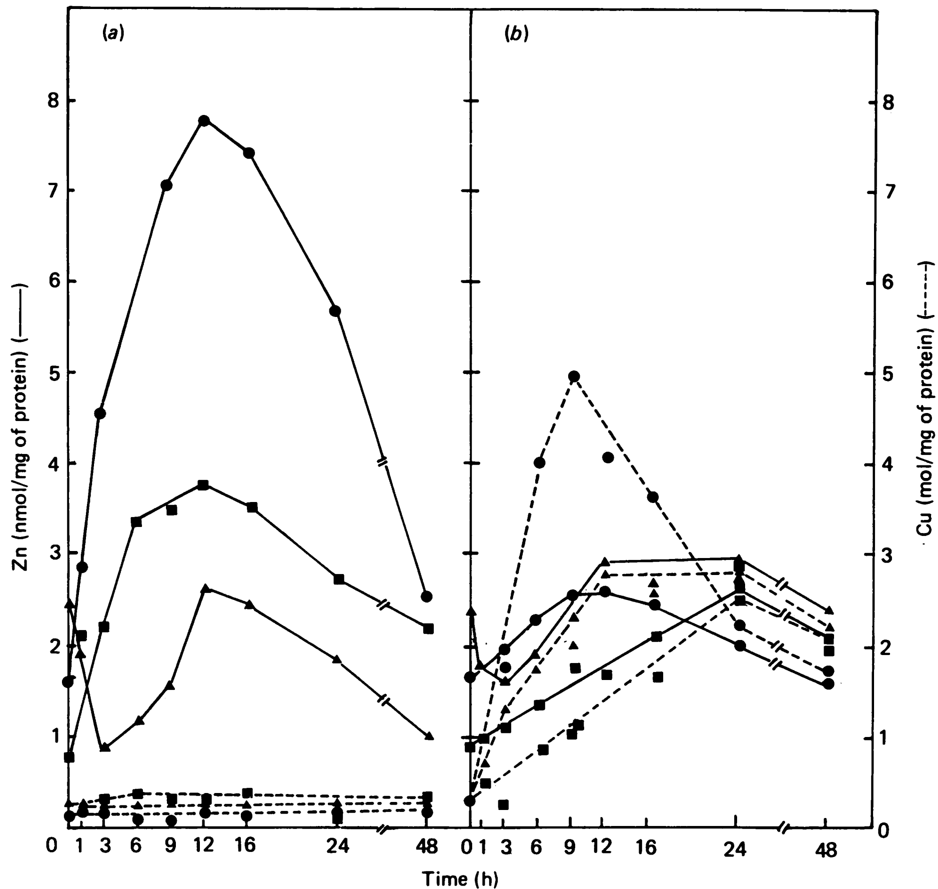

Fig. 1. Accumulation of Zn and Cu in cells and liver from Zn-treated tumour-bearing mice

The tumour-bearing mice and normal (tumour-free) mice were injected i.p. with 5 μg of Zn^{2+}/g (*a*) and 5 μg of Cu^{2+}/g (*b*). At various time intervals the animals were killed and the cells and livers removed from tumour-bearing mice. The livers (6 g wet wt.) and tumour cells (5×10^8 cells) from six animals were homogenized as described in the Materials and methods section. Portions (3 ml) of the cytosols were digested with HNO_3 and Zn (——) and Cu (- - - -) levels were assayed by atomic-absorption spectrometry. ●, Ascites sarcoma S180A cells; ▲, host livers; ■, normal livers.

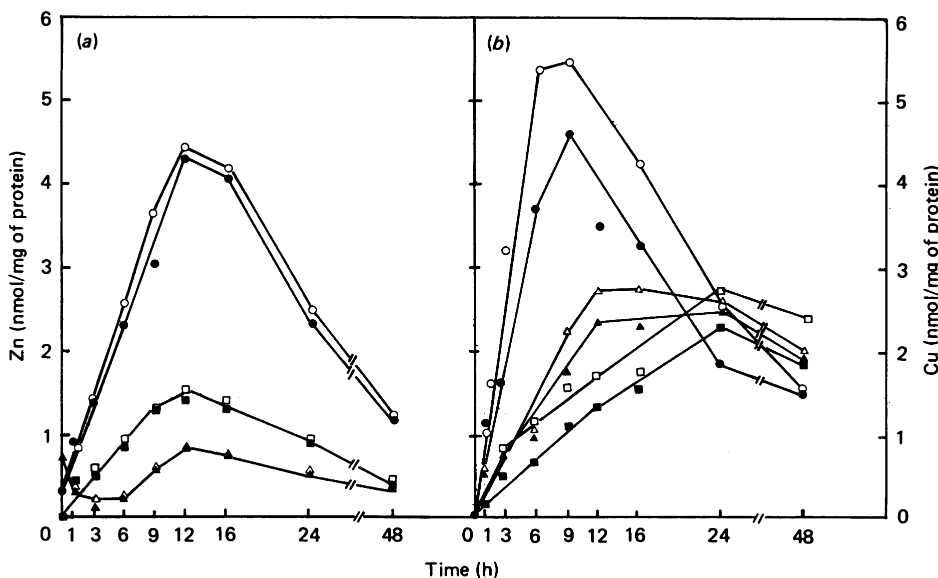

Fig. 2. Accumulation of metals binding MT in the cells and liver from Zn- or Cu-treated tumour-bearing mice

The ascites-tumour-bearing mice were injected i.p. with 5 μg of Zn^{2+} (*a*) or Cu^{2+} (*b*)/g. Portions (3 ml) of cytosols were applied to a Sephadex G-75 column. The amounts of MT-binding metals were expressed as the amounts of Zn plus Cu (open symbols) and Zn or Cu (closed symbols) in the MT fraction by a gel filtration ($V_e/V_o = 2.0$)/mg of cytosol protein. ○●, Tumour cells; △▲, host liver; □■, normal (tumour-free) liver.

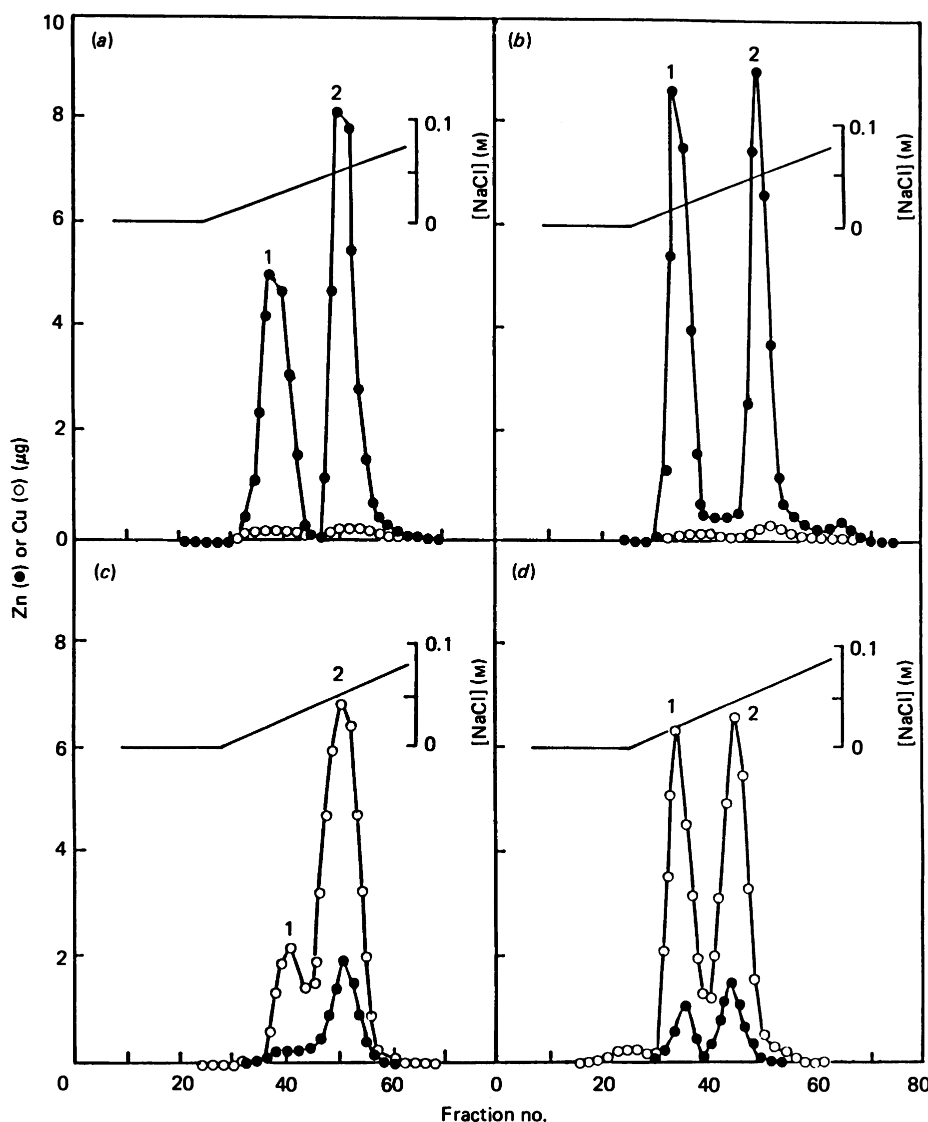

Fig. 3. DEAE-Sephadex A25 ion-exchange-chromatography profiles of MT fractions

The MT fractions (obtained by gel filtration) in cytosols of cells treated with 5 µg of Zn^{2+} (*a*) or Cu^{2+} (*c*)/g body weight and liver treated with 5 µg of Zn^{2+} (*b*) or Cu^{2+} (*d*)/g body weight for 15 h were applied to a DEAE-Sephadex A25 column equilibrated with 0.01 M-Tris/HCl buffer, pH 7.5. A linear gradient of NaCl (0–200 mM) in 0.01 M-Tris/HCl buffer, pH 7.5, was used as the eluent.

fraction. After 12 h the maximum Zn level associated with the MT fraction in the tumour cells was about four times that in the host liver.

The amount of Cu associated with MT was also proportional to such dose levels accumulated in the ascites cells, host liver and normal liver; 90 % of the Cu in each cytosol was associated with the MT fraction (Fig. 2*b*). The maximum level of Cu associated with the MT fraction in the cells was twice that in the host liver and control liver respectively.

Isolation of MTs accumulated in tumour cells

The cytosols of cells and livers obtained from metal-treated tumour-bearing mice were chromatographed on a Sephadex G-75 column and the MT fractions subsequently purified by anion-exchange chromatography on a DEAE-Sephadex A25 column. As shown in Figs. 3(*a*)–3(*d*), the elution profiles were very similar to each other, each showing two Zn- or Cu-binding subfractions; Zn- or Cu-MT-1 was eluted at 20–30 mM-NaCl and Zn- or Cu-MT-2 was eluted at 50–60 mM-NaCl.

The ratio of Zn-MT-1 (or Cu-MT-1) to Zn-MT-2 (or Cu-MT-2) in the host liver was 1.0 (or 0.1), but in the cells the ratio was 0.7 (or 0.25) respectively, suggesting that the amounts of MT-2 induced in the tumour cells are higher than those of MT-1.

Electrophoretic analysis of MT subfractions

The subfraction MT-1 or MT-2 from Zn-treated tumour cells, host liver and normal liver showed a single visible band on the gel (results not shown). The electrophoretic mobilities of MT-1 from the cells, host liver and control liver were 0.6, 0.61 and 0.61 respectively relative to Bromophenol Blue, whereas that of MT-2 from cells, host liver and control liver were 0.74, 0.73 and 0.73 respectively.

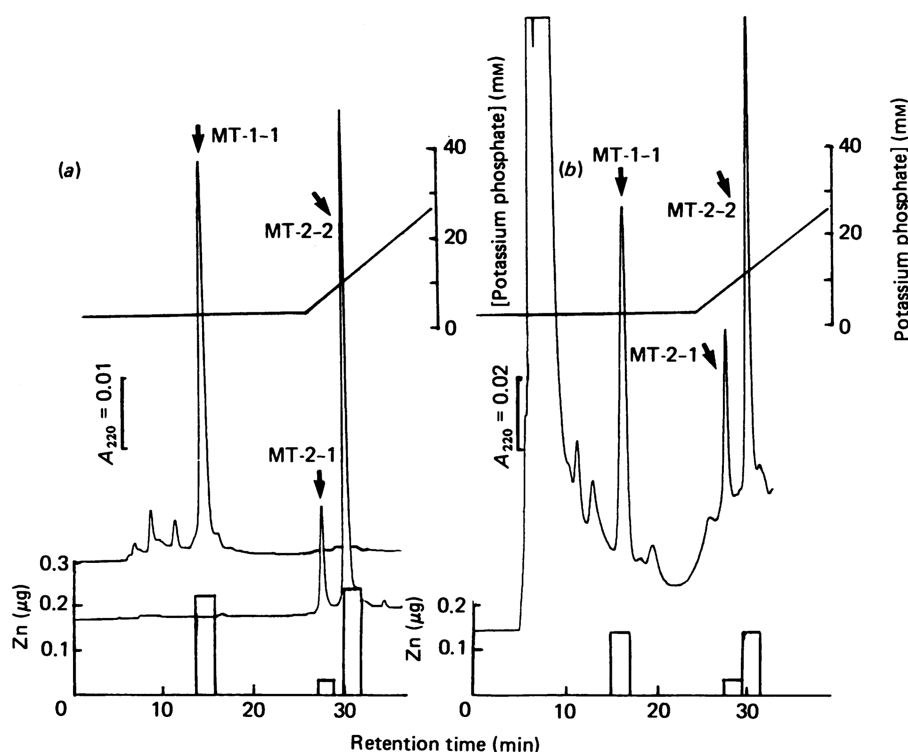

Fig. 4. Elution profiles of MT subfractions (Zn-MT-1 and Zn-MT-2) submitted to an anion-exchange column/h.p.l.c. procedure

To analyse iso-MTs, 10 µg of Zn^{2+}/g body weight was injected i.p. into control mice and livers were removed from mice after 15 h. The MT subfractions, MT-1 and MT-2, were purified by gel filtration and anion-exchange chromatography. (*a*) A 10 µl solution of Zn-MT-1 (2.5 µg of Zn) and Zn-MT-2 (3.0 µg of Zn) were applied to Asahipak ES-502N column (7.5 mm × 100 mm). (*b*) A 10 µl solution of the crude MT fraction obtained from Zn-treated mice by gel filtration (3.75 µg of Zn) was applied to an anion-exchange column. The ion-exchange column was eluted with a linear concentration gradient of phosphate buffer (pH 7.5) by mixing starting buffer (4 mM) and limiting buffer (50 mM) up to a concentration of 52 mM over 30 min at a flow rate of 0.5 min/ml. Iso-MTs were identified by measuring the Zn concentration of each peak by its A_{220} (open bars).

Amino acid composition of MT subfractions

The amino acid compositions of MT-1 and MT-2 from the cells and host liver were very similar to that of the MT subfractions from Zn-treated control liver (results not shown). Each contained a high proportion of cysteine, namely 19–20 residues/molecule. There also existed a substantial amount of lysine (7–8 residues/molecule), and tyrosine, tryptophan, phenylalanine and histidine were absent from these subfractions.

Analysis of MT isoforms in tumour cells by h.p.l.c.

To analyse MT isoforms the Zn-MT-1 and -MT-2 from Zn-treated ddY-mouse livers (10 µg of Zn^{2+}/g body wt. injected i.p. after 24 h) isolated by gel filtration and anion-exchange chromatography were chromatographed over an anion-exchange h.p.l.c. column. The MT-1 and MT-2 subfractions showed a single fraction (MT-1-1) and two fractions (MT-2-1 and MT-2-2) respectively; MT-1-1, MT-2-1 and MT-2-2 were eluted at retention times of 15 min (4 mM-potassium phosphate buffer), 28 min (7–8 mM-potassium phosphate buffer) and 31–32 min (11–12 mM-potassium phosphate buffer) respectively (Fig. 4*a*). The total recoveries of Zn for MT-1 and MT-2 were 90 and 80 % respectively. The total-A_{220}/total-Zn-content ratios for the individual fractions, MT-1-1, MT-2-1 and MT-2-2, had the same value. These results indicate that the MTs induced in mouse liver contained three isoforms: MT-1-1, MT-2-1 and MT-2-2.

The crude MT fraction obtained by gel filtration in mouse liver treated with 10 µg of Zn^{2+}/g body weight after 15 h was analysed for MT isoforms with an anion-exchange h.p.l.c. column (Fig. 4*b*). The Zn-MT fraction in mouse liver was separated into three Zn-containing fractions; the retention time for each peak at 15 min, 28 min and 31 min respectively corresponded to those of the purified mouse MT subfractions Zn-MT-1-1, -MT-2-1 and -MT-2-2. The amounts of Zn bound to MT-1-1, MT-2-1 and MT-2-2 fractions in the Zn-treated liver were 34, 11 and 30 % of that in the crude MT fraction. The total-A_{220}/total-Zn-content ratios for the individual subfractions approximated to those of the isolated mouse iso-MTs (results not shown).

The crude Zn-MT fraction (obtained by gel filtration) from the tumour cells also showed three Zn-containing subfractions on an anion-exchange h.p.l.c. column. The elution profiles showed the same retention times as iso-MTs obtained from the crude MT fraction in Zn-treated liver (Fig. 5). The recoveries of Zn for MT-1-1, MT-2-1 and MT-2-2 were 22, 27 and 25 % respectively. The MT-2-1/MT-2-2 ratio in the Zn-treated liver or tumour cells was 0.37 or 1.08; in the tumour cells the amounts of Zn bound to MT-2-1 were three times that in the Zn-treated normal mice. These results suggest that MT in tumour cells has the same three isoforms as that of liver; moreover, the MT-2-1 isoform in tumour cells is induced in markedly higher amounts than that in the liver.

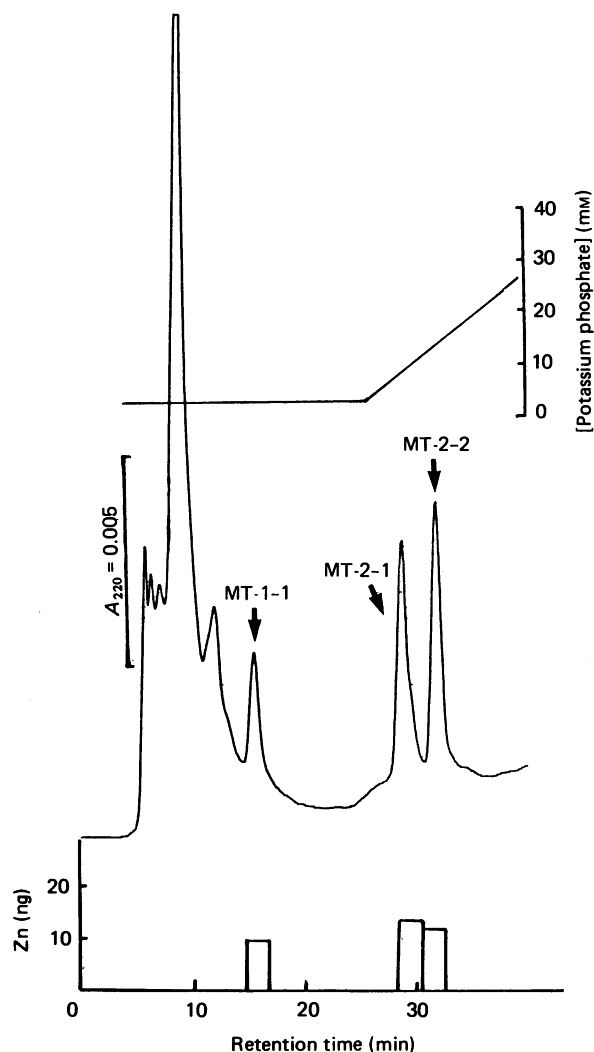

Fig. 5. H.p.l.c. elution profiles of MT fractions from ascites-tumour cells

A 10 μl solution of the MT fraction of ascites sarcoma S180A cells obtained from Zn-treated tumour-bearing mice by gel filtration (65 ng of Zn) was applied to an Asahipak ES-502N column. The chromatographic conditions were described in the Materials and methods section. Iso-MTs were identified by measuring the Zn concentration of each peak by its A_{220} (open bars).

DISCUSSION

Before metal administration, the endogenous level of Zn in cells and liver from tumour-bearing mice were 2 and 3 times that in the normal mice liver, and approx. 30–40 % of the Zn in cytosols was associated with the MT fraction (Fig. 1*a*). These results suggest that Zn redistribution occurs from plasma into liver and cells in response to tumour infection and the increased Zn bound to newly synthesized MT (Kraker & Petering, 1983; Ujjani *et al.*, 1986). After metal administration, the levels of Zn and Zn-MT in the cells increased more than those in the host liver and normal liver, in spite of the fact that liver is a target organ for Zn. These results suggest that Zn is preferentially incorporated into growing cells (tumour cells) compared with post-mitotic cells (adult hepatic cells), and the metal increases correspond to MT synthesis. In newborn-mouse liver and adult regenerating liver there also is an increase in liver Zn and a concomitant synthesis of MT (Ohtake *et al.*, 1978; Bell, 1980; Bell & Waalkes, 1982). Thus MT may be acting as a storage site for Zn in cell-growth processes, and MT induction may relate to cell growth. Also, the Cu and Cu-MT levels in the tumour cells from the Cu-treated mice bearing sarcoma S180A cells were higher than in the host liver and control liver, suggesting that there may be a high level of Cu and MT induction in malignant cells, but the mechanism is not clear.

During the experimental period the Zn level in the host liver was lower than that in the normal liver; the Zn level declined rapidly during 3 h after administration. On the other hand, the Cu level in the host liver increased rapidly to reach a maximum at 9 h after administration. We have demonstrated that the level of Zn the human liver cells required for initiating production of MT is three times that of Cu (Kobayashi *et al.*, 1985*b*). Thus the difference between Zn and Cu accumulation patterns in the host liver may be due to differences in the form of metal (free metal or bound metal to MT). In the host liver most Zn presumably exists as the free ion or is bound to a peptide molecule smaller than MT, whereas the Cu binds to MT. Free Zn ion in the host liver might move from liver into the cells. When ^{64}Cu was administered i.p. to mice bearing Krebs ascites cells, a large proportion of the radioisotope was recovered in the ascites cells themselves, and the amount of radioisotope in the host liver was lower than in the control liver (Apelgot *et al.*, 1981). In the present experiment, if a lower Cu concentration than that for initiating production of MT is administered to mice bearing ascites sarcoma S180A cells, Cu in the host liver might move to the tumour cells.

MT from the tumour cells was separated into two components, MT-1 and MT-2, by ion-exchange chromatography as well as in other mammals. The amount of Zn- or Cu-MT-2 in the tumour cells was higher than that of -MT-1, but in the host liver the amounts of Zn- or Cu-MT-1 and -2 were equal. Similar findings were noted for regenerating liver, in which the amount of MT-2 is twice that of MT-1 (Cain & Griffiths, 1984). Moreover, a higher content of MT-2 compared with MT-1 was also observed in hepatoma cells from Cd-treated tumour-bearing mice (Hidalgo *et al.*, 1978) and in the liver of mice bearing Ehrlich ascites tumour (Ujjani *et al.*, 1986). It is possible, therefore, that MT-2 has functions that are related to cancerous stress and cell growth.

MT from Zn-treated liver contained three isoforms (MT-1-1, MT-2-1 and MT-2-2). Similar findings were made by Suzuki *et al.* (1984, 1985), who analysed a rat liver Cd-MT by h.p.l.c.–atomic-absorption spectrophotometry with on-line switching from gel filtration to ion-exchange columns. In the present experiments, the h.p.l.c. profile of a crude MT fraction obtained by gel filtration from the tumour cells also showed three Zn-associated peaks that corresponded to MT-1-1, MT-2-1 and MT-2-2 from the isolated MT-1 and MT-2 subfractions in the Zn-treated liver, but the relative amounts of the iso-MTs in the cells are clearly different from that in liver; the amount of MT-2-1 in the tumour cells increased three times compared with that in the Zn-treated liver. Although we are unable to explain the difference in iso-MTs between the tumour cells and liver,

it is interesting that the MT-2-1 isoform, which is a minor component in post-mitotic cells, increases in growing cells. The MT-2-1 isoform might be related to cell growth or cancerous stress.

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