Hepatic zinc in metallothionein-null mice following zinc challenge: *in vivo* and *in vitro* studies

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Hepatic zinc uptake and accumulation were compared in freshly isolated and cultured hepatocytes prepared from control (MT+/+) and metallothionein (MT)-null (MT-/-) mice. In freshly isolated hepatocytes, rapid (10–15 min) exchange of ⁶⁵Zn was proportional to the Zn concentration in the medium and occurred to the same extent in hepatocytes from MT+/+ and MT-/- mice. In 24 h culture experiments with MT+/+ and MT-/- hepatocytes it was shown that approx. 40% of newly acquired cell-associated Zn was attached to the cell surface and not internalized. In MT+/+ and MT-/- hepatocyte cultures, internalized Zn (*intZn*) increased in proportion to extracellular Zn. Zn accumulation in MT-/- hepatocytes was only 60% that of MT+/+ cells. Addition of 1 µM dexamethasone (Dex) and recombinant mouse interleukin-6 (IL-6; 100 units/ml) increased MT accumulation by 8.6-fold in MT+/+ hepatocytes (at 50 µM Zn) and there was an associated parallel increase in

intZn. Dex and IL-6 did not increase *intZn* in the MT-/- hepatocytes. At 16 h after an intraperitoneal injection of 5 µg/g Zn, plasma and urine Zn concentrations were 69 ± 10 µM and 86 ± 25 µM respectively in MT-/- mice (n = 10) and 27 ± 1 µM and 23 ± 4 µM respectively in MT+/+ controls (n = 9) (P < 0.001, plasma; P < 0.05, urine). Hepatic cytosolic Zn concentrations doubled in MT+/+ mice and increased by a significant 15% in MT-/- mice. There was no increase in hepatic Zn (dry wt.) concentrations or in total hepatic Zn, demonstrating that the increase in cytosolic Zn in MT-/- mice was due to hepatic water loss rather than net Zn uptake. It appears that even at extreme plasma concentrations of Zn, little if any accumulates within the liver when there is no MT available for its sequestration. That this is not fully demonstrated *in vitro* is probably due to nature of cell culture, where organ architecture is lost and the external protein binding milieu is less complex.

INTRODUCTION

Zn is an essential trace element with a diverse range of functions in the cell. It confers structure and/or catalytic function to some 300 enzymes involved in metabolism and cell growth [1,2]. Clinical signs of Zn deficiency are manifested only when supply of Zn is limited and when growth or repair is rapid or has accelerated following tissue damage [3,4]. In health, whole-body homeostasis of Zn is efficiently controlled by a balance between gastrointestinal absorption and excretion [5]. In infection and inflammation, however, the liver is regarded as the main site of Zn regulation due mainly to the induction of metallothionein (MT), an intracellular Zn-binding protein [6–8]. After two decades of research, the essential role of MT in Zn homeostasis remains unclear [7–9].

Hepatic MT may provide a storage reserve for Zn in inflammation; potentially important when dietary Zn is limited. In addition, MT may be needed to maintain surplus hepatic Zn, which can be directed to metalloenzymes required to sustain changes in protein synthesis (acute phase response) and energy metabolism [9]. Alternatively, MT may provide an intracellular buffer, protecting the cell from excessive ionized Zn while at the same time removing Zn from the circulation [10]. A low plasma Zn concentration may be important in macrophage activation [11] whilst lowering the free Zn available to an invading pathogen. After an inflammatory event has subsided, Zn-MT is degraded in the liver to release Zn back into the circulation, presumably to support cell differentiation and proliferation in tissues undergoing repair.

A fundamental question remains: how does MT influence hepatic Zn uptake and accumulation? In the unstimulated liver

the level of MT is low, accounting for less than 1% of hepatic Zn in rodents, and thus is unlikely to play a significant part in Zn transport or storage [12]. However, with the immediate rise (within hours) of MT following induction by exposure to inflammatory stimuli [13–15], MT exerts a significant influence on hepatic Zn uptake and accumulation, mainly due to its obligatory requirement for Zn incorporation and its binding capacity of up to 7 g-atoms per mol [16,17]. It is also possible that inflammatory mediators directly stimulate a hepatic Zn transporter. In this latter case, MT may act solely as a Zn-binding protein without a transport role.

The availability of an MT-null (MT-/-) mouse model [18] enables mechanisms of Zn uptake and accumulation to be studied in the presence of inflammatory mediators and independent of hepatic MT synthesis. Here we have investigated the exchange of Zn between the medium and freshly isolated hepatocytes, and the accumulation of Zn in the presence of combinations of Zn, dexamethasone (Dex) and interleukin-6 (IL-6) in cultured hepatocytes prepared from MT+/+ and MT-/- mice. In addition, the influence of parenteral Zn loading on hepatic Zn accumulation was investigated in both MT+/+ and MT-/- mice.

MATERIALS AND METHODS

Materials

Materials used and their suppliers were: William's medium E (Gibco); fetal calf serum (Cytosystems Pty. Ltd., Castle Hill, N.S.W., Australia); sodium EGTA and dexamethasone (Sigma Chemical Co.); mouse recombinant IL-6, fatty-acid-free BSA

Abbreviations used: MT, metallothionein; Dex, dexamethasone; IL-6, interleukin-6; *tcZn*, total cell-associated Zn; *intZn*, internalized Zn; Zn50 (etc.), medium containing 50 µM Zn (etc.).

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and collagenase H (Boehringer Mannheim, Castle Hill, N.S.W., Australia); ^{65}Zn as zinc chloride in 500 mM HCl (specific radioactivity 581 Bq/g of Zn; NEM Research Products, Biotechnology Division, Du Pont, Wilmington, DE, U.S.A.); [^3H]inulin (Amersham Australia, North Ryde, N.S.W., Australia).

Animals

MT-I- and MT-II-deficient mice (MT $^{-/-}$) were produced and supplied by A. Michalska and K. H. A. Choo (Murdoch Institute, Royal Children's Hospital, Melbourne, Vic., Australia) [18]. The mice were of a mixed genetic background of OLA129 and C57BL6 strains. Control mice (MT $^{+/+}$) were of the C57BL6 strain, supplied by the Animal Resource Centre, Canning Vale, Western Australia. All mice were housed on sawdust in stainless steel or plastic cages in an animal house at 22 °C with a 14 h light/10 h dark cycle and were fed a commercial pelleted diet [19]. All experimentation was approved by the IMVS Animal Ethics Committee.

Mouse hepatocyte preparations

Hepatocytes were isolated after liver perfusion with collagenase by a modification of the method outlined by Maslansky and Williams [20]. Mice weighing 25–30 g were anaesthetized with sodium pentobarbitone (Boehringer Ingelheim, Artarmon, Australia; 200 mg/kg body wt.; intraperitoneal injection of a solution in water). The abdominal cavity was opened, and 400 units of sodium heparin (Delta West, Bentley, Western Australia; porcine mucous) was injected via the subhepatic inferior vena cava which was then ligated. The portal vein was cannulated with a 22-gauge $\times$ 25 mm Teflon catheter (Jelco; Johnson and Johnson Medical K.K., Tokyo, Japan) and the thoracic inferior vena cava was severed to allow solution to escape. The liver was washed with 50 ml of a 37 °C perfusion buffer (NaCl, 130 mM; KCl, 4.7 mM; MgSO_4 , 1.2 mM; KH_2PO_4 , 1.2 mM; Hepes, 10 mM; NaHCO_3 , 24 mM; pH 7.4; gassed with an atmosphere of 5% CO_2 in oxygen) at a perfusion rate of 8–10 ml/min. Sodium EGTA was added to the perfusion buffer to a final concentration of 0.5 mM and infusion was continued for 7 min. The buffer solution was then changed to original perfusion buffer to which was added CaCl_2 (4 mM) and collagenase H (50 mg/100 ml). This final perfusion was carried out for 20 min at the same rate as above, and recirculation of the buffer was included to reduce the amount of collagenase required. The isolation and washing of mouse hepatocytes was the same as that previously outlined for rat hepatocytes [13,21]. Viability of hepatocytes as determined by Trypan Blue exclusion was in the range 80–90%.

Zinc uptake in isolated hepatocytes

Hepatocytes were resuspended in William's medium E with Hepes (10 mM), bicarbonate (24 mM), pH 7.4, and fatty-acid-free BSA (0.5%, w/v) at a cell density of approx. 2×10^6 cells/ml. Where the rate of Zn uptake was investigated over 30 min, 5 ml aliquots of hepatocyte suspension were gassed (IL Model 237 tonometer; Instrumentation Laboratory) at 37 °C in an atmosphere of 5% CO_2 in O_2 . A 5 min period was allowed for the cells to achieve equilibrium after which ^{65}Zn (2 μCi) was added to the incubations. Hepatocyte ^{65}Zn incorporation was determined at 0, 5, 10, 20, 30 and 60 min after addition of radiochemical using a modification of the one-step micro-centrifugation separation technique as described by Barritt and co-workers [22]. A sample of incubation mixture was added to a

1.5 ml Eppendorf tube to produce a three-phase system comprising the sample (0.3 ml) in the top layer, a middle layer of silicone oil (0.4 ml) (silicone oil is a 1:7 mixture of 200:550 fluid; Dow Corning Australia, Blacktown, N.S.W., Australia) and a bottom layer of perchloric acid (0.1 ml; 1.5 M). The tubes were immediately centrifuged at 10000 g for 1 min after which the side of the uncapped tube was punctured with a red-hot needle and a sample (75 μl) of the acid layer taken by microsyringe; radioactivity was counted using an LKB 1260 Multigamma counter. Total counts were determined by counting an aliquot (0.3 ml) of the incubation mixture. The volume of extracellular fluid associated with the cells after centrifugation through silicone oil was estimated using [^3H]inulin (0.5 $\mu\text{Ci}/\text{ml}$) in a separate incubation. This value was routinely found to be $0.24 \pm 0.09\%$ of the 0.3 ml sample volume. The basal Zn concentration in the control medium with fatty-acid-free BSA (0.5%, w/v) and that following the addition of ^{65}Zn was $< 1 \mu\text{M}$ Zn by atomic absorption spectroscopy.

Where the effect of zinc concentration on zinc uptake was investigated, incubations were carried out for 30 min in 20 ml glass scintillation vials previously sialanized with dimethyldichlorosilane. The cell suspensions were purged with an atmosphere of 5% CO_2 in O_2 and the vials capped and then placed in a reciprocating (80 oscillations/min) water bath at 37 °C.

Cell culture

Liver cells were washed ($\times 3$) in William's medium E containing Hepes buffer (20 mM), bicarbonate (10 mM), pH 7.4, penicillin (240 mg/l) and gentamycin (160 mg/l) and resuspended in the same medium with the addition of 10% (v/v) foetal calf serum and insulin (25 units/l). Approx. 1×10^6 hepatocytes in 2 ml of medium were seeded on to collagen-coated flat-bottom culture wells (35 mm diameter; Corning) and allowed to adhere to the surface at 37 °C in an atmosphere of 5% CO_2 in air. After 4 h the medium was replaced with 2 ml of fresh medium without added insulin and the cells were cultured for 20 h. The culture medium was then replaced with 2 ml of William's medium E containing Hepes (20 mM), bicarbonate (10 mM), pH 7.4, penicillin (240 mg/l), gentamycin (160 mg/l) and fatty-acid-free BSA (0.5%, w/v). Zn, Dex and IL-6 were then added and the cells were incubated for a further 24 h. Zn added to the culture medium was bound to fatty-acid-free BSA as previously described [13].

Measurements of cellular Zn

In studies measuring cellular Zn concentrations, the medium was discarded and the following procedures were used to wash the hepatocytes. (1) For total cell-associated Zn (*tcZn*) estimations, the hepatocytes were washed three times in 2 ml of PBS (NaCl, 137 mM; KCl, 2.7 mM; KH_2PO_4 , 1.5 mM; Na_2HPO_4 , 8.1 mM; pH 7.4) and digested for 30 min at room temperature in 0.5 ml of 16 M nitric acid (Aristar) before measuring the Zn concentration. Cell-associated Zn is that which is non-specifically attached to the cell membrane as well as Zn which is internalized. (2) For internalized Zn (*intZn*) estimations, the cells were washed once with 2 ml of PBS, and then twice in the same volume of 10 mM EDTA buffered in PBS before being digested in acid as above. The EDTA wash was described by Failla and Cousins [23] and was used to remove residual Zn from the medium and that loosely bound to the cell surface. A direct measurement of membrane-bound Zn was also obtained. Cells were washed in PBS and then scraped from the plastic surface of the culture well with a rubber policeman. The cells were lysed by rapid freezing and thawing ($\times 3$) and centrifuged at 10000 g for 10 min. The

membrane-rich pellets were then digested in acid as described above. Zn was measured in the acid digests to determine membrane-bound Zn, and in the cell lysate supernatants to determine soluble cytoplasmic Zn.

MT was analysed using a modified Cd/haemoglobin affinity assay [17] in supernatants from cell lysates that had been boiled for 2 min to coagulate the heat-labile protein, which was then pelleted by centrifugation at 10000 *g* for 1 min. The MT concentration in hepatocyte cultures is reported as nmol of Cd bound/10⁷ cells.

Cytosolic hepatic Zn uptake following intraperitoneal Zn administration

MT+/+ and MT−/− mice weighing 28.2±0.9 g (*n* = 9 per group) were given intraperitoneal injections of 5 µg/g Zn as ZnSO₄. After 16 h, mice were anaesthetized with halothane and blood was withdrawn by cardiac puncture, urine collected directly from the bladder and the mice killed by cervical luxation. Control mice were divided into two groups, uninjected and injected with 0.15 ml of saline 16 h before being killed. There were no differences in the measured analytes between these two control groups and the data have consequently been pooled.

The livers were excised, weighed and homogenized in 4 vol. of 0.01 M Tris/HCl buffer, pH 8.2, using a motor-driven Potter/Elvehjem homogenizer and then centrifuged (×2) at 30000 *g* for 30 min (S30). Zn was measured in the S30 supernatants (cytosolic), bladder urine and plasma samples. One bladder urine was unobtainable (MT−/−; +Zn). Portions (1 ml) of the homogenates were dried at 70 °C for 48 h, digested in 2 ml of nitric acid (Aristar), evaporated at 140 °C and redissolved in 3 ml of de-ionized water prior to Zn analysis. MT was assayed in the hepatic cytosol only. The molecular-mass profiles of several supernatants were determined by Sephadex G-75 gel filtration. A 0.2 ml sample of supernatant was eluted with 40 mM phosphate buffer, pH 7.4, from a 150 mm-length × 10 mm-diameter column, and 30 × 0.5 ml fractions were collected for Zn analysis. A Perkin-Elmer model 3030 atomic absorption spectrophotometer was used for Zn analyses.

Statistics

Results are reported as the means±S.E.M. and significant differences were assessed using Student's *t* test for independent means. Relationships between parameters were assessed using Minitab software of Cricket Graph.

RESULTS

MT accumulation in hepatocytes

The basal MT concentration in control cultures from MT+/+ mice was 4.88±0.23 nmol of Cd bound/10⁷ cells (Table 1). MT accumulation increased in proportion [$y = 3.632 + 0.272x$; $r = 0.980$; y = MT (nmol of Cd bound/10⁷ cells); x = Zn (µM)] with increasing external Zn concentration. Zn and Dex combined gave a 2.5-fold greater response than Zn alone, and MT was 22% greater with the combination of Zn+Dex+IL-6 than with Zn+Dex alone. The response with Zn+Dex+IL-6 was 8.6-fold higher than in the control cultures. In MT−/− control cultures, MT measurements were at background Cd binding (0.24±0.02 nmol of Cd bound/10⁷ cells; *n* = 3), which was less than 5% of that obtained in MT+/+ controls. As expected, the MT response was not significantly altered by the presence of combinations of Zn, Dex or IL-6 in MT−/− hepatocytes.

Table 1 Effects of Zn, Zn + Dex and Zn + Dex + IL-6 on MT accumulation in 24 h cultures of hepatocytes from MT+/+ and MT−/− mice

The concentration in cultures of the various inducers was 10, 50 or 100 µM Zn (Zn10, Zn50 and Zn100), 1 µM Dex and 10⁵ units/l IL-6 (1 unit of IL-6 is the amount required to mediate half-maximal proliferation with 7TD1 cells). Further details are given in the Materials and methods section. The results are the means±S.E.M. of three independent culture experiments performed in triplicate.

Conditions	MT concn.	
	MT +/+	MT −/−
Control	4.88±0.23	0.24±0.02
Zn10	7.00±0.11	0.32±0.01
Zn50	13.58±0.53	0.34±0.08
Zn100	32.61±0.92	0.26±0.15
Zn50 + Dex	34.30±0.35	0.39±0.12
Zn50 + Dex + IL-6	41.80±0.74	0.43±0.08

Zn uptake by freshly isolated hepatocytes

The rate of Zn exchange was investigated in freshly prepared hepatocytes from MT+/+ and MT−/− mice. Cells were incubated for up to 60 min in medium containing ⁶⁵Zn. A rapid one-step microcentrifugation technique was used to separate ⁶⁵Zn incorporated into the cells from that remaining in the incubation medium. In MT+/+ hepatocytes, Zn exchange with the medium was 90% complete after 10 min, and reached a maximum uptake of 10.5% of the total counts after 30 min of incubation at 37 °C. The rate of cellular Zn association was similar in hepatocytes prepared from MT−/− mice, and regression equations for Zn incorporation plotted against time are shown in Table 2.

The effect of Zn concentration on Zn exchange between the medium and hepatocytes was investigated in incubations with freshly prepared cells at 37 °C. Radiolabelled Zn concentrations at equilibrium (30 min) increased in direct proportion to Zn concentration, to the same extent in hepatocytes from both MT+/+ and MT−/− mice (see Table 2).

Zn uptake by cultured hepatocytes

In 24 h culture experiments with hepatocytes from MT+/+ and MT−/− mice, the effects of combinations of Zn, Dex and IL-6 on the change in intracellular Zn concentration were investigated. To discriminate between internalized Zn (*intZn*) and total cell-associated Zn (*tcZn*) (loosely bound membrane Zn plus internalized Zn), cultured hepatocytes were washed with buffered EDTA, to remove residual medium and cell-surface-bound Zn, or with PBS respectively. Hepatocyte Zn measured after either wash was compared with the Zn-MT concentration. The Cd-haem assay used has a 1:1 stoichiometry between Zn incorporated into MT and the displacing Cd. Copper is the only other cation in the culture medium available for MT incorporation. Since intracellular Cu concentrations were a constant 10.7±1.1 nmol/10⁷ cells (*n* = 9) in all groups, and Cd does not displace Cu from MT, the reported changes in hepatic MT concentrations refer specifically to Zn-MT.

In MT+/+ hepatocytes, *tcZn* increased in proportion to the Zn concentration in the culture medium (Figure 1). Zn at 100 µM gave a 4-fold increase above the controls (15.2±1.4 nmol/10⁷ cells). The addition of Dex and Dex + IL-6 to the 50 µM Zn medium ('Zn50') resulted in increases in the *tcZn* of 39% and 48% respectively above Zn50 alone. All of this

Table 2 Relationship of cellular Zn pools to extracellular Zn in isolated and cultured hepatocytes from MT +/+ and MT -/- mice

The experimental details are given in the Materials and methods section. The units are expressed as nmol/10⁷ cells for all cellular Zn pools, μ M for extracellular Zn and min for incubation time. nm = not measured. The regression equations chosen gave the highest regression coefficients. Values in parentheses are the numbers of independent observations.

Comparison	MT +/+		MT -/-	
	Regression equation	<i>r</i>	Regression equation	<i>r</i>
⁶⁵ Zn uptake versus time	$y = 0.41 + 0.40 \log x$	0.965 (3)	$y = 0.45 + 0.43 \log x$	0.943 (3)
⁶⁵ Zn uptake versus Zn	$y = 1.41 + 0.46x$	0.979 (3)	$y = 0.80 + 0.48x$	0.987 (3)
Cell-associated Zn versus Zn	$y = 16.90 + 0.58x$	0.975 (5)	$y = 15.86 + 0.57x$	0.966 (5)
Internalized Zn versus Zn	$y = 13.51 + 0.25x$	0.968 (3)	$y = 12.51 + 0.15x$	0.895 (3)
Membrane-bound Zn versus Zn	nm	nm	$y = 7.62 \pm 0.34x$	0.902 (3)
Soluble cytosolic Zn versus Zn	nm	nm	$y = 6.41 + 0.15x$	0.900 (3)

increase could be accounted for by the change in Zn-MT, and the trend in *tcZn* was the same as that for Zn-MT in the presence of combinations of Zn, Dex and IL-6. A linear relationship between *intZn* and Zn concentration in the medium (Table 2) was closely reflected in increased Zn-MT, indicating that the newly translocated Zn was associated with Zn in the MT pool. A strong linear relationship was obtained between *intZn* and MT [$y = 10.4 + 0.91x$; $r = 0.991$; $x = \text{MT (nmol of Cd bound/10}^7 \text{ cells)}$; $y = \text{intZn (nmol/10}^7 \text{ cells)}$]. Combinations of Zn, Dex and IL-6 resulted in increased *intZn* concentrations, attributable to increased Zn-MT. Washing with buffered EDTA did not affect the basal *intZn* concentration of the controls (13.4 ± 0.3 nmol/10⁷ cells), which was the same as that in hepatocytes washed with PBS (15.2 ± 1.4 nmol/10⁷ cells). These levels were significantly higher than the corresponding Zn-MT concentration (5.0 ± 0.2 nmol/10⁷ cells), indicating that Zn-MT accounts for a minor portion of the total *intZn* in the basal state. In MT +/+ hepatocytes, incubation with 100 μ M Zn, Zn50+Dex or Zn50+Dex+IL-6 trebled the *intZn* concentrations.

Comparable changes in *tcZn* were obtained in hepatocytes from MT -/- and MT +/+ mice cultured in media with increasing Zn concentrations, although *tcZn* was 24 % and 27 % lower with Zn50+Dex and Zn50+Dex+IL-6 in MT -/- hepatocytes due to the absence of Zn-MT. The MT assay background in MT -/- hepatocytes was 0.25 ± 0.02 nmol of Cd bound/10⁷ cells ($n = 5$), which was 5 % of that in the MT +/+ controls. The 'MT' concentrations remained at background levels (i.e. no MT) in the presence of combinations of Zn, Dex and IL-6. In MT -/- hepatocytes, the increases in *intZn* were substantially lower than those in *tcZn* in all treatment groups. *intZn* in the MT -/- controls was 12.7 ± 2.1 nmol/10⁷ cells, the same as that in MT +/+ controls. In the absence of MT, the *intZn* concentration increased in proportion to the Zn concentration in the medium, reaching twice the control *intZn* concentration at 100 μ M Zn. In sharp contrast to the findings in MT +/+ hepatocytes, there was no increase in *intZn* when Dex or Dex+IL-6 were added to the Zn50 medium. Averaged across all treatment groups, the increase in *intZn* in MT -/- cells was 29.5 ± 1.5 % ($n = 5$) that of *tcZn*.

A direct measurement of membrane-bound Zn was obtained for MT -/- hepatocytes washed in PBS. In this experiment, the cells were lysed by freeze-thawing, centrifuged and the membrane-rich pellet was digested in acid prior to Zn analysis. In addition, the cytoplasmic soluble Zn was determined in the supernatants of the cell lysates. Increases in membrane-bound Zn were one-third of Zn concentration increases in the medium (see equation in Table 2). The amount bound to the membrane

Figure 1 Effect of Zn, Zn+Dex and Zn+Dex+IL-6 on cell-associated, internalized and MT-bound Zn in 24 h cultures of hepatocytes from (a) MT +/+ and (b) MT -/- mice

The concentration in cultures of the various inducers was 10, 50 or 100 μ M Zn, 1 μ M Dex and 10⁵ units/l IL-6. Cell-associated Zn was determined in cells washed with PBS; internalized Zn was determined in cells washed with EDTA; MT-bound Zn was calculated from the MT concentration using 1:1 stoichiometry between Zn incorporated into MT and the displaced Cd in the Cd-haem assay. Further details are given in the Materials and methods section. The results are the means $\pm$ S.E.M. of 3–5 independent culture experiments performed in triplicate.

Table 3 Effect of intraperitoneal Zn on plasma, urine and liver Zn and on hepatic MT concentrations 16 h after Zn administration to MT+/+ and MT−/− mice

The experimental details are given in the Materials and methods section. Significant differences from the control group (−Zn): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. nd = not detected. Values in parentheses are the numbers of animals in each group. With the exception of the body weight change, MT+/+ controls (−Zn) were not significantly different from MT−/− controls.

Parameter	MT+/+		MT−/−	
	−Zn	+Zn	−Zn	+Zn
Plasma Zn (μM)	13.8 $\pm$ 0.4 (9)	26.8 $\pm$ 0.6 (9)***	12.9 $\pm$ 0.3 (12)	68.5 $\pm$ 9.8 (9)***
Urine Zn (μM)	7.9 $\pm$ 1.9 (9)	23.4 $\pm$ 3.8 (9)**	10.8 $\pm$ 2.2 (12)	85.5 $\pm$ 24.7 (8)*
Liver MT (nmol of Cd bound/g wet wt.)	6.6 $\pm$ 0.9 (9)	171.4 $\pm$ 12.8 (9)***	nd	nd
Liver Zn (nmol/g wet wt.)	400 $\pm$ 8 (9)	718 $\pm$ 26 (9)***	407 $\pm$ 7 (12)	453 $\pm$ 6 (9)***
Liver cytosolic Zn (nmol/g wet wt.)	317 $\pm$ 4 (9)	604 $\pm$ 27 (9)***	313 $\pm$ 8 (12)	360 $\pm$ 7 (9)*
Liver Zn (nmol/g dry wt.)	1536 $\pm$ 86 (9)	2453 $\pm$ 151 (9)***	1391 $\pm$ 72 (12)	1422 $\pm$ 44 (9)
Total liver Zn (nmol)	548 $\pm$ 33 (9)	883 $\pm$ 38 (9)***	540 $\pm$ 39 (12)	557 $\pm$ 56 (9)
Change in body wt. (g)	2.13 $\pm$ 0.18 (9)	0.78 $\pm$ 0.22 (9)***	0.25 $\pm$ 0.31 (12)	−0.98 $\pm$ 0.34 (9)***

was the same with Zn50, Zn50+Dex and Zn50+Dex+IL6. Increases in soluble cytosolic Zn were less than one-half of increases in membrane-bound Zn (Table 2) and, like membrane-bound Zn, the Zn concentration in the soluble cytosolic fraction was the same for Zn50, Zn50+Dex and Zn50+Dex+IL6. There was no change in the proportion of membrane-bound or soluble cytoplasmic Zn with increasing Zn concentration or with combinations of Zn, Dex and IL-6 in the culture medium. The average proportion of *tcZn* attached to the membrane was $56.5 \pm 3.1\%$ ($n = 6$), while that in the soluble cytoplasmic fraction was $37.8 \pm 6.7\%$ ($n = 6$).

The concentration of fatty-acid-free albumin in the medium markedly affected the binding of Zn to the hepatocyte membrane. In MT−/− hepatocytes cultured in the presence of $50 \mu\text{M}$ Zn, *tcZn* decreased in a logarithmic relationship ($y = 32.8 - 10.9 \log x$; $r = 0.97$; $n = 3$) with fatty-acid-free BSA in the medium, at albumin concentrations ranging from 0.5 to 40 g/l.

Hepatic Zn uptake *in vivo*

In order to examine the influence of elevated extracellular fluid Zn concentration of hepatic Zn *in vivo*, both MT−/− and MT+/+ mice were injected intraperitoneally with an amount of ZnSO_4 that doubled the hepatic zinc concentration in MT+/+ mice within 16 h (Table 3). At this time plasma Zn was $68.5 \pm 9.8 \mu\text{M}$ in MT−/− mice, 2.6 times its counterpart in MT+/+ mice and 5.3 times the pre-injection level. The corresponding values for urinary Zn were $85.5 \pm 24.7 \mu\text{M}$ (3.7-fold and 7.9-fold increases respectively), demonstrating a more pronounced increase in Zn excretion in the MT−/− mice. Substantial increases were observed in all indices of hepatic Zn in MT+/+ mice, with cytosolic Zn almost doubling. In MT−/− mice the changes in hepatic Zn were much more modest, with significant 15% and 10% increases ($P < 0.001$) in cytosolic and wet liver Zn. Interestingly, the changes in dry Zn (+2.2%) and total Zn (+3.1%) were not significant, and thus, taking all four hepatic Zn indices together, it is likely that the rise in cytosolic Zn (and wet hepatic Zn) in MT−/− mice is caused by a decrease in intracellular hydration rather than an influx of Zn from the extracellular compartment.

Hepatic cytosol preparations were analysed by Sephadex G-75 gel filtration, and Zn was measured in the eluent fractions (Figure 2). The protein profiles from homogenates of livers removed 16 h after intraperitoneal Zn injection in MT+/+ mice were bimodal, with Zn binding to high-molecular-mass proteins

Figure 2 Typical Sephadex G-75 gel-filtration elution profile of the cytosolic fractions from liver homogenates from MT+/+ and MT−/− mice, 16 h after intraperitoneal injection with ZnSO_4

MT+/+ and MT−/− mice were injected intraperitoneally with ZnSO_4 (5 mg/g) and hepatic cytosolic fractions were prepared 16 h post-injection. An uninjected MT−/− mouse was used as the zero time control. The molecular-mass profile of the hepatic cytosolic fraction was determined by Sephadex G-75 gel filtration and analysis of the Zn content in the collected fractions. Further details are given in the Materials and methods section.

which eluted between fractions 6 and 9 and to low-molecular-mass proteins eluting between fractions 10 and 19. The low-molecular-mass peak centred at fraction 14 is characteristic of MT. The Zn elution profiles for MT−/− liver homogenates taken 0, 8 and 16 h post-injection do not show any trace of an MT peak, with Zn entirely bound to high-molecular-mass proteins which were quantitatively similar to the Zn-binding proteins in the MT+/+ liver cytosols.

DISCUSSION

Combinations of Zn, Dex and IL-6 have been shown to induce MT synthesis in cultured rat hepatocytes [15,19]. Here, a similar effect has been noted in mouse hepatocytes, with the greatest

response to Zn + Dex + IL-6 being an 8.6-fold increase over that of the control cultures. This was less than that previously obtained in rat hepatocytes (22.4-fold stimulation) due, in part, to higher basal levels of MT in mouse hepatocytes (4.88 ± 0.23 nmol of Cd bound/ 10^7 cells) than rat hepatocytes (3.0 ± 0.3 nmol of Cd bound/ 10^7 cells). Recombinant IL-6 (*Escherichia coli*) of mouse or human origin caused the same accumulation of MT in mouse hepatocytes when used in combination with Zn (50 μ M) and Dex (1 μ M).

We have previously observed that Zn-MT accounts for only 0.6% of hepatic Zn in normal control mice [24]. After intraperitoneal lipopolysaccharide injection, however, the amount of Zn associated with MT increased to 10% of total liver Zn, representing approx. 60% of hepatic Zn uptake [24]. Clearly, the expression of the MT gene, and the capacity of MT to bind Zn in the liver, are greatly enhanced by inflammatory stimuli. In the present *in vitro* study, using hepatocytes from MT+/+ mice, Zn-MT accounted for 37% of the Zn in unstimulated cells, increasing to 96% after culture with Zn50 + Dex + IL-6. The cell culture system thus reflects the direction, but not the magnitude, of the changes observed *in vivo*. The significantly greater proportion of Zn bound to MT *in vitro* may follow MT induction by stress hormones released during the handling and anaesthetization of the mice or result from events triggered by isolation and culture of hepatocytes. Moreover, in isolated cells, Zn exchange may occur across membranes to which plasma access is restricted *in vivo* by the hepatic architecture, e.g. the tight junctions between cells and the external environment of the sinusoid. The different physiological availability of Zn in each system must also be considered. About two-thirds of Zn in plasma is loosely bound to albumin with an association constant of 10^6 M⁻¹ [5], while the free Zn concentration in plasma may be less than 15 nM [25]. Zn in William's medium with 5 g/l fatty-acid-free BSA would be more available than Zn in normal plasma with an albumin concentration of approx. 40 g/l. In MT-/- hepatocytes, the uptake of 50 μ M Zn with 40 g/l albumin in the medium was 22% of that with an albumin concentration of 5 g/l. Similar findings were obtained in studies where rat hepatocytes were incubated for 15 min at 37 °C with ⁶⁵Zn (35 μ M) (P. Coyle, unpublished work). In those studies, Zn exchange from medium containing 40 g/l albumin was 35% of that with 5 g/l albumin. The combined data indicate that at least 4-fold more Zn is free to exchange at 5 g/l albumin than at normal plasma protein concentrations.

MT mRNA and protein are detected within 4 h of adjuvant administration to rats and as early as 2–3 h in response to various inflammation-released factors in freshly isolated and cultured hepatocyte systems [13,15,19]. Here, ⁶⁵Zn exchange in freshly isolated hepatocytes from MT+/+ and MT-/- mice, both prior to Zn challenge as well as in response to increasing external Zn, demonstrated a rapid (10–15 min) transfer of Zn from the medium to the cells. The final concentration of *tcZn* was the same in hepatocytes from both MT+/+ and MT-/- mice, and ⁶⁵Zn exchange increased in proportion to the Zn concentration in the medium up to 100 μ M. Atomic absorption spectrometry was used to confirm these findings in cultured hepatocytes, and also demonstrated a proportional increase in *tcZn* with increasing Zn concentration in the medium. Moreover, the increments in *tcZn* were similar for hepatocytes prepared from MT+/+ and MT-/- mice. Washing the cells with buffered 10 mM EDTA, which removes Zn non-specifically associated with the cell surface, showed, however, that at least 36% of the *tcZn* was attached to the cell surface, not internalized by the hepatocyte. Similar levels of non-specific binding (approx. 25%) of Zn to rat hepatocytes have been reported by Failla and Cousins [23]. Direct measurement of the membrane-bound

fraction in MT-/- hepatocytes indicated that as much as 56% was attached to the cell surface, with 38% present in the cytosolic fraction. It should be noted that membrane-bound Zn not only includes non-specifically attached Zn but also that which is structurally bound and necessary for the integrity of the membrane. Based upon these findings, it is likely that in the short-term incubations with ⁶⁵Zn, approx. 40% of the *tcZn* was non-specifically bound to the cell surface. The remainder is presumably tightly bound to the membrane or translocated to the cell interior. The membrane may contain the primary source of Zn for exchange with intracellular Zn pools and could be equally important in co-ordinating Zn transfer to metalloproteins which control many cellular functions. In this regard, it has been shown that Zn can affect the phagocytic activity of macrophages [10] and inhibit the adenylate cyclase activity of hepatocyte membranes [26].

The hepatic response to increased extracellular Zn concentrations in the absence of MT was tested *in vivo*. At 16 h after an intraperitoneal injection of 5 μ g/g Zn, a dose capable of doubling hepatic cytosolic Zn concentrations in normal mice, the total liver Zn remained unchanged in MT-/- mice, although cytosolic Zn concentrations rose by 15% because of hepatic water loss. Plasma and urinary Zn concentrations in those mice remained elevated 2.5- and 3.7-fold above those in their MT+/+ counterparts, demonstrating the extent to which intracellular MT synthesis can remove Zn from the circulation. In the absence of MT there is a saturation of urinary Zn excretion, resulting in sustained elevations in extracellular Zn concentrations. It appears that potent homeostatic mechanisms act to keep excess Zn from accumulating in the liver when there is no MT available for its sequestration (in a harmless form). Indeed, gel filtration chromatography clearly demonstrated that no Zn was associated with fractions below molecular mass 10 kDa in liver cytosols from MT-/- mice, and the Zn distribution on the higher-molecular-mass proteins was similar to that of MT+/+ mice with the same Zn loading. The MT+/+ mice were, however, able to double their hepatic Zn by incorporating virtually all of the hepatic Zn uptake into MT.

Availability of Zn in the liver is thought to be controlled largely by changes in MT gene expression, but there may also be changes in plasma membrane activity [5,27]. The liver can also sustain its Zn content during periods of dietary Zn deficiency. Zn concentrations have been shown to be maintained in hepatocytes when cultured in basal medium containing 1 μ M Zn by balancing lowered uptake with reduced efflux [28]. The present study demonstrates that, in culture, Zn enters the hepatocyte and equilibrates with the external milieu. However, accumulation of Zn in the liver is unlikely to occur against a concentration gradient in the absence of MT. In cultures of MT-/- hepatocytes, Zn accumulation was proportional to the extracellular Zn concentration and was approx. 60% of that obtained in MT+/+ hepatocytes. Zn accumulation in MT-/- hepatocytes appears to be specific to cells in culture, since there was no increase in hepatic Zn when MT-/- mice were given intraperitoneal injections of ZnSO₄ resulting in plasma Zn levels as high as 68.5 μ M at 16 h after Zn administration. That the magnitude of the difference in hepatic Zn accumulation between MT-/- and MT+/+ mice *in vivo* was not fully reflected *in vitro* is perhaps not surprising in view of reasons outlined previously. On the other hand, hepatocyte culture was fully able to demonstrate an obligatory role of MT synthesis in Dex- and IL-6-stimulated hepatic Zn accumulation.

The linear relationship between Zn accumulation in MT-null hepatocytes and Zn concentration in the medium suggests a mass-action mechanism of Zn transfer across the cell membrane

rather than induction of a specific Zn transporter. While no specific Zn transport mechanism has been elucidated, the kinetics of hepatic Zn uptake and accumulation have been described [29,30]. In rat hepatocytes it has been shown that Zn is taken up and exchanges with the intracellular compartment which includes a small (approx. 10%) rapidly turning over intracellular pool. This pool is not associated with MT but serves as a precursor for Zn-MT. In addition, efflux from the hepatocyte occurred from both Zn-MT and the 'rapid-turnover' pools, but not from a 'slow-turnover' pool which is presumably tightly bound cellular Zn. Zn accumulation in MT^{-/-} hepatocytes may reflect the rapid turnover pool. The question remains as to whether in MT^{-/-} hepatocytes a greater proportion of free ionized Zn exists in this pool or whether it is mainly an internal store of non-specifically bound Zn. Sephadex G-75 column chromatography of hepatic cytosol preparations from ZnSO₄-treated mice provided no evidence of extra Zn eluting in the low-molecular-mass fractions or those associated with free Zn. These findings indicate that mammalian cells lacking functional MT genes do not accumulate Zn by expressing other low-molecular-mass ligands. This is different from what has been found in yeast systems, where phytochelatin [(γ EC)_nG isopeptides] protects the cells from cadmium salts when MT genes are absent or not expressed [10].

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