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Hepatic Metallothionein as a Source of Zinc and Cysteine during the First Year of Life

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ABSTRACT. Metallothionein, a high cysteine-containing protein, can bind with both essential and nonessential metals and thus play an important role as a metal storage protein and also in the detoxification of toxic metals. Although in the human fetus, levels of trace minerals and metallothionein are very high, their postnatal changes are not well documented. The purpose of the present investigation, therefore, was to quantify the accumulation of metallothionein in premature and full-term infants during the first year of life and to identify factors affecting its accumulation. From 47 postmortem samples, it was determined that hepatic metallothionein levels were highest in newborn premature and full-term infants falling to levels found in older children by 4.4 months of age. Hepatic zinc levels were also highest in the youngest infants, falling with increasing postnatal age. There was a significant positive correlation between zinc and metallothionein at all ages. However, there was a negative correlation between hepatic metallothionein levels and cystathionase activity. Hepatic copper and metallothionein levels were unrelated. The renal concentration of metallothionein, zinc, and copper were significantly lower than corresponding hepatic levels. The fall in hepatic levels of zinc and metallothionein during the first months of life correspond to a period of negative zinc balance and low endogenous cysteine production in the newborn. Thus metallothionein may play an important role as a storage depot for these two essential nutrients during this critical period of active growth. (*Pediatr Res* 24: 326–329, 1988)

During the last trimester of pregnancy the mature placenta regulates the transfer of nutrients and protects the fetus from potentially toxic substances. With premature birth, the newborn infant is at risk of both nutrient deficiencies and toxicities for at least five reasons. *1*) Nutrient intake requirements may be unknown thus too much or too little may be provided; *2*) homeostatic mechanisms in the functionally immature preterm are poorly developed; *3*) the immature blood-brain barrier is more permeable to any elements present in the plasma; *4*) growth is extremely rapid, thus nutrient needs are highest at the same time that nutrient stores are very low; and *5*) postnatal adaptation may be prolonged for months even in the absence of overt disease. This combination of factors demand that both environmental and nutritional factors be rigidly controlled for the newborn premature infant.

It has been established that most of the minerals of the human fetus are layed down during the last trimester of pregnancy (1). For example, two-thirds of the zinc present in the full-term infant accumulate during the final 12–15 wk of gestation. The major zinc-binding protein of the liver is metallothionein (2). Metallothioneins are a group of structurally similar low molecular weight intracellular proteins with high content (30%) of cysteinyl residues and complete absence of aromatic amino acids and histidine. They have a high affinity for essential divalent metals such as zinc and copper and nonessential metals such as cadmium and mercury. Inasmuch as this protein can bind with various metals, both essential and nonessential, it may have an important function in regulating the metabolism and toxicity of these metals (3).

Richards and Cousins (4) demonstrated that intraperitoneal administration of zinc resulted in a marked increase in hepatic zinc uptake. Because the increase in hepatic zinc uptake occurred simultaneously with the incorporation of labeled amino acids into metallothionein, it was proposed that zinc uptake into the liver under these conditions involved increased metallothionein synthesis. Notable in these studies was the observation that zinc uptake was prevented when rats had received prior administration of actinomycon D, a known inhibitor of mRNA synthesis. Although tissue levels of metallothionein are low in adults, its synthesis can be induced by different metals such as cadmium, zinc, copper, and mercury thus explaining their putative role as protective and storage proteins (5–8). For example, synthesis of the thioneins takes place in liver, kidney, and spleen when animals are treated with sublethal doses of cadmium and certain other metallic salts (9). High endogenous levels of metallothionein bound to zinc and copper are present in human fetal livers and it is decreased to low levels after birth (10, 11).

When intravenous nutrients are used to feed preterm infants, a reasonable goal is the provision of nutrients, including trace metals, at dosages that will result in duplication of intrauterine accretion rates (12). Growth rates are extremely high during the last trimester of gestation (15 g/kg/day), thus, intake dosages are also very high (13). Inasmuch as the exact biological fate of parenterally infused metals is not known, the high intakes create the potential for toxicity. The role of metallothionein, therefore, in both storage and protection for the parenterally fed preterm infant has potential importance.

Since 30% of the amino acids of metallothionein are cysteine, the availability of cysteine to the fetus and preterm infant may affect the capacity of the infant to synthesize this protein. The fact that cystathionase activity is absent from the fetus (14) and that all three transsulfuration enzymes are absent from the placenta (15), suggests that the fetus is entirely dependent on the mother for its supply of cysteine. Inasmuch as cystathionase does not become active until some time after birth, the newborn, especially the newborn premature infant, is dependent on a dietary source of cysteine for at least the first few weeks of life, in order to ensure an adequate intake (16). The majority of parenteral amino acid formulations currently used to intravenously feed the premature infant contain no cysteine (because of

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the insolubility of cystine), thus, the infant who is parenterally fed will be dependent on endogenous cysteine synthesis. It is our contention that the use of parenteral feeding solutions that combine high intake doses of trace metals without cysteine may limit the young infant's ability to synthesize metallothionein, predisposing the infant to potential toxicity from the infused metals.

The purpose of the present investigation, therefore, was to quantify the accumulation of metallothionein in premature and full-term infants during the first year of life and to identify factors affecting its accumulation.

MATERIALS AND METHODS

Subjects. Forty-seven samples of human liver tissue were obtained during postmortem examination. In addition, a small number of samples of kidney was also obtained. Samples were divided into four groups based on their gestational and postnatal ages. The three study groups consisted of newborn preterm and full-term infants and infants less than 1 yr of age (Table 1). Infants older than 1 yr were considered to be the control group. At the time of death, all premature and full-term infants were less than 2 wk old. The mean postnatal age of the older infants was 4.4 ± 3.7 months. By chance, more male than female subjects were included. The clinical diagnosis of the infants studied included prematurity and respiratory distress syndrome, congenital heart disease, other congenital anomalies, birth asphyxia, sudden infant death syndrome, and other pulmonary-related causes. The control group consisted of samples of children who died when they were more than 1 yr old. Their cause of death was unrelated to primary hepatic or renal pathology.

Handling of samples. Organ samples from autopsies completed with 48 h of death (mean time = 25 h) were collected and immediately frozen at −20° C until the assay for metallothionein and metals (Zn and Cu) were made. Within 4 h of collection, a portion of the sample was added in a 10:1 ratio (ml/g wet weight) to cold 0.03 M potassium phosphate buffer, pH 6.9, and immediately homogenized in a glass grinding chamber using a Teflon pestile and a Tri-Rotir-R (model K43) stirring apparatus (Tri-R Instruments, Rockville Centre, NY). The homogenates were centrifuged at 5000 × *g* for 7 min and their supernatant fluid either immediately assayed for cystathionase activity or stored at −70° C. All steps were performed at less than 4° C.

Estimation of cystathionase activity. Cystathionase activity was determined using a modification of the method of Gaull *et al.* (16, 17) as previously described.

Estimation of metallothionein and metals (Zn and Cu). Approximately 0.5 g of tissue from liver or kidney was dissected and homogenized in an ice bath in 4 vol of 0.25 M sucrose solution with a polytron (Tecmar Co., Cincinnati, OH). Duplicates were available for most samples, except a few kidney samples where the sample size was too small. The homogenates were centrifuged at 10,000 × *g* for 20 min and the supernatant fractions were assayed for metallothionein by a silver-Hb method as previously described (18). In brief, aliquots of 0.2 ml of the supernatants were adjusted to a sample volume of 1.2 ml with 0.5 M glycine-Tris buffer solution, pH 8.5. The samples were mixed with 1 ml of radioactive silver nitrate reagent (^{110m}Ag, 5 nCi; 20 ppm of solution). The samples were allowed to mix at room temperature for 10 min. The excess silver was removed by addition of rat hemolysate and heated in a water bath for 2 min. Centrifugation steps were repeated twice. The heated supernatant samples contained radioactive ^{110m}Ag exclusively bound to metallothionein. To obtain the background level, a control tube with glycine buffer alone (not containing sample) was processed. The radioactivity in the samples and control was measured in a 1270 Rackgamma II LKB Counter (Wallac, Turku, Finland) with a counting efficiency of more than 80%. The radioactivity from the control tube was subtracted from samples to calculate the silver bound to metallothionein. The amount of metallothionein in samples were expressed as μg/g wet tissue, assuming 17 g atoms of silver was bound to 1 mol of metallothionein.

Table 1. *Clinical data*

	Gestational age (wk)	Postnatal age (days)	Sex (male:female)
Premature (n = 10)	30.6 ± 2.9*	4.1 ± 5.4	9:1
Full-term (n = 13)	39.6 ± 1.8	4.0 ± 5.2	10:2†
Infants (n = 15)	Full-term	4.4 ± 3.7 mo	10:4†
Controls‡ (n = 9)	N.A.	7.9 ± 4.4 yr	6:3

* Mean ± SD.

† The sex of one subject from each group was unavailable.

‡ All control subjects died from accidents and were older than 1 yr of age.

For analysis of zinc and copper, tissue samples (about 0.2 g) were digested as follows (19). Metal-free nitric acid (1 ml) was added to each sample and maintained at room temperature for at least 24 h. The nitric acid digests were heated to 60° C for 5 min to dissolve small particle. The total volumes were then adjusted to 2 ml with deionized distilled water. The concentrations of metals were estimated by atomic absorption spectrophotometry in a Varian Spectra 30 (Varian, Toronto, Canada) equipped with an air-acetylene flame and autosampler. Recover was >95% by standard addition. The detection levels for zinc and copper were 0.1 μg/g wet tissue and for metallothionein the levels were 5 μg/g wet tissue. Special precautions were taken both in the storage of tissue samples in plastic containers and in the analysis to avoid any possible external contamination.

Statistics. Group means were compared using Student's t test. In addition, regression analysis was used to determine the relationship between the predictor values and the outcome variables. The suitability of the linear equations determined by this method were checked using residual analysis, quadratic transformation of the predictor variable, and logarithmic transformation of the dependent variable. By implementing the procedures described above, it was determined that a nonlinear model was most appropriate to mathematically describe the relationship between metallothionein, zinc, and copper concentration and postnatal age (20). To study the relationship between zinc, metallothionein, and postnatal age, a "best fitting" curve of the form $y = A/x$ using PROC NLIN in SAS was used (20). Differences in the results with a $p < 0.05$ were considered to be statistically significant.

RESULTS

Concentrations of metallothionein, zinc, and copper in hepatic tissue. There was no difference in the concentration of metallothionein either between premature and full-term infants, or between older infants and control values (Table 2). However, the concentration of metallothionein was significantly lower in older infants than newborns. The hepatic zinc concentration was significantly different in each of the three groups. The highest values were in the premature group. Interestingly, similar to data for concentrations of zinc in other body tissues, including plasma, the lowest levels were in the older infants, with concentrations increasing in the older control group (21, 22). Copper levels were similar in the three groups, but significantly higher than control values. As we have previously reported, cystathionase levels were lowest in the premature group (16).

The renal concentration of metallothionein (7.2 ± 8.6 μg/g), zinc, and copper (35.6 ± 11.8; 3.2 ± 1.2 μg/g) were significantly lower than corresponding hepatic levels.

Zinc, copper, and metallothionein correlations. There was a significant positive correlation between hepatic zinc and metallothionein concentrations ($r = 0.76$, $p < 0.0001$) (Fig. 1). Although data in Figure 1 is a combination of the three groups, the correlation remains strongly positive when each of the three groups (and the control group) are evaluated independently. Hepatic copper and metallothionein concentrations were unrelated ($r = 0.19$).

Effect of postnatal age on hepatic concentrations of zinc, copper,

Table 2. *Hepatic metallothionein, zinc, copper concentrations, and hepatic cystathionase activity (mean ± SD)*

	Metallothionein	Zinc (μg/g wet wt)	Copper	Cystathionase activity (nmol cyst/mg protein/h)
Premature	234.5 ± 214.6*[a]	226.0 ± 85.6[a]	58.0 ± 20.4[cd]	54.5 ± 32.4[a]
Full-term	217.6 ± 218.0[a]	142.2 ± 60.8[b]	64.3 ± 32.5[c]	85.1 ± 44.2[b]
Infants	52.2 ± 47.0[b]	49.8 ± 14.6[c]	38.2 ± 33.3[d]	133.6 ± 45.6[c]
Controls	54.9 ± 37.6[b]	64.2 ± 14.8[d]	9.0 ± 2.7[e]	179.0 ± 60.7[d]

* Means in each vertical column not sharing a common superscript are significantly different.

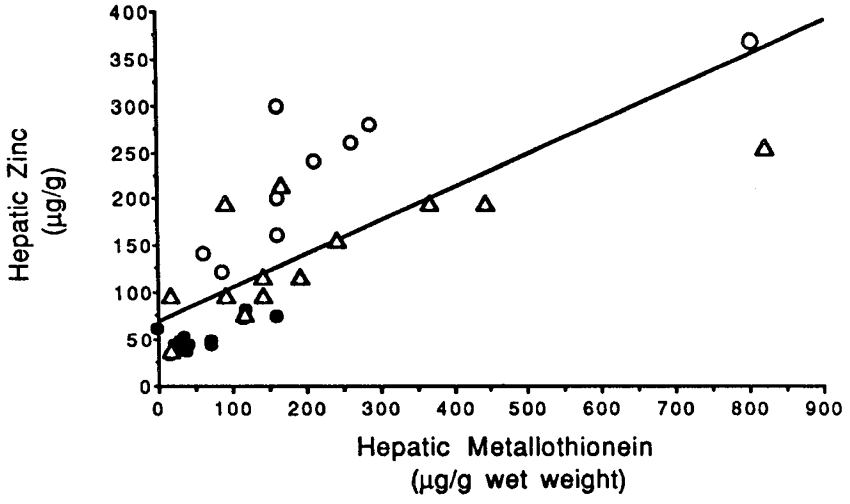

Fig. 1. The relationship between hepatic zinc and metallothionein concentrations. Each point represents an individual data measurement. *Open circles* represent data from preterm infants; *open triangles* from full-term infants; and *closed circles* from older infants. There was a significant positive correlation between hepatic zinc and metallothionein concentrations ($r = 0.75$, $p < 0.0001$).

and metallothionein. During the first year of life, there was a significant negative correlation between postnatal age and hepatic metallothionein concentration, and postnatal age and hepatic zinc concentration. By plotting the graphs of postnatal age and metallothionein (and zinc), the best fit obtained was an exponential curve. The graph of postnatal age *versus* metallothionein is shown in Figure 2.

Metallothionein and cystathionase activity. There was a significant negative correlation between hepatic cystathionase activity and metallothionein concentration (Fig. 3). This finding is in keeping with the previously observed positive correlation between postnatal age and cystathionase activity, and the negative correlation between postnatal age and metallothionein concentration (Fig. 2).

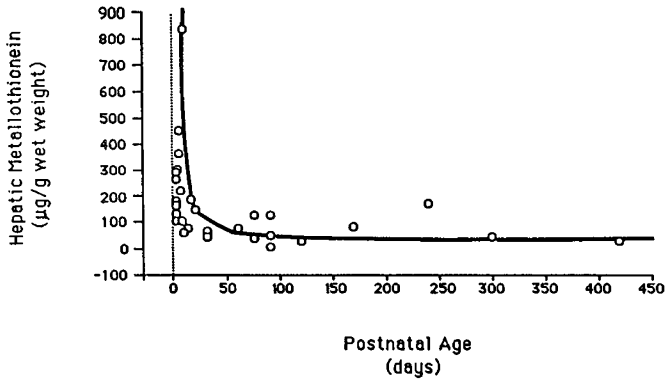

Fig. 2. The relationship between hepatic metallothionein concentration and postnatal age. Each point represents a data measurement from a single subject. A best fitting curve of the form y = A/x using PROC NLIN in SAS was used to generate the line (20).

DISCUSSION

Although it has been suggested that metallothionein is involved in human zinc homeostasis, this study documents in human premature and full-term tissue, concurrent measurements of zinc, copper, and metallothionein. In the perinatal rat liver, the great accumulation of cytosolic zinc-bound metallothionein peaks at or soon after birth (23, 24). This has been interpreted to indicate that the liver is the main site of regulation of zinc metabolism (and prevention of zinc toxicity). In the human fetal liver, metallothionein concentration is extremely high with the highest levels found between 14–23 wk of gestation (2500 μg/g) (25). We have determined that during the last trimester of gestation, metallothionein concentrations fall from the high fetal values, but are still elevated compared to older infants. However, hepatic zinc levels remained as high in the last trimester as described in the younger fetus (25). Once again, similar to other mammalian species and to results from young human fetuses, there was a strong correlation between the concentration of zinc and metallothionein in hepatic tissue.

Despite a difference of 10 wk in gestational age between the preterm and full-term infants included in the current study, within the first week of life, hepatic metallothionein concentrations were similarly high. It has been suggested that a primary role for metallothionein is as a storage depot for zinc and that zinc bound to metallothionein can be reutilized in the body thus protecting the body during periods of limited access to exogenous zinc (2). Consistent with the theory that metallothionein is a storage depot for zinc is the described negative zinc balance in preterm and full-term infants during the first months of life (26, 27). Thus at a time when zinc balance is negative, hepatic metallothionein/zinc levels are at their highest and are likely able to contribute zinc to maintain the high rates of tissue synthesis that occur during the newborn period.

It has not been established if fetal metallothionein increased because of elevated fetal zinc or copper or if induction via a hormonal stimulus leads to increased metal accumulation. The

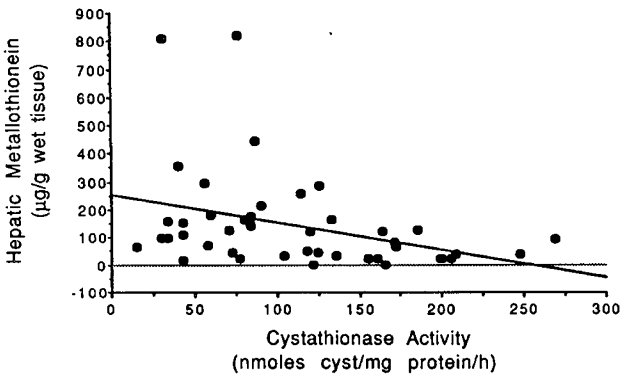

Fig. 3. The relationship between hepatic metallothionein and cystathionase activity. Each *closed circle* represents an individual data measurement (control values were included). There was a significant negative correlation between the two variables ($r = 0.36$, $p < 0.01$).

fetus seems to be totally dependent on maternal transfer of cysteine because of absent cystathionase activity both in the fetus and placenta (14, 15). Unlike other amino acids, the concentration of cysteine is lower in the fetal than the maternal circulation (28). Despite the low fetal plasma concentration of cysteine, high metallothionein concentrations corresponding to the hepatic zinc concentrations imply that adequate substrate is available for metallothionein synthesis.

We demonstrated in the current study that metallothionein levels are high in the first weeks of life in both the preterm and full-term infant. We had previously demonstrated that cystathionase activity is inducible immediately after birth, even in the preterm infant, but remains below mature levels until around 3 months of age (16). It is likely therefore, that during the first months of life, the decrease in the high concentration of hepatic metallothionein will contribute not only zinc, but also cysteine to the growing infant.

Because 30% of metallothionein is cysteine, the premature infant receiving cyst(e)ine-free, trace metal-containing parenteral nutrition may be particularly vulnerable to metal toxicity. At a time when cystathionase activity is low, parenteral formulations containing high levels of trace metals including zinc, copper, manganese, chromium, and selenium are infused directly into the systemic circulation bypassing the protective mechanisms present in the gut. A combination of decreased endogenous cysteine production and no exogenous intake may limit the infants ability to synthesize metallothionein, thus predisposing the infant to potential trace metal toxicity. The lack of cysteine may also decrease the infants' ability to store trace metals. Although toxicity to metals has not been observed in newborns receiving total parenteral nutrition, increased urinary trace metal excretion, as we have previosuly described, may indicate limited hepatic storage capacity (29). Neither the newborns nor the older infants in the current study received parenteral nutrients, thus we were unable to determine if the parenteral infusion of trace metals influenced metallothionein concentrations. Because tissue from live infants is most often impossible to obtain, the answer to this question will not be readily available.

In the current study we demonstrated that full-term and preterm newborns have higher hepatic stores of metallothionein than older infants, and, as described in other species, there is a high correlation between concentrations of zinc and metallothionein in the liver. These observations are important for at least two reasons. First, because of serious concurrent illnessess, premature infants often receive few nutrients for the first 7–10 days of life with little obvious detrimental affect. Stores of zinc, as zinc bound to metallothionein, are present even in the premature infant and may act to maintain zinc homeostasis even in the total absence of dietary zinc intake. In addition to the stores of cysteine from glutathione, metallothionein stores may also contribute cysteine to the infant at a time when endogenous cysteine production from methionine is limited. Second, premature infants often receive nutrients directly into their systemic venous circulation, bypassing the protective mechanisms present in the gut for trace metal homeostasis, with no obvious indications of toxicity. The presence of hepatic metallothionein may act to protect the infant from potentially toxic effects even when the gut is bypassed.

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