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Erythrocyte metallothionein as an index of zinc status in humans

(nutrition/trace elements/induction/reticulocytes)

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ABSTRACT **Metallothionein concentrations in erythrocyte lysates derived from human subjects were measured by an ELISA procedure. IgG obtained from serum of sheep injected with human metallothionein 1 was used in this competitive assay. Subjects were fed a semipurified zinc-deficient diet (0.7 mg of zinc per kg of diet) for an 8-day depletion period after 3 days of acclimation. Fasting plasma zinc concentrations were reduced ≈7%. Metallothionein in the erythrocyte lysates was significantly decreased to 59% of the initial level by the end of the depletion period. Supplementation of these depleted subjects with zinc (50 mg) did not increase erythrocyte metallothionein levels within 24 hr. Daily supplementation of control subjects with zinc (50 mg/day) increased erythrocyte metallothionein to a 7-fold maximum within 7 days. These levels were reduced by 61% within 14 days after zinc supplementation was terminated. Incubation of rat [^{35}S]metallothionein with human erythrocyte lysate showed a time-dependent increase in ^{35}S soluble in 20% trichloroacetic acid, indicating degradation of the labeled protein, presumably via protease activity in the lysate. It is proposed that zinc supplementation induces erythrocyte metallothionein during erythropoiesis and that low zinc intake decreases synthesis and/or accelerates degradation of the protein in reticulocytes/erythrocytes. Metallothionein levels in erythrocytes may provide a useful index upon which to assess zinc status in humans.**

Methods currently available for the assessment of zinc status in humans are unsatisfactory. These methods include measuring hair and urine zinc concentrations, antibody-mediated immune responses, and platelet aggregation (1). The most common of these is the measurement of plasma zinc concentrations by atomic absorption spectrophotometry. Plasma zinc levels decrease in normal individuals fed a zinc-deficient diet (1). However, factors related to infection, disease, and pregnancy also depress the plasma zinc concentration (1). The cytokine interleukin 1 and glucocorticoid hormones are among the mediators of these metabolic effects that appear linked to expression of the metallothionein gene (2, 3). The decrease in plasma zinc concentration in these conditions may not necessarily indicate low zinc status. Rather, it represents a redistribution to some tissues, particularly the liver and bone marrow (3, 4), presumably for functions related to host defense (1).

One possible measure of zinc status is metallothionein, a zinc-binding protein involved in various aspects of zinc metabolism and/or function (2). Expression of the metallothionein gene is sensitive to the dietary zinc supply in rats (5). Furthermore, RIA data indicate that plasma and erythrocyte metallothionein 1 concentrations were reduced to very low levels within days in rats fed a zinc-deficient diet (6). In addition, although endotoxin produces a large increase in the plasma metallothionein concentration, bone marrow and erythrocyte metallothionein 1 are increased only slightly (<2-fold) (7). Therefore, erythrocyte metallothionein appears much less responsive to stress and infection than either plasma metallothionein or plasma zinc in experimental animals and may be a more stable indicator of zinc status in humans.

Recently, we described an ELISA for human metallothionein (8). In this paper we report the use of this ELISA to measure changes in human erythrocyte metallothionein concentrations influenced by dietary zinc intake.

MATERIALS AND METHODS

Human Subjects. This study was approved by the Institutional Review Board of the University of Florida College of Medicine. The subjects used were males between 25 and 32 years of age. A total of 13 subjects participated: 7 in the zinc deficiency experiment and 6 to study the effects of zinc supplementation. Informed consent was obtained from each subject before participation in this study. The blood chemistry profile of each subject was normal. Subjects were nonsmokers with normal medical histories, who currently were not taking prescription or over-the-counter medications and did not exercise excessively.

Protocol for Zinc Depletion and Zinc Supplementation. The egg white-based semipurified diet, deficient in zinc (0.7 mg of zinc per kg of diet; Research Diets, New Brunswick, NJ), was comparable to that described by Turnlund *et al.* (9). To verify the low zinc content of this diet, male Sprague–Dawley strain rats (Harlan–Sprague–Dawley) were fed the diet for 3 days as described (5) before measuring the serum zinc concentration.

To help limit zinc absorption in human subjects during zinc depletion, sodium phytate from corn (Sigma) was added to the diet to provide 2.1 g/kg of body weight (9, 10). A multivitamin tablet was fed each day as a separate supplement. Doubly distilled H_2O or Diet 7-Up (8 ng of zinc per ml) was given ad libitum. Except for zinc, each of the seven subjects received recommended levels of all known essential nutrients. Supplemental biotin (2 mg) was given daily to counteract the effects of biotin binding by avidin present in egg albumin. Subjects received a 42-kcal (1 cal = 4.18 J) diet per kg of body weight per day. This diet provided 0.46 mg of zinc daily on a 70-kg body weight basis. The diet was fed as a liquid mixture in doubly distilled H_2O at 0700, 1200, 1700, and 2200 hr. Blood was drawn at 0630 and 1130 hr. The study was divided into a 3-day period of acclimation to the diet and an 8-day study period. During the first day of the acclimation period, 4 mg of zinc as $ZnCO_3$ (1 mg of zinc per meal) was given. On the seventh and eighth day of the study period, 50 mg of zinc as $ZnCO_3$ was given at the first meal.

Each of the six volunteers in the 63-day supplementation experiment consumed normal diets except that oysters and other foods with exceptionally high zinc content were prohibited. A zinc supplement (50 mg of zinc as zinc gluconate) was consumed daily 30 min before the first meal.

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Analytical Procedures. Venous blood samples were drawn in trace element-free-evacuated tubes containing EDTA for the preparation of plasma and erythrocytes. Whole blood was centrifuged at 600 × *g* for 5 min to separate the cells from plasma. The buffy coat was removed from the erythrocyte pellet, and an equal volume of ice-cold 0.9% NaCl was added. After being inverted several times, the tubes were centrifuged again; this process was repeated twice. The washed cells were lysed by addition of ice-cold doubly distilled H_2O (1:1.4); this solution was the erythrocyte lysate used for the ELISA. All samples were stored at −80°C. Plasma and erythrocyte lysate were diluted 5-fold and 15-fold, respectively, in doubly distilled H_2O for measurement of zinc concentrations by flame atomic absorption spectrophotometry and were compared against standards diluted in 1% HCl/5% glycerol. Serum ferritin was measured by RIA (11). Protein concentrations of the erythrocyte lysate were determined by the Lowry method (12).

Metallothionein concentrations were determined by a human metallothionein ELISA developed in our laboratory (8). The method is a competitive assay using IgG raised in sheep injected with human liver metallothionein 1 purified by gel filtration, ultrafiltration, and HPLC on a Pharmacia Mono Q column. The purified metallothionein 1 was used to coat 96-well microtiter plates. Serial dilutions of erythrocyte lysate (1.4-fold to 180-fold) and metallothionein were added followed by sheep anti-human metallothionein-1 IgG, donkey anti-sheep IgG, and *p*-nitrophenyl phosphate with appropriate washing and incubation steps. Absorbance was measured at 405 nm. Concentrations were determined by linear regression after logit transformation. Dilutions that were parallel to the purified human metallothionein-1 standard curve were used in determining the metallothionein concentration.

Metallothionein Degradation in Erythrocyte Lysates. To prepare ^{35}S-labeled metallothionein, rats (described above) were injected with 77 μmol of zinc i.p., and 2 hr later with 50 μCi of [^{35}S]cystine (1 Ci = 37 GBq) (188 Ci/mmol; DuPont/NEN). This injection pattern was repeated on 2 consecutive days. Labeled metallothionein isoforms were purified from liver by HPLC as reported (8). The method for the preparation of erythrocyte extracts for measuring protein degradation was modified from Davies and Goldberg (13). Whole blood from a human volunteer was obtained, and cells were prepared as described above. Cells (4 ml) were suspended in 6 ml of doubly distilled H_2O, mixed by inversion, and centrifuged at 40,000 × *g* for 90 min to remove cell fragments. The supernatant was dialyzed (3.5-kDa cutoff) for 20 hr at 4°C against 300 vol of 5 mM $MgCl_2$/10 mM Na_2HPO_4, pH 7.8, with three buffer changes. Protein concentrations were determined by the Lowry method (12). The lysate was stored at −80°C.

Proteolytic susceptibility of metallothionein *in vitro* was measured as described (14). Rat [^{35}S]metallothionein (1260 cpm; specific activity of 270 cpm/μg) was mixed with 6.7 mg of lysate protein in phosphate buffer ± 0.5 mM dithiothreitol ± 175 μM Zn^{2+} in a total vol of 100 μl. The solutions were incubated at 37°C for 15 min, 2 hr, or 4 hr. Lysate was omitted from control incubations. Reactions were stopped by adding 40 μl of 3% bovine serum albumin and then 460 μl of 20% trichloroacetic acid; all tubes were placed on ice for 10 min. Precipitated proteins were separated by centrifugation at 10,000 × *g* for 5 min. An aliquot of the supernatant (400 μl) was added to 5 ml of Scintiverse scintillation cocktail (Fisher) to measure acid-soluble ^{35}S by liquid scintillation counting (Beckmann LS 7500).

Statistical Analysis. Tests for significance were performed by paired comparison *t* test (SAS Institute, Cary, NC) (15).

RESULTS

Measurement of Human Erythrocyte Metallothionein by ELISA. Metallothionein concentrations obtained from serial dilutions of erythrocyte lysates were parallel to the standard curve for purified human metallothionein 1. Results from two representative lysates are shown in Fig. 1. The interassay coefficient of variation, determined from repeated measurements of normal erythrocyte lysates, was 13% and the intraassay coefficient of variation was 4.2%.

Reduction in Erythrocyte Metallothionein with Dietary Zinc Restriction. Rats were fed the zinc-deficient diet for 3 days to verify its effect on serum zinc levels. Those rats exhibited a 65% decrease (38 μg of zinc/dl) in serum zinc concentration compared to control rats (110 μg of zinc/dl). This shows the zinc content of the diet is sufficiently low to produce metabolic changes in animals. Human subjects fed the deficient diet exhibited a slight (7%), but nonsignificant, decrease in their fasting plasma zinc concentration by day 7 of the study period [from 77 ± 2 to 72 ± 5 μg/dl (SE)]. Postprandial plasma zinc concentrations, measured 5 hr after the morning meal, averaged 14% lower than fasting levels throughout the study (comparative data not shown). Repletion of the subjects with 50 mg of zinc on day 7 resulted in an average 17% increase in fasting plasma zinc concentration the following day, raising the concentration to that seen in subjects fed conventional diets [87 ± 4 μg of zinc/dl (SE)]. The concentration of zinc in the erythrocyte lysate from fasting subjects was 15.5 times higher than the plasma zinc concentration. However, erythrocyte zinc concentrations did not change during the study period.

In contrast to plasma zinc concentration, metallothionein in erythrocyte lysate exhibited a definitive response to consumption of the zinc-deficient diet (Fig. 2). Initial erythrocyte metallothionein concentrations in this group of subjects averaged 34 ± 6 μg of metallothionein per g of protein (SE). Concentrations fell significantly to 68% of the initial value by day 7 [23 ± 4 μg of metallothionein per g of protein (SE); $P = 0.033$] and to 59% by day 8 [20 ± 4 μg of metallothionein per g of protein (SE); $P = 0.015$]. The erythrocyte metallothionein concentrations on day 8 did not reflect the 24-hr response to zinc supplementation, as did the plasma zinc concentration. In comparison, serum ferritin concentrations did not change during the study period (data not shown).

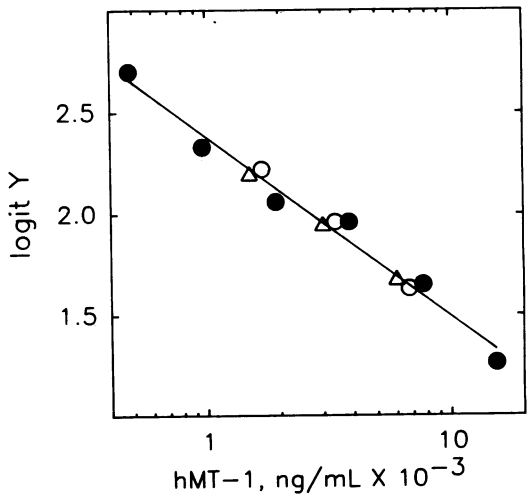

FIG. 1. Serial dilutions of human erythrocyte lysates compared with a purified human metallothionein 1 (hMT-1) standard curve. Dilutions were from 1.4-fold to 5.6-fold for erythrocyte lysate from two unsupplemented subjects (△, ○). Dilutions of known amounts (●) of human metallothionein 1 ($y = -0.89x + 5.04$; $r = 0.989$).

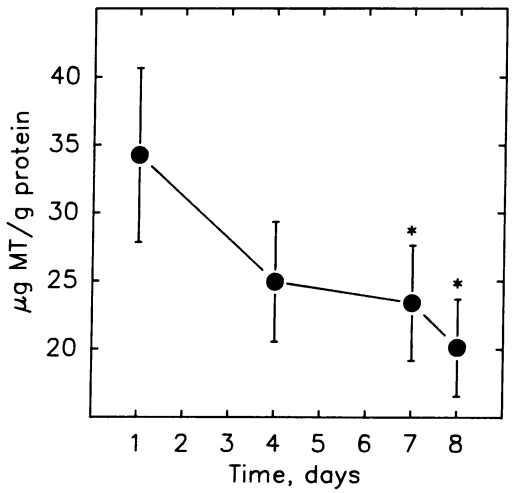

FIG. 2. Effects of dietary zinc deficiency on erythrocyte metallothionein (MT) concentration over time. *, Significantly different ($P < 0.05$) from day 1. Values are expressed as mean ± SE ($n = 7$).

Increase in Erythrocyte Metallothionein with Zinc Supplementation. To examine the response of metallothionein to zinc supplementation, a 63-day supplementation study was done with a separate volunteer group. Fig. 3 shows that erythrocyte metallothionein concentrations increased 7-fold [from 40 ± 6 μg of metallothionein per g of protein (SE) to 273 ± 85 μg of metallothionein per g of protein (SE)] by 7 days of supplementation at 50 mg of zinc per day. This concentration was maintained throughout the supplementation period, suggesting that once metallothionein is induced in marrow cells, a steady state of turnover of the protein in erythrocytes precludes a continous linear increase in concentration. At least 3–4 days of supplementation are required before any increase in metallothionein is seen (data not shown). A decrease in erythrocyte metallothionein was seen at 7 days, the first time samples were obtained after supplementation was terminated. At least 14 days are needed to observe a 61% reduction in these concentrations in a subset of the population ($n = 3$) after supplementation is terminated [day 63: 241 ± 42 μg of metallothionein per g of protein (SE); day 77: 93 ± 43 μg of metallothionein per g of protein (SE)].

Metallothionein Degradation by Erythrocyte Extract. The marked decrease in erythrocyte metallothionein during zinc depletion and limited production during zinc supplementation suggest that in humans, turnover of this protein is determined by zinc. To examine the susceptibility of metallothionein to proteolysis under these conditions, rat [^{35}S]metallothionein was incubated with human erythrocyte lysate. Metallothionein degradation was measured as the increase in trichloroacetic acid-soluble ^{35}S over a 4-hr incubation period. Fig. 4 shows that all ^{35}S was insoluble in control incubations without erythrocyte lysate, indicating that little metallothionein degradation occurred. A progressive, time-dependent increase in soluble ^{35}S was seen upon incubation with human erythrocyte lysate. At 4 hr, *in vitro* degradation of [^{35}S]metallothionein was 34%. Addition of dithiothreitol prevented proteolytic activity of erythrocyte extract toward rat [^{35}S]-metallothionein.

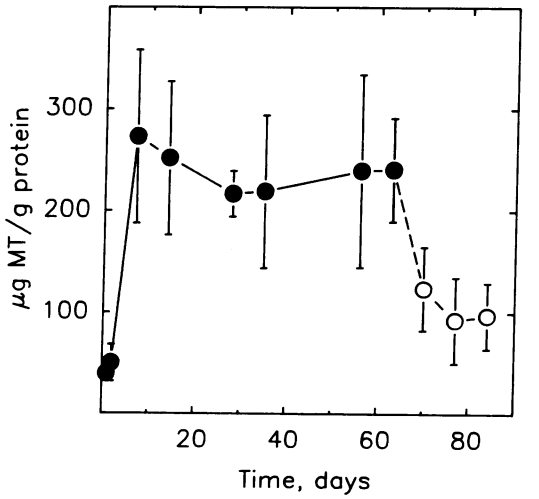

FIG. 3. Effects of zinc supplementation (50 mg of zinc per day) on erythrocyte metallothionein concentration over time. ●, 50 mg of zinc supplement per day to day 63 ($n = 4$–6); ○, supplement withdrawn after day 63 ($n = 3$). Values are expressed as mean ± SE.

DISCUSSION

Plasma zinc concentration was monitored as a comparative indicator of responsiveness to the zinc-deficient diet. Gordon *et al.* (10) found that fasting plasma zinc levels from two of three human subjects fed a zinc-deficient semi-purified diet, nearly identical to that used in our study, reached a minimum by 4 days. The subjects in the present study did not exhibit a significant decrease in plasma zinc levels during the 8-day study period. A plethora of factors besides the dietary zinc supply can influence plasma zinc concentration, which seriously hampers its value for assessment of zinc status (1, 16).

Human erythrocyte metallothionein levels were effectively determined by the human metallothionein 1 ELISA that we recently described in detail (8). This assay is not as sensitive as the RIA for rat metallothionein 1 reported by others (6). Nevertheless, we could measure metallothionein levels in human erythrocyte lysates. Because the ELISA measures both isoforms of the protein, we assume these measurements represent total metallothionein. The metallothionein concentrations in human erythrocytes were greater than those seen in the rat. This disparity is probably attributable to species differences because metallothionein concentrations in normal human liver have consistently been reported higher compared with those for rat liver (8, 17, 18).

The significant decrease in metallothionein concentrations observed by us in the erythrocyte lysate in response to short-term zinc depletion indicates that this parameter could

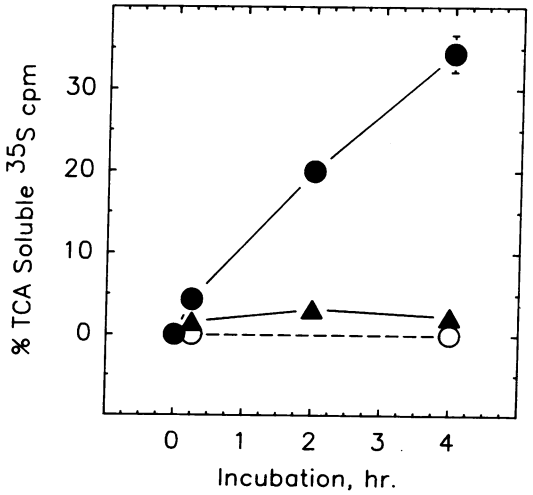

FIG. 4. Degradation of rat [^{35}S]metallothionein when incubated with human erythrocyte extract. ○, No erythrocyte extract (control, $n = 11$); ●, erythrocyte extract ($n = 7$); ▲, erythrocyte extract plus 0.5 mM dithiothreitol ($n = 4$). Values are expressed as mean ± SE; error bars are generally smaller than symbol size. TCA, trichloroacetic acid.

serve as an index for assessment of human zinc deficiency. The levels decreased within the depletion period much faster than could be accounted for by loss of older erythrocytes from the circulation, which in humans have an average life-span of 120 days (19). Results from rat studies indicate that reticulocytes contain most metallothionein found in the erythrocyte population (20). Furthermore, rat erythrocyte metallothionein also appears to be rapidly removed from the circulation—with its decrease in concentration correlated with decrease in dietary zinc intake (6). Binding of plasma metallothionein to the erythrocyte apparently does not account for the presence of this protein in these cells (7). Therefore, as the erythrocyte ages, metallothionein appears to be degraded rather than retained within the cell and lost with the older cells when they are removed from the circulation.

The apparent regulation of human erythrocyte metallothionein is of particular interest. Our hypothesis to explain the effect of dietary zinc on erythrocyte metallothionein involves the regulation of synthesis and degradation in different regions of the hematopoietic system. Zinc has been shown to regulate metallothionein gene expression in bone marrow progenitor cells as in other cell types, such as hepatocytes (3, 21). If expression is lowered upon dietary zinc restriction, lower levels of metallothionein will be produced, and less metallothionein will eventually appear in the systemic erythrocyte. Similarly, higher dietary zinc intake should increase gene expression. This idea is supported by our supplementation data. The maximum increase in erythrocyte metallothionein attained was 7-fold over the basal level. In addition, at least 3–4 days of supplementation were required before an increase in erythrocyte metallothionein was seen—a lag consistent with a mechanism whereby metallothionein production is augmented in the marrow before release of developing cells into the circulation. No function for metallothionein in marrow cells has been defined; possible functions include response to a metabolic demand for zinc to support rapid proliferation and differentiation of various blood cells (22) or to protect DNA from hydroxyl-radical damage (23).

Once metallothionein-containing reticulocytes are released, the potential exists for protein degradation. Recently erythrocyte lysates have been convincingly shown to degrade proteins (13). Individual proteins differ markedly in susceptibility to proteolysis (24). Furthermore, damage from oxygen radicals, particularly the hydroxyl radical, increases protein susceptibility to proteolysis (13). Metal loss and/or damage due to oxygen radicals could increase the susceptibility of metallothionein to proteolysis in a way dependent on zinc status.

In past studies, we showed that apometallothionein was much more easily degraded *in vitro* than the holoprotein (14). If zinc from metallothionein is released for requisite cellular functions in erythrocytes—e.g., to maintain carbonic anhydrase activity (25) or for membrane stabilization (26), the apoprotein could degrade before the individual erythrocyte. To meet metabolic demands for zinc during dietary zinc restriction, degradation of the protein might increase. In support of this hypothesis are our findings that (*i*) erythrocyte metallothionein decreases when dietary zinc intake is limited and (*ii*) the maximum metallothionein level during zinc supplementation is limited to a 7-fold increase rather than a continuous increase. This sequence suggests that further increases in erythrocyte metallothionein with continuing synthesis in marrow are precluded by continuous degradation. Separate studies *in vitro* have shown that adding Zn^{2+} to the erythrocyte lysate prevents [^{35}S]metallothionein degradation (data not shown). This result would suggest that release of zinc from erythrocyte metallothionein increases degradation of the protein. Alternatively, as has been shown for other erythrocyte proteins (13), hydroxyl radicals may damage metallothionein and increase its proteolysis. Metallothionein, under *in vitro* conditions, is a hydroxyl-radical scavenger through a mechanism that could involve metal loss and sulfhydryl oxidation (27).

Collectively, these data show that metallothionein levels in human subjects are sensitive to dietary zinc intake. The presence of significant amounts of metallothionein in the erythrocyte will provide a basis around which both experimental and field studies can be configured for assessment of zinc nutritional status. Furthermore, functional aspects of metallothionein and, perhaps, zinc in human erythroid cells are suggested from these data.

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