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Magnesium Deficiency Inhibits Biosynthesis of Blood Glutathione and Tumor Growth in the Rat (42260)

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Abstract. Previously we found that blood glutathione (GSH) levels increase in response to tumor growth in the rat and that this increase is not prevented with zinc deficiency. We also found that zinc deficiency which inhibited tumor growth did not prevent this increase in blood GSH. Therefore, the objectives of this study were to determine the effects of another nutritional modification, namely magnesium deficiency, on blood GSH status and on tumor growth. Magnesium was selected because it is an obligatory cofactor in GSH synthesis and in all biosynthetic reactions involving ATP. To this end, magnesium- and zinc-deficient rats with and without tumors were compared to pair-fed control rats with and without tumors. After 32 days of depletion, the rats were killed, and blood samples were analyzed for nonprotein sulfhydryls (SH) and specifically for GSH. The key finding was that in magnesium-deficient rats with or without tumors, blood GSH levels were low and SH levels were normal indicating a decrease in GSH biosynthesis. In contrast, zinc deficiency affected SH and GSH in parallel. Thus, these two deficiencies must act by different mechanisms. The zinc data verified our earlier results obtained with a different tumor type and rat strain, for blood GSH levels increased in tumor-bearing rats fed control diets, and zinc deficiency did not prevent this increase. Depletion of magnesium or zinc was equally effective in inhibiting tumor growth. These results provide *in vivo* evidence of a magnesium requirement for GSH biosynthesis in rat erythrocytes. Further, the results suggest that magnesium deficiency may inhibit tumor growth by limiting GSH synthesis from SH precursors.

In previous work using the rapidly growing Walker 256/M1 tumor in Sprague–Dawley rats, we found differential effects of tumor and zinc deficiency on blood glutathione (GSH) levels (1). Compared with that of control rats, the level was increased as a result of tumor alone and decreased as a result of zinc deficiency alone. Moreover, in rats that were both tumor bearing and zinc deficient, the decrease due to zinc deficiency was overcome, and blood GSH increased significantly over control levels as a result of the tumor. Positive correlations between final tumor weights and the concentrations of blood GSH suggested a possible role of GSH in tumor growth. These findings led us to the hypothesis that high GSH levels promote tumor growth and that a nutritional modification that limits GSH synthesis *in vivo* will also inhibit tumor growth rate.

Therefore, the objectives of the present research were to determine the specific effects of magnesium deficiency on blood GSH levels and tumor growth and to investigate the relationship of GSH to overall sulfhydryl (SH) changes. Magnesium was selected as it is an obligatory cofactor in the enzyme reactions of GSH synthesis (2) and in all biosynthetic enzyme reactions involving ATP.

To determine if effects were mineral specific, both magnesium- and zinc-deficient rats with and without tumors were compared to pair-fed control rats with and without tumors. The experimental protocol differed from our previous tumor growth studies in several ways. First, tumors were established *before* magnesium and zinc depletions were initiated. Second, the slower growing R3230AC mammary adenocarcinoma was selected to allow sufficient time for the deficiencies to be effective. Finally, the blood samples were analyzed for both total nonprotein sulfhydryls (SH) (3) and for glutathione by a specific enzymatic cycling method (4) in order to determine whether SH content reflected levels of GSH. The SH/GSH ratio served as an indicator of the utilization of SH precursors for GSH biosynthesis.

These glutathione studies were done in conjunction with previously reported experiments which demonstrated that dietary magnesium and zinc depletions inhibited the

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growth of established tumors (5, 6), and a preliminary account of this work has been presented (7).

Materials and Methods. *Animal model and protocol.* Male Fischer 344 rats (National Cancer Institute, Mammalian Genetic and Production Section and Charles River Breeding Laboratories, Kingston, N.Y.) were implanted with R3230AC mammary adenocarcinomas (E. E. & G. Mason Research Institute, Worcester, Mass.). Another group of rats received sham implants of Hanks' solution. All rats were fed a complete control diet for 13 days when positive tumors were detected by palpation. Both tumor-bearing and sham-implanted rats were divided into groups matched by weight and degree of tumor development; pair-fed either a control, a zinc-deficient, or a magnesium-deficient diet for 32 days; and were killed. In the three zinc experiments the rats were 60, 76, and 124 days of age, and their initial weights in grams (means ± SEM) were 129 ± 2.53, 193 ± 2.25 and 241 ± 6.12, respectively. In the magnesium experiment the rats were 76 days of age and weighed 174 ± 3.19 g.

In the diets for the zinc experiments (Ralston Purina Co., Richmond, Ind.) the protein was egg albumin; the zinc content ranged from 35–50 ppm for the control diets and <4 ppm for the deficient diets. Diets for the magnesium experiment (Teklad Test Diets, Madison, Wis.) contained casein as the protein source; the magnesium content was 655 ppm for the control diet and 23 ppm for the deficient diet. Details of the experimental protocol, diet compositions, housing, and necropsy procedures have been described previously (6, 8).

Blood analyses. Hemolysates (9.1%, v/v) of freshly drawn blood were obtained by intracardiac puncture using heparinized syringes, and deproteinized by the addition of 10% (w/v) metaphosphoric acid (Fisher Scientific Co., Fairlawn, N.J.) in the ratio of 1.43 ml acid to 1 ml hemolysate. The mixture was kept at room temperature for 15 min and then filtered using Whatman No. 1 paper. The acid filtrates were immediately frozen and assayed within 14 days. We had previously determined that samples processed in this way were stable for at least 25 days at −10°C. For hemoglobin concentration, 0.10 ml of hemolysate was added to 5.0 ml of a ferricyanide–cyanide solution, and its absorbance at 540 nm was determined. Hematocrits were measured in capillary tubes after centrifugation by standard methods, and red blood cells were counted using a Coulter counter.

Undiluted acid filtrates were used to determine total nonprotein sulfhydryls by the method of Beutler *et al.* (1, 3). The filtrates were diluted 1/10 for the assay of total glutathione using the specific enzymatic cycling method of Tietze (4, 9). Standards and blanks were prepared using the same concentrations of metaphosphoric acid as the samples. In both assays, two levels of filtrate were analyzed routinely to ensure proportionality. Since reduced glutathione (GSH) in blood comprises over 97% of the total glutathione (10), the abbreviation, GSH, is near-equivalent to total glutathione (reduced plus oxidized).

Concentrations of SH and GSH were expressed per gram hemoglobin which we validated as an index of erythrocyte number. In the zinc experiments, hematocrits were correlated with hemoglobin concentrations ($r = 0.95$, $P < 0.0005$) and with red cell numbers ($r = 0.92$, $P < 0.0005$). Mean cell volumes were the same in all groups. In the magnesium experiment, hematocrits were correlated with hemoglobin concentrations, $r = 0.92$ ($P < 0.0005$). In order to compare concentrations of GSH with SH, microequivalents based on the molecular weight of GSH were used to express results.

Mineral concentrations in plasma. Plasma obtained by centrifugation of heparinized blood at 500*g* for 20 min was diluted 1/5 with deionized water for zinc analysis and 1/50 for magnesium analysis. Zinc and magnesium standards in 5% (v/v) glycerol were diluted 1/5 and 1/50 with deionized water. Samples were then analyzed using a Perkin–Elmer Model 603 atomic absorption spectrophotometer (Perkin–Elmer Corp., Norwalk, Conn.) (8).

Statistical analysis. Data from the three zinc experiments were pooled. Results were analyzed using the Student *t* test (11) and a test of independence (12).

Results. *Magnesium experiment.* Blood GSH and SH levels were both significantly higher (32 and 27%) in control rats with tumors (Table I). However, GSH levels were about 20% lower in both magnesium-deficient groups, with or without tumors. Since SH

TABLE I. EFFECT OF TUMOR AND MAGNESIUM AND ZINC DEFICIENCIES ON BLOOD GLUTATHIONE AND SULFHYDRYL LEVELS

Dietary group	Glutathione (μeq/g Hb)	% of control	SH (μeq/g Hb)	% of control	SH/GSH
Magnesium experiment					
Control	3.48 ± 0.229 (6)	(100)	5.13 ± 0.0978 (6)	(100)	1.50 ± 0.0908 (6)
Control + tumor	4.61 ± 0.289 (7)*	132	6.53 ± 0.613 (7)*	127	1.41 ± 0.0722 (7)
Deficient	2.76 ± 0.214 (5)*	79	4.84 ± 0.304 (5)	94	1.77 ± 0.0762 (5)*
Deficient + tumor	2.82 ± 0.241 (9)*	81	4.76 ± 0.388 (9)	93	1.70 ± 0.0638 (9)*
Zinc experiments					
Control	3.04 ± 0.254 (16)	(100)	4.20 ± 0.146 (12)	(100)	1.35 ± 0.0885 (12)
Control + tumor	4.34 ± 0.392 (24)*	143	6.35 ± 0.523 (17)*	151	1.35 ± 0.0908 (17)
Deficient	2.17 ± 0.163 (10)*	71	3.08 ± 0.168 (6)*	73	1.35 ± 0.0466 (6)
Deficient + tumor	3.44 ± 0.328 (22)	113	4.54 ± 0.346 (16)	108	1.20 ± 0.0431 (16)

Note. Data are expressed as means ± SEM for the number of animals in parentheses. Data from three zinc experiments are pooled.

* Significantly different from control groups ($P < 0.05$).

concentrations were the same as the controls, the SH/GSH ratios were significantly higher due to the decrease in GSH.

Plasma magnesium levels in deficient rats were 66% lower than control values ($P < 0.0005$), and in rats that were both deficient and tumor bearing, levels were 82% lower ($P < 0.0005$). There was also a significant correlation between plasma magnesium levels and blood GSH concentrations ($r = 0.69$, $P < 0.001$). Plasma zinc levels were about 40% lower ($P < 0.0005$) in tumor-bearing rats. These lower zinc levels were tumor specific since they were not found as a result of magnesium deficiency alone.

Tumor-bearing rats in both control and deficient groups had significantly lower hematocrits and hemoglobin concentrations than control rats without tumors (Table II). Deficient rats without tumors had decreases of about 10% in both parameters. Although the mean corpuscular hemoglobin concentration (MCHC) was only 8% lower in control rats with tumors, the decrease was significant ($P < 0.05$).

Zinc experiments. These experiments confirmed our previous finding that blood GSH concentrations increased with rat age (13). In control rats, final mean GSH concentrations were 2.22, 2.60, and 4.02 μeq/g hemoglobin

TABLE II. EFFECT OF TUMOR AND MAGNESIUM AND ZINC DEFICIENCIES ON HEMATOLOGICAL PARAMETERS

Dietary group	Hemoglobin (g/dl)	Hematocrit (%)	Red cell no. (10^6/mm^3)	MCV (μ^3)	MCHC (g/dl)
Magnesium experiment					
Control	16.7 ± 0.274 (6)	45.4 ± 0.484 (5)	—	—	36.9 ± 0.581 (5)
Control + tumor	12.3 ± 0.970 (7)*	36.0 ± 1.900 (7)*	—	—	33.9 ± 1.15 (7)*
Deficient	15.0 ± 1.180 (5)	40.2 ± 2.452 (5)*	—	—	37.3 ± 0.861 (5)
Deficient + tumor	13.7 ± 0.777 (9)*	37.2 ± 0.741 (9)*	—	—	36.6 ± 1.41 (9)
Zinc experiments					
Control	15.6 ± 0.430 (16)	44.3 ± 1.03 (12)	6.64 ± 0.174 (6)	64.4 ± 1.04 (6)	33.7 ± 0.634 (12)
Control + tumor	11.7 ± 0.768 (24)*	31.1 ± 2.45 (17)*	5.23 ± 0.389 (9)*	65.4 ± 1.07 (9)	33.2 ± 0.997 (17)
Deficient	18.4 ± 0.386 (10)*	47.5 ± 0.707 (6)*	—	—	37.4 ± 0.664 (6)*
Deficient + tumor	13.2 ± 0.654 (22)*	36.4 ± 1.68 (16)*	5.58 ± 0.509 (8)	67.1 ± 3.89 (8)	33.7 ± 0.709 (16)

Note. Data are expressed as means ± SEM for the number of animals in parentheses. Data from three zinc experiments are pooled.

* Significantly different from control groups ($P < 0.05$).

in rats that were 105, 121, and 169 days of age, respectively. Since the direction of changes was the same in the three experiments, the results have been pooled.

Blood GSH and SH levels were both significantly higher (43 and 51%) in tumor-bearing rats fed the complete control diet (Table I). Since the changes in both moieties were similar, the SH/GSH ratio was not altered. In rats that were only zinc deficient, both GSH and SH were lower (29 and 27%) and the SH/GSH ratio was the same as in the control group. Although GSH and SH levels in tumor-bearing, zinc-deficient rats were not significantly different from controls, they were 58 and 47% higher compared to zinc-deficient rats without tumors.

Zinc concentrations in plasma were 40% ($P < 0.0005$) lower as a result of tumor presence and 46% lower ($P < 0.0005$) with zinc deficiency. Plasma zinc levels were even lower (31% of control levels) ($P < 0.0005$) in rats that were both zinc deficient and tumor bearing.

Both tumor-bearing groups were anemic with significant decreases in hemoglobin concentration, hematocrit, and red cell number (Table II). Zinc-deficient rats without tumors had increases in hemoglobin concentration, hematocrit, and MCHC.

Other effects of mineral depletion. A major result was the inhibition of tumor growth in depleted rats. The mean weight (±SEM) of tumors in magnesium-deficient rats was 9.89 ± 1.90 g compared to 18.4 ± 4.09 g in rats fed control diets (6). This is a significant decrease of 46% ($P < 0.05$). Tumors were 35% lower ($P < 0.05$) in zinc-deficient rats, 13.7 ± 2.38 g compared to 21.1 ± 4.30 g in rats fed control diets (5). Food intake was the same for all groups.

When final tumor weights were plotted against blood GSH concentrations for each rat, there were significant positive correlations ($P < 0.01$) in both the magnesium and zinc experiments (Fig. 1).

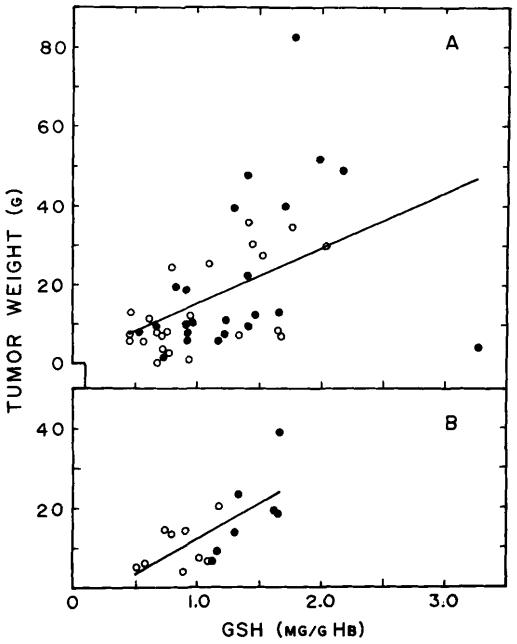

FIG. 1. Relationship between blood GSH concentration and tumor weight. Data from each rat were plotted, and the regression lines computed by the method of least squares were drawn. Rats from the different dietary groups are identified as follows: ●, control; ○, deficient. In the zinc experiment (A), $r = 0.46$; $P < 0.01$. In the magnesium experiment (B), $r = 0.70$; $P < 0.01$.

Discussion. These results demonstrate a unique role of magnesium in the regulation of GSH status and the growth of tumors. Indeed, these data specifically support our hypothesis that nutritional modifications that limit GSH synthesis will also inhibit tumor growth. The rationale for magnesium may be its essential role as a cofactor for the enzyme reactions of GSH biosynthesis (2) and for ATP-linked reactions (14). Substantiation of this possibility will require future investigation of GSH synthesis and degradation in magnesium-deficiency states.

Magnesium-deficient rats have been reported to have lower levels of erythrocyte GSH and ATP (15). However, in contrast to our results, the SH/GSH ratio in erythrocytes was unchanged. This difference in findings may be due to our specific determination of GSH with the enzymatic cycling method of Tietze whereas Hsu *et al.* used a nonspecific alloxan procedure. Moreover, we followed rapid processing and proper storage of samples which are essential to minimize autooxidation and to achieve quantitative recovery of samples supplemented with known amounts of GSH.

Elevation in blood GSH levels may be a generalized phenomenon in tumor-bearing animals. The high levels of GSH and SH we found in tumor-bearing control rats in the

magnesium experiment were not due merely to the 8% decrease in the MCHC of red cells since the magnitude of increase was three- to fourfold greater. Also, in our zinc experiments there was no decrease in the MCHC, and the GSH and SH levels were 43 and 51% higher than the controls. Moreover, the lower GSH and SH levels in zinc-deficient rats without tumors were not the result of an MCHC increase.

Elevated GSH levels in blood have been observed in both humans and in animals with a variety of malignancies (16–21). Moreover, these conditions were found in patients with tumors not derived from lymphoid or bone marrow tissue. The reason for this increase in blood GSH has not been identified although a number of altered erythrocyte enzyme activities indirectly related to GSH have been associated with tumor growth (22).

It seems likely that the higher levels of GSH in whole blood of tumor-bearing rats were derived from erythrocytes since the observed changes were of millimolar proportions and not micromolar levels as found in plasma (23). However, this does not rule out the possibility that some of the GSH may have come from liver or tumor tissues which have higher GSH levels compared with those of their normal counterparts (24–27). Considerable interorgan transport of GSH in plasma has been demonstrated (28, 29), but in our study plasma concentrations could not account for the increased GSH levels in whole blood.

Although glutathione has been known since the mid-1920s, investigation of its multiple metabolic roles has undergone a renaissance that has been extensively reviewed recently by Meister (30, 31). However, the relationship of GSH and tumor growth has not yet been elucidated. It can be argued from experimental data that GSH has a host protective function or that it has a role in supporting tumor growth. There is little doubt that GSH is important for the detoxification of electrophilic molecules that may be carcinogenic and thus plays a role in the prevention of cancer.

The increased metabolism of glutathione observed during tumor growth suggests that GSH may be a tumor growth factor. Not only were higher levels of GSH found in blood and in a variety of tumors, but high activity levels of γ-glutamyl transpeptidase, a key GSH-linked enzyme, have been observed in tumor tissue, in serum, and in uninvolved tissues of tumor-bearing hosts (32–37). Many early studies indicated that GSH plays an important role in normal cell division and growth (38). Recent investigation in our laboratory of GSH concentrations throughout the life span of the mosquito revealed that the highest GSH concentrations coincided with the period of maximal DNA biosynthesis, DNA polymerase activity and DNA, RNA, and protein content (39).

Several lines of experimental evidence indicate that GSH may provide an important antioxidant defense mechanism for the tumor and thereby enhance its growth. Studies on mice demonstrated an essentiality of thiols for tumor cell transplantation and proliferation (40). Recent findings indicate that SH inhibitors have a greater toxicity for cancer cells than for normal cells (41) and also increase the radiosensitivity of tumor cells *in vivo* (42).

Tumor growth may be enhanced when high levels of antioxidant (e.g., GSH) detoxify hydrogen peroxide produced by invading macrophages, thus preventing the lysis of tumor cells. A wide range of susceptibility to lysis by hydrogen peroxide was found in a variety of tumor cell types. In four different detoxification mechanisms tested (catalase, glutathione reductase, glutathione peroxidase, and GSH) only GSH levels in tumors correlated with susceptibility to lysis (43). Moreover, inhibition of glutathione reductase markedly increased susceptibility. Glutathione synthetase activity was maximal in the resistant Novikoff tumor and minimal in the more sensitive MC-1A tumor cells (44). It may be the differential capacities of tumor cells to generate GSH that in large part determine their potential for continued growth and response to chemotherapeutic agents.

Our own data suggest that GSH may be a factor in increasing tumor growth. This concept is supported by the significant positive correlation between blood GSH concentrations and final tumor weights in both the zinc and magnesium experiments. The different effects of tumor presence on blood GSH concentrations in zinc- or magnesium-deficient rats indicate that the inhibition of tumor growth in these deficient animals occurred by different mechanisms. As postulated earlier, zinc would be required for tumor growth be-

cause of its well-known involvement in DNA, RNA, and protein synthesis and also as an intrinsic component of collagenase. However, the present experiments demonstrated that in contrast to magnesium deficiency, zinc deficiency did not lower the GSH level in tumor-bearing rats. These new findings suggest the possibility that magnesium deficiency may mediate tumor growth inhibition by decreasing GSH concentrations.

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