Zinc in Human Health: Effect of Zinc on Immune Cells

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Although the essentiality of zinc for plants and animals has been known for many decades, the essentiality of zinc for humans was recognized only 40 years ago in the Middle East. The zinc-deficient patients had severe immune dysfunctions, inasmuch as they died of intercurrent infections by the time they were 25 years of age. In our studies in an experimental human model of zinc deficiency, we documented decreased serum testosterone level, oligospermia, severe immune dysfunctions mainly affecting T helper cells, hyperammonemia, neurosensory disorders, and decreased lean body mass. It appears that zinc deficiency is prevalent in the developing world and as many as two billion subjects may be growth retarded due to zinc deficiency. Besides growth retardation and immune dysfunctions, cognitive impairment due to zinc deficiency also has been reported recently. Our studies in the cell culture models showed that the activation of many zinc-dependent enzymes and transcription factors were adversely affected due to zinc deficiency. In HUT-78 (T helper 0 (Th_0) cell line), we showed that a decrease in gene expression of interleukin-2 (IL-2) and IL-2 receptor α (IL-2Rα) were due to decreased activation of nuclear factor-κB (NF-κB) in zinc deficient cells. Decreased NF-κB activation in HUT-78 due to zinc deficiency was due to decreased binding of NF-κB to DNA, decreased level of NF-κB p105 (the precursor of NF-κB p50) mRNA, decreased κB inhibitory protein (IκB) phosphorylation, and decreased Iκκ. These effects of zinc were cell specific. Zinc also is an antioxidant and has anti-inflammatory actions. The therapeutic roles of zinc in acute infantile diarrhea, acrodermatitis enteropathica, prevention of blindness in patients with age-related macular degeneration, and treatment of common cold with zinc have been reported. In HL-60 cells (promyelocytic leukemia cell line), zinc enhances the upregulation of A20 mRNA, which, via TRAF pathway, decreases NF-κB activation, leading to decreased gene expression and generation of tumor necrosis factor-α (TNF-α), IL-1β, and IL-8. We have reported recently that in both young adults and elderly subjects, zinc supplementation decreased oxidative stress markers and generation of inflammatory cytokines.

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INTRODUCTION

In 1869, Raulin (1) first showed that zinc was required for the growth of Aspergillus niger. In 1934, Todd *et al*. (2) reported that zinc was essential for the growth of rats. Although the essentiality of zinc for animals was established, its ubiquity made it seem improbable that zinc deficiency in humans could lead to any significant clinical problem. During the past 40 years, however, it has become apparent that deficiency of zinc in humans is quite prevalent and may affect over two billion subjects in the developing world (3–5). Here, we review some of the clinical manifestations of zinc deficiency in humans, with particular emphasis on our observations in experimental human models of this syndrome.

CLINICAL MANIFESTATIONS OF ZINC DEFICIENCY

During the past four decades, a spectrum of clinical deficiency of zinc in human subjects has emerged (6). On the one hand, the manifestations of zinc deficiency may be severe; and, on the other end of the spectrum, zinc deficiency may be mild or marginal. A severe deficiency of zinc has been reported to occur in patients with acrodermatitis enteropathica (a genetic disorder), following total parenteral nutrition (TPN) without zinc, following excessive use of alcohol, and following penicillamine therapy. The manifestations of severe zinc deficiency in humans include bullous pustular dermatitis, alopecia, diarrhea, emotional disorder, weight loss, intercurrent infections due to cell-mediated immune dysfunctions, hypogonadism in males, neurosensory disorders, and problems with healing of ulcers. If this condition is unrecognized and untreated, it becomes fatal.

The manifestations of a moderate deficiency of zinc include growth retardation and male hypogonadism in adolescents, rough skin, poor appetite, mental lethargy, delayed wound healing, cell-mediated immune dysfunctions, and abnormal neurosensory changes.

In our studies in the experimental human model in whom only a mild deficiency of zinc in males was induced by dietary means, decreased serum testosterone level, oligospermia, decreased natural killer (NK) cell activity, decreased interleukin-2 (IL-2) production, decreased thymulin activity, hyperammonemia, hypogeusia, de-

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creased dark adaptation, and decreased lean body mass were observed. It is, therefore, clear that even a mild deficiency of zinc in humans affects clinical, biochemical, and immunological functions adversely (7–9).

Nutritional deficiency of zinc in the developing countries is caused by ingestion of high cereal protein intake, rich in phytate (an organic phosphate compound), which makes zinc unavailable for absorption. Other causes of zinc deficiency include malabsorption syndrome, hyperzincuria as seen in cirrhosis of the liver and sickle cell disease, blood loss due to hookworm infection, and excessive sweating in hot tropical climates.

In 1974, a landmark decision to establish recommended dietary allowances (RDAs) for humans for zinc (15 mg elemental zinc daily for adult men and women) was made by the Food and Nutrition Board of the National Research Council of the United States of America National Academy of Sciences.

ZINC AND IMMUNITY

Zinc affects multiple aspects of the immune system (10). Zinc is crucial for normal development and function of cells mediating innate immunity, neutrophils, and NK cells. Macrophages also are affected by zinc deficiency. Phagocytosis, intracellular killing, and cytokine production all are affected by zinc deficiency. Zinc deficiency adversely affects the growth and function of T and B cells. The ability of zinc to function as an antioxidant and stabilize membranes suggests that it has a role in the prevention of free radical-induced injury during inflammatory processes.

STUDIES OF IMMUNE FUNCTIONS IN EXPERIMENTAL HUMAN MODEL

During our studies in the Middle East, we observed that most of the zinc deficient dwarfs did not live beyond the age of 25 years. The cause of death appeared to be infections. Parasitic infections were common. Viral and bacterial infections, although prevalent, remained undocumented, however. The possibility that zinc deficiency may have played a role in immune dysfunctions in the zinc-deficient dwarfs was considered, but lack of proper facilities prevented us from gathering meaningful data on immune functions in those patients in the Middle East.

In our experimental human model studies, when zinc deficiency was very mild (3 to 5.0 mg Zn intake during the zinc-restricted period), the plasma zinc concentration remained more or less within the normal range and it decreased only after 4–5 months of zinc restriction. On the other hand, zinc concentrations in lymphocytes, granulocytes, and platelets decreased within 8–12 weeks, suggesting that the assay of cellular zinc provided a more sensitive criterion for diagnosing mild deficiency of zinc (7–9).

We assayed serum thymulin activity in mildly zinc-deficient human subjects (8). Thymulin is a thymus-specific hormone and it requires the presence of zinc for its biological activity to be expressed. Thymulin binds to high-affinity receptors on T cells, induces several T-cell markers, and promotes T-cell function, including allogenic cytotoxicity, suppressor functions, and interleukin-2 (IL-2) production. As a result of mild deficiency of zinc, the activity of thymulin in serum was decreased significantly and was corrected by both *in vivo* and *in vitro* zinc supplementation (7). The *in vitro* supplementation studies indicated that the inactive thymulin peptide was present in the serum in zinc-deficient subjects and was activated by the addition of zinc.

For the quantification of T cells, beads coated with T_{101} Mab and, for B lymphocytes, beads coated with anti human immunoglobulin were used, (Quantigen, Bio-Rad laboratories, Richmond, CA, USA) 1×10^6 mononuclear cells (MNC) were incubated with 200 μL of one or two microbeads preparation. Cells with no beads were counted as T_{101}–, slg–. An increase in T_{101}–, slg- cells, a decrease in the ratio of $CD4^+$ to $CD8^+$, and decreased IL-2 activity (proliferation assay [7]) were observed in the experimental human model during the zinc-depletion phase, all of which were corrected after repletion with zinc. We previously had reported that NK cells' activity was also sensitive to zinc restriction, thus it appears that zinc may play a very important and critical role in the functions of T cells in humans (6,10). Our studies in the experimental human model showed for the first time that the production of IFN-γ was decreased, whereas the production of IL-4, IL-6 and IL-10 was not affected due to zinc deficiency (8,9). IFN-γ is known to downregulate the T helper 2 (Th_2) clone, and IL-10 may downregulate the T helper 1 (Th_1) clone. An imbalance between Th_1 and Th_2 responses in patients with human immunodeficiency virus infection has been implicated in the immune dysregulation in these patients, and it has been proposed that resistance to infection and/or progression to acquired immunodeficiency syndrome is dependent on a $Th_1 > Th_2$ dominance. Our data in experimental human models suggest that cell-mediated immune dysfunctions in human zinc deficiency may be due to an imbalance between Th_1 and Th_2 cell functions.

Th_1 cells are known to promote macrophage activation and production of complement fixing and opsonizing antibodies. IFN-γ is the major component of the Th_1 response panel, and it upregulates major histocompatibility complex class I antigen expression. Inasmuch as we have observed that IFN-γ production is decreased as a result of zinc deficiency, our studies provide a possible mechanism by which zinc deficiency may affect cell-mediated immunity adversely.

Several studies have shown the benefits of zinc supplementation on infectious diseases in humans. In double-blind placebo-controlled trials of zinc supplementation, zinc reduced the incidence and duration of acute and chronic diarrhea and acute lower respiratory tract infections in infants and children (11,12). Zinc supplementation of sickle cell anemia patients in a placebo-controlled trial resulted in decreased incidence of staphylococcus aureus pneumonia, streptococcus pneumonia tonsillitis, and *E. coli* urinary tract infections (13). Our recent studies

have shown that zinc supplementation to elderly subjects results in a significant decrease in the incidence of infections (14).

ZINC ACTIVATES NF-κB IN HUT-78 CELLS

The current estimate is that over 2,000 transcription factors may be zinc dependent. However, the present dogma is that the amount of zinc required for the integrity of these transcription factors is so small that, in zinc-deficient cells, one would not see any change.

Because zinc deficiency affects IL-2 production and T-cell activation adversely, we have investigated the role of zinc on NF-κB (nuclear factor-κB) activation in HUT-78, a T helper 0 (Th_0) human malignant lymphoblastoid cell line. We showed for the first time that, in zinc deficient HUT-78 cells, the activation of NF-κB was affected adversely (15).

PHOSPHORYLATION AND UBIQUITINATION OF IκB

Intracellular zinc concentration was decreased by 40% in zinc-deficient HUT-78 cells in comparison to the zinc-sufficient cells after 4 d of incubation. To examine the effect of zinc on κB inhibitory protein (IκB) in HUT-78 cells, we measured phosphorylated IκB-α, Iκκ-α (IκB kinase-α), and ubiquitinated IκB-α in zinc-treated cells. Zinc deficiency caused a 60% decrease in phosphorylated IκB-α in non-stimulated and 40% decrease in phorbol-12 myristate 13 acetate (PMA)/phytohemagglutinin-p (PHA-p)–stimulated cells, compared with zinc-sufficient cells. Zinc deficiency decreased Iκκ-α in PMA/PHA-p–stimulated cells by 30% in comparison to zinc-sufficient cells. Zinc deficiency caused a 50% reduction of ubiquitinated IκB-α after PMA/PHA-p stimulation (15).

We showed that the phosphorylation of IκB and Iκκ, translocation of NF-κB, and its binding to DNA in HUT-78 cells were all zinc dependent (15). The activation of NF-κB was considerably reduced in zinc-deficient HUT-78 cells. Additionally, zinc deficiency decreased the level of NF-κB p105 (the precursor of NF-κB p50) mRNA. All these effects of zinc were cell specific.

ZINC ENHANCES THE EXPRESSION OF IL-2 AND IL-2 RECEPTORS IN HUT-78 CELLS VIA NF-κB ACTIVATION

The production of interleukin-2 (IL-2) is a key and early event in the activation of T-lymphocytes. IL-2 triggers peripheral T-lymphocytes to enter the S phase of the cell cycle and to divide. This is probably a result of the suppressive effect of IL-2 on cell cycle inhibitors, which interfere with the activity of cyclin-dependent kinases (cdks) at checkpoints of the cell cycle (16). IL-2 also is involved in the differentiation of thymocytes, peripheral T- and B-lymphocytes and other cells of hematopoietic origin (16).

A short segment of DNA, 275 base pairs in the promoter area of IL-2 gene, integrates numerous signaling pathways leading to IL-2 synthesis and the activation and proliferation of T-lymphocytes (16). Both the murine and human IL-2 promoters contain one binding site for genuine Rel/NF-κB factors. The sequence of this site, GGGATTTCAC, is identical for both promoters. NF-κB factors are induced rapidly by a variety of stimuli activating T cells. Almost every stimulus leading to T cell activation also activates NF-κB. Induction of IL-2Rα gene expression also is mediated by the induced nuclear expression of NF-κB (17,18).

IL-2 AND SOLUBLE IL-2Rα PRODUCTION

The details of cell culture, zinc assay, and other methods have been published elsewhere (19). We used an Enzyme-Linked ImmunoSorbent Assay (ELISA) to measure the concentration of IL-2 produced by the cells. Unstimulated HUT-78 cells incubated in zinc-deficient (1 μmol/L zinc) and zinc-sufficient (15 μmol/L zinc) media for 4 d produced a very small amount of IL-2. Following PMA/PHA-p stimulation, the IL-2 production and IL-2 mRNA were significantly greater in zinc-sufficient cells in comparison to the zinc-deficient cells (19). Similar results were seen for soluble IL-2 receptor α (soluble IL-2Rα) production and gene expression (19).

ZINC DECREASES OXIDATIVE STRESS AND INFLAMMATORY CYTOKINES

The role of zinc in modulating oxidative stress has recently been recognized. Oxidative stress is an important contributing factor in several chronic human diseases, such as atherosclerosis and related vascular diseases, mutagenesis and cancer, neurodegeneration, immunologic disorders, and the aging process (20–22). Together $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$ are known as reactive oxygen species (ROS), and these are produced continuously *in vivo* under aerobic conditions. The NADPH oxidases are a group of plasma membrane associated enzymes, which catalyze the production of $O_2^{\cdot-}$ from oxygen by using NADPH as the electron donor. Zinc is an inhibitor of this enzyme. The dismutation of $O_2^{\cdot-}$ to H_2O_2 is catalyzed by an enzyme super oxide dismutase (SOD), which contains both copper and zinc. Zinc is known to induce the production of metallothionein, which is very rich in cysteine, and is an excellent scavenger of $^{\cdot}OH$ (6). Iron and copper ions catalyze the production of $^{\cdot}OH$ from H_2O_2. Zinc is known to compete with both iron and copper for binding to cell membrane, thus decreasing the production of $^{\cdot}OH$ (6).

A few investigators have reported that inflammatory cytokines such as TNF-α (tumor necrosis factor-α) and IL-1β, generated by activated monocytes-macrophages, also are known to produce increased amounts of ROS (23,24). Increases in these cytokines are associated with decreased zinc status in patients.

NF-κB is involved in the expression of a variety of responsive specific genes and is activated by several stimuli such as cytokines, radiation, and oxidative stress. *In vitro* activation of NF-κB by TNF-α in MNC has been shown to be an excellent model of oxidative stress-sensitive transactivating factor, and has been used to evaluate the efficacy of compounds in protecting cells from oxidative stress (21). Zinc has been shown to inhibit NF-κB activation in prostate cancer cells, thus enhancing anti-cancer therapy (25), bovine cerebral epithelial

cells (26), as well as reducing increased levels of activated NF-κB in diabetic CD1 mice (27) and zinc deficient cultured human hepatocellular carcinoma-derived cell line (28).

The induction of NF-κB activation pathway appears to be cell specific and is counterbalanced by concomitant activation of NF-κB activation inhibitors. One such inhibitor of NF-κB activation is A20, a zinc finger-transactivating factor which also binds to DNA, producing the A20 protein which inhibits TNF-α-induced NF-κB activation (29–33). A20 plays an important role in reducing IL-1β- and TNF-α-induced NF-κB activation (29–33).

Our data showed that zinc supplementation to normal healthy subjects a) lowers the oxidative stress-related by-products MDA (malondialdehyde), 4-hydroxyalkenals (HAE), and 8-hydroxy deoxyguanine (8-OHdG) generated by cells and released into the plasma, b) inhibits the induction of TNF-α and IL-1β mRNA in MNCs, and c) exhibits a protective effect against TNF-α-induced NF-κB activation in isolated MNCs (34). In addition, we provided evidence to show that, in the human pro-myelocytic leukemia cell line HL-60 which differentiates to the monocyte-macrophage phenotype by PMA, zinc increased the expression of A20 and the binding of A20 transactivating factor to DNA, thereby enhancing inhibition of induced NF-κB activation (34).

The role of zinc in regulation of the gene expression of IL-1β and TNF-α has not been defined. The zinc finger protein A20 has been shown to inhibit NF-κB signaling by TNF-α and IL-1β via TNF-receptor gene (TRAF pathways) in endothelial cells (31,35). A20 is expressed in various types of cells in response to a number of stimuli such as TNF-α, IL-1β, LPS (lipopolysaccharide), PMA, Epstein-Barr virus latent membrane protein, as well as other stimuli (32). A20 expression primarily protects cells from TNF-α-induced cytotoxicity by decreasing the activation of NF-κB, which leads to decreased IL-1β and TNF-α gene expression as has been demonstrated in endothelial cells. We propose that a similar effect of zinc supplementation on the A20 pathway occurs in primary cells. Further studies are ongoing in our laboratory to aid in the understanding the mechanisms of zinc action.

Our study provides molecular evidence for an anti-oxidant effect of zinc in human subjects and shows that zinc supplementation *in vivo* protected MNC against oxidative stress (34). Although there are several possible biochemical mechanisms by which zinc may decrease oxidative stress in cells, our study shows that zinc negatively regulates gene expression of inflammatory cytokines such as TNF-α and IL-1β, which are known to generate ROS and this may be one additional mechanism by which zinc may be functioning as an antioxidant in humans. Thus, our study provides rationale for use of zinc in therapeutic trials either alone or in conjunction with other modalities in chronic diseases, including chemo-prevention of cancer in which oxidative stress is known to play an important role.

The immunological hallmarks of zinc deficiency in humans and higher animals include thymic atrophy, lymphopenia, and compromised cell- and antibody-mediated responses that result in increased incidences of infections (36). In chronic zinc deficiency, a reprogramming of the immune system occurs, beginning with the activation of the stress axis and chronic production of glucocorticoids that accelerate apoptosis of pre-B and pre-T cells (36), resulting in reduced lymphopoeisis and atrophy of thymus. In contrast, myelopoesis is preserved. Changes in the gene expression for cytokines, DNA repair enzymes, zinc transporters, and signaling molecules, suggest that the cells of the immune system are attempting to adapt to the stress of suboptimal zinc (36).

We have observed that, as a result of zinc deficiency, the macrophages-monocytes are stressed and they generate inflammatory cytokines such as TNF-α and IL-1β (14,34). In a recent study, we reported that, in comparison to the younger adults, the elderly subjects had lower plasma zinc, increased oxidative markers, and increased generation of inflammatory cytokines (14). Following zinc supplementation to the elderly subjects, the plasma zinc increased, oxidative stress markers decreased, and generation of inflammatory cytokine decreased, in comparison to the placebo group.

In a large study organized by the National Eye Institute, NIH, (Bethesda, MD, USA) it was reported that zinc and antioxidants (vitamin C, vitamin E, and β carotene) significantly reduced the odds of developing advanced age related macular degeneration (AMD), and prevented blindness in the high-risk group of elderly subjects (37). Also, it was reported that longevity was increased in the zinc supplemented group (38). Although the mechanism of zinc effect was not defined, one may hypothesize that zinc reduced the oxidative stress and thus was beneficial in AMD.

ROLE OF ZINC IN SUPPRESSING ALLOGENEIC REACTION WITHOUT AFFECTING THE ANTIGENIC RESPONSE

Faber *et al*.(39) have shown that *in vivo* zinc supplementation in therapeutic levels resulted in blocking allogeneic response while maintaining the antigenic potency. This observation may have future implication in transplantation subjects where a selective suppression of allogeneic response is desired.

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