Marginal Zinc Deficiency Exacerbates Experimental Colitis Induced by Dextran Sulfate Sodium in Rats

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Abstract

We investigated the impact of Zn status on the maintenance of mucosal homeostasis. Rats were fed diets containing different amounts of Zn (30, 10, 5, <1 mg Zn/kg diet) for 21 d. Serum Zn concentrations were lower in rats fed marginally Zn-deficient (MZD; 5 mg Zn/kg diet) and severely Zn-deficient (<1 mg/kg) diets but not in those fed the marginally Zn-adequate diet (10 mg/kg) or the Zn-adequate (ZA; 30 mg/kg) group (P < 0.05). However, organ weights, colonic epithelial cell proliferation, and crypt fission did not differ between the MZD and ZA groups. We then evaluated whether MZD modulated dextran sulfate sodium (DSS)-induced colonic inflammation by administering 2% DSS to the MZD and ZA groups for 7 d. Myeloperoxidase activity and TNFα production increased in response to DSS in the MZD group (P < 0.03). Colonic permeability in the 2 groups did not differ after DSS administration. In a culture experiment using isolated mesenteric leukocytes, TNFα production was higher (P < 0.05) and TNF receptor type I (TNFR1) expression was detected in culture medium containing 20 and 30 μmol/L of Zn compared with culture medium lacking Zn supplementation. These results suggest that MZD exacerbated colitis by modulating the immune response through the impairment of TNFα production and TNFR1 expression rather than through the impairment of epithelial barrier function.

Introduction

Marginal Zn deficiency is prevalent in infants, children, women, and elderly people due to high nutrient requirements or compromised absorptive functions. A recent study demonstrated that ~20% of the Japanese population was marginally Zn deficient (MZD) and 10% was severely Zn deficient (ZD). Over 25% of the global population is at high risk of ZD, which constitutes a public health problem. Epidemiological studies have revealed associations between low blood Zn concentrations and increased risk of cancer. Reduced dietary intake, impaired absorption, and/or increased excretion of Zn have been observed in patients suffering from inflammatory bowel disease (IBD). These symptoms are also found in ZD, although the precise contribution of Zn to IBD has not been determined to date.

Dextran sulfate sodium (DSS)-induced colitis exhibits many morphological and pathophysiological features that are similar to IBD, such as mucosal damage, ulceration, leukocyte infiltration, and inflammatory cytokine production. The migration of polymorphonuclear neutrophils (PMN) into the mucosa is a pathological hallmark of IBD that is commonly observed in the lamina propria and epithelial layer. The pathophysiological roles of TNF receptors type I (TNFR1) and type II (TNFR2) in innate immune pathways differ during acute colitis. TNFR2-mediated activation of innate immune cells in the colon contributes to the exacerbation of colitis, and TNFR1 expression in lamina propria cells is required for recovery following acute intestinal injury.

The oral or intrarectal administration of Zn has been shown to ameliorate colonic inflammation in experimental colitis. Although the effects of Zn supplementation on IBD have been investigated, the influence of ZD on colitis remains unclear. Furthermore, ZD has been shown to enhance lipid peroxide production and reduce metallothionein levels in rat livers. It has also been shown to increase oxidative damage, as assessed by the free radical-mediated oxidation of arachidonic acid. These processes suggest that Zn protects tissues from oxidative damage. These results must be carefully interpreted, because severe ZD may trigger complex and diverse responses in the body, such as anorexia with retarded growth. In a previous study, we demonstrated that rats fed an MZD diet (5 mg Zn/kg...
diet) had low levels of serum Zn despite body weight gains similar to rats fed a Zn-adequate (ZA) diet (17). The present study evaluated whether marginal Zn deficiency played a role in the pathogenesis of DSS-induced colitis, with a focus on immune responses and intestinal epithelial homeostasis.

Materials and Methods

Rats and diets. Three-week-old male WKAH/Hktm Scl rats (Japan SLC) were individually housed in stainless-steel cages with wire-mesh bottoms. The cages were located in a room with controlled temperature (22 ± 2°C), relative humidity (40–60%), and lighting (lights on 0800–2000 h) throughout the study. The diets were based on modified AIN-93G rodent diets (18) for growing rats, formulated with egg whites and ZnCO₃ (Wako Pure Chemical Industries) (Supplemental Table 1). The rats had free access to food and deionized water. This study was approved by the Hokkaido University Animal Committee, and the rats were maintained in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

Organ weight, Zn concentrations, and epithelial homeostasis in response to dietary Zn. After 7 d of adaptation, the rats (n = 6/group) were randomly assigned to the following 4 dietary groups: a ZA diet (30 mg Zn/kg diet), an MZD diet (5 mg/kg), and a severely ZD diet (≤1 mg/kg). Additionally, a group of rats [pair-fed (PF)] was fed the ZA diet to match the mean food intake of ZD rats on the previous day. Body weight and food intake were measured for 21 d. On the last day of the experimental period, the rats were killed using pentobarbital anesthesia [Nembutal (sodium pentobarbital), 50 mg/kg body weight; Abbott Laboratories].

Tissue and blood collection. Blood samples were collected from the tail vein, and the serum was frozen at −80°C until analysis. The liver, kidney, thymus, femur, small intestine, cecum, and colon were weighed. Liver samples were immediately frozen at −80°C pending analysis of their Zn concentrations.

Serum and liver Zn concentrations. Serum Zn concentrations were determined with a Wako Zn test (Wako Pure Chemical Industries) and liver Zn concentrations were measured with an atomic absorption spectrophotometer (Z-5310; Hitachi Kyowa Engineering). First, the freeze-dried livers were weighed and milled to a fine powder. Then, samples (1.0 g) of the powdered livers were dry-ashed for 30 h in a muffle furnace (EYELA, TMF-3200; Tokyo Rikakikai), heated with 20% HCl samples (1.0 g) of the powdered livers were dry-ashed for 30 h in a muffle furnace (EYELA, TMF-3200; Tokyo Rikakikai), heated with 20% HCl in deionized water for 7 d (22). Briefly, the DSS colitis using a previously described qualitative disease activity index (DAI) scoring procedure (22). The tissues were transferred to a slide glass, gently flattened beneath a cover slip, and examined under a microscope. The number of fissioning crypts in a sample of 200 crypts was scored to determine the fission rate.

Crypt fission. Fissioning crypts were determined as previously reported (21). Small pieces of colon were fixed overnight with Carnoy's fluid. The specimens were cut open along the mesenteric border to expose a circular area (0.64 cm²) of epithelium. The serosal and mucosal sides of the specimens were bathed in 1 mL HBSS at pH 7.4 and 37°C to mimic physiological conditions and the medium was continuously exposed to oxygen gas. After stabilization for 10 min, the medium was replaced with HBSS. Lucifer yellow (LY; final concentration of 100 μmol/L) was added to the mucosal chamber at the beginning of incubation. At 2 h after incubation, the concentration of LY in the serosal chamber was determined by measuring its fluorescence (CAB-110; JASCO International). The mucosal flux was calculated as nmol/h · cm⁻² surface area).

Expression of TNFα mRNA in colonic mucosa. The colonic mucosa was scraped with a glass slide and kept at −80°C until extraction of RNA. Total RNA was isolated from the mucosa with an RNeasy mini kit (QIAGEN) and converted to cDNA using a random hexamer (Toyobo Biologics) and ReverScript 1 (Wako Pure Chemical Industries). The cDNA was amplified using a specific primer pair for GAPDH (NM_0170008; forward: 5'-acctgtggtcatcacaagc-3', reverse: 5'-caccatacacaacaagg-3', annealing temperature 51°C, 360 bp) or TNFα (NM_012775; forward: 5'-caccatacacaacggctgtac-3', reverse: 5'-acctggtgttctgacagc-3', annealing temperature 47.5°C, 172 bp) to make a standard curve for the cDNA or for RT-PCR analyses. In a separate experiment, the cDNA templates were filtered with a MicroSpin S-300 HR column (Amersham Pharmacia Biotech) before qPCR analysis. qPCR was performed using a Mx3000P real-time PCR system (Stratagene) with TaqMan Gene Expression Assays for TNFα (assay ID: Rn00562055_m1) and GAPDH (assay ID: Rn99999916_s1) as an endogenous control according to the manufacturer’s instructions. The PCR consisted of one 10-min cycle at 95°C, followed by 50 30-s denaturation cycles at 95°C, 40 s of primer annealing at 35°C, and 45 s of extension at 72°C. Relative expression levels were calculated for each sample after normalization against GAPDH using the standard-curve method.

Leukocyte isolation from mesenteric lymph nodes. Leukocytes were collected as previously described (26). In brief, mesenteric lymph nodes were removed and homogenized in a glass homogenizer with a complete RPMI1640 medium (Gibco; Invitrogen) supplemented with

MPO activity. MPO activity was determined as previously described (23) and expressed as the initial velocity of absorbance increase at 655 nm (A₆₅₅/min·g pellet).

Giemsa staining in colon. Frozen sections of colon were fixed in methanol for 15 min and stained with Giemsa staining solution (Wako Pure Chemical Industries) for 1 h at room temperature. Slides were then dehydrated and mounted.

TNFα production. Colonic mucosa was homogenized in 1 mL of lysis buffer containing 10 mmol/L HEPES, 1 mmol/L EDTA, 5 mmol/L EGTA, 10 mmol/L MgCl₂, 10 mmol/L K₂HPO₄, 150 mmol/L NaCl, 100 mmol/L NaF, and 10 g/L Triton-100 and then centrifuged for 10 min at 5700 × g and 4°C. The supernatants were used to conduct ELISA analyses (RT TNF Alpha US ELISA kit; Invitrogen) according to the manufacturer’s instructions.

Permeability of colonic tissue. The Ussing chamber technique was used to investigate the effect of Zn on permeability in colonic tissues as previously described (24,25). Briefly, rats that had been administered deionized water or 2% DSS were killed and the colons were immediately removed. Each specimen was cut open along the mesenteric border to produce a flat sheet and then rinsed with ice-cold HBSS (pH 7.4) containing 117 mmol/L NaCl, 5.4 mmol/L KCl, 0.4 mmol/L KH₂PO₄, 4.2 mmol/L NaHCO₃, 0.3 mmol/L Na₂HPO₄, 1.3 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, 0.4 mmol/L MgSO₄, 10 mmol/L HEPES, 10 mmol/L D-glucose, and 4 mmol/L L-glutamin. The colonic tissues were mounted onto chambers (Coster) to expose a circular area (0.64 cm²) of epithelium. The serosal and mucosal sides of the specimens were bathed in 1 mL HBSS at pH 7.4 and 37°C to mimic physiological conditions and the medium was continuously exposed to oxygen gas. After stabilization for 10 min, the medium was replaced with HBSS. Lucifer yellow (LY; final concentration of 100 μmol/L) was added to the mucosal chamber at the beginning of incubation. At 2 h after incubation, the concentration of LY in the serosal chamber was determined by measuring its fluorescence (CAB-110; JASCO International). The mucosal flux was calculated as nmol/h · cm⁻² surface area).

Leukocyte isolation from mesenteric lymph nodes. Leukocytes were collected as previously described (26). In brief, mesenteric lymph nodes were removed and homogenized in a glass homogenizer with a complete RPMI1640 medium (Gibco; Invitrogen) supplemented with
Production and mRNA expression of TNFα in ML. ML (6–7 × 10⁶ cells/well) were seeded onto 6-well plates and cultured for 24 h in complete RPMI medium containing 5% FBS and supplemented with ZnCl₂ to adjust Zn concentrations (10, 20, 30, 50 μmol/L). The cells were cultured under a humidified atmosphere of 5% CO₂. The complete RPMI medium contained 5 μmol/l of Zn, nearly the same serum Zn concentration as used for MZD rats. The complete media containing ML were collected in 1.5-mL tubes and centrifuged for 5 min at 400 g at 4 °C. The resulting cell pellets were suspended in 4 mL of 40% Percoll solution, underlayed with PBS. The viability of isolated mesenteric leukocytes (ML) was assessed immediately by Trypan-blue exclusion.

Statistical analyses. Statistical analyses were performed with JMP software (ver. 5.0; SAS Institute). Differences in food intake, body weight, serum Zn concentrations, and organ wet weight compared with the ZA control group were determined using Dunnett’s test. A 2-way ANOVA was used to evaluate in vivo differences in DAI scores (day and Zn), colon lengths, MPO activity, TNFα production, LY permeability, and TNFα expression (Zn and DSS). Differences in TNFα production in the culture experiment were determined with Tukey-Kramer tests. Differences were considered significant at P < 0.05. All data are shown as mean ± SEM.

Results

Body weight and food intake. Food intake was lower in the ZD group beginning on d 4 (Fig. 1A) and body weight was lower on d 6 (Fig. 1B) compared with the ZA group (P < 0.05). Final body weights and daily food intakes were lower in the ZD and PF groups than in the ZA group. At 21 d, body weights in the ZD and PF groups were 49.3 and 52.7% those of the ZA group, respectively (Fig. 1B). Notably, food intake and body weight did not differ between the MZD and ZA groups.

Serum and liver Zn concentrations. Serum Zn concentrations were lower in the ZD and MZD groups than in the ZA group (P < 0.05; Fig. 2). PF did not affect serum Zn concentrations. Although dietary Zn level affected serum concentrations, liver Zn concentrations did not differ among groups (data not shown). We also investigated colonic epithelial cell proliferation with BrdU immunostaining and isolated crypts. BrdU incorporation and crypt fission in the colonic mucosa did not differ among groups (data not shown). Weights of liver, kidney, thymus, femur, small intestine, cecum, and colon were lower in the ZD and PF groups than in the ZA group (P < 0.05). In contrast, organ weights in the MZD and MZA groups did not differ significantly from those in the ZA group (Table 1).

DAI score. In response to DSS administration, DAI scores in the MZD and ZA groups were elevated from baseline values on d 5 (P < 0.05). This elevation was accompanied by an increased frequency of loose stools and occult bleeding (Fig. 3). However, the increase in DAI values was greater in the MZD group than in the ZA group (P < 0.0001).

MPO activity, TNFα production, and LY permeability. DSS treatment decreased colon lengths in the ZA and MZD groups (P < 0.05; Fig. 4A). Colon length was shorter in the MZD group than in the ZA group (P < 0.05). MPO activity increased in response to DSS administration (P < 0.05) but was not affected by dietary ZA (Fig. 4B). TNFα production decreased in the MZD group (Fig. 4C). MZD diet and DSS administration suppressed TNFα mRNA expression in the colonic mucosa (P < 0.05). In particular, DSS treatment decreased TNFα expression in the ZA group (P < 0.05; Fig. 4D). To elucidate the role of Zn in intestinal barrier function, we evaluated LY permeability using isolated colonic tissues from MZD and ZA rats. The addition of DSS to the mucosal side of the chamber increased LY permeability to a larger extent in the MZD group than in the ZA group (Fig. 4D).

FIGURE 1 Food intake (A) and body weight (B) in ZA, MZA, MZD, ZD, and PF rats. Values are means ± SEM, n = 6. *Different from ZA at that time, P < 0.05.

FIGURE 2 Serum Zn concentrations in ZA, MZA, MZD, ZD, and PF rats. Values are means ± SEM, n = 5–6. *Different from ZA, P < 0.05.
Table 1

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<th>ZD</th>
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<td>1.59 ± 0.06</td>
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<td>Femur</td>
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<td>Small intestine</td>
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Values are means ± SEM, n = 6. *Different from ZA, P < 0.05.

Discussion

Zn is an indispensable mineral for gut immunity and free-radical protection and both systems are impaired under ZD conditions (27). In particular, marginal Zn deficiency is physiologically relevant to human Zn deficiency (28). In this study, we demonstrated that in vivo marginal Zn deficiency aggravated colonic inflammation and caused massive PMN accumulation in the colonic mucosa in response to DSS. Lower dietary calcium levels have been shown to increase disease activity in DSS-induced colitis, accompanied by epithelial damage through the markedly increased expression of colonic caspases 3 and 9 (29). In contrast, marginal Zn deficiency did not affect any tested parameter of intestinal epithelial homeostasis, such as epithelial proliferation or mucosal permeability, in the present study.

Experimental studies using growing rats have shown that Zn is stored in skeletal muscle, even when the rats are fed ZD diets (30,31). In vivo, Zn is transiently pooled in bone and/or skeletal muscle and redistributed to other tissues. A reduction of Zn concentrations treated with N,N,N',N'-tetrakis (2-phridylmethyl) ethylenediamine in culture medium has been shown to inhibit cell growth and induce apoptosis in Caco-2 cells (32). Optimal conditions for the maintenance, growth, and functioning of cells have been achieved within a narrow range of intracellular Zn2+ concentrations (33), demonstrating that cell proliferation and death were regulated by Zn concentration in the surrounding environment. These observations indicate that Zn deficiency disturbs cellular function as a signaling molecule. In the present study, an MZD diet resulted in decreased serum Zn concentrations in rats without affecting growth or food intake. Tissue homeostasis outside of the immune system thus appeared to be stable under marginal Zn deficiency. We demonstrated that the MZD diet did not affect BrdU incorporation or the number of crypt epithelial cells. In Ussing chamber analysis, transepithelial electrical resistance and LY permeability in colonic tissues did not differ significantly among the 5 groups (data not shown). These results suggest that barrier functions were maintained in intestinal epithelia, even under MZD or severe ZD conditions.

The immune response may be affected by marginal Zn deficiency in a challenging environment, such as that produced by experimental colitis. The inflammatory process in experimental colitis is often characterized by an increase in colonic permeability, which amplifies the recruitment and activation of PMN (34). Zn is an intracellular signaling molecule (35) involved in immune functions, such as the development and functioning of innate immunity-mediated cells (e.g. PMN, macrophages, NK cells) (36). However, the precise mechanism of immune regulation in the intestinal mucosa under marginal Zn deficiency remains largely unknown. We analyzed the effect of an MZD diet on mucosal immune function in DSS-induced colitis. Large amounts of MPO are stored in azurophilic granules in PMN (37). Massive PMN recruitment into the colonic tissue was further confirmed by increased MPO activity. Additionally, apparent granulocyte infiltration into the lamina propria was observed in MZD rats in response to DSS, although fewer resident immune cells were present in these rats without DSS treatment (Supplemental Fig. 1). Marginal Zn deficiency may have induced an inappropriate immune response characterized by intestinal inflammation. Furthermore, the present study demonstrated that Zn levels affected TNFR1 mRNA expression but not TNFR2 expression. Mice lacking recombinease-activating genes (RAG) and TNFR1 have been shown to exhibit higher mortality rates than RAG-knockout and RAG × TNFR2 double-knockout mice, suggesting that TNFR1-mediated activation of the innate immune response contributed to the prevention of intestinal damage-associated mortality (11). Taken together, these findings suggest that marginal Zn deficiency may impair TNFR1-mediated activation of innate immune pathways and lead to the exacerbation of DSS-induced colitis.
The migration of PMN into inflamed sites depends on several factors, including the presence of various chemokines, upregulation of integrins, and reorganization of the cellular cytoskeleton (38–40). SCFA, produced mainly by the commensal flora of the gut, are recognized by GPR43 (41,42). In vitro experiments revealed a dose-dependent, SCFA-dependent induction of chemotaxis in human PMN expressing GPR43 (43). Increased neutrophil, eosinophil, and monocyte counts in blood were reported in rats fed a ZD diet for 3 wk (44). We confirmed GPR43 expression even under MZD conditions, suggesting that DSS treatment provoked the uncontrolled recruitment of PMN into intestinal mucosa through the activation of GPR43 in response to SCFA under MZD conditions. Furthermore, the absence of metallothionein has been shown to be beneficial in the suppression of colitis in metallothionein−/− mice receiving DSS, suggesting that the presence of metallothionein may have promoted the induction of colitis (14). Marginal Zn deficiency may have modulated metallothionein concentrations and contributed to DSS-induced colitis in our study.

The activity of NF-κB, an important transcription factor involved in the regulation of TNFα expression, decreases with Zn deficiency (45). A Zn-finger transactivating factor, A20 protein, binds to DNA and inhibits TNFα-induced NF-κB activation (46,47). In the present study, we found that marginal Zn deficiency suppressed TNFα production and mRNA expression in the colonic mucosa without DSS treatment. Additionally, IL-1β and IL-6 mRNA expression was lower in the MZD group than in the ZA group (Supplemental Fig. 2). These results suggest that marginal Zn deficiency transcriptionally suppressed TNFα expression. Such dysregulation of the immune response in IBD may induce a shift/imbalance in cytokine profiles at different stages of the disease process.

In summary, we demonstrated that marginal Zn deficiency exacerbated DSS-induced colitis without deterioration of intestinal epithelial cell proliferation. The dysregulation of the immune system under MZD conditions was responsible for the disease process.

Acknowledgments
S.I., K.H., and H.H. designed the research procedures; H.I., M.K., A.S., and J.L. conducted the research; H.I. and S.I. analyzed the data and wrote the manuscript; and S.I. had primary responsibility for its final content. All authors read and approved the final manuscript.

Literature Cited


