Zinc deficiency induces dysregulation of cytokine productions in an experimental colitis of rats

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ABSTRACT
Dextran sulfate sodium (DSS)-induced colitis is an experimental model of ulcerative colitis, although the precise mechanism has not yet been elucidated. We investigate whether Zn deficiency affects the pathogenesis of colitis induced by DSS with a focus on immune responses. Male WKAH/Hkm Slc rats were fed either a Zn-adequate (ZA, 30 mg Zn/kg diet) as a control or Zn-deficient (ZD, 5 mg Zn/kg diet) diet for 21 days and then treated with 2% DSS via deionized drinking water for 7 days. The disease activity index (DAI) was recorded daily throughout DSS treatment. Serum Zn concentrations were significantly lowered in rats fed the ZD diet than those fed the ZA diet at day 7 and 14. Surprisingly, DSS treatment considerably reduced the serum Zn in both groups. The rats fed the ZD diet showed exacerbated colitis based on clinical outcomes, including weight loss, increased DAI, and shortened colon length. An in vitro study corroborated these results, showing that a large amount of TNFα was induced by rat mesenteric leukocytes in response to lipopolysaccharide in ZD medium, but not in ZA medium. These results indicate that modulation of TNFα production due to Zn deficiency influences disease activity in DSS-induced colitis. In addition, more attention should be given to Zn for prevention of colitis.

Inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn’s disease, are considered chronic inflammatory disorders of the intestine (3, 23). The pathogenic mechanism of IBD is presumably the dysregulation of intestinal immune responses to intestinal environmental antigens, such as intestinal microflora. This immune response is characterized by the activation of lymphocytes, macrophages, enterocytes, and endothelial cells, which induce the production of inflammatory mediators, such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, and IL-6 (1, 21). However, the fundamental role of nutrition in relation to IBD pathology is not well understood.

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Zinc (Zn) is an essential nutrient that plays multiple roles in immune responses, including anti-inflammatory effects (26). Zn also acts as growth cofactor, anti-apoptotic, and cytoprotectant with antioxidant (29). Zn deficits in IBD patients result in reduced dietary intake, absorption impairment, and/or increased Zn excretion (8, 16). Epidemiological studies have revealed associations between low blood Zn concentrations and an increased risk of sepsis (2) and cancer (28). Consistent with literature reporting human IBD, experimental animals with marginal Zn deficiency after chronic exercise exhibit oxidative DNA damage in the prostate (25). It has also been shown that marginally Zn-deficient rats experience increased susceptibility to acute lipopolysaccharide (LPS)-induced liver injury (24). Nevertheless, the precise contribution of Zn deficiency to IBD is still not completely understood.

An increasing number of associations have been drawn between diseases and Zn status. Similarly, normal health has been attributed to Zn and its po-
tential efficacy in preventing certain conditions; Zn compounds show pharmacologic promise (15). Thus, the aim of the present study is to investigate changes in the Zn status under the pathogenesis of colitis induced by dextran sulfate sodium (DSS). Furthermore, leukocytes isolated from rat mesenteric lymph nodes were examined to elucidate the direct response to LPS exposure under different Zn concentrations, focusing on cytokine productions.

MATERIALS AND METHODS

Animals and diets. WKAH/Hkm Slc male rats (3 weeks old; Japan SLC, Hamamatsu, Japan) were individually housed in stainless steel cages with wire mesh bottoms. The cages were located in a room with controlled temperature (23 ± 1°C), relative humidity (55 ± 5%), and lighting (lights on from 8:00–20:00 h) throughout the study. Test diets were modified based on AIN-93G rodent diets (22), formulated with egg whites and ZnCO₃ (Wako Pure Chemical Industries, Osaka, Japan). The rats (n = 6/group) were divided into the following 2 groups based on body weight (Table 1): Zn-adequate (ZA) diet as a control group and Zn-deficient (ZD) diet. The rats had free access to each test diet for 28 days. Body weight and food intake were measured daily. On the last day of the experiment, blood was drawn from the abdominal aorta under anesthesia (Somnopentyl: sodium pentobarbital, 0.77 mL/kg body weight; Kyoritsu Seiyaku Corporation, Tokyo, Japan) for the measurement of serum Zn concentrations, and the rats were then sacrificed by exsanguination. Colon length was measured using an experimental ruler. The mucosa of the colon was scraped using a sterilized glass slide and subjected to preparations of myeloperoxidase (MPO) activity assay. The liver, spleen, femur, and cecum were weighed and immediately frozen at −80°C. In a separate experiment, isolated leukocytes from WKAH/Hkm Slc male rats (5 weeks old; Japan SLC) were analyzed to evaluate cytokine productions in response to stimulants under different Zn concentrations in vitro. All experimental procedures in this study were approved by Hokkaido University Animal Committee, and were conducted in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

Induction of colitis. To induce colitis, all rats consumed 2% (w/v) DSS (MW 36,000–50,000; MP Biomedicals, Tokyo, Japan) dissolved in deionized drinking water between day 21 and day 28 after a 21-day adaptation to the ZA or ZD diet. New DSS solution was prepared every 2 days. Weight loss, stool consistency, and presence of fecal bleeding were recorded daily for each rat. These parameters were each assigned a score according to the criteria proposed by Murthy et al. (17), which was used to calculate the disease activity index (DAI).

Measurement of Zn concentrations in serum, liver, and femur. Serum was separated from blood collected from the tail vein at day 7 and 14 and from the abdominal aorta at the end of the experiment (day 28). Serum Zn concentration was determined using a commercially available kit (Wako Zn test, Wako Pure Chemical Industries). Zn concentrations in organs (liver and femur) were measured with an atomic absorption spectrophotometer (Z-5310; Hitachi Kyowa Engineering, Ibaraki, Japan). Freeze-dried organs were weighed and subsequently ashed for 30 h in a muffle furnace (TMF-3200; EYELA, Tokyo, Japan). The resulting samples were further heated with 20% HCl at 120°C until evaporated, and they were then dissolved with 3% HCl solution. Zn concentration in the samples was measured following appropriate dilution.

MPO activity. MPO activity was determined as

<p>| Table 1 Composition of test diets |
|-------------------------------|----------------|</p>
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>ZA diet (control)</th>
<th>ZD diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg white</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Dextrin</td>
<td>600.5</td>
<td>600.5</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Crystalline cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture (Zn free)</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Biotin-sucrose mixture</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Zn-sucrose mixture</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>94</td>
<td>99</td>
</tr>
</tbody>
</table>

Abbreviations: ZA diet = Zn adequate diet; ZD diet = Zn deficient diet

1 Taiyo Kagaku, Yokkaichi, Japan
2 TK-16; Matsutani Chemical Industry, Itami, Japan
3 I-Oil Mills, Tokyo, Japan
4 Ceolus PH102; Asahi Chemical Industry, Tokyo, Japan
5 ZnCO₃ 5 g/kg sucrose
6 Nippon Beet Sugar Manufacturing, Tokyo, Japan
previously described (4). In brief, the scraped colonic mucosa was placed in 10 times its volume of 100 mmol/L potassium phosphate buffer (pH 7.4) and homogenized with a hand-held homogenizer. The homogenate was centrifuged at 20,000 × g for 20 min, and the supernatant was removed. A 10 times homogenate volume of 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and 10 mmol/L ethylenediaminetetraacetic acid was added to the pellet and homogenized. The resulting homogenates were frozen and stored at −80°C until analysis. The reaction mixture for the MPO activity assay contained 80 mmol/L PBS (pH 5.4) with 0.5% HETAB (420 μL), 10 mmol/L of 3,3′,5,5′-tetramethylbenzidine dissolved with N,N-dimethylformamide (80 μL), and 15% hydrogen peroxide solution (50 μL). The reaction mixture was added to the homogenate (50 μL) and then incubated for 3 min at 37°C. After the incubation period, the reaction was stopped by adding 2 mL of 0.2 mol/L cold sodium acetate buffer (pH 3.0) to the mixture. Following the same procedure, a control that did not contain colonic mucosa was prepared. MPO activity was expressed as the initial velocity of increasing absorbance at 655 nm [A_{655}/(min • g pellet)].

Preparation of leukocytes isolated from mesenteric lymph nodes. After an acclimation period, the rat was decapitated (10:00–11:00 h) under diethyl ether (Kanto Chemical Co., Inc., Tokyo, Japan) anesthesia, and the mesentery was removed. Leukocyte isolation was performed as previously described (14) with a slight modification. In brief, mesenteric lymph nodes were collected and homogenized in a glass homogenizer with RPMI-1640 medium (pH 7.4; Gibco, Invitrogen) containing 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 23.8 mM sodium bicarbonate, 0.17 mM streptomycin sulfate, 0.01 mM penicillin G potassium, and 50 μM 2-mercaptoethanol. Cell suspensions were then passed through a cell strainer (40 μm nylon mesh; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged at 400 × g for 5 min at 4°C. The resulting cell pellets were suspended in 3 mL of 40% Percoll solution, underlaid with 2 mL of 75% percoll solution, and centrifuged at 1,900 × g for 20 min at room temperature. Following density gradient centrifugation, the leukocytes located within the interface between 40% and 75% percoll solutions were collected and washed with RPMI-1640 medium. The viability of isolated mesenteric leukocytes was assessed immediately by trypan blue exclusion. The purity of the leukocytes was also evaluated using a FACSCalibur (Becton, Dickinson and Company), and the data (300,000 total events) were analyzed using the flow cytometry analysis software FlowJo (version 7.2.5; TreeStar Inc., Ashland, OR, USA).

Cytokine productions by mesenteric leukocytes in response to stimuli under various Zn concentrations. Isolated leukocytes (1 × 10⁶ cells/mL) were further cultured in sterile 1.5 mL microcentrifuge tubes at 37°C and 5% CO₂ in the following stimulants-containing medium: 0.1 μg/mL LPS (Escherichia coli 0111:B4, Sigma) or, for the unstimulated control, complete RPMI-1640 medium (pH 7.4; Gibco, Invitrogen, Camarillo, CA, USA) containing 5% (v/v) heat-inactivated fetal bovine serum (FBS). To assign the ZA or ZD condition as seen in the in vivo study, each culture medium was supplemented with 50 μM ZnCl₂. All culture media were based on complete RPMI-1640 containing 5% FBS. After a 48 h incubation period, the supernatants were collected for the measurement of cytokine productions including TNFα, IL-10, IL-1β, and interferon gamma (IFNγ). Each cytokine was assayed according to the ELISA kits manufacturer’s instructions. ELISA kits for the following cytokines were used in this study: TNFα (Rat TNFα, Thermo Fisher Scientific Inc., Rockford, IL, USA), IL-10 (Rat IL-10, Thermo Fisher Scientific), IL-1β (Rat IL-1β, Thermo Fisher Scientific), and IFNγ (Rat IFNγ, Invitrogen).

Statistical analysis. Statistical analysis was performed with JMP software (version 5.0; SAS Institute). Differences in the following variables compared with the ZA control group were determined using Student’s t-test: differences in dietary Zn, body weight, food intake, Zn intake, organ wet weight, serum Zn concentrations at different time points, colon length, MPO activity, Zn level in medium, and cytokine production in culture medium. In addition, a Dunnett’s test was used to compare serum Zn concentrations at different time points against day 7 and cytokine production in culture medium compared to the control group. A two-way analysis of variance (ANOVA; Zn treatment and time) was used to evaluate differences in DAI score. A difference with P < 0.05 was considered significant. All values are expressed as the mean ± SEM.

RESULTS

Body weight, food intake, and organ weights
The in vivo study was conducted to examine the im-
Table 2  Dietary Zn, initial body weight, final body weight, food intake, Zn intake, liver weight, and spleen weight

<table>
<thead>
<tr>
<th>Diet</th>
<th>Dietary Zn μmol/g diet</th>
<th>Body weight (BW)</th>
<th>Food intake</th>
<th>Zn intake</th>
<th>Liver weight</th>
<th>Spleen weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-day</td>
<td>7-day</td>
<td>14-day</td>
<td>28-day (DSS)</td>
<td>g/day</td>
</tr>
<tr>
<td>ZA</td>
<td>0.27 ± 0.01</td>
<td>47.1 ± 1.3</td>
<td>73 ± 2</td>
<td>105 ± 2</td>
<td>153 ± 3</td>
<td>10.5 ± 0.4</td>
</tr>
<tr>
<td>ZD</td>
<td>0.05 ± 0.01*</td>
<td>46.8 ± 1.5</td>
<td>65 ± 2*</td>
<td>80 ± 2*</td>
<td>87 ± 4*</td>
<td>7.1 ± 0.2*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM, n = 4–6. * vs. the ZA value (P < 0.05, Student’s t-test)

Fig. 1  Serum Zn concentrations in rats fed ZA and ZD diets. Zn concentrations in the serum collected from the tail vein at day 7 and 14 and from the abdominal aorta at day 28 were measured after the start of feeding. Administration of DSS was applied from day 21 to 28. Each value represents the mean ± SEM, n = 4–6. * vs. the value at day 7 in ZA and ZD groups (P < 0.05, Dunnett’s test). # vs. the ZA value (P < 0.05, Student’s t-test).

Impact of a ZD diet and acute treatment with 2% DSS to induce colitis. Body weight (at day 7, 14, and 28), food intake, and Zn intake were lower in the rats fed the ZD diet than in those fed the ZA control diet (P < 0.05, Student’s t-test, Table 2). Although the dietary Zn level affected body weight, the ZD group showed a similar liver weight to the ZA control group. Additionally, cecal weight (g/100 g BW) did not differ between the ZD and the ZA groups (data not shown). In contrast, spleen weight was much lower in the rats fed the ZD diet than in those fed the control diet. On the last day of the experimental period, 2 rats from the ZD group were confirmed to be in postmortem state due to colonic inflammation.

Zn concentrations in serum, liver, and femur

The ZD diet gradually decreased serum Zn concentrations during the experimental period (Fig. 1). Serum Zn concentration at day 7 of the ZD group was significantly 2.0-fold lower than the ZA control group. After 2 weeks of ZD diet feeding, the serum Zn concentration dropped to 3.3-fold lower than the control rats, confirming the development of Zn deficiency in the ZD rats. Surprisingly, DSS treatment considerably reduced serum Zn by up to 51% as measured from day 14 not only in the rats fed the ZD diet, but also in those fed the ZA control diet. Such a reduction in serum Zn also affected Zn homeostasis in the liver and femur. Zn concentrations in the liver and femur of ZD rats were 2.4- and 3.8-fold lower, respectively, than those of the corresponding ZA control rats (data not shown).

DAI, colon length, and MPO activity

The induction of colitis significantly altered food intake, body weight, stool consistency, and fecal bleeding. In response to DSS administration, DAI score in the ZD group was first elevated from baseline at day 22. DAI score elevation was attributed to a lack of appetite resulting in weight loss (Fig. 2A). Both groups showed a progressively increasing DAI from day 25 until the end of the study (day 28). During colitis induction, the ZD group tended to be similar to the ZA control group with respect to rectal bleeding; however, weight loss and stool consistency were more extreme for the ZD rats (data not shown). Eventually, the increase in DAI score was greater in the rats fed the ZD diet than in those fed the control diet (P < 0.05, two-way ANOVA, Fig. 2A). A prior result has shown that low serum Zn (less than 10 μmol/L) is observed in the rats fed the ZD diet (Fig. 1), suggesting that increased DAI score are inversely related to decreased serum Zn. In fact, when a regression slope test between these variables was carried out, a distinct correlation between decreased serum Zn and increased DAI score (R² = 0.42, P = 0.043) was observed (Fig. 3).

Shortening of colon length, one of the macroscopic signs of colitis, was more evident in the rats fed the ZD diet (P < 0.05, Student’s t-test, Fig. 2B). No difference was observed in MPO activity for either group (Fig. 2C).

Cytokine productions in culture media under various Zn concentrations

The in vitro study was conducted to clarify the di-
Zn deficiency and cytokines in colitis

Fig. 2 Clinical parameters following DSS administration from day 21 to day 28 in rats fed ZA and ZD diets. Changes in DAI score (A) [combined scores of weight loss, stool consistency, and rectal bleeding] based on Murthy et al. (17) criteria. Probability was shown in Zn treatment (Zn) and time (Day) estimated by two-way ANOVA. N.S., not significant. Colon length (B) and mucosal MPO activity (C) in rats treated with DSS. Each value represents the mean ± SEM, n = 4–6. *vs. the ZA value (P < 0.05, Student’s t-test).

DISCUSSION

Zn has a variety of effects on the immune systems in vivo and in vitro, and these effects depend primarily on the Zn concentration in the cellular environment (6). It is reported that all kinds of immune cells show decreased function after Zn depletion (11). In this study, we showed that ZD modulated immune responses, resulting in aggravation of experimental colitis induced by DSS. We further proved with in vitro study that the aggravation of colitis was closely associated with dysregulated cytokine production, especially with respect to TNFα signaling.

A previous study from our laboratory has shown that rats fed a marginally Zn-deficient diet had low levels of serum Zn, despite body weight gains that were similar to control or rats fed adequate Zn (9). In this study, feeding the rats a ZD diet resulted in a decreased level of serum Zn. Interestingly, DSS exposure also substantially reduced serum Zn in both the rats fed the ZD diet and in those fed the ZA control diet. The reduction rates were nearly the same in both groups. Prolonged low Zn intake deprives an organism of potential localized Zn benefits, including interactions with oxidative free radicals and nitric oxide metabolism (27).

All rats demonstrated diarrhea and rectal bleeding, which characterized the severity of colitis symptoms. We found that this bleeding and stool inconsistency was amplified in the ZD rats; being associated with the higher elevated DAI observed between day 25 and day 28. Moreover, 2 rats from the ZD group were found in a postmortem state prior to sacrifice at the end of the study. The DAI evaluation showed lower values in the ZA control group, suggesting that Zn may act as an intracellular signaling molecule (13) in immune activation, including the development and functioning of innate immunity-mediated cells, for instance, polymorphonuclear neutrophils (PMN), macrophages, and natural killer cells (20). However, the precise mechanism by which Zn regulates immune function during the disease activity remains largely unknown.

In the present study, a clear correlation was drawn between increased serum Zn concentrations and decreased DAI scores. To the best of our knowledge,
find any difference in MPO activity in either the ZA control or ZD groups. Additionally, granulocyte infiltration in the intestinal mucosa appeared nearly the same in both groups (data not shown). These results suggest that the number of granulocytes recruited to the inflamed mucosal site may be the same, but the immune response between the groups has to be totally different.

To explore the mechanisms underlying ZD diet-aggravated experimental colitis, we evaluated the response of immune cells against LPS in various Zn concentrations. Cell culture experiments confirmed that even though the number of immune cells was the same, the response of immune cells against the stimulants was different. These different responses are strongly dependent on the surrounding Zn condition. We clearly demonstrated that LPS-stimulated leukocytes dramatically increased TNFα production in the ZD medium, but not in the ZA medium. The result corroborates those of a previous report showing that marginal Zn deficiency exacerbates experimental colitis (12). Additional studies (5, 10) have shown that Zn supplementation decreases LPS-, reactive oxygen species (ROS)-, or TNFα-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in endothelial and cancer cells. Prasad and co-workers (18) also reported that, compared with placebo-treated volunteers, healthy volunteers supplemented with 45 mg Zn/day had
significantly reduced levels of TNFα and IL-1β mRNA expressions as well as TNFα-induced NF-kB DNA binding in isolated peripheral mononuclear cells; and Zn upregulated the expression of A20, a Zn finger-transactivating factor, in human promyelocytic leukemia cells (HL-60).

In this study, TNFα signaling impaired by ZD medium was seen concomitantly with an increase in production of IL-10, a prominent immunoregulator in the gastrointestinal tract. This result is consistent with another study (19), which demonstrated a decrease in IL-10 production was found in Zn-supplemented elderly subjects, and that this decrease may also have an effect of increasing IL-2 production. Taken together, the simultaneous increase in the TNFα and IL-10 production is a potential cause for the considerable immune dysfunction observed in the rats fed the ZD diet.

In conclusion, although the requirement of Zn in the immune system has been investigated for decades, recent work has broadened our knowledge about the unique capacities of Zn in regulating the immune response to various extents. Our findings provide evidence that feeding rats a ZD diet accompanied by an induction of DSS exacerbates acute colitis. Clinical outcomes included weight loss, reduction of serum Zn, increased DAI, and a shortening of colon length. Exacerbation of colitis may have been mediated by dysregulated cytokine productions through promotion of TNFα signaling. In addition, Zn concentration should be taken into account whenever complex alterations of immune functions are perceived in vivo or in vitro.

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REFERENCES


