Zinc Deficiency Induces Membrane Barrier Damage and Increases Neutrophil Transmigration in Caco-2 Cells\textsuperscript{1,2}

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Abstract

Zinc may contribute to the host defense by maintaining the membrane barrier. In this study, we questioned whether zinc deficiency affects the membrane function and junctional structure of intestinal epithelial cells, causing increased neutrophil migration. We used the Caco-2 cell line grown in control (C), zinc-deficient, or zinc-replete medium until differentiation. Zinc deprivation induced a decrease of transepithelial electrical resistance and alterations to tight and adherens junctions, with delocalization of zonula occludens (ZO-1), occludin, β-catenin, and E-cadherin. Disorganization of F-actin and β-tubulin was also found in zinc deficiency. These changes were associated with a loss of the amounts of ZO-1, occluding, and β-tubulin. In addition, zinc deficiency caused a dephosphorylation of occludin and hyperphosphorylation of β-catenin and ZO-1. Disruption of membrane barrier integrity led to increased migration of neutrophils. In addition, zinc deficiency induced an increase in the secretion of interleukin-8, epithelial neutrophil activating peptide-78, and growth-regulated oncogene-α, alterations that were not found when culture medium was replete with zinc. These results provide new information on the critical role played by dietary zinc in the maintenance of membrane barrier integrity and in controlling inflammatory cell infiltration. J. Nutr. 138: 1664–1670, 2008.

Introduction

Zinc, a trace element present in all body tissues and fluids, is essential for the survival and function of cells. Its importance is emphasized in conditions of zinc deficiency, when the gut, brain, and immune system may be impaired, depending on the severity of the deficiency (1,2). Zinc depletion may contribute to the severity of infectious diseases and mortality in malnourished children and zinc supplementation has been shown to reduce the severity of diarrhea and the incidence of infections (3). Zinc deficiency is estimated not only to be present in developing countries but also to occur frequently throughout the world as mild to moderate deficiency in the elderly, in subjects suffering from cancer or sickle cell anemia, in patients during the acute-phase response to infection and inflammation, and in some chronic diseases such as asthma and diabetes (4–6). In addition, low zinc concentrations have frequently been associated with inflammatory bowel disease (IBD)\textsuperscript{5} and in Helicobacter pylori-induced gastric mucosa inflammation (7,8).

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\textsuperscript{5}Abbreviations used: AJ, adherent junction; C, control; ENA-78, epithelial neutrophil activating peptide-78; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FMLP, N-formyl-methionyl-leucyl-phenyl-alanine peptide; GRO-α, growth-regulated oncogene-α; IBD, inflammatory bowel disease; IL, interleukin; PMN, polymorphomononuclear cells; RIPA, cold radio-immune precipitation buffer; TEER, transepithelial electrical resistance; TJ, tight junctions; ZD, zinc-deficient cells; ZD-R, zinc-replete cells; ZO, Zonula occludens.

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Zinc may contribute to the host defense by maintaining the structure and function of the membrane barrier (7,9–11) and this is particularly important in the intestine, which is continuously exposed to a myriad of pathogens and noxious agents. The manner in which the intestinal epithelium constitutes a barrier involves intercellular junctional complexes between neighboring cells that provide a continuous seal around the apical region of the cells (12,13). These complexes are composed of several units, including the tight junctions (TJ) and adherens junctions (AJ) that form circumferential zones of contact between adjacent cells. Zonula occludens (ZO) proteins are the major TJ plaque proteins that bind to the transmembrane protein occludin and these interactions are crucial for maintaining TJ structure (14–16). E-cadherin is the main transmembrane adhesion molecule localized at the AJ and its binding to β-catenin is fundamental for appropriate AJ organization (17,18). Bundles of actin form a ring in the apical zone of the cell and link with the TJ and AJ (19). Previous studies have shown that zinc deficiency alters the membrane barrier permeability of endothelial and lung epithelial cells (9) and causes ulcerations of the small intestine (20,21). A recent study has shown that zinc depletion in combination with proinflammatory cytokines enhances degradation of E-cadherin and β-catenin proteins of lung epithelial cells (9). However, it is not clear whether zinc is crucial for the preservation of junctional complexes.

Other than constituting the epithelial barrier, TJ may regulate the passage of polymorphomononuclear cells (PMN), consisting essentially of neutrophils, which are immune cells for the protection against pathogen infection. These cells are...
recruited at sites of injury or inflammation and transmigrate across mucosal epithelia by a process involving remodeling of TJ structure and/or localization of its proteins (19,22). Although the migration of neutrophils represents a first line of defense, a massive and prolonged infiltration of these cells may perpetuate inflammation and ultimately lead to cell damage by releasing mediators such as proteases and cytokines (23,24). Indeed, clinical studies have shown a neutrophil accumulation within epithelial crypts and in the intestinal lumen associated with intestinal disease and epithelial injury (25,26). In previous studies, we have shown that gut membrane damage caused by zinc deficiency is associated with inflammatory cell infiltration (20,21). Interestingly, patients with chronic intestinal permeability disturbances have been shown to have a reduced level of mucosal zinc (27).

Based on these observations, we hypothesized that zinc deficiency may affect the TJ structure of intestinal epithelial cells and consequently allow a more extensive migration of neutrophils. By using an in vitro model of intestinal cells, Caco2 cells, grown in a zinc-deficient (ZD) medium, we found that depletion of zinc strongly affects membrane barrier function and integrity and induces an increase in neutrophil transmigration and an upregulation of chemokines that plays a role in neutrophil migration and inflammatory development.

Materials and Methods

ZD and zinc-replete culture media. To prepare a ZD serum, fetal bovine serum (FBS) was stirred with 10% (wt/v) Chelex-100 resin (Sigma) overnight at 4°C, according to Tate et al. (28). An aliquot of the chelated serum was analyzed for zinc, copper, iron, magnesium, and calcium content by flame atomic absorption spectrometry. Three different media were prepared and analyzed for mineral content. The control (C) medium was the DMEM containing 10% heat-inactivated FBS, 3.7 g NaHCO3/L, 4 mmol glutamine/L, 10 g/L nonessential amino acids, 10^5 U/L penicillin, and 100 g/mL streptomycin). The ZD medium was the C medium in which ZD serum substituted FBS and contained 0.070 ± 0.01 mg/L zinc. The CuCl2, FeSO4, CaCl2, and MgSO4 were added to adjust the mineral concentration to that of the C medium (Cu, 0.02 ± 0.004 mg/L; Fe, 0.26 ± 0.025 mg/L; Ca, 64.5 ± 7 mg/L; Mg, 14.1 ± 1 mg/L). The zinc-replete (ZR) medium was the ZD medium in which ZnSO4 was added to adjust the zinc concentration to that present in the C medium (0.92 ± 0.21 mg/L). All reagents were from Biochrom.

Epithelial cell culture. The human intestinal Caco-2 cells were routinely grown in the C medium and maintained at 37°C in an atmosphere of 5% CO2:95% air at 90% relative humidity. In all experiments, Caco-2 cells were seeded (1.5 × 10^5 cells) on Transwell filters (polyethylene terephthalate filter inserts for cell culture, 3.0-µm pore diameter; Becton Dickinson) and grown in C, ZD, or ZD-R medium. They were named C, ZD, or ZD-R cells, respectively. After confluency, cells were maintained for 18 d to allow differentiation.

Membrane permeability. Membrane barrier permeability of cells grown in the C, ZD, or ZD-R medium was tested by measuring the transepithelial electrical resistance (TEER), according to Ferruzza et al. (29). TEER was monitored every day until differentiation to test the effect of the different media using Millicell Electrical Resistance System (Millipore). TEER was expressed as Ohm (resistance) × cm² (surface area of the monolayer) after subtracting the filter resistance value.

Neutrophil transmigration. Caco-2 cells were differentiated as inverted monolayer on Transwell filters to allow the physiological transmigration of neutrophils from basolateral to apical compartment. We measured neutrophil transmigration as previously described (30). Briefly, neutrophils were isolated from whole blood of healthy volunteers by Ficoll gradient centrifugation, added (1 × 10^6 cells/well) to the basolateral compartment (upper reservoir) of the Transwell filters, and induced to transmigrate by the addition of 1 × 10^-2 mol/L bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma) for 1.5 h. All transmigrated (within the monolayer and apical compartment) and nontransmigrated (basolateral compartment) neutrophils were measured by myeloperoxidase activity. All experiments were performed in HBSS, which eliminates the induction of neutrophil transmigration by the eventual chemokines secreted in the medium. Experiments on human volunteers were approved by the National Ethics Committee. Informed consent was obtained from all participants.

Immunolocalization. Proteins of TJ (ZO-1, occludin), AJ (β-catenin and E-cadherin), and cytoskeleton (F-actin and β-tubulin) were analyzed by immunofluorescence analysis as described previously (31) using a laser scanning confocal microscope. Briefly, for junctional proteins, cells were fixed in ice-cold absolute methanol and incubated for 1 h with rabbit anti-ZO-1, mouse anti-occludin, mouse anti-β-catenin, or rabbit anti-E-cadherin antibodies (Zymed Laboratories). For secondary detection, the cells were incubated with fluorescein isothiocyanate (FITC) or rhodamine conjugated secondary antibodies (Jackson ImmunoResearch) added to the cells for 1 h. For β-tubulin immunolocalization, cells were fixed with 1,4-piperazinediethanesulfonic acid buffer (10 mmol/L, 1,4-piperazine-diethanesulfonic acid, 5 mmol/L EGTA, 1% paraformaldehyde, 0.2% Triton, 2 mmol/L MgCl2) for 30 min and then with cold ethanol for 3 min. Cells were incubated with mouse anti-human β-tubulin (1 mg/L, Zymed Laboratories) for 1 h followed by FITC-conjugated secondary antibody incubation for 1 h. For F-actin localization, cells were fixed with 4% paraformaldehyde-0.2% Triton for 30 min and incubated with 0.4 mg/L FITC-conjugated phallolidin (Sigma). Nuclei were labeled with propidium iodide (1 mg/L, Sigma) after digestion of cytoplasmic RNA with 50 mg/L RNase (Roche Diagnostics) at 37°C for 30 min. Stained monolayers were mounted on glass slides in vextashields (Vector Laboratories). The slides were examined under a confocal scanning laser microscope (Sarastro 2000, Molecular Dynamics) using an argon ion laser as light source. Negative controls were set by exposing the serial sections under similar conditions omitting the primary antibody.

Western blot. Caco-2 cells were analyzed for ZO-1, occludin, β-catenin, E-cadherin, and β-tubulin amounts according to Roselli et al. (32). Cells were washed with ice-cold PBS and lysed in 0.5 mL of cold radio-immune precipitation buffer (RIPA) containing 1 mmol/L of phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Complete Mini, Roche). Equal amount of proteins, measured by Bradford assay (Bio-Rad), were analyzed by SDS-PAGE (4-20% precast gel, Cambrex) and electrophoretically transferred to nitrocellulose sheets (Schleicher and Schuell, Bioscience) using transfer buffer (25 mmol/L Tris-192 mmol/L glycine, pH 8.3, 20% methanol, or 5% in the case of ZO-1) at 4°C for 1 h. The membranes were incubated with primary antibodies (2 mg/L in 3% bovine serum albumin, Zymed Laboratories) for 1 h. Anti-β-actin (Sigma) was also used as loading control. Preliminary experiments showed that β-actin was not affected by zinc deficiency (data not shown). After incubation with appropriate horse-radish peroxidase secondary antibody (1:10,000), blots were incubated with Luminol Reagent (Tebu-bio) for 1 min to visualize the immunoreactive protein bands and exposed to Hyperfilm ECL (Amersham). The band intensity was measured by Scion image software.

Phosphorylation assay. Tyrosine phosphorylation of ZO-1, occludin, and β-catenin was analyzed by Western blot of immunoprecipitated proteins, as previously described (32). Cells were lysed in 0.5 mL of RIPA containing 1 mmol/L of phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Complete Mini, Roche). Proteins were immunoprecipitated by adding 3 µg of anti-occludin, anti-β-catenin, or anti-ZO-1 antibodies to total proteins (300 µg protein) diluted in 1 mL of RIPA using an ExactaCruz F kit (Tebu-bio) according to the company instructions. Supernatants were used to detect β-actin level as control for sample loading and the immunoprecipitates were divided into 2 aliquots for detection of protein and phosphotyrosine level. Samples were analyzed by Western blot as described above. We reported the results as the ratio of protein:β-actin and phosphorylated protein:protein.
Chemokine measurements. Interleukin (IL)-8, epithelial neutrophil activating peptide-78 (ENA-78), and growth-regulated oncogene-a (GRO-a) levels were assayed in culture medium of Caco-2 cells by ELISA using a commercial kit (R&D System). The culture medium was collected at the end of the differentiation time before the neutrophil transmigration experiments and stored at −80°C until usage.

Statistical analysis. The significance of the differences was evaluated by 1-way ANOVA followed by Tukey’s post hoc test. Significance was set at $P < 0.05$. All statistical analyses were performed with SPSS software program (version 8.0).

Results

Zinc deficiency affects membrane function. The TEER of the ZD cells was significantly lower than that of C cells after 18 d of culture. The supplementation of zinc in the ZD medium prevented the increase in TJ permeability (Fig. 1).

Zinc deficiency causes alterations to TJ, AJ, and cytoskeleton protein localization. Immunolocalization of junctional proteins was performed to investigate whether zinc deficiency affected the correct distribution of these proteins. Immunofluorescence of TJ proteins (Fig. 2) shows a uniform distribution of ZO-1 and occludin in the C cells almost exclusively at the level of cell boundaries. On the contrary, the ZO-1 and occludin immunostaining of the ZD cells was less homogeneous, with numerous fluorescence interruptions in the plasma membrane. The AJ proteins β-catenin and E-cadherin (Fig. 3) were properly localized on the periphery of C cells, whereas a delocalization of these proteins was detected in the ZD cells as indicated by a diffuse staining inside the cells and loss of continuous staining around cell boundaries. The immunolocalization of both TJ and AJ proteins in the ZD-R cells did not differ from that in C cells, indicating that the alterations were strictly associated with zinc deprivation. The staining of cytoskeleton proteins (Fig. 4) shows a bright and dense fluorescence of F-actin in the C cells at the apical level, corresponding to microvilli, and a rich network of actin filaments on the inner surface of the cell membrane close to the junctional belt. In contrast, a reduction in subcortical and perijunctional F-actin staining was visible in the ZD cells, whereas the organization of F-actin at the microvilli level appears unaltered, as shown by vertical sections. In addition, the propidium iodine staining of nuclei showed an irregular alignment in the ZD cells compared with C cells, as highlighted by the vertical sections. A dramatic disorganization of the microtubular cytoskeleton was caused by zinc deficiency, together with a strong decrease in β-tubulin labeling. The ZD-R cells showed characteristics similar to those of C cells.

Zinc deficiency affects the amounts of junctional and cytoskeleton proteins and phosphorylation level. To investigate whether the altered junctional and cytoskeletal protein localization was associated with alterations in their levels, we performed Western blot experiments. Occludin, ZO-1, and β-tubulin levels were lower in ZD cells compared with C cells, whereas those of E-cadherin and β-catenin did not differ. The levels of all these proteins in ZD-R cells did not differ from those of C cells (Fig. 5). In addition, the level of phosphorylation was
evaluated for those proteins whose activity and/or localization are known to be deeply affected by changes in phosphorylation state. The tyrosine residue phosphorylation of β-catenin and ZO-1 was strongly increased in ZD cells compared with C and ZD-R cells. In contrast, the phosphorylation of occludin was lower in ZD cells than in C and ZD-R cells (Fig. 6).

**Zinc deficiency induces increased neutrophil migration.**

We then assessed whether disruption of membrane barrier integrity caused by zinc deficiency led to an increase in neutrophil migration (Fig. 7). At 60 min after treatment with the chemoattractant, most of the neutrophils in C and ZD-R cells were located in the basolateral compartment and in the filter. At the same time after fMLP addition, a higher transmigration of neutrophils occurred in ZD cells than in C and ZD-R cells, as indicated by the higher number of neutrophils in the apical compartment.

**Increased chemokine secretion induced by zinc depletion.**

We further investigated whether the increased passage of neutrophils was associated with a higher level of chemotactic cytokines by assaying the amounts of IL-8, ENA-78, and GRO-α secreted in the culture media. Higher quantities of IL-8, ENA-78, and GRO-α were secreted by ZD cells than by C cells (Fig. 8). The amount of chemokines released by ZD-R cells did not differ from that of C cells.

**Discussion**

In physiological conditions, the small intestine represents an efficient barrier to noxious antigens and pathogens and disruption of the intestinal barrier has been considered a major factor in several inflammatory intestinal diseases (33). We and other authors have previously shown that zinc deficiency induces intestinal membrane damage and inflammatory cell infiltration (9,21). Here, we provide new evidence on the role of zinc in the maintenance of membrane barrier integrity and prevention of massive neutrophil infiltration, showing that zinc deficiency impairs the membrane permeability, the integrity of the apical junction complexes, and the cytoskeleton organization of intestinal cells, favoring neutrophil migration through the paracellular space. These effects were caused by the deficiency of zinc, because zinc supplementation in the ZD culture medium was highly effective in preserving the membrane barrier. This finding is in agreement with previous studies reporting that the addition of zinc to endothelial or lung epithelial cells restores membrane barrier integrity (9,34). Moreover, Sturniolo et al. (7)

![Figure 3](https://example.com/fig3.png)

**FIGURE 3** Zinc deficiency induced alterations in AJ protein localization in Caco-2 cells. Immunofluorescence of E-cadherin and β-catenin was analyzed in cells grown in C, ZD, or ZD-R medium by confocal microscope. The figure is representative of at least of 6 independent experiments (bar = 10 μm).

![Figure 4](https://example.com/fig4.png)

**FIGURE 4** Zinc deficiency induces a rearrangement of cytoskeleton proteins in Caco-2 cells. Immunofluorescence of F-actin and β-tubulin was analyzed in cells grown in C, ZD, or ZD-R medium by confocal microscope. Vertical sections are reported at the bottom of each panel. The images are representative of at least 6 independent experiments (bar = 5 μm).
showed that individuals with chronic intestinal permeability disturbances had low plasma zinc concentrations and that zinc therapy restored the intestinal permeability associated with increased plasma zinc levels. The importance of zinc for membrane barrier function has also been highlighted by previous studies showing that zinc-deprived airway epithelial cells undergo proteolysis to E-cadherin and β-catenin (9). However, in this study, the ZD culture medium was combined with tumor necrosis factor-α, interferon-γ, and Fas receptor ligand, making it impossible to separate precisely the effects of zinc alone. Here, we show that zinc depletion caused disruption of TJ and AJ, as indicated by the rearrangement throughout the cell of occludin, ZO-1, E-cadherin, and β-catenin as well as by the dramatic disorganization of the cytoskeletal tubulin and actin. Because the association of occludin and ZO-1 is critical for the integrity of the TJ (13), the decrease in these proteins indicated by Western blot analysis is further evidence of TJ disruption. In agreement with these findings, an altered expression of occludin in enterocytes of patients with IBD, such as Crohn’s disease and ulcerative colitis, has been shown to coexist with altered expression of ZO-1. Interestingly, only the expression of ZO-1, and not of ZO-2, was affected in inflamed mucosal tissues, suggesting a specialized function of ZO-1 in the rearrangement of TJ structure in an inflamed milieu (35).

Tyrosine phosphorylation of occludin is necessary for the stabilization of this protein on the TJ (36). A recent study has

FIGURE 6  Zinc deficiency causes changes in phosphorylation level of TJ and AJ proteins in Caco-2 cells. The level of occludin, ZO-1, and β-catenin was analyzed in cells grown in C, ZD, or ZD-R medium by Western blot (top) and the densitometric values of bands were normalized to β-actin (bottom). The figure is representative of 4 independent assays. Values are means ± SD. Within each ratio, values without a common letter differ, \( P < 0.01 \).

FIGURE 7  Zinc deficiency induces increased neutrophil transmigration in Caco-2 cells. The peptide fMLP (\( 1 \times 10^{-5} \) mol/L) was used to induce neutrophil transmigration. The number of transmigrated neutrophils was measured in cells grown in C, ZD, or ZD-R medium and quantified by myeloperoxidase assay. Values are means ± SD of 4 independent experiments. Within each column, values without a common letter differ, \( P < 0.01 \).

FIGURE 8  Zinc deficiency induced an increase in chemokine secretion in Caco-2 cells. The amount of IL-8, ENA-78, and GRO-α secreted in culture medium of cells grown in C, ZD, or ZD-R medium was analyzed by ELISA. Values are means ± SD of 4 independent assays. Within each column, values without a common letter differ, \( P < 0.01 \).
shown that the protein phosphatase 2A interacts with occludin and modulates its phosphorylation status, inducing a strong reduction in tyrosine residue phosphorylation during the disassembly of TJ and an increase in phosphorylation during the reassembly (37). On the other hand, hyperphosphorylation of ZO-1 was associated with alterations of ZO-1 localization in Caco 2 cells (38). The interaction of β-catenin with the intracellular domain of E-cadherin is also regulated by tyrosine phosphorylation of β-catenin and hyperphosphorylation of β-catenin results in the loss of cadherin-based cell-cell adhesion (39–41). A recent study has further highlighted that the dissociation of protein tyrosine phosphatase 1B from the E-cadherin-β-catenin complex is accompanied by an increase in tyrosine phosphorylation of β-catenin and by a loss of its interactions with E-cadherin (37). Consistent with these data, we report a decrease in tyrosine residue phosphorylation of occludin and an increase in phosphorylation of ZO-1 and β-catenin associated with zinc deficiency, suggesting that the phosphorylation state of these proteins might play a pivotal role in their dissociation and translocation from the junctional complexes to intracellular compartments, giving rise to the disruption of barrier integrity.

In this study, we report that the alterations to the structure of TJ and AJ favor the passage of neutrophils. In agreement with our results, Kucharzik et al. (26) have shown a down-regulation of occludin in regions of actively transmigrating PMN, together with a decrease in ZO-1, claudin-1, β-catenin, and E-cadherin in epithelial cells immediately adjacent to transmigrating neutrophils in colonic mucosa of IBD patients. The importance of occludin in modulating the migration of neutrophils has also been shown by Huber et al. (42). Other authors have demonstrated that hyperpermeability associated with PMN transmigration occurs concomitantly with tyrosine hyperphosphorylation of β-catenin and loss of this protein at the cell membrane (43). In addition, dephosphorylation of occludin and degradation of ZO-1 have been shown to facilitate the transepithelial passage of neutrophils in intestinal cells infected with a pathogen (44).

It could be argued that the enhanced neutrophil migration was due to an increase in chemokines rather than the leaking barrier, because we show that depletion of zinc induces an increased secretion of IL-8, ENA78, and Gro-α, which are known to induce neutrophil migration (45). However, this increase was not responsible for the observed enhanced neutrophil migration, because the neutrophil migration assay was performed in a chemokine-free culture medium and there was a very low level of transmigration without the addition of the chemotacticant fMLP in both the C and ZD cells. Nevertheless, our results suggests that a condition of hypozincemia may cause uncontrolled migration of neutrophils through both the disruption of junctional complexes and the induction of chemokines, which may lead to the development or exacerbation of inflammation and mucosal damage by releasing factors such as inflammatory cytokines or proteases that contribute further to intestinal damage. Indeed, an extensive neutrophil migration across the epithelium has been shown to be associated with epithelial injury and intestinal disease (46,47). An additional contribution to the neutrophil migration in hypozincemia may derive from an increased apoptosis, because previous studies have shown that zinc deprivation increases susceptibility to apoptosis and facilitates barrier disruption in epithelial cells (9,48).

In conclusion, our results provide new information on the critical role played by dietary zinc in the maintenance of membrane barrier function and in controlling inflammatory reactions by showing that the depletion of zinc causes phosphorylation-mediated disruption of junctional complexes and cytoskeleton disorganization, thus promoting the migration of neutrophils.

Although the present study was conducted in vitro, our results may provide an explanation of the findings that patients with IBD and low mucosal zinc concentration typically present an accumulation of neutrophils in epithelial crypts and intestinal lumen resulting in the formation of crypt abscesses (25–27).

**Literature Cited**

1670 Finamore et al.


