Small intestine barrier function failure induces systemic inflammation in monosodium glutamate-induced chronically obese mice

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Small intestine barrier function failure induces systemic inflammation in monosodium glutamate-induced chronically obese mice

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Abstract

Chronic obesity has increased worldwide, in conjunction with Type 2 diabetes. Chronic obesity causes systemic inflammation that may result in functional deterioration of the gastrointestinal barrier. However, gastrointestinal conditions associated with chronic obesity have not been comprehensively investigated. The purpose of this study was to evaluate morphological changes in small intestine barrier structures during chronic obesity. A mouse model of chronic obesity induced by monosodium glutamate treatment was established. At postnatal week 15, pathological changes including in small intestinal epithelial cells were analyzed in chronically obese mice compared with controls. Numerous gaps were identified between small intestinal epithelial cells in chronically obese mice, and levels of both desmosomal and tight junction proteins were significantly lower in their small intestinal epithelial cells. Additionally, numbers of intestinal inflammatory cells, particularly macrophages, were significantly increased in chronically obese mice, as were levels of the inflammatory factors, TNF-alpha and IL1-beta, in blood samples from the mouse model. These findings suggest that functional deterioration of adhesion structures between small intestinal epithelial cells causes gastrointestinal barrier function failure, leading to a rise in intestinal permeability to blood vessels and consequent systemic inflammation, characterized by macrophage infiltration.

Keywords: desmosome; electron microscopy; macrophage; rough endoplasmic reticulum; small intestine; systemic inflammation

Introduction

In recent years, chronic obesity has become the primary factor causing metabolic syndrome worldwide. Most chronic obesity leads to a range of health problems later in life, such as circulatory system disease and type 2 diabetes (Freedman et al. 2009). Chronic obesity is also one of the primary factors driving nonalcoholic fatty liver disease (NAFLD) (Kotronen et al. 2008). Many patients with NAFLD resulting from chronic obesity display comorbid hyperlipidemia, hypertension, and insulin resistance. The pathogenesis of NAFLD remains unclear; however, inflammation and liver fibrosis are thought to result from a two-phase process (Malaguarnera et al. 2009): first, there is an increase in fatty acid biosynthesis or a blockade of fatty acid combustion, and second, causal factors such as oxidative stress and/or lipid peroxidation are increased. It has been suggested that lifestyle factors, such as overeating, alter the quality and/or quantity of the bacterial flora in the small intestine. This can result in the translocation of gram-negative bacteria from the small intestine into the portal vein, which activates resident liver macrophages, resulting in an immune response.

Typically, the intestinal epithelium has nutritional absorption and defense mechanisms to prevent the invasion of pathogenic microbes such as bacteria. However, these mechanisms can fail when the small intestinal epithelium permeability is increased following overeating

(Enomoto et al. 1998). The transport of material through the small intestinal epithelium occurs by either transcellular transport (intracellular pathway) or paracellular transport (intercellular pathway). The transcellular pathway is involved in nutrient uptake and is regulated by numerous transporters and channels on the small intestinal epithelium membrane, while the paracellular pathway is involved in the absorption of minerals such as calcium. Failure of these intestinal membrane mechanisms underlies numerous pathological conditions. Liver damage is caused by an inflow of bacteria and endotoxins through the intestinal tract lining into the portal vein. Hence, the defense mechanisms of the small intestinal epithelium are critical in preventing liver damage and systemic infection (Enomoto et al. 1998; Ikejima et al. 1998).

Little is known about the pathological changes in the small intestine that are associated with chronic obesity. However, we recently reported that the villi of the small intestine are elongated (Nakadate et al. 2016). We also reported a significant decrease in rough endoplasmic reticulum (rER) density in the small intestinal epithelium during chronic obesity (Nakadate et al. 2016). This report showed that chronic obesity causes pathological intracellular changes in the small intestinal epithelium. Moreover, we found evidence that small intestine epithelia tight junctions exhibited some damage in monosodium glutamate (MSG)-induced chronically obese mice. However, it was unclear whether dysfunction of small intestinal epithelium-mediated defense mechanisms, such as protein synthesis to prevent pathogenic microbe invasion, also occurred. Here, we investigated the junctional adhesion structures between small intestinal epithelial cells during chronic obesity and determined whether chronic obesity induces systemic inflammation. These pathological changes in small intestinal epithelial cells are thought to be the basis for not only systemic inflammatory reactions but also research on nutritional absorption by the small intestine.

Materials and Methods

Animals

We analyzed 34 C57BL/6J male mice (Charles River, Kohoku-ku, Japan). All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the committee of Meiji Pharmaceutical University approved these experiments. All efforts were made to minimize animal suffering and reduce the number of animals used in the study.

Obesity induction

MSG was used to induce chronic obesity in mice, as previously described (Nakadate et al. 2014, 2015). On postnatal days 1, 2, 4, 6, 8, and 10, twenty male C57BL/6J mice were subcutaneously injected with MSG (2 mg/g body weight, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Another fourteen male mice were subcutaneously injected with saline on the same days as controls. After weaning, MSG-treated mice and control mice were housed under temperature- and humidity-controlled conditions with a 12:12-h light-dark cycle and free access to normal food and water. The body weight of mice was measured once a week.

Intestinal permeability measurement

According to a previous report (Cifarelli et al. 2017), fluorescein isothiocyanate (FITC)-conjugated dextran (10 kDa) (Thermo Fisher Scientific, Waltham, MA, USA; cat. #D1820) was intragastrically administered to mice (n=3/group) sedated initially with 4% isoflurane and maintained with 1% isoflurane. After 3 h, the intestine was collected under deep

anesthesia with an overdose of sodium pentobarbiturate (50 mg/kg, administered intraperitoneally; Nembutal®, Abbott Laboratories, Chicago, IL, USA) and quickly frozen. Using a freezing microtome, intestines were cut into 30- μ m-thick sections, and the level of FITC-dextran was measured (BZ-X700, Keyence, Osaka, Japan).

Tissue preparation

We examined the proximal ileum of the small intestine, according to our previously described methods (Nakadate et al. 2014, 2015). At postnatal weeks 3 and 15, four mice from each group were deeply anesthetized with an overdose of sodium pentobarbiturate (50 mg/kg, administered intraperitoneally; Nembutal®), and the small intestines quickly removed. For histological analyses, the small intestines from four animals per group (postnatal weeks 3 and 15) were immersed in a fixative containing 4% paraformaldehyde for 2 days at 4°C. After immersion, the small intestine was dehydrated through graded concentrations of ethanol, cleared in Lemosol (Wako Pure Chemical Industries, Ltd.), and then embedded in paraffin. For ultrastructural analysis, the small intestines from four animals per group (postnatal week 15) were immersed in a fixative containing 4% paraformaldehyde and 2% glutaraldehyde for 2 days at 4°C. After washing, the small intestines were immersed in 1% osmium tetroxide solution for 2 h at 4°C and dehydrated through graded concentrations of ethanol and propylene oxide. Small intestines were then embedded in Epon-812 resin (TAAB Co., Berkshire, UK).

Histological and immunohistochemical analysis

For hematoxylin-eosin (HE) staining and immunohistochemical analysis, paraffin-embedded small intestine blocks were cut into 3- μ m-thick sections on a sliding microtome (REM-710, Yamato Kohki Industrial, Asaka, Japan). All sections were mounted on

glass slides, deparaffinized with Lemosol, and hydrated with ethanol. For morphological analysis, sections were stained with HE solution.

For immunohistochemical analysis, sections were incubated in 10 mM sodium citrate buffer solution (pH 6.0) for 60 min at 95°C. Sections were rinsed in phosphate-buffered saline (PBS) containing Triton-X 100 and incubated with 10% normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) at room temperature for 2 h, except for those incubated with primary antibodies raised in mouse, in which case we used a Vector M.O.M. Fluorescent Kit (Vector Laboratories, Inc.). Sections were incubated with rabbit anti-pan cadherin antibody (diluted 1:1,000, Abcam Inc., Cambridge, MA, USA), rabbit anti-LI-cadherin (cadherin 17) antibody (diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-CD45 antibody (diluted 1:300, Abcam Inc.), rabbit anti-CD4 antibody (diluted 1:500, Abcam Inc.), rabbit anti-Iba1 antibody (diluted 1:2,000, Wako Chemical Co., Japan), rabbit anti-myeloperoxidase (MPO) antibody (diluted 1:100, Abcam Inc.), or mouse anti-F4/80 antibody (diluted 1:100, eBioscience, San Diego, CA, USA). After several washes with PBS, sections were incubated in Alexa 488-conjugated goat anti-rabbit IgG (1:250, Abcam Inc.) or Alexa 488-conjugated goat anti-mouse IgG (1:250, Vector Laboratories, Inc.) and then incubated with Hoechst 33342 solution.

After dehydration and overlaying with a cover-slip, images of HE- and antibody-stained sections were captured using a CCD camera (BZ-X700, Keyence).

Electron microscope analysis

Small intestines embedded in Epon-812 resin were trimmed, and ultrathin sections (70 nm thick) cut with an MT-XL ultramicrotome (Research and Manufacturing Company, Tucson, AZ, USA). Electron-stained ultrathin sections were examined by transmission electron

microscopy (JEM-1011, JEOL, LTD, Tokyo, Japan), and images captured using a CCD camera.

Western blot analysis

We examined protein levels in small intestine and blood samples by western blotting; proteins were prepared according to previously described methods (Nakadate 2015; Nakadate et al. 2006). After being deeply anesthetized, blood was collected from four mice from each group. Then, small intestines from all mice were rapidly removed. All samples were homogenized in ice-cold homogenate buffer (20 mM Tris-HCl at pH 7.5, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 150 mM NaCl) containing protease inhibitors (1 tablet/10 mL homogenate buffer, Complete™ Mini, Roche Diagnostics, Basel, Switzerland). Homogenates were then centrifuged at $500 \times g$ for 5 min at 4°C, and supernatants dissolved in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 3% SDS, 5% glycerol, and 2% 2-mercaptoethanol) and then boiled for 5 min. According to the Bradford method, protein concentrations were measured using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis and western blotting were performed using the ECL-Plus immunoblotting detection system (GE Healthcare Life Sciences, Buckinghamshire, UK). Proteins (75 µg per lane) were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred, at 50 V for 1 h, onto a polyvinylidene difluoride membranes (Immobilon™-P, EMD Millipore, Billerica, MA, USA). After blocking with 10% (w/v) skim milk (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in PBS containing 0.1% Tween 20 (TPBS, pH 7.5) for 45 min at room temperature (RT), membranes were washed and

incubated with rabbit anti-LI-cadherin (cadherin 17) antibody (diluted 1:1,000, Santa Cruz Biotechnology), rabbit anti-desmoglein 2 antibody (diluted 1:1,000, Abcam Inc.), anti-TNF-alpha antibody (diluted 1:1,000, Abcam Inc.), or anti-IL1-beta antibody (diluted 1:1,000, Abcam Inc.) for 1 h at RT. Following incubation with a horseradish peroxidase-conjugated anti-rabbit antibody (diluted 1:2,000, LI-COR Corporate, Lincoln, NE, USA) for 45 min at RT, immunoreactive bands were detected using WesternSure PREMIUM chemiluminescent substrate (LI-COR Corporate) with a LI-COR C-DiGit chemiluminescence western blot scanner (LI-COR Corporate). Following assays of target proteins, western blotting was repeated using a primary antibody against α -tubulin (diluted 1:2,000, Sigma Aldrich, St. Louis, MO, USA) or albumin (diluted 1:2,000, Abcam Inc.) as an internal control.

Measurements and data analysis

For optical microscopy, the gap frequency between intestinal epithelia cells was measured using Image J software (National Institutes of Health, Bethesda, MD, USA). For these measurements, different villi were chosen (5–10 villi/section from each animal, measurement position: 50–200 μ m from the top of the villi). To detect the frequency of immunostained cells in the lamina propria, immunopositive cells and Hoechst 33342-stained nuclei from different villi (5–10 villi/section from each animal, measurement position: 50–200 μ m from the top of the villi) were also counted using Image J software. Immunoblot bands were captured and analyzed with Image J software. Data were collected from four animals per group and are expressed as means $\pm$ standard deviation. Statistical analysis was performed using StatView statistical software (SAS Institute Inc., Cary, NC, USA). The significance of differences between control and MSG-treated chronically obese mice was determined by Student's t-test. Values of $p < 0.05$ were considered statistically significant.

Results

Histopathological changes to the intercellular structures between small intestine epithelial cells during chronic obesity

To examine the histopathological changes to small intestinal epithelia in chronically obese mice, we determined the pathological changes in small intestines from control and chronically obese mice. Similar to our previous studies, obesity was induced in mice using MSG (Nakadate et al. 2014, 2015). MSG-induced obese mice were significantly heavier at 15 weeks of age than age-matched controls (44.3 ± 3.1 g vs. 31.6 ± 2.6 g) (Figure 1A). The histomorphology of the small intestines of control mice at postnatal week 15 was healthy (Figure 1B and D); however, in chronically obese mice of the same age (Figure 1C and E), we detected areas with a swollen appearance (gaps) in the small intestine epithelia (arrows in Figure 1E). These gaps between small intestinal epithelial cells were more frequently observed in the basal, rather than the apical, portion of the epithelium. We calculated the frequency of gaps under an optical microscope. The results demonstrated that the gap frequency in MSG-induced chronically obese mice was significantly increased compared with controls (Figure 1F). We also evaluated whether gaps could be detected between the epithelial cells in small intestines from animals administered MSG before obesity onset (postnatal 3 weeks). No gaps were detected between epithelial cells in small intestines from MSG-administered animals before obesity relative to age-matched control mice (data not shown).

Ultrastructural changes to the intercellular structures between small intestinal epithelial cells during chronic obesity

To evaluate the intercellular structures between small intestinal epithelial cells in detail, we examined ultrastructural changes using transmission electron microscopy. Small intestinal epithelia of control animals were healthy, and we could clearly identify adhesion structures between the epithelial cells (Figure 2A). Furthermore, no apparent gaps were identified between the small intestinal epithelial cells in the apical or basal regions (Figure 2B–D). In contrast, in MSG-induced chronically obese mice, we observed two types of small intestinal epithelial cells: those that were healthy and contained, for example, numerous rER; and others characterized by rER loss, consistent with our previous study (Nakadate et al. 2016). The ultrastructural analysis revealed two distinct morphological structures between intestinal epithelial cells. First, normally functioning adhesion structures were observed between the small intestinal epithelia (arrowheads in Figure 2E). These normal cell adhesion structures were only observed between pairs of healthy epithelial cells. The second type of adhesion structure exhibited an apparent gap between epithelial cells (arrows in Figure 2E). These apparent gap structures were observed between epithelial cells where either one or both displayed decreased rER density and were also observed more often in the basal, rather than the apical, regions of epithelia, consistent with observations by light microscopy (arrows in Figure 2G–I). Small gaps were also identified in the apical portion, close to tight junctions (arrow in Figure 2F). Lipid or oil droplet-like structures were observed in the gaps between epithelial cells (arrowheads in Figure 2H and I).

Changes in junctional structures between epithelial cells

Next, we evaluated the expression of cell–cell adhesion molecules in MSG-induced chronically obese mouse small intestines. Cadherin, an important component of adherens junctions, which functions to bind cells, was primarily distributed along the lateral membranes and in cell bodies in control mouse small intestines (Figure 3A). No changes in the distribution of cadherin were observed in the small intestines of MSG-induced chronically obese animals; however, the expression of cadherin was slightly decreased compared with that in control mice (Figure 3B). Immunohistochemical evaluation of the distribution of LI-cadherin (cadherin 17) demonstrated that it was specifically localized in the liver and intestine, with changes observed in small intestinal epithelial cells. Intense LI-cadherin protein staining was localized to intercellular structures in control small intestinal epithelial cells (Figure 3C). Again, no changes were observed in the distribution of LI-cadherin in MSG-induced chronically obese mice; however, levels of this protein were decreased (Figure 3D). Using western blot analysis, we evaluated levels LI-cadherin protein in MSG-induced chronically obese mice and found they were decreased compared with those in control mice (Figure 3E). Moreover, we investigated whether the expression of desmoglein 2 protein was altered in MSG-induced chronically obese mice, based on the hypothesis that the function of desmosomes may be compromised in this animal model. In the small intestines of MSG-induced chronically obese animals, desmoglein 2 expression was significantly reduced compared with that in control mice (Figure 3F). These data suggest that cell adhesion proteins were unable to form adhesions between small intestinal epithelial cells and dysfunction of the small intestinal epithelium-mediated defense mechanism occurred. In order to clarify whether leakage occurred between the small intestinal epithelium, we intragastrically administered FITC-conjugated dextran (10 kDa) to mice and investigated whether it infiltrated across the small intestinal

epithelium. FITC-dextran fluorescence was only detected inside the villi of the small intestines of MSG-induced chronically obese animals (Figure 3G and H).

Induction of immune cells in MSG-induced chronically obese mice

We next investigated whether immune cells exhibited increased migration in the small intestines of MSG-induced chronically obese mice using an anti-CD45 antibody. The few CD45-immunopositive leukocytes in the small intestines of control mice were distributed in the lamina propria (Figure 4A). In contrast, many CD45-immunopositive leukocytes in MSG-induced chronically obese animals were detected in the lamina propria of the small intestines (Figure 4B). Quantification data indicated that there were significantly higher levels of CD45-immunopositive leukocyte migration in MSG-induced chronically obese animals than in control animals (Table 1). Immunostaining using an anti-F4/80 antibody indicated that there was increased migration of macrophages into the lamina propria of the small intestines in MSG-induced chronically obese animals compared with that observed in control animals (Figure 4C vs. D and Table 1). However, immunostaining using anti-CD4 (data not shown) and anti-Iba1 (Figure 4E vs. F and Table 1) antibodies did not reveal any differences between control and MSG-induced chronically obese mice. Next, by MPO staining, we determined whether neutrophil infiltration was increased in the small intestines of chronically obese mice. The few MPO-immunopositive neutrophilic leukocytes in the small intestines of control mice were distributed in the lamina propria (Figure 4G). In contrast, many MPO-immunopositive neutrophilic leukocytes in MSG-induced chronically obese animals were detected in the lamina propria of the small intestines (Figure 4H).

Finally, we investigated whether the observed systemic inflammation was induced by chronic obesity. Using western blot analysis, we evaluated changes in the expression of

TNF-alpha (Figure 5A) and active IL1-beta (Figure 5B) in MSG-induced chronically obese mice. The expression of TNF-alpha in MSG-induced chronically obese mice was significantly increased compared with that in control mice (Figure 5A). Moreover, the expression of IL1-beta was also significantly higher in MSG-induced chronically obese mice than in controls (Figure 5B).

Discussion

The objective of this study was to determine whether the junctional adhesion structures between small intestinal epithelial cells exhibited pathological changes associated with chronic obesity. We induced obesity in mice soon after birth using MSG, a previously reported model of early-onset obesity (Nakadate et al. 2014, 2015, 2016). Early postnatal administration of MSG in rodents produces lesions in the preoptic and arcuate nuclei of the hypothalamus, as well as the median eminence (Olney 1969, 1971). These MSG-induced lesions cause a reduction in growth hormone production and alterations in the autonomic nervous system, with an increase in parasympathetic activity and a reduction in sympathetic activity (Scomparin et al. 2009). These neuroendocrine alterations contribute to increased body fat accumulation in this type of obesity, since these animals are hypo or normophagic (Balbo et al. 2000; Nardelli et al. 2011). However, our previous data showed that there was no change in food intake after maturity to adulthood, but there was a significant increase in food intake during the developmental period (Nakadate et al. 2014b). In addition, based on clinical evidence in chronically obese patients and data from our previous studies, we hypothesized that pathological changes affecting intestinal functions, such as nutrient absorption and small

intestinal barrier function, would be observed in these mice; however, structural changes in the small intestinal epithelium have not been established to be associated with chronic obesity. Therefore, we investigated the structural and ultrastructural components of junctional adhesion structures of the small intestinal epithelia during chronic obesity using an MSG-induced chronically obese mouse model.

First, we investigated histopathological changes in the small intestinal epithelia in chronically obese mice. The small intestines of young obese animals were as healthy as those of age-matched control mice; however, in chronically obese mice, we detected areas with a swollen appearance (gaps) between cells of the epithelia of the small intestine, revealing age-related differences in the small intestine epithelia of MSG-induced chronically obese mice. Moreover, ultrastructural analysis revealed a reduction in rER density and shortening of rER in the small intestinal epithelium in chronically obese mice, as previously reported (Nakadate et al. 2016). The rER has numerous functions, including protein folding in the cisternae and the transport of synthesized proteins in vesicles to the Golgi apparatus. These pathological changes of the small intestinal epithelium observed during chronic obesity suggest some loss of function in this tissue. The primary functions of the small intestine are nutrient absorption and defense against the invasion of pathogenic microbes, such as bacteria. It has been previously reported that MSG-induced chronically obese mice exhibit an imbalance between energy supply and energy consumption, leading to increased body weight after weaning (Bunyan et al. 1976; Nakadate et al. 2014, 2015; Tanaka et al. 1978). MSG-induced chronically obese animals also display lower levels of spontaneous motor activity rhythm (Nakagawa et al. 2000; Nemeroff et al. 1977a) and diminished levels of growth and thyroid hormones in the blood (Bakke et al. 1978; Nemeroff et al. 1977a, 1977b, 1978; Redding et al. 1971), leading to reduced basal metabolism.

In addition to changes in absorption, it is possible that the defense function of the small intestinal epithelia is also impaired in the obese mouse model. Reduced defense functions could allow pathogens to pass through the intestinal epithelium into the blood. Pathogens in the portal vein are one of the primary causes of liver damage, resulting in systemic inflammation. Indeed, a previous study reported that serum CRP and IL-6 concentrations were higher in obese mice than in controls (Khazaei and Tahergorabi 2015). To determine whether defense functions were impaired in our obese mouse model, we performed an ultrastructural analysis. Areas between epithelial cells of the small intestine appeared swollen, particularly in the basal region. Additionally, these areas of swollen appearance were primarily observed in the intercellular space adjacent to rER-decreased small intestinal epithelial cells. As proinflammatory agents are transported paracellularly, the small intestinal epithelia in which rER density is reduced would be expected to exhibit impaired defense function during chronic obesity. Therefore, defense function impairment may result from the effects of proinflammatory agents released from the small intestine into the circulation. Indeed, we determined that the leakage between epithelial cells, CD45-immunopositive cells, particularly macrophages, and MPO-immunopositive neutrophilic leukocytes was induced in the intestines of mice with chronic obesity in the present study. It is possible that this functional deterioration of the intestinal epithelial paracellular pathway during chronic obesity causes a rise in intestinal permeability to blood vessels, leading to systemic inflammation.

For paracellular transport, the tight junction proteins, zonula occludens-1 and occludin, contribute to the barrier function of the intestinal mucosa. Zonula adherens and desmosomes are also necessary for binding small intestinal epithelial cells (Felinski and Antonetti 2005; Forster 2008; Itoh and Bissell 2003). Tight junctions and zonula adherens localize to the apical portion of the epithelium of the small intestine; however, desmosomes are distributed between

the apical and basal portions. In the present study, the decreased expression of the cadherin and desmosome constitutive proteins in small intestinal epithelial cells from chronically obese mice resulted in a loss of barrier function. In particular, the expression of desmoglein 2, a desmosome constituent protein, was decreased. Desmoglein 2 is also markedly reduced in the small intestinal mucosa of patients with Crohn's disease (Spindler et al. 2015).

TNF-alpha can cause epithelial cell adhesion loss. In addition, stabilization of desmosomal adhesion can mitigate reductions in barrier function. In this study, in addition to the observation of decreased levels of desmosomal proteins, we also detected a significant increase in TNF-alpha in blood from obese mice. Hence, it is possible that, during chronic obesity, the barrier function of small intestinal epithelia is stabilized via desmosomal adhesion. Zonula occludens-1, which is a tight junction component protein, is induced by glucagon-like peptide-2 when small intestinal permeability is increased (e.g., during endotoxemia) (Cani et al. 2009). Additionally, a lipoglycan-like lipopolysaccharide found on the cell surface of gram-negative bacteria interacts with toll-like receptor 4 and initiates the release of proinflammatory cytokines (Akash et al. 2013; Lu et al. 2008). The loss of desmosome function results in proinflammatory cytokines entering the circulation. In the present study, we detected significant increases in TNF-alpha and IL1-beta in blood from obese mice. Moreover, consistent with our previous report, sinusoidal dilatation and swollen fenestrations of the sinusoid were observed, and the number of inflammatory cells was increased in the liver of chronically obese mice (Nakadate et al. 2015); however, circulating plasma TNF-alpha and IL1-beta may be secreted by other tissues affected by the metabolic disruption induced by chronic obesity. Caetano et al. reported lower TNF-alpha levels and no changes in plasma IL1-beta levels in MSG-obese rats (Caetano et al. 2017). Hence, it is possible that plasma

cytokines are not directly related to the small intestine. Future studies to perform detailed analysis of plasma cytokines derived from other tissues are required.

In the present study, it is possible that chronic obesity led to decreased rER density in the small intestinal epithelia, resulting in dysfunction of this tissue, specifically loss of desmosomal adhesion stabilization, and induction of systematic inflammation.

In conclusion, we identified gaps between small intestinal epithelial cells in rER density, induced by chronic obesity. We further proved that the expression levels of junction proteins were reduced in intestinal epithelial cell membranes. Moreover, immune cell migration was observed in the small intestines and levels of cytokines in the blood were induced by chronic obesity. These findings suggested that barrier function failure of small intestines caused by chronic obesity induces systemic inflammation via paracellular transport.

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Conflicts of Interest: The authors declare no conflict of interest.

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Figure Captions

Figure 1. Histopathological changes in cell–cell interactions between small intestinal epithelial cells.

(A) Plot of body weight from 3 weeks to 15 weeks of age for monosodium glutamate (MSG)-induced obese mice (white squares) and control mice (black circles). The mean $\pm$ standard deviation is shown for each group. * $P < 0.05$ compared with the control. (B) Low- and (D) high-magnification view of an HE-stained section of the small intestine from a control mouse. (C) Low- and (E) high-magnification view of an HE-stained section of the small intestine from an MSG-induced chronically obese mouse. Arrows in E indicate separation between small intestine epithelial cells. Scale bars in B and C = 50 μm , and D and E = 10 μm . (F) Frequency of gaps between intestinal epithelial cells determined by optical microscopy (200 cells/animal, 4 animals). Data are presented as mean $\pm$ S. D. * $P < 0.05$ compared with the control.

Figure 2. Ultrastructural changes of cell–cell interactions between small intestinal epithelial cells.

(A) Low-magnification view of epithelial cells in control mouse small intestine. The three white boxes in A indicate the areas enlarged in B to D. (E) Low-magnification view of epithelial cells in MSG-induced obese mouse small intestine. The four white boxes in E indicate the areas enlarged in F to I. Arrows in E to I indicate cell–cell separation between small intestine epithelial cells. Arrowheads in E indicate normal gap structures between

epithelia. Arrowheads in H and I indicate oil droplet-like structures. Scale bars: A = 5 μm , E = 3 μm , B–D and F–I = 1 μm .

Figure 3. Expression patterns of epithelial cell adhesion molecules in the small intestine.

(A and B) High-magnification fluorescence microscopy images demonstrating the distribution of cadherin (green) in small intestinal epithelial cells from control and MSG-induced obese mice, respectively. (C and D) High-magnification views of LI-cadherin (cadherin 17)-stained small intestine sections from control and MSG-induced obese mice, respectively. Hoechst 33342 (blue) staining indicates cell nuclei. Scale bars = 10 μm . (E and F) Western blot analysis of the levels of LI-Cadherin (E) or Desmoglein 2 (F) expression in the small intestine of control and MSG-induced chronically obese mice. Data are presented as mean $\pm$ S. D. * $P < 0.05$, compared with the control group. Tubulin = internal standard. (G and H) Low-magnification views of FITC-conjugated 10-kDa dextran-stained small intestine sections from control and MSG-induced obese mice, respectively. Hoechst 33342 (blue) staining indicates cell nuclei. Scale bars = 100 μm .

Figure 4. Distribution of immune cells in the small intestine.

High-magnification views of fluorescence microscopy images of CD45 (A and B), F4/80 (C and D), Iba1 (E and F), and myeloperoxidase (MPO) (G and H) in the small intestines are shown. The lamina propria of the small intestines from control (A, C, E, and G) and MSG-induced obese mice (B, D, F, and H) are shown. Hoechst 33342 (blue) staining indicates cell nuclei. Scale bars = 10 μm .

Figure 5. Changes in TNF-alpha and IL1-beta expression in the blood of MSG-induced obese mice.

(A) Western blot analysis to evaluate changes in TNF-alpha expression in the blood of control and MSG-induced chronically obese mice. (B) Western blot analysis to evaluate changes in active IL1-beta (17.5 kDa) expression in the blood of control and MSG-induced chronically obese mice. Albumin = internal standard. Data are presented as mean $\pm$ S. D.

*P < 0.05 compared with the control group.

Draft

Table 1. Frequencies of immunostained cells in the lamina propria

	CD45	Iba1	F4/80
Control	5.2 % $\pm$ 5.1 %	2.8 % $\pm$ 10.7 %	3.8 % $\pm$ 6.7 %
Chronic obesity mice	30.5 % $\pm$ 10.4 % *	7.9 % $\pm$ 12.2 %	29.1 % $\pm$ 11.2 % *

Data are expressed as mean $\pm$ standard deviation values. * $p < 0.05$ compared with the control.

Figure 1. Histopathological changes in cell-cell interactions between small intestinal epithelial cells. (A) Plot of body weight from 3 weeks to 15 weeks of age for monosodium glutamate (MSG)-induced obese mice (white squares) and control mice (black circles). The mean $\pm$ standard deviation is shown for each group. * $P < 0.05$ compared with the control. (B) Low- and (D) high-magnification view of an HE-stained section of the small intestine from a control mouse. (C) Low- and (E) high-magnification view of an HE-stained section of the small intestine from an MSG-induced chronically obese mouse. Arrows in E indicate separation between small intestine epithelial cells. Scale bars in B and C = 50 μm , and D and E = 10 μm . (F) Frequency of gaps between intestinal epithelial cells determined by optical microscopy (200 cells/animal, 4 animals). Data are presented as mean $\pm$ S. D. * $P < 0.05$ compared with the control.

698x1388mm (72 x 72 DPI)

Figure 2. Ultrastructural changes of cell-cell interactions between small intestinal epithelial cells. (A) Low-magnification view of epithelial cells in control mouse small intestine. The three white boxes in A indicate the areas enlarged in B to D. (E) Low-magnification view of epithelial cells in MSG-induced obese mouse small intestine. The four white boxes in E indicate the areas enlarged in F to I. Arrows in E to I indicate cell-cell separation between small intestine epithelial cells. Arrowheads in E indicate normal gap structures between epithelia. Arrowheads in H and I indicate oil droplet-like structures. Scale bars: A = 5 μ m, E = 3 μ m, B-D and F-I = 1 μ m.

136x230mm (300 x 300 DPI)

Figure 3. Expression patterns of epithelial cell adhesion molecules in the small intestine. (A and B) High-magnification fluorescence microscopy images demonstrating the distribution of cadherin (green) in small intestinal epithelial cells from control and MSG-induced obese mice, respectively. (C and D) High-magnification views of LI-cadherin (cadherin 17)-stained small intestine sections from control and MSG-induced obese mice, respectively. Hoechst 33342 (blue) staining indicates cell nuclei. Scale bars = 10 μ m. (E and F) Western blot analysis of the levels of LI-Cadherin (E) or Desmoglein 2 (F) expression in the small intestine of control and MSG-induced chronically obese mice. Data are presented as mean $\pm$ S. D. * P < 0.05, compared with the control group. Tubulin = internal standard. (G and H) Low-magnification views of FITC-conjugated 10-kDa dextran-stained small intestine sections from control and MSG-induced obese mice, respectively. Hoechst 33342 (blue) staining indicates cell nuclei. Scale bars = 100 μ m.

129x250mm (300 x 300 DPI)

Figure 4. Distribution of immune cells in the small intestine. High-magnification views of fluorescence microscopy images of CD45 (A and B), F4/80 (C and D), Iba1 (E and F), and myeloperoxidase (MPO) (G and H) in the small intestines are shown. The lamina propria of the small intestines from control (A, C, E, and G) and MSG-induced obese mice (B, D, F, and H) are shown. Hoechst 33342 (blue) staining indicates cell nuclei. Scale bars = 10 μm.

180x134mm (300 x 300 DPI)

Figure 5. Changes in TNF-alpha and IL1-beta expression in the blood of MSG-induced obese mice. (A) Western blot analysis to evaluate changes in TNF-alpha expression in the blood of control and MSG-induced chronically obese mice. (B) Western blot analysis to evaluate changes in active IL1-beta (17.5 kDa) expression in the blood of control and MSG-induced chronically obese mice. Albumin = internal standard. Data are presented as mean $\pm$ S. D. *P < 0.05 compared with the control group.

159x179mm (300 x 300 DPI)