1. Introduction

The loss of a large part of the small bowel in infants, owing to surgical removal or a congenital defect, leads to a condition called short bowel syndrome. The 3 most common causes of short bowel syndrome in children are necrotizing enterocolitis, intestinal atresia, and midgut volvulus [22]. When a large part of the small intestine is lost, the functional ability of the remaining intestine is often inadequate to support growth and hydration, and prolonged parenteral nutritional support is required. Children with a short bowel are at risk for many life-threatening complications such as sepsis due to catheter-related blood stream infection and parenteral nutrition-associated liver disease even when these children are under total parenteral nutrition. In the clinic, decisions about the optimal management of pediatric short bowel syndrome are often based on repeated trial-and-error treatments, depending on the condition of a specific patient. Therefore, there is an urgent need for a new therapy to compensate for the lost functionality of the small intestine.

Fundamentally, when a large section of the small intestine is lost, the reduction in nutritional absorption is compensated gradually by an increase in the mucosal surface area of the remaining bowel, accompanied by increases in the villus height and crypt cell proliferation rates. This process is known as adaptation [32]. The regulation and augmentation of the function of the remaining intestine is induced through a complex interaction of many different factors, including luminal nutrients and gastrointestinal hormones [22,28]. Physiologically, bowel adaptation is supposed to occur only in response to oral feeding [32]. In this study, we investigated the levels of 3

gastrointestinal hormones, acyl ghrelin, des-acyl ghrelin, and glucagon-like peptide-2 (GLP-2).

Ghrelin is secreted by the X/A-like cells of the stomach and the proximal small intestine. Two major molecular forms of ghrelin exist, of which acyl ghrelin with n-octanoylated modification appears to serve multiple functions [7,15,27], including exerting positive effects on food intake, growth hormone secretory action, glucose and lipid metabolism, gastrointestinal motility, cell proliferation, and hemodynamics, all of which may contribute to intestinal adaptation after massive small bowel resection. On the other hand, non-acylated des-acyl ghrelin induces a negative energy balance by decreasing food intake and delaying gastric emptying [2]. Furthermore, des-acyl ghrelin suppresses acyl ghrelin-induced food intake [10]; a continuous infusion of des-acyl ghrelin is reported to reduce weight gain [21].

GLP-2 is secreted by the intestinal L-cells of the distal ileum and proximal colon in response to both direct stimulation of luminal nutrients and vagally mediated pathways, which are activated by the presence of nutrients in the proximal bowel [12]. GLP-2 is best known for its beneficial role in intestinal adaptation and has become a focus of studies on short bowel syndrome [32]. A randomized placebo-controlled study of teduglutide, a GLP-2 analogue, showed a potential reduction in the dependency on parenteral support of adult patients with short bowel syndrome [11]. However, this treatment has not been applied clinically in children.

The purpose of this study was to clarify the trends in the secretion of endogenous acyl ghrelin, des-acyl ghrelin, and GLP-2 following massive small bowel resection in order to obtain basic data for the future

investigation of a new treatment that may induce efficient intestinal adaptation in patients with short bowel syndrome.

2. Materials and methods

2.1. Animals

Sixty-three 7-week-old male Sprague-Dawley rats weighing 200-240 g (purchased from KYUDO Co., LTD., Saga, Japan) were used in this experiment. The animals were individually housed in cages with free access to standard rat chow and water, and maintained under standardized temperature (23℃ ± 1℃), humidity (50% ± 10%), and 12-h light-dark cycles (lights on at 7:00 a.m.).

All experimental procedures were approved by the Laboratory Animal Committees of Kagoshima University Graduate School and were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals."

2.2. Study Design

Sixty animals were randomized to either the 80% small bowel resection (SBR) group or the transection and re-anastomosis operation (sham) group and allowed to acclimatize to their environment for 6 days before experimentation. Changes in body weight, food intake, water intake, amount of stool, and amount of urine were measured from 7:00 to 8:00 a.m. throughout the experimental period. Preprandial plasma acyl ghrelin and des-acyl ghrelin levels, postprandial plasma GLP-2 levels, and intestinal morphology were assessed at days 1, 4, 7, 11, and 15 after the operation (6 animals per day for the 2 operation groups). As a control, the same measurements described above were assessed in 3 animals at day 0. Plasma

acyl and des-acyl ghrelin levels are known to fluctuate according to psychological or physical stresses [16,34]; therefore, the environments of the experimental animals were noted to be uniform. The 80% SBR animal model has been well established [18,37]. Adaptive response in an 80% SBR rat model was reported to be most pronounced in the first week [18], and the morphological changes reached a plateau (equivalent to 30-postoperative-day levels) within 12 days in a 70% SBR rat model [9]. Thus, we set 15 days as the experimental period for this study. The promotion of functional alterations in response to morphological adaptations following massive small bowel resection has been previously reported [19,26,37].

2.3. Surgical methods

The animals were fasted overnight, anesthetized with isoflurane (1.5% inhalation by mask), and explored through a midline laparotomy under sterile conditions. Intestinal length was measured in a standardized fashion, and 80% SBR was performed, leaving 15 cm of the ileum above the ileocecal valve anastomosed to the jejunum 5 cm below the ligament of Treitz [13]. Bowel anastomoses were completed with the aid of an operating microscope, using interrupted 6-0 silk sutures (Alfresa Pharma Corporation, Tokyo, Japan), and the abdominal incision was closed with 3-0 polyglycolic sutures (Johnson & Johnson K.K., Tokyo, Japan). Sham-operated rats were transected at 15 cm above the ileocecal valve and re-anastomosed [13].

All animals received cefazolin (50 mg/kg per dose subcutaneously; Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) to prevent postoperative infection and buprenorphine (0.01 mg/kg per dose subcutaneously; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for analgesia [13]. Additionally, a

subcutaneous injection of 10 mL isotonic saline was given to prevent postoperative dehydration. The animals were allowed free access to water immediately after surgery and standard rat chow ad libitum at the dark cycle of the first postoperative day.

2.4. Measurement of plasma acyl ghrelin, des-acyl ghrelin, and GLP-2 levels

After an overnight fast, rats were anesthetized by isoflurane inhalation. Blood was obtained from the tail between 10:00 and 12:00 a.m. then immediately centrifuged at 1500 × *g* for 15 min at 4℃. All plasma samples were stored at -80℃ until assayed.

2.4.1. Preprandial acyl ghrelin and des-acyl ghrelin

For the acyl ghrelin and des-acyl ghrelin assay, blood samples were drawn into chilled polypropylene tubes containing 0.2 M ethylenediaminetetraacetic acid, disodium salt (EDTA・2Na) (20 μL/1 mL blood sample) and aprotinin (0.3-0.8 trypsin inhibitor unit/1 mL of blood sample) and then centrifuged. Aliquots of plasma were acidified with 1 N hydrogen chloride and then stored. Plasma acyl ghrelin and des-acyl ghrelin levels were measured using an enzyme-linked immunosorbent assay kit (Mitsubishi Chemical Medicine Corporation, Tokyo, Japan).

2.4.2. Postprandial GLP-2

One hour after gavage of the animals with 2 mL of a liquid meal (ENSURE H; Abbott Japan Co., LTD., Tokyo, Japan), blood samples were drawn into chilled polypropylene tubes containing 0.2 M EDTA・2Na (20 μL/1 mL blood sample) and centrifuged. Aliquots of plasma were stored. Plasma GLP-2 levels were measured by an enzyme immunoassay kit (Yanaihara Institute Inc., Shizuoka, Japan).

2.5. Gross intestinal morphology and histology

After the collection of blood for the GLP-2 assay, the animals were euthanized by exsanguination. The total small intestine was harvested for gross and microscopic morphological analysis.

The mesentery was removed, and the total length of the small bowel was measured from the ligament of Treitz to the ileocecal valve along the antimesenteric border. The harvested small intestine was quickly opened along the mesenteric border, rinsed in cold saline, and then weighed. Bowel width was measured at the middle point of the opened jejunum and ileum. Samples for microscopic analysis were harvested from the jejunum (2.5 cm below the ligament of Treitz), the proximal ileum (12.5 cm above the ileocecal valve, i.e., 2.5 cm below the anastomotic line), and the distal ileum (2.5 cm above the ileocecal valve) and fixed in a 10% formaldehyde neutral buffer solution for 24 h. Mucosal scrapings from the residual jejunum and ileum were weighed. Paraffin sections of formalin-fixed tissue were cut at 3-μm thickness and stained with hematoxylin and eosin. For each sample slide, microscopic measurements of the villus height, villus width, crypt depth, and number of villi per 1 mm were recorded from 5 well-oriented villi/crypt units. The quantification was performed with the help of an expert pathologist. The absorptive mucosal surface area per 1 cm^2 of the intestine was calculated using methods discussed previously. In brief, the mucosal surface area was calculated by first considering the intestine as a cylinder and then multiplying the added mucosal surface area contributed by the villi, considering each villus as a cone [19,23].

2.6. Statistical analysis

Data are presented as the mean values ± standard error (SE). Statistical analyse between groups and time courses were performed by 2-factor factorial analysis of variance (ANOVA) followed by Tukey's multiple-comparison posttest. Comparisons with controls were performed by Dunnett's test. Comparisons between the experimental groups at similar time points were performed by Student's *t*-test. Statistical analysis was completed using Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo). All results were considered statistically significant when *P* values were <0.05.

3. Results

3.1. Changes in daily assessment data

The body weight of animals in the 80% SBR group returned to preoperative levels within 4 days, and continued to increase steadily (Fig. 1). Food intake in the 80% SBR animals recovered to preoperative levels on postoperative day 4. After day 4, roughly equivalent intake was maintained between the 2 operative groups, the 80% SBR animals and the sham-operated animals (Fig. 2). The sham-operated animals showed higher water intake, amount of stool, and amount of urine than the 80% SBR animals until postoperative day 4. Subsequently, these measures remained almost the same in both groups (data not shown). Loose stools were observed in the 80% SBR animals several days after the operation; however, watery or muddy stools were not observed.

3.2. Changes in intestinal morphology

Gross morphological changes in bowel weight and mucosal weight were evident in the 80% SBR animals after postoperative day 4 compared with

controls. Moreover, these changes were significantly different in comparisons with the sham-operated animals at similar time points (Table 1). The increase in villus height of the 80% SBR animals was more evident than that of the sham-operated animals after postoperative day 4 (Fig. 3). In the microscopic quantification, villus height and crypt depth were significantly increased after day 4 in the 80% SBR animals (Table 2). There were no significant differences in the changes of villus width or the number of villi per 1 mm (data not shown). The increase in absorptive mucosal surface per unit area was based on the growth of the villus height. The expansion of absorptive mucosal surface was observed starting on day 4 (Table 2).

3.3. Changes in gastrointestinal hormone levels

The levels of all 3 gastrointestinal hormones immediately increased following massive small bowel resection compared with the controls.

In 80% SBR animals, preprandial plasma acyl ghrelin peaked on day 4, with a significant difference versus controls (means ± standard error, 104.7 ± 14.1 fmol/mL, $P = 0.049$). The time when acyl ghrelin reached its peak level accorded with the time when the body weight and food intake recovered to the preoperative levels. It also matched the timing when the morphology of the remaining intestine began to change significantly. Interestingly, the peak of preprandial plasma des-acyl ghrelin was observed on day 1 (1021.6 ± 93.1 fmol/mL, $P = 0.0007$ vs control). Concerning preprandial acyl ghrelin and des-acyl ghrelin, equivalent plasma levels were maintained in 80% SBR animals, i.e., under short bowel conditions, and in sham-operated animals, i.e., under native small bowel length conditions ($P = 0.09$ and $P = 0.70$).

The postprandial plasma GLP-2 concentration in 80% SBR animals

peaked on day 4, with a significant difference versus controls (4.3 ± 0.8 ng/mL, P = 0.009). GLP-2 levels were maintained at significantly higher levels under short bowel conditions than in native small bowel length conditions ($P = 2.25 \times 10^{-6}$, Table 3).

4. Discussion

Ideally, the progression of intestinal adaptation in infants with short bowel syndrome would occur gradually over 1 to 2 years [32]. In the first 1 to 2 weeks after resection, ileus occurs in the remaining bowel. The next 1 to 6 months are characterized by hypersecretion. Fluid and electrolytes are lost owing to a large amount of watery stool excretion. Subsequently, morphological and functional adaptations occur, such as an increase in the absorptive mucosal surface area. Adaptation is known to result from an increase in villus height and in the rate of crypt cell proliferation [32]. After 1 to 2 years, the compensated absorptive mucosa could perform adequate nutrition and fluid absorption. However, we have encountered some cases in which ideal adaptation cannot be obtained. The clinical challenge is to induce intestinal adaptation early and effectively among children with short bowel syndrome. Postresection intestinal adaptation is supposed to occur only in response to feeding [32]. It was reported that adaptation was impaired in the absence of luminal nutrients in case of total parenteral nutrition [17]. Moreover, only 25% reduction in oral intake caused significantly lower enterocyte production in the crypts [6]. Here, we considered a management strategy to achieve both an orexigenic effect, which promotes food intake, and a trophic effect, which increases the absorptive mucosal surface. Among several gastrointestinal hormones, we

focused on ghrelin and GLP-2, which are known to actively bring about these 2 effects [2,12,18,24]. They may provide a clue about how to solve the clinical problem of short bowel syndrome.

In short bowel environments, acyl ghrelin was maintained at an equivalent level compared with the native bowel length conditions. Acyl ghrelin levels increased immediately in the early postoperative period (Table 3). The timie when acyl ghrelin reached its peak on day 4 accorded with the time when the body weight and food intake recovered to the preoperative levels (Figs. 1 and 2). It also matched the start of the morphological adaptation of the remaining intestine. Among the multiple functions of acyl ghrelin [15], here we want to focus on the regulation of food intake. Numerous peptides secreted from the gastrointestinal tract decrease food intake; only one, acyl ghrelin has a promoting effect [35]. Luminal nutrients ingested orally can stimulate intestinal peristalsis, mucosal blood flow, and endogenous secretions of various hormones and growth factors [15,26,28]. The significant increase in endogenous acyl ghrelin levels in our 80% SBR animals within the first 4 postoperative days may suggest the necessity of ensuring the presence of luminal nutrients to initiate the adaptation of the residual intestine. Interestingly, a significant increase in endogenous des-acyl ghrelin level was seen on postoperative day 1, before a peak in the acyl ghrelin levels was reached on day 4. It was reported that the metabolic machinery that induces intestinal adaptation had already been turned on within the first 24 h in the 80% SBR model rats [37]; however, intestinal permeability, which may be detrimental to the organism, was also increasing at the same time [37]. Des-acyl ghrelin induces an anorexigenic effect by

decreasing food intake and delaying gastric emptying [2,10]. Therfore, des-acyl ghrelin secretion may suppress the intake of luminal nutrients until the intestinal condition becomes well regulated. It is thus reasonable to say that the control of intake is an indispensable factor for intestinal compensation.

As was found in previous reports [18,19], significantly higher levels of GLP-2 were secreted under short bowel conditions compared with native bowel length conditions. GLP-2 levels increased immediately after the operation (Table 3). The peak GLP-2 level in the 80% SBR animals was reached at the same time as the start of the expansion of the absorptive mucosal surface on day 4. Subsequent steady gain in body weight implied the production of functionally mature enterocytes [29]. Both in the preclinical and clinical models, GLP-2 is a well-studied intestinotrophic factor that enhances nutrient and fluid absorption in the context of massive small bowel resection [11,22,28]. Exogenous GLP-2 stimulates crypt cell proliferation and results in a significant increase in the absorptive mucosal area due to villus lengthening [8,20,31]. Additionally, it promotes nutrient transporter expression, intestinal blood flow [20,31], and intestinal barrier function [5]. The significant increase in endogenous GLP-2 in our 80% SBR animals within the first 4 postoperative days may suggest the necessity of ensuring enough stimulation to initiate the expansion of the absorptive mucosal surface. GLP-2 secretion is stimulated by luminal nutrients [12,19,25]. It was interesting that both acyl ghrelin and GLP-2 increased immediately at the same time and peaked at the time when morphological adaptation became evident.

From our laboratory findings, we could imagine a more effective method of inducing residual intestinal adaptation after massive small bowel resection. In a previous study, GLP-2 receptor expression was shown to have significantly increased by postoperative day 3 in 90% SBR model rats [13]. Moreover, continued intravenous administration of GLP-2 during the first postoperative week was required to maximize the adaptation of the remaining bowel in rats [12,13]. These findings suggest that early treatment with a continuous infusion of GLP-2 would be clinically beneficial for short bowel syndrome patients. However, it has been reported that exogenous GLP-2 suppresses ghrelin secretion by nearly 10% in humans [4]. In addition, GLP-2 administration led to a significant elevation of glucagon level [33]. Glucagon is a powerful inhibitor of ghrelin in humans [1]. In rats, intravenous glucagon has been reported to upregulate the synthesis and release of des-acyl ghrelin [14]. The regulation of food intake with exogenous GLP-2 is controversial. Both peripheral [3] and central [36] administration of GLP-2 inhibited food intake in rodents. In healthy humans, the physiologic [33] and pharmacologic [30] GLP-2 doses given intravenously for 3 to 4.5 h had no effect. Yet, the effects of the sustained administration of GLP-2 on food intake in humans are still unknown. These reports imply that a combined administration of acyl ghrelin and GLP-2 would be more useful. As an example of a possible clinical application of a combination that reinforces physiological hormone secretion patterns, the therapeutic schema may be as follows. Under total parenteral nutritional support, a continuous intravenous administration of GLP-2 should be initiated to induce augmentation of the absorptive mucosal area. Additionally, oral food intake

should be started as early as possible, and GLP-2 supplementation should be continued in the postprandial period with administration of acyl ghrelin in the preprandial period to stimulate adequate compensation in the remaining small intestine.

Further studies on the relation between acyl ghrelin, des-acyl ghrelin and GLP-2 after massive small bowel resection are necessary to gain concrete information that may aid in developing a more effective method to induce remnant intestinal adaptation.

5. Conclusion

This is the first report to show the trends of endogenous preprandial plasma acyl ghrelin, des-acyl ghrelin and postprandial plasma GLP-2 in the context of massive small bowel loss. The expansion of the absorptive mucosal surface area became evident after postoperative day 4. All the 3 gastrointestinal hormones studied were elevated immediately after resection. The acyl ghrelin and GLP-2 levels were peaked at the same time as when body weight and food intake recovered to the preoperative levels and as when the remnant intestinal adaptation started. A management strategy that could achieve active orexigenic and trophic effects at the same time may provide a clue as to the development of a new therapy for inducing intestinal compensation in short bowel syndrome patients.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Legends:

Fig. 1. Changes in body weight.

Body weight was set at 100% at the time of the operation. Data are reported as the means ± standard error. A significant difference ($P < 0.01$) in body weight was found between the 80% SBR group and the sham operation group throughout the postoperative period, as analyzed by Student's *t*-test. 80% SBR, 80% small bowel resection.

Fig. 2. Changes in the amount of food intake.

Data are shown as the mean value of intake per 100 g of body weight ± standard error. The statistical differences between the groups were analyzed by Student's *t*-test. ††$P < 0.01$ and †$P < 0.05$ versus the sham operation group at similar time points. 80% SBR, 80% small bowel resection.

Fig. 3. Changes in intestinal morphology in the proximal ileum.

Morphological changes were evident after day 4 in the 80% SBR animals but not in sham-operated animals. This tendency was seen in both the remaining jejunum and ileum. The changes in microscopic morphology in the proximal ileum (12.5 cm above the ileocecal valve) are shown. (A) Post 80% SBR on day 1; (B) post 80% SBR on day 4; (C) post 80% SBR on day 15; (D) post sham operation on day 1; (E) post sham operation on day 4; (F) post sham operation on day 15. Bar, 200 μm. 80% SBR, 80% small bowel resection.