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The protective and anti-inflammatory effects of glucagon-like peptide-2 in an experimental rat model of necrotizing enterocolitis

Kazuhiko Nakame[a,*], Tatsuru Kaji[a], Motoi Mukai[a], Shin Shinyama[a], Hiroshi Matsufuji[b]

[a] Department of Pediatric Surgery, Kagoshima University Graduate, School of Medical and Dental Sciences 8-35-1, Kagoshima shi, Kagoshima 890-8520, Japan
[b] Department of Pediatric Surgery, St Luke's International Hospital 9-1, Akashi-cho, Chuo-ku, Tokyo 104-8560, Japan

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ABSTRACT

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal disease, that affects premature infants. Glucagon-like peptide-2 (GLP-2) is an intestinotrophic hormone and reduces the inflammation. We suspected that GLP-2 would have protective and anti-inflammatory effects in an experimental rat model of NEC. NEC was induced in newborn rats by enteral feeding with hyperosmolar formula, asphyxial stress and enteral administration of lipopolysaccharide (LPS). Rats were randomly divided into the following four groups: dam-fed, NEC, NEC + GLP-2(L) given 80 μg/kg/day of GLP-2, and NEC + GLP-2(H) given 800 μg/kg/day of GLP-2. GLP-2 was administered subcutaneously every 6 h before stress. All animals surviving beyond 96 h or any that developed signs of distress were euthanized. The clinical sickness score in the NEC + GLP-2(H) group was significantly lower than that in the NEC group. The NEC score and the survival rate in the NEC + GLP-2(H) group was significantly improved compared with those in the NEC and the NEC + GLP-2(L) groups. Villous height and crypt depth in both the GLP-2 treatment groups were significantly increased compared with those in the NEC group. There were no significant differences in the crypt cell proliferation indices among the groups. Ileal interstitial TNF-α and IL-6 level in the NEC + GLP-2(H) group was decreased to the same levels in the dam-fed group. High dose GLP-2 administration improved the incidence and survival rate for NEC. It also decreased mucosal inflammatory cytokine production. These results support a potential therapeutic role for GLP-2 in the treatment of NEC.

1. Introduction

Necrotizing enterocolitis (NEC) is the most common surgical emergency and fatal gastrointestinal disorder of premature infants [6,19]. Despite aggressive management leading to the salvage of premature infants, the incidence of NEC continues to increase, presenting at a rate of 0.5–5 cases per 1000 live births each year [22,39]. Mortality rates from NEC range from 15% to 30% and are inversely related to gestational age and birth weight [18,23,35]. We need a novel therapy to improve the mortality in NEC patients.

Epidemiological studies have identified multiple factors that increase the risk of NEC, with prematurity, enteral formula feeding except breast milk, intestinal hypoxia/ischemia, and bacterial colonization thought to play important roles in its pathogenesis [7,8]. Thus, NEC has a multifactorial etiology, with a common final pathway of intestinal inflammation and necrosis [30]. The histology of NEC is characterized by widespread mucosal loss in the gastrointestinal tract, especially the ileum.

Intestinal mucosal surface repair against the injury occurs in two phases [9,12], the first of which involves adjacent epithelial cells migrating into the injury site and the second of which involves cell proliferation. New enterocytes arise from stem cells in the crypts in the cell proliferation. Wang J. presented that putrescine and proline improved epithelial restitution in piglets [41]. However, Richter et al. showed that lipopolysaccharide binding protein, the novel candidate agent for the rat model of NEC, improve the intestinal epithelial restitution, but did not decrease the degree of intestinal damage [33]. Therefore, cell proliferation would be a requisite for epithelial repair and recovery of intestinal function in NEC. We focused on the mechanism of the cell proliferation after mucosal injury. Additionally, Impaired gut barrier integrity allows bacterial translocation and systemic circulation, which leads to NEC via perinatal hypoxia and mucosal damage. Formula feeding and bacterial proliferation allow the attachment of bacteria to the damaged

Abbreviations: GLP-2, glucagon-like peptide-2; NEC, necrotizing enterocolitis; TNF-α, tumor necrosis factor; IL-6, interleukine-6.

* Corresponding author. Fax: +81 99 275 2628.
E-mail address: knakame@m3.kufm.kagoshima-u.ac.jp (K. Nakame).

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and immature mucosal barrier, which in turn triggers the release of some cytokines, especially tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). Consequently, increased epithelial permeability enables the translocation of bacteria and bacterial products from the intestinal lumen [14,27,32].

We speculate that the novel candidate agents of treatments for NEC should have the anti-inflammatory and epithelial proliferation effects. Glucagon-like peptide-1 stimulates the insulin secretion and decreased the blood glucose level, and reduce the pro-inflammatory cytokines, although, in the only pancreatic islets [17]. Ghrelin is secreted by the X/A-like cells of the stomach and the proximal small intestine. It exerts positive effects on food intake, growth hormone secretory action, and intestinal cell proliferation and inhibits pro-inflammatory cytokines [20]. However, the effectiveness of ghrelin for the improvements of intestinal mucosal damage is not apparent. In contrast, Glucagon-like peptide-2 (GLP-2) is derived from the L cells of the small and large intestine in response to both proximal enteric neuronal signaling and the presence of luminal nutrients [34,36]. Previous studies have demonstrated that GLP-2 administration results in intestinal hypertrophy by increasing the crypt cell proliferation rate, which results in increased villous height, crypt depth, and an overall increase in small intestinal length and weight [10,25,37]. GLP-2 is primarily viewed as a trophic regulator of mucosal function in the small intestine [24]. The peptide has been shown to reduce inflammatory mucosal lesions in a rat model of inflammatory bowel disease by decreasing the expression of the inflammatory mediator TNF-α [1]. Furthermore, in our previous experiment, we identified that GLP-2 administration increased the crypt cell proliferation rate and decreased the level of inflammatory cytokines in a rat model of inflammatory bowel disease [38].

We hypothesized that GLP-2 would work as an intestinotrophic agent, maintaining mucosal health and reducing inflammatory cytokine production in the small intestinal mucosa in an experimental rat model of NEC.

2. Materials and methods

2.1. Animal model and study design

This study followed guidelines approved by the Institutional Animal Care and Use Committee of Kagoshima University (MD08069). Time-mated pregnant Sprague-Dawley rats were delivered by cesarean section under anesthesia on day 21 of gestation. Neonatal rats were placed into a neonatal incubator for control of temperature (30 °C), humidity (50 %) and 12 h light-dark cycles. The dose of GLP-2 was decided referring the previous study for NEC experiment [5]. Their study showed that 100 μg/kg/day of GLP-2 was not enough to prevent the onset of NEC. Therefore, we determined the 80 μg/kg/day as low dose and 800 μg/kg/day of GLP-2 as high dose. Rats were assigned to four groups: (1) Dam-fed (n = 18) rats left with their mother, breast fed ad libitum, and not exposed to stress; (2) NEC rats (n = 22) given 5 mL/kg of normal saline (NS) without GLP-2 subcutaneously every 6 h before stress; (3) NEC + GLP-2(L) rats (n = 16) given 20 μg of GLP-2 in 5 mL/kg NS subcutaneously every 6 h before stress; and (4) NEC + GLP-2(H) rats (n = 19) given 200 μg of GLP-2 in 5 mL/kg NS subcutaneously every 6 h before stress.The method initially described by Barlow et al. [4] and modified by Zani et al [42] was used to induce the rat model of NEC. Briefly, four hours after caesarian section, newborn rats from groups (excluding those from the dam-fed group) were gavaged with hyperosmolar rat milk every 6 h and exposed to asphyxia stress for 5-min induced by 100% nitrogen. In addition, on the first and the second days of life, the NEC rats were force-fed with 4 mg/kg/d of *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) (Sigma–Aldrich Company Ltd, UK). A special rodent formula was prepared using 15 g of Balance milk (ICREO CO. LTD, Tokyo, Japan) in a 75-mL Esbilac canine supplement (Pet-Ag Inc, Hampshire, Ill), based on the method described by Feng et al. [13], and rats ware force-fed using a 1.7 French catheter (Excelsior microcatheter, Boston Scientific Target, Bayside Parkway, USA). Human synthetic GLP-2 (1-33, Bachem Ag, Bubendorf, Switzerland) was administered subcutaneously every 6 h before milk in each of the GLP-2 treatment groups.

2.2. In vivo assessment

All rats were inspected and evaluated at each feeding point for the presence or absence of huddling. We evaluated behavior using a clinical sickness score for neonatal rats (Table 1) [42]. This evaluated rats by appearance, response to touch, natural activity and colour. Every endpoint has score from zero to three per measure. Each score was added to calculate to the total score, with lower scores being favorable. Animals that developed distress (lethargy, abdominal distention, bloody diarrhea) or imminent death before 96 h were euthanized by cervical dislocation. After 96 h, all surviving animals were euthanized in same manner. The number of deaths and approximate time of death were recorded daily for all groups and calculated as the survival rate.

2.3. Intestinal morphology and histology

After rats had been euthanized, their intestines were removed, washed by phosphate buffered saline (PBS). A 3 cm section of the distal ileum was harvested and fixed in 10% formalin, embedded in paraffin, sectioned at 4-μm thickness, and stained with Hematoxylin and eosin for histological evaluation of NEC, using the modified histologic scoring system described by Nadler et al. [29] and Dvorak et al. [11]. Histological changes in the intestines were graded as follows: grade 0, no damage; grade 1, slight submucosal and/or lamina propria separation without villous core separation; grade 2, moderate submucosal and/or lamina propria separation with villous core separation; grade 3, severe submucosal and/or lamina propria separation and epithelial sloughing of the villi; and grade 4, loss of villi with transmural necrosis. To determine the incidence of NEC, tissue damage with histological injury scores of grade 2 or greater were considered positive for NEC. Microscopy measurements of villous height, and crypt depth were recorded from

Table 1
Clinical sickness score for neonatal rats.

Appearance	
0	The pup rat is tonic and well hydrated
1	The pup rat is slimmer but still tonic and hydrated
2	The pup rat is skinny, floppy, and dehydrated
3	The pup rat is gasping and in agony
Response to touch	
0	The pup rat is alert (without stimulation)
1	The pup rat responds to mild stimulation
2	The pup rat responds to vigorous stimulation
3	The pup rat is unresponsive notwithstanding a vigorous stimulation
Natural activity	
0	The pup rat moves normally in the cage
1	The pup rat, if put supine, is able to wriggle
2	The pup rat, if put supine, is not able to wriggle
3	The pup rat does not move its limbs and lays still
Colour	
0	The pup rat skin colour is pink
1	The pup rat skin colour is pale (just at the extremities)
2	The pup rat skin colour is pale (whole body)
3	The pup rat skin colour is grey

Excerpted from ref Zani.