

Glucagon-like Peptide 2: A New Treatment for Chemotherapy-Induced Enteritis¹

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Background. Glucagon-like peptide 2 (GLP-2) is a recently identified intestinal epithelium-specific growth factor that has been shown to reduce the severity of inflammatory disorders of the intestine in rodent models. We hypothesized that GLP-2 administration would be beneficial in chemotherapy-induced enteritis either by preventing injury or by promoting recovery.

Material and methods. Rats received no drug (control), chemotherapy alone [5-fluorouracil (5-FU), 190 mg/kg, ip] (Chemo), 5-FU followed by 3 days of GLP-2 analog (ALX-0600, 0.1 µg, sc twice daily) (CH-G), or GLP-2 analog for 6 days prior to 5-FU and for 3 days afterward (G-CH-G). Animals were pair fed. Rats received 5-bromo-2-deoxyuridine (Br-dU, 50 mg/kg, 2.5 h prior to sacrifice on Day 3 postchemotherapy) for immunohistochemical assessment of cellular proliferation.

Results. Chemotherapy induced significant reductions in body weight, villus height, and crypt depth compared with controls. Intestinal wet weight, villus height, and crypt depth were significantly higher for the CH-G group compared with the Chemo group. The CH-G group also showed a significant improvement in villus height compared with the G-CH-G group. Crypt depth, but not jejunal wet weight or villus height, was significantly improved in the G-CH-G group compared with the Chemo group. The percentage of Br-dU-labeled cells in the intestinal crypts did not differ among the groups.

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Conclusions. These results suggest, for the first time, that GLP-2 treatment initiated after chemotherapy administration enhances intestinal recovery. In contrast, GLP-2 treatment initiated prior to chemotherapy administration to prevent injury has less beneficial effect. GLP-2 administration may be beneficial to patients suffering from chemotherapy-induced enteritis. © 2000 Academic Press

Key Words: glucagon-like peptide 2; chemotherapy; 5-fluorouracil; intestine.

INTRODUCTION

5-Fluorouracil (5-FU) has become the mainstay of adjuvant therapy for colorectal and other gastrointestinal cancers, with several clinical trials confirming improvements in disease-free and overall survival in select groups [1–3]. 5-FU acts by inhibiting the enzyme thymidine synthase [4] or by incorporation into cellular RNA, yielding fraudulent RNA [5]. Both pathways result in reduced cellular proliferation. As with other chemotherapy agents, 5-FU targets rapidly dividing cells and thus can produce intestinal mucosal damage as an unwanted side effect. Diarrhea is a common complication following 5-FU administration, with a reported incidence of up to 40% [3, 6]. This gastrointestinal side effect is more than a mere inconvenience as it can result in dehydration, requiring hospitalization for intravenous hydration. These adverse intestinal effects may also necessitate 5-FU dose reduction, thus reducing the treatment efficacy. In a recent retrospective review, 31% of patients receiving 5-FU for metastatic colorectal cancer required hospitalization for “5-FU toxicity,” often secondary to gastroenteritis and

dehydration. This toxicity-related inpatient care was estimated to cost \$2700 per patient [7]. There has been a substantial body of work aimed at finding specific therapeutic agents to treat or prevent 5-FU associated intestinal toxicity [8, 9]. Yet no standard treatment protocols exist for treating chemotherapy-induced enteritis, and further research into new therapeutic avenues aimed at treatment or prevention of this unwanted side effect is needed [10].

Glucagon-like peptide 2 (GLP-2) is a recently identified 33-amino-acid peptide secreted by the L-type enteroendocrine cells of the distal small intestine and colon. It is a potent intestinotrophic hormone which increases bowel weight, villus height, crypt depth, and crypt cell proliferation rate and reduces apoptosis in the small intestinal epithelium [11]. Acute or chronic GLP-2 administration induces functional changes in the bowel, improving the absorptive capacity for various nutrients including glucose and amino acids [12–14]. GLP-2 treatment has been shown to augment intestinal adaptation following massive small bowel resection [15], prevent TPN-induced mucosal atrophy [16], and reduce the severity of colitis [17] and indomethacin-induced enteritis [18, 19] in rodent models.

As 5-FU acts by interrupting DNA synthesis and increasing cellular apoptosis in the small intestine [20], and GLP-2 promotes intestinal crypt cell proliferation and reduces cellular apoptosis, we hypothesized that GLP-2 would be beneficial in the treatment of chemotherapy-induced intestinal damage. GLP-2 could exert a beneficial effect by either preventing treatment-associated intestinal injury or enhancing postinjury recovery. We tested both hypotheses. In these experiments we used the GLP-2 analog r[Gly²]GLP-2 with an alanine to glycine substitution at position 2. This alteration in the structure of the peptide prevents cleavage of the hormone by dipeptidyl peptidase IV (DPP-IV) in the rat, prolonging its serum half-life and enhancing its intestinotrophic action in this rodent [21]. Our results demonstrate that GLP-2 treatment following chemotherapy administration promotes intestinal recovery and thus could be of therapeutic value to patients suffering from chemotherapy-induced enteritis.

MATERIAL AND METHODS

Chemicals. GLP-2 analog was obtained from Allelix Biopharmaceuticals inc., Mississauga, Ontario, Canada. 5-FU was purchased from American Pharmaceutical Partners, Los Angeles, California. Other chemicals were obtained from Sigma, St. Louis, Missouri.

Animals. The study protocol was approved by the Harvard Medical Area Standing Committee on Animals. Male Sprague–Dawley rats (330–430 g, Taconic Farms, Germantown, NY) were acclimated for at least 3 days, during which they were maintained at a 12-h light–dark cycle. They were given a diet of standard Purina rat chow and had free access to water. On the first day of the experiments

(Day 1), the rats were weighed and divided into four groups, each containing eight animals. Control (C) rats received an intraperitoneal injection of saline (2 ml) followed by twice daily subcutaneous injections of saline for 3 days, before being sacrificed on Day 4. The chemotherapy group (Chemo) was given an intraperitoneal injection of 5-FU (190 mg/kg), followed by twice daily subcutaneous injections of saline for 3 days, and then were sacrificed on Day 4. The third group (G-CH-G) received twice daily subcutaneous injections of GLP-2 analog, ALX-0600, at a dose of 0.1 μ g/g for 6 days. On the morning of Day 7 rats received the above chemotherapy regimen and continued with GLP-2 treatment for a further 3 days before being sacrificed on Day 10. The last group of animals (CH-G) had the intraperitoneal injection of 5-FU, followed by their first injection of GLP-2 2 h later. The GLP-2 treatment continued for 3 days as above and animals were sacrificed on the morning of Day 4.

Food intake. Chemotherapy is associated with significant reduction in daily food intake. In our preliminary set of experiments, the above chemotherapy regimen resulted in a $72 \pm 7\%$ reduction in daily food intake. To eliminate the effect of this difference in oral intake on the experimental outcomes, all animals were pair fed. All the experimental animals were kept in groups of four animals per cage, and each cage received 40, 40, and 15 g of standard chow on Days 1, 2, and 3 postchemotherapy respectively. All groups finished all the food provided to them each day. Rats had free access to water.

Tissue harvesting. Two and one-half hours before sacrifice, all rats received an intraperitoneal injection of 5-bromo-2-deoxyuridine (Br-dU) (50 mg/kg). Rats were weighed and anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg). A mid-line laparotomy was performed, and the first 8 cm of jejunum, starting at the ligament of Treitz, was collected and measured under constant tension. A paperclip was used as the standard weight to produce tissue tension, and the measurements were carried out by the same individual (P.A.) to avoid observer variation. The first 5-cm length of the intestine was used for measurement of intestinal wet weight. A 1-cm segment of the remaining proximal jejunum was fixed in 10% neutral buffered formalin embedded in paraffin, and 10- μ m sections were prepared. H&E-stained slides were used for histological assessment, with plain slides for BrdU immunohistochemistry.

Histological measurements. Two blinded observers (N.K. and an independent observer) measured six crypts and villi per slide under a microscope with $\times 10$ magnification, using a measuring micrometer.

BrdU staining and data analysis. Proliferating intestinal cells were detected by immunostaining for an S-phase marker, BrdU. Deparaffinized and rehydrated 10- μ m-thick sections were unmasked using microwave antigen retrieval for 10 min in a pH 6.0, 0.1 M citrate buffer. Sections were rinsed, then quenched for endogenous peroxidase with 80% methanol and 0.6% hydrogen peroxide for 20 min. After a rinse in 0.01 M phosphate-buffered saline + 0.05% Tween 20 (PBST) slides were placed in an automated immunostainer (Shandon Lipshaw) for precise application of immunostaining reagents. Sections were applied with 10% normal horse serum in phosphate-buffered saline (PBS) for 30 min. The primary antibody was applied for 60 min using a dilution of 1:50 mouse antibromodeoxyuridine (DAKO) in 1% normal horse serum in PBS. Negative controls were used on sections by substituting the primary antibody with 1 μ g/ml mouse IgG (Vector Laboratories). Next the sections were rinsed with PBST and applied with 1:200 biotinylated horse anti-mouse IgG (Vector Laboratories) in 1% normal horse serum in PBS for 30 min. Detection was performed with avidin–biotin–peroxidase and diaminobenzidine chromagen-substrate kits (Vector Laboratories) according to instructions. Slides were rinsed, counterstained with Mayer's hematoxylin, then dehydrated and mounted. The slides were reviewed under a microscope with $\times 40$ magnification, and the number of brown BrdU stained nuclei was calculated as a fraction of the total number of cells per crypt and expressed as a percentage. In total 20 crypts per slide were counted by two blinded observers.

TABLE 1
Summary of Collected Data^a

	Control	Chemo	G-CH-G	CH-G
% Weight loss	8.5 ± 1.2	12.0 ± 0.8 ^b	11.3 ± 0.8 ^b	11.7 ± 0.9 ^b
Jejunal weight (mg)	0.21 ± 0.004	0.17 ± 0.01	0.20 ± 0.02	0.23 ± 0.03 ^c
Villus height (μm)	491.9 ± 9.9	384.1 ± 8.8 ^b	379.9 ± 10.8 ^b	453.4 ± 11.7 ^{b-d}
Crypt depth (μm)	169.8 ± 4.1	134.3 ± 5.0 ^b	155.3 ± 6.6 ^c	168.6 ± 10.0 ^c
% Br-dU-stained cells in the crypt	65.6 ± 0.7	69.0 ± 0.9	68.9 ± 1.0	64.2 ± 0.9

^a Data presented as means ± SEM.

^b $P < 0.05$ by ANOVA compared with controls.

^c $P < 0.05$ compared with the Chemo group.

^d $P < 0.05$ compared with the G-CH-G group.

Statistics. Data are presented as means ± SEM. Analysis of variance (ANOVA) was performed for comparison of means among groups using a commercially available statistics package (Statistica, StatSoft Inc., Tulsa, OK). Significance was taken as a $P < 0.05$.

RESULTS

Animal morbidity and weight. There were no mortalities in any of the experimental groups. Three animals in the G-CH-G group developed diarrhea. Control group animals had a mean weight loss of $8.5 \pm 1.2\%$ due to the limited quantity of food provided to them during pair feeding. Animals in each of the other groups receiving chemotherapy had significantly greater weight losses (Chemo: $12.0 \pm 0.8\%$, G-CH-G: $11.3 \pm 0.8\%$, CH-G: $11.7 \pm 0.9\%$, $P < 0.05$ compared with controls) (Table 1). GLP-2 treatment had no effect on the animal weight loss.

Jejunal wet weights. GLP-2 treatments after administration of chemotherapy resulted in a significant improvement in jejunal wet weight compared with the Chemo group: (CH-G) 0.23 ± 0.03 g vs (Chemo) 0.17 ± 0.01 g, $P < 0.05$. GLP-2 treatment starting before administration of 5-FU did not result in a significant improvement in jejunal wet weight (G-CH-G: 0.20 ± 0.01 g) (Table 1).

Crypt depth and villus height. 5-FU treatment resulted in a significant shortening of the villi, (Chemo) 384.1 ± 8.8 μm vs (C) 491.9 ± 9.9 μm, $P < 0.05$, and a decrease in crypt depth, (Chemo) 134.3 ± 5.0 μm vs (C) 169.8 ± 4.1 μm, $P < 0.05$. GLP-2 treatment instigated before chemotherapy administration had no beneficial effect on villus height (G-CH-G: 379.9 ± 10.8 μm) but did increase crypt depth (G-CH-G: 155.3 ± 6.6 μm, $P < 0.05$ compared with Chemo). GLP-2 treatment started after chemotherapy administration resulted in significant improvements in villus height (CH-G: 453.4 ± 11.7 μm) and crypt depth (CH-G: 168.6 ± 10.0 μm) relative to the Chemo group ($P < 0.05$). Villus height in the CH-G group was significantly

higher compared with the G-CH-G group ($P < 0.05$) (Table 1), (Figs. 1 and 2).

Br-dU staining. The percentage of crypt cells stained with Br-dU was similar in all groups (C: $65.6 \pm 0.7\%$, Chemo: $69.0 \pm 0.9\%$, G-CH-G: $68.9 \pm 1.0\%$, CH-G: $64.2 \pm 0.9\%$) (Table 1).

DISCUSSION

GLP-2 is a recently identified and potent intestinotrophic hormone [22] that has therapeutic effects in several models of intestinal injury. Several groups have reported the therapeutic benefits of GLP-2 treatment in rodent models of colitis and nonsteroidal anti-inflammatory drug-induced enteritis [17–19], but po-

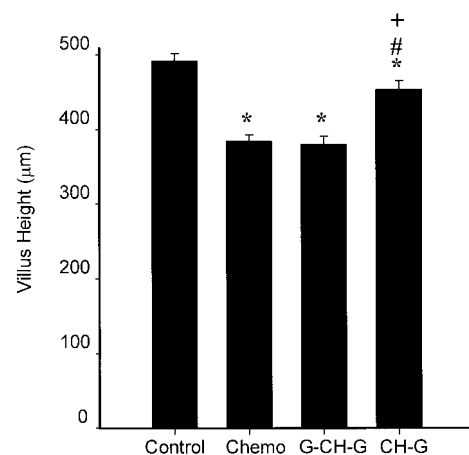


FIG. 1. Villus height. Control animals received an intraperitoneal injection of saline. The Chemo group received an intraperitoneal injection of 5-FU (190 mg/kg, ip). G-CH-G animals received 6 days of GLP-2 treatment before administration of chemotherapy, followed by another 3 days of GLP-2 treatment. CH-G animals received chemotherapy followed by 3 days of GLP-2 treatment. Data presented as means ± SEM. * $P < 0.05$ compared with control. # $P < 0.05$ compared with Chemo group. + $P < 0.05$ compared with G-CH-G group.

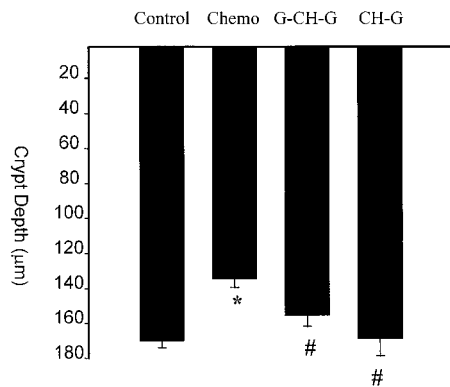


FIG. 2. Crypt depth. Control animals received an intraperitoneal injection of saline. Chemo group animals received an intraperitoneal injection of 5-FU (190 mg/kg, ip). G-CH-G animals received 6 days of GLP-2 treatment before administration of chemotherapy, followed by another 3 days of GLP-2 treatment. CH-G animals received chemotherapy followed by 3 days of GLP-2 treatment. Data presented as means \pm SEM. * $P < 0.05$ compared with control. # $P < 0.05$ compared with Chemo group.

tential benefits of this peptide in ameliorating chemotherapy-induced enteritis have not been reported before. Our data show that GLP-2 treatment started after but not before chemotherapy administration results in significant improvements in the small intestine morphology. Twice daily administration of GLP-2 for 3 days resulted in an increase in jejunal wet weight, crypt depth, and villus height compared with the chemotherapy group. GLP-2 treatment following chemotherapy resulted in a significant improvement in villus height compared not only with the 5-FU group, but also with the group that was treated with GLP-2 for 6 days prior to 5-FU administration.

Our findings are consistent with previous reports emphasizing the importance of temporal relationships between growth factor administration and chemotherapy treatment in resolving chemotherapy-associated complications. It has been reported that infusion of insulin-like growth factor 1 (IGF-1) after, but not concurrent with, chemotherapy stimulates intestine recovery [23]. Various hypotheses have been put forward to explain this observation. It has been suggested that coadministration of mitogenic peptides increases the number of proliferating cells which are more susceptible to chemotherapy toxicity, and thus concurrent therapy with such agents may, in fact, worsen the degree of injury. In support of this hypothesis, it has been reported that coadministration of epidermal growth factor (EGF) with chemotherapy results in increased breakdown of oral mucosa in hamsters [24]. Another hypothesis is a change in the peptide receptor density following chemotherapy. Chemotherapy following pretreatment with the peptide may result in downregulation of the hormone receptor postchemotherapy, making the hormone treatment less efficacious [23]. Our

data did not show a difference in proliferation rates following GLP-2 treatment and thus the explanation for the temporal relationship between treatments may lie in alterations in receptor density.

We examined histological and proliferation parameters in animals 3 days after 5-FU administration. This time point was selected based on previous studies that had shown the most severe morphological changes 3–5 days after administration of a single toxic dose of 5-FU, following which the bowel starts to recover and begins to return to normal morphology [25, 26]. It is not surprising that GLP-2 would demonstrate significant benefits within this treatment period, as other groups have demonstrated evident intestinotrophic effect in the small bowel within 4 days of commencing GLP-2 treatment [27].

We used Br-dU for immunohistochemical staining of intestinal crypt cells in the S phase of the cell cycle. Br-dU is a thymidine analog that is incorporated into the cellular DNA during its synthesis. The percentages of intestinal crypt cells stained with Br-dU among the four experimental groups were similar. Although it has been shown that cellular proliferation and DNA synthesis are greatly reduced after the administration of a toxic dose of 5-FU, this reduction reaches a maximum within 24 h, and by 70 h following chemotherapy administration DNA synthesis returns to normal rates [20, 28]. It is consistent with these observations that we saw similar percentages of Br-dU-stained cells in the crypts of the chemotherapy and control groups. Using proliferating cell nuclear antigen (PCNA) as a cellular proliferation marker, Drucker and colleagues have reported that GLP-2 administration increases crypt cell proliferation rates [11, 27]. However, such an increase in the percentage of Br-dU-stained cells was not evident in our experimental groups that received GLP-2. There are several potential explanations for this difference. Unless appropriate conditions are applied, inferences made using PCNA as a marker of cellular proliferation can differ from conclusions reached using other markers such as Br-dU [29]. PCNA is present in late G₁ and G₂ phases of the cell cycle, as well as the S phase when its concentration peaks [30], whereas Br-dU specifically stains S-phase cells. Previous reports of increased cellular proliferation come from data collected from mouse small intestine while our data are based on chemotherapy-damaged rat jejunum. As there is no obvious difference in the proliferation rates among the different groups, perhaps GLP-2 acts by altering the rate of enterocyte apoptosis or enterocyte migration along the crypt-villus axis. It is possible that GLP-2 exerts its intestinotrophic actions via different pathways, either promoting cellular proliferation, reducing cellular apoptosis, or altering enterocyte migration rates based

on the species and the injury model studied. Further study is necessary to test this hypothesis.

The presented data confirm an important potential role for GLP-2 in reversal of chemotherapy-induced intestinal damage. Our data are based on a rodent model where administration of 5-FU results in small intestinal mucosal injury. The effect of 5-FU administration on human small bowel is less well documented. Some groups have observed an inflammatory response with a reduction in villus:crypt ratio and flattening of villus height [9, 31], while others have not demonstrated any marked or consistent morphological changes [32, 33]. The likely explanation for this discrepancy is the different doses of 5-FU used and variation in the chemotherapy agents used concurrently. The increasing severity of intestinal damage with higher doses of 5-FU may account for the worsening of the intestinal side effects reported at such doses. With the increasingly important role of antineoplastic adjuvant therapy in the treatment of malignant disease, this action of GLP-2 could be of clinical value to a large population of patients.

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