Maintaining Gut Integrity During Parenteral Nutrition of Tumor-Bearing Rats: Effects of Glucagon-Like Peptide 2

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Abstract: Maintaining tumor-bearing rats on total parenteral nutrition (TPN) for eight days significantly reduced mass, protein, and DNA in small intestine and colon. Coinfusion of glucagon-like peptide 2 (GLP-2) significantly increased each of these variables in the duodenum, jejunum, and ileum, but not in the colon. Histological analysis of tissue revealed normal mucosa thickness and villus height in the small intestine of GLP-2-treated rats, whereas nontreated rats maintained on TPN exhibited villus shortening and thinning of the mucosa. Compared with TPN alone, no significant effects of GLP-2 were noted on tumor growth, liver weight, or heart weight. Coinfusion of GLP-2 with TPN had no significant effect on TPN-associated immunosuppression, as measured by mitogen-induced proliferation of cultured splenocytes. Although translocation of bacteria to the mesenteric lymph nodes appeared to be reduced in GLP-2-treated rats, the difference between groups was not statistically significant. These results suggest that hormonal alterations may be more important than an absence of luminal nutrition in TPN-associated mucosa changes in tumor-bearing rats. Additionally, maintenance of gut integrity during TPN does not appear to be a sufficient condition for the avoidance of the negative sequelae associated with this route of supplemental nutrition.

Introduction

As early as 1930, Warren (1) identified nutritional depletion as a major cause of death of cancer patients. In addition to increased mortality, anorexia/cachexia contributes significantly to cancer morbidity, with malnourished patients responding less well to therapy (2,3). Therefore, the aggressive use of available cancer therapy may also be severely limited in anorectic/cachectic patients (4).

A solution to the problem of cancer anorexia and cachexia would appear to be the utilization of nutritional support. In experimental and clinical studies conducted over the past two decades, however, nutritional support has generally not produced positive results. Although the goal of nutritional support is to reduce cachexia and improve survival, clinical studies (5,6) have not generally demonstrated improved survival or increased protein accrual subsequent to total parenteral nutrition (TPN).

In addition to the lack of significant protein accrual (5), specific problems associated with TPN include intestinal atrophy, at least in experimental studies (7,8), and increased infection rate (9,10). This increased rate of infection appears to be due in part to decreased immunologic status (11,12) as well as to increased translocation of bacteria across the intestinal barrier (13). Alteration in immune status may occur through changes in cell- (11) and gut-mediated immunity involving secretory immunoglobulin A (12). The secretory immunoglobulin A immune system is particularly important for bacterial translocation, since this immunoglobulin reduces bacterial adherence to gut mucosa.

Although the use of liquid enteral nutrition may spare the small intestine the depleting effects of TPN, studies utilizing enteral nutrition to replete tumor-bearing (TB) organisms have also not consistently demonstrated improvement in host response (14). In addition, in certain circumstances, such as cancer involving the gastrointestinal system, TPN must be employed for a period of time. Because immunosuppression is characteristic of cancer patients, particularly if they are receiving chemotherapy or radiation, additional suppression of the immune response by TPN may lead to further deterioration of the patient. Therefore, the determination of nutritional and pharmacological methods to limit intestinal atrophy and stimulate the immune response during TPN remains a goal of high priority.

Recent results indicate that a peptide fragment of the proglucagon hormone glucagon-like peptide 2 (GLP-2) has intestinal growth-promoting effects in mice (15). Thus, treating mice for 10 days with GLP-2 at 12.5 µg/day sc elevated mass, protein, and DNA in the small intestine by 50%. We have also observed that confusing GLP-2 with TPN in
normal rats at a daily dose of 150 µg/kg totally prevented the gut atrophy normally associated with this form of nutritional support (16). In the present study we sought to extend these observations to nutritional support of TB organisms and report that conferring GLP-2 with TPN also prevented gut hypoplasia in TB rats.

**Methods**

After one week of habituation to the laboratory environment, methylcholanthrene sarcoma tissue (50 mg) was inoculated subcutaneously into the midscapular region of 18 male (250–300 g) Fischer 344 rats (Charles River Laboratories, Wilmington, MA). The fresh tumor tissue, which was maintained by serial transplantation every 30 days for several years, was provided by a donor rat taken from our tumor colony. These tumor inoculations were accomplished using a 4-mm-diameter trocar after anesthetization with halothane (Halocarbon Laboratories, River Edge, NJ). An additional six rats received sham inoculations, which involved inserting the empty trocar. Eighteen days later, after anesthetization with ketamine and xylazine (80 and 10 mg/kg im, respectively), Silastic catheters (Dow Corning 602-155) were surgically implanted into the external jugular veins of 12 of these TB rats. An additional six TB and six non-TB (NTB, control) rats received sham operations involving unilateral occlusion of the external jugular vein. These operations were conducted aseptically, according to our previously published report (17).

The catheters were connected to syringe pumps (Harvard Apparatus) by 22-gauge feed-through swivels (Harvard Apparatus) that allowed free movement of the rats inside the stainless steel metabolic cages. Normal saline was infused through the catheters for the first three days after surgery at a rate of 2 ml/h. After this period of adaptation, the rats received our standard TPN solution, which is isocaloric and isonitrogenous to the macronutrient content of rat chow. This TPN formulation was constituted as 6% amino acids (Freamine III), 21.5% dextrose, and 1.5% lipid and supplies 1.1 kcal/ml (17). In six of the catheterized rats, GLP-2 (human proglucagon 126–159, American Peptide, Sunnyvale, CA) was added to the TPN solution at a concentration of 0.5 µg/ml, which was infused at a rate to equal the caloric intake of the chow-fed rats (2.8 ml/h). The remaining catheterized rats were maintained on TPN only, at an identical flow rate, whereas all sham-operated rats continued to be maintained on rat chow. Rats were maintained on TPN for a total of eight days, then they were euthanized by decapitation.

After the animals were euthanized, the entire small intestine and colon were removed and flushed with ice-cold normal saline. The small intestine was stretched at a constant force over a chilled dissection plate and sectioned into duodenum (pylorus to ligament of Treitz), jejunum (proximal 20 cm), and ileum (terminal 20 cm), which were frozen in liquid nitrogen. The colon was also removed and flushed, and 10 cm were frozen in liquid nitrogen. Small sections of each segment were also taken, fixed in 10% buffered formalin, and stained with hematoxylin-eosin for visualization of morphological changes by observers who were blinded to treatment conditions. Protein (18) and DNA (19) content of the gut sections were also determined. Tumors were removed and weighed, and the gastrocnemius muscle was taken for protein determination as an index of cachexia. To permit determination of organ specificity of GLP-2 effects, the liver, stomach, and heart were also removed and weighed.

As an estimate of cell-mediated immunity, the mitogen response of isolated splenocytes was determined according to the methods published by Ogle and co-workers (20). The spleens were rapidly removed aseptically and placed in RPMI 1640. Splenocytes were isolated and adjusted to a concentration of 2 × 10⁸ cells/ml in 5% heat-inactivated bovine serum albumin and 1% penicillin-streptomycin. Aliquots (100 µl) of each cellular suspension were incubated at 37°C in 5% CO₂, and 10 µl (5 µg/ml) of pokeweed mitogen, phytohemagglutinin, concanavalin A, or control mitogen were added to each well. The culture plates were incubated for 24 hours at 37°C (5% CO₂) and pulsed with 0.5 µCi of tritiated thymidine in 10 µl of medium per well 18 hours before cells were harvested and counted. Reduced response to the mitogens was considered evidence of immunosuppression.

The mesenteric lymph nodes were also removed aseptically and cultured for 24 hours on agar plates to provide evidence of the translocation of bacteria from gut sources.

Statistical evaluations were determined using a one-way analysis of variance, with individual means being compared post hoc by Tukey’s corrected t-test. All procedures were reviewed and approved by the animal care committees at the respective institutions.

**Results**

As illustrated in Figure 1, the rats maintained on TPN and the TB group maintained on chow (TB-chow) grew at a similar rate. All TB groups gained body weight at a faster rate than did the NTB chow-fed (NTB-chow) rats, a common observation with this animal model of cachexia. This difference in body weight was primarily due to the presence of the tumor, with nontumor body weight being decreased significantly in TB-chow rats (Figure 2). Although tumor weights at sacrifice tended to be reduced in the rats maintained on TPN (47 ± 7 g) compared with the NTB chow-fed rats (66 ± 5 g), the difference was not statistically significant. Nontumor body weight, however, was reduced significantly (p < 0.05) in the TB-chow group and restored to a near-normal level in TB groups maintained on TPN (TB-TPN; Figure 2). The TB-chow group also exhibited significant (p < 0.05) anorexia (22.0 ± 1.2 vs. 14.3 ± 2.4 kcal/100 g body wt) by the third infusion day, which was also 25 days after tumor inoculation. Determination of protein content of the gastrocnemius muscle revealed significant (p < 0.01) depletion in TB-chow rats (251 ± 23 vs. 320 ± 10 mg/muscle), with the content of the gastrocnemius...
muscle of TB-TPN rats (273 ± 23 mg/muscle) or TB-TPN/GLP-2 (283 ± 11 mg/muscle) not statistically significant from that of NTB-chow or TB-chow.

As summarized in Figure 3, there was significant loss of small intestine mass in the rats maintained on TPN. Addition of GLP-2 to the TPN formulation, however, increased the weight of the small intestine beyond that observed in the NTB-chow rats (p < 0.01). Although colon mass was also decreased significantly in the TPN group compared with the NTB-chow rats, GLP-2 treatment had no significant effect in this area of the gut. Stomach mass was reduced (p < 0.01) in all TB groups, whereas heart mass was not affected significantly by any of the treatments. Liver weight was increased in the TB-TPN + GLP-2 group, but only in comparison with the NTB-chow (p < 0.01) and TB-chow (p < 0.05) groups.

Determination of protein concentration in the four segments of the gut (Figure 4) revealed significant (p < 0.01) loss of protein in the duodenum and jejunum of TB-TPN rats. As observed with intestinal mass, coinfusing GLP-2 with the TPN completely prevented the loss of protein in each of these segments. Protein concentration in the ileum was also increased significantly (p < 0.01) in the TB-TPN + GLP-2 group. No significant alterations in protein concentration were observed in the colon in any treatment groups.

The concentrations of DNA in the duodenum and jejunum were also reduced significantly in TB-TPN rats (Figure 5). Consistent with the observations for protein measurements, adding GLP-2 to the treatment increased DNA concentrations in each segment of the small intestine to levels

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**Figure 1.** Body weights of tumor-bearing (TB) and control [non-TB (NTB)] rats maintained on chow or total parenteral nutrition (TPN) and treated with glucagon-like peptide 2 (GLP-2). Values are means ± SE.

**Figure 2.** Tumor weight and nontumor body weight (BW) of TB and NTB rats maintained on chow or TPN with and without GLP-2 for 8 days. Values are means ± SE.

**Figure 3.** Mass of small intestine, colon, and other organs from TB and NTB rats maintained on chow or TPN and treated with GLP-2. Values are means ± SE. gpps, Groups.

**Figure 4.** Protein concentrations in segments of small intestine and colon from TB and NTB rats maintained on chow, TPN, or TPN + GLP-2. Values are means ± SE.

**Figure 5.** DNA concentrations in small intestine segments from TB and NTB rats maintained on chow or TPN and treated with GLP-2. Values are means ± SE.
greater than those observed in chow-fed rats. DNA concentration was not altered in the colon by any experimental treatments.

Histological analysis of sections of duodenum, jejunum, and ileum supported the positive effect of GLP-2 on gut mucosa in rats maintained on TPN. Representative sections of jejunum and ileum are presented in Figure 6. In both areas of the gut, the primary effect in GLP-2-treated rats was to increase mucosal thickness, with villus height being more pronounced in these animals. Ileal villi in the TB rats maintained on chow or TPN appeared to be flattened, whereas the villi in the TB-TPN + GLP-2 group appeared better developed and exhibited more typical apical morphology. Villus height and total thickness were increased significantly ($p < 0.01$) in each section of the small intestine, compared with any other group, in the GLP-2-treated rats (Figure 7). Significant ($p < 0.05$) decreases were observed in villus height and total thickness of TB-TPN rats only in the jejunum. Although crypt depth was not affected significantly in any group, the villus height-to-crypt depth ratio was also increased in TB-TPN + GLP-2 rats compared with all other groups for the duodenum ($p < 0.01$) and ileum ($p < 0.05$). There was no difference in this ratio between any other groups for any sections of the gut.

Figure 8 illustrates the mitogenic response of cultured splenocytes to phytohemagglutinin, pokeweed mitogen, and concanavalin A. Each of the TB groups of rats exhibited a significantly reduced growth response to each of the mitogens, suggesting suppression of cell-mediated immunity. Administration of TPN or TPN + GLP-2 did not improve the growth response to any of the mitogens. Assessment of translocation of bacteria to the mesenteric lymph nodes gave ambiguous results, with all groups exhibiting some colony-forming units in the node cultures (NTB-chow = 2/6, TB-chow = 3/5, TB-TPN = 5/5, TB-TPN + GLP-2 = 2/6). Although the mean number of colony-forming units appeared to be greater in the TB-TPN group (131 ± 69) than in the TB-chow (2.6 ± 1.5) or TB-TPN + GLP-2 (0.7 ± 0.5) group, the degree of variability precluded any statistical significance.

Discussion

The development of malnutrition is a significant risk factor for patients with malignancies. Complicating the treatment of cancer patients is the reality that chemotherapy and radiation often induce an additional anorectic stimulus and always present a greater metabolic stress burden. Because unexplained weight loss may be a presenting sign of a malignancy and better nutritional status generally correlates well with positive postsurgical outcome (2,3,21), the availability of an adequate method to replete cancer patients before and after treatment is important.

A solution to the problem of cancer cachexia would appear to be found in the utilization of nutritional support. However, in experimental and clinical studies conducted over the past two decades, nutritional support of cachectic cancer patients employing TPN has generally not produced positive results in terms of repleting protein or increasing survival (5,6). Because in certain situations, such as cancer of the gastrointestinal system, parenteral nutrition must be employed, delineation of methods to increase its efficacy would be helpful.

Although much of the research describing problems associated with TPN has been done in experimental animals (7,8,11–13), evidence of morphological and biochemical changes has also been reported in humans after a period of TPN (22,23). In addition, reports of complications, infections (9,10,24), and, apart from pediatric cases, general absence of clinical improvement (5,6) suggest that TPN may be inappropriate for repleting cancer patients. Because a portion of the problem with TPN has been thought to be secondary to mucosal depletion and translocation of bacteria to sites outside the gut (13), it was hypothesized that treatments that spared the mucosa would reduce bacterial translocation and improve immunosuppression.

The present results demonstrate complete normalization of gut mass, protein, DNA, and morphology in TB rats maintained on TPN and treated with GLP-2. Histological examination of the tissues indicated that the mucosal area of the gut is the site of savings, with the muscular tissue being largely unaffected by GLP-2. The increase in tissue DNA content indicates that the effect is true hyperplasia and not merely cellular enlargement or nonspecific protein synthesis. An absence of statistically significant increases in the mass of heart, liver, or stomach compared with the TPN-alone group suggests that the hyperplastic effect of GLP-2 is highly tissue specific. This observation is in agreement with previous reports of the specificity of GLP-2-induced hyperplasia to the small intestine (15,16).

In past studies conducted in our laboratory, no significant alteration in colon mass, protein, or DNA was observed in rats maintained on TPN and treated with GLP-2. In the present study, although colon mass was not increased significantly after GLP-2 infusion, the significant decrease in rats treated with TPN alone was obviated. A similar trend was observed for colon DNA (data not shown). A significant effect of GLP-2 or of the protease-resistant analog [Gly3]GLP-2, has been reported for mouse colon tissue (25,26). In these mouse studies the dose of GLP-2 (5 µg/mouse) was typically twice as large on a body weight basis (~200 vs. 100 µg/kg/day) as that used in the present study. In addition, the protease-resistant analog increased colon mass and protein in situations where native GLP-2 was without effect (26). Therefore, the effect of GLP-2 on the colon appears to occur at dosages that are higher than that required for significant hyperplastic effect of the native compound on the small intestine.

Despite the normalization of gut mucosa, however, immunosuppression did not appear to be reduced in TB rats maintained on TPN and treated with GLP-2. Thus cell-mediated immunity, as estimated by the mitogenic response of cultured splenocytes, was decreased in each group of TB rats. Maintaining the rats on TPN with or without GLP-2 had
Figure 6. Representative histology sections of jejunum (Rows A and B) and ileum (Rows C and D) from chow-fed control (Rows A and C, Column 1) and TB rats maintained on chow (Rows A and C, Column 2) or TPN (Rows B and D, Column 1) and treated with GLP-2 (Rows B and D, Column 2). Magnification ×34.
no effect on the immunosuppression. Similarly, translocation of bacteria to the mesenteric lymph nodes was not reduced significantly by the GLP-2 treatment. Although a suggestion of decreased translocation was present in GLP-2-treated rats, the degree of variability precluded any chance of statistical significance. These results are similar to the observation of Helton and Garcia (27), who reported preservation of gut mass, protein, and DNA in rats maintained on TPN and treated with oral prostaglandin E2. As in the present study, mass of the small intestine was normalized, but translocation of bacteria was not affected.

A few other treatments have been shown to produce significant savings in gut mucosa of mammals maintained on TPN. The addition of glutamine to the TPN formula has been shown to elicit a small savings in intestine protein in rats, with the effect being potentiated by concomitant epidermal growth factor treatment (28). In humans, adding glutamine to TPN increased villus height and decreased permeability (22). Treatment with urogastrone-epidermal growth factor has been reported to increase gut mass in rats maintained on TPN (29). Coinfusing peptide YY with TPN has also been shown to induce modest savings in rats maintained on TPN (29). Coinfusing peptide YY with TPN has also been shown to induce modest savings in rats maintained on TPN (30). In addition, gastrin (31), cholecystokinin (31), secretin (31), neurotensin (32), and a nucleotide-nucleoside mix (33) have been shown to reduce TPN-induced gut atrophy to some degree. Recent studies have shown that treatment of rats maintained on TPN with insulin-like growth factor-I (IGF-I) preserves gut mu cosa, while also increasing mass of other organs (34–36). Thus the specificity of GLP-2 for small intestine was not observed with other hyperplastic agents, such as IGF-I (37) or neurotensin (38,39), which may cause growth in other organs or produce tumors when administered along with TPN. A recent study (26) compared GLP-2, the protease-resistant analog [Gly2]GLP-2, epidermal growth factor, IGF-I, IGF-II, and human growth hormone for efficacy in increasing bowel mass in mice. The GLP-2 analog was the most potent of the compounds tested, and a degree of synergy appeared to exist when [Gly2]GLP-2 was administered with IGF-I or growth hormone. Furthermore, the greatest response was observed when all the compounds were administered together, suggesting separate mechanisms of action of the various factors that may potentiate each other.

In addition to increased mucosa growth, elevated functional activity appears to accompany GLP-2 treatment. Thus absorption of sugars (40) and amino acids (40) in the small intestine of rats was increased after 14 days of GLP-2 intravenous infusion. The activities of brush-border enzymes were also reported to be increased in mouse duodenum after ten days of GLP-2 treatment (41). Suggestions of small intestine hyperplasia due to elevated levels of endogenous GLP-2 come from reports of gut hypertrophy in patients (42) and mice (15) with tumors that overproduce proglucagon, from which GLP-2 is derived after translation primarily in the gut (43). In addition, proglucagon gene expression is increased (44) and circulating levels of enteroglucagon are elevated (45) after the development of small bowel resection-induced gut hypertrophy in the rat. This resection-induced gut hypertrophy was increased further by treating the animals with [Gly2]GLP-2 (46). Furthermore, diabetes-induced gut hypertrophy has been associated with elevated levels of GLP-2 (47). These studies, along with the many demonstrations of the effectiveness of GLP-2 in stimulating growth of the small bowel, suggest strongly that it may function normally to maintain continued growth of this organ.

The GLP-2 receptor has been recently cloned (48), and the distribution of the receptors appears to correspond to locations where GLP-2 exhibits its greatest effects. Thus GLP-2 receptor RNA concentrations were highest in the jejunum, with sequentially lower levels being found in the duodenum, ileum, colon, and stomach. In agreement with morphological results, no GLP-2 receptor RNA was detected in the brain, heart, kidney, liver, lung, muscle, or spleen. On the basis of the differential hyperplastic effects, the receptors in the proximal small intestine would appear to be most sensitive to GLP-2. However, it is not known whether the protease-resistant analog [Gly2]GLP-2 bound to the high-affinity binding site (48) because no value was supplied for this test.

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**Figure 7.** Villus height (VH) and total thickness (TT) of small intestine histology sections from TB and NTB rats maintained on chow or TPN and treated with GLP-2. Values are means ± SE.

**Figure 8.** Mitogenic response of cultured splenocytes to phytohemagglutinin (PHA), pokeweed mitogen (PWM), or concanavalin A (ConA). Spleens were taken from TB and NTB rats maintained on chow, TPN, or TPN + GLP-2 for 8 days. CPM, counts per minute.
However, similar to native GLP-2, this analog did stimulate cAMP systems and promoted intestinal mucosa growth. Clearly, further investigation of high-affinity binding of native GLP-2 and the protease-resistant analog is required to answer questions concerning whether this receptor is primary for the hyperplastic effects of GLP-2.

These results suggest that indirect hormonal effects, rather than direct stimulation of the gut by food, are responsible for maintaining gut integrity. Thus, even though the gut lumen was not exposed to food for at least eight days, the mucosa appeared normal from biochemical and morphological assessments. However, improvement in immunosuppression or bacterial translocation was not found after GLP-2 treatment, suggesting that mechanisms other than depletion of gut mucosa may be involved in these negative sequelae of TPN. This conclusion is supported by other reports (49,50) suggesting that immunosuppression and infections are associated with additional factors, including bacterial overgrowth and viability.

Regardless of whether GLP-2 alone will restore the immune system and reduce infection, utilization of this peptide for a variety of diseases or trauma that involve intestinal depletion or require TPN appears promising at this time. In addition, induction of tumors and nonspecific stimulation of organ growth may not present a problem for utilization of this peptide to preserve gut mucosa. Therefore, it appears that GLP-2 treatment may be one additional putative therapeutic intervention in the nutritional management of absorption, permeability, and nutrition problems in a variety of patients.

Acknowledgments and Notes

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