Induction of intestinal epithelial proliferation by glucagon-like peptide 2

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ABSTRACT Injury, inflammation, or resection of the small intestine results in severe compromise of intestinal function. Nevertheless, therapeutic strategies for enhancing growth and repair of the intestinal mucosal epithelium are currently not available. We demonstrate that nude mice bearing subcutaneous proglucagon-producing tumors exhibit marked proliferation of the small intestinal epithelium. The factor responsible for inducing intestinal proliferation was identified as glucagon-like peptide 2 (GLP-2), a 33-aa peptide with no previously ascribed biological function. GLP-2 stimulated crypt cell proliferation and consistently induced a marked increase in bowel weight and villus growth of the jejunum and ileum that was evident within 4 days after initiation of GLP-2 administration. These observations define a novel biological role for GLP-2 as an intestinal-derived peptide stimulator of small bowel epithelial proliferation.

The proliferative compartment of the small bowel epithelium contains subpopulations of cells that exhibit rapid turnover in a highly regulated manner. Intestinal stem cells reside within the crypts of the bowel mucosa and give rise to specialized epithelial cells that migrate upward to constitute functional villi important for nutrient and macromolecule absorption. Several molecules important for the modulation of intestinal epithelial growth are peptide growth factors, such as epidermal growth factor, transforming growth factor α, and insulin-like growth factor 1 (IGF-1), that are produced locally (1) and may regulate proliferation and differentiation. Intestinal epithelium also contains receptors for the IGFs, and IGF-1 has been shown to stimulate small bowel growth and enhance mucosal adaptation after small bowel resection (2, 3).

Peptide hormones have also been implicated in the regulation of intestinal epithelial proliferation. Regulatory peptides, such as neurotensin, cholecystokinin, bombesin, and peptide YY, have been associated with the control of small bowel growth in vivo (4, 5). These peptides are produced locally in specialized enteroendocrine cells distributed along the length of the small and large bowel epithelium. The close proximity of the enteroendocrine cells in relation to the proliferating compartment of epithelial stem cells provides further indirect evidence in support of an association between one or more intestinal peptides and proliferation of the intestinal epithelium (6, 7).

Considerable evidence supports a relationship between expression of the products of the proglucagon gene and bowel growth and regeneration (8). The proglucagon-derived peptides (PGDPs) are synthesized in the L cells of the small and large intestine, and tissue-specific posttranslational processing of proglucagon in the intestine liberates a number of PGDPs, including glicentin, oxyntomodulin, glucagon-like peptide 1 (GLP-1), GLP-2, and several spacer or intervening peptides (Fig. 1). The report of two human patients presenting with PGDP-producing tumors in association with elevated plasma levels of the PGDPs and small bowel hypertrophy suggested a link between peptide products derived from proglucagon and the control of small bowel epithelial proliferation (9, 10). Injection of human tumor cells into mice produced intestinal enlargement in vivo, and semipurified glicentin was reported to stimulate intestinal cell growth in vitro (8). Since these initial reports, a considerable body of experimental evidence supports an association between increased levels of the PGDPs and intestinal growth and regeneration in human and experimental models (3, 8, 11, 12). To explore the physiological consequences of increased proglucagon gene expression in vivo, we generated glucagon-SV40 T antigen (GLUTag) transgenic mice that develop proglucagon-producing tumors and elevated plasma levels of the PGDPs (13, 14). In the course of studying these animals, we observed prominence of the small bowel in GLUTag mice. As the intestinal epithelium of GLUTag mice contains cells expressing the SV40 T antigen transgene, it was not possible to definitively elucidate the mechanisms responsible for small bowel growth in GLUTag mice. Therefore, to ascertain whether elevated levels of the PGDPs stimulate small bowel growth in the absence of intestinal SV 40 Tag expression in vivo, we studied intestinal growth in nude mice carrying three different proglucagon-producing tumors subcutaneously.

METHODS

Propagation of Tumors in Nude Mice. The characterization of the mice and the three rodent proglucagon-producing cell lines InR1-G9 (15), RIN1056A (16), and STC-1(17), has been described (18). Approximately 10 million cells per animal were injected subcutaneously. The nude mice were age-matched females (n = 10–15 per group) and were not restricted by diet or activity during the experiment. Each mouse was killed 30 ± 2 days after injection. Controls were age- and sex-matched (n = 8) animals that were sham-injected. No growth of nude mouse small bowel was demonstrated in previous experiments using subcutaneous v-jun-transformed fibrosarcomas (19). After the mice were killed, the peritoneal and thoracic cavities were inspected for gross evidence of tumor. No evidence of intestinal, mesenteric, peritoneal, or pulmonary seeding was found. The small bowel was then removed from the animal from the pylorus to the cecum, cleaned of feces with 0.9% (vol/vol) normal saline, weighed, and measured. All animal experimentation was carried out in accordance with guidelines approved by the Toronto Hospital Animal Care Committee.

Peptide Injection. Peptides were dissolved in PBS or acetic acid, and the pH was adjusted to 7, after which peptides were suspended in a 16% gelatin solution or in PBS (for time course experiments).

Abbreviations: IGF-1, insulin-like growth factor 1; PGDP, proglucagon-derived peptide; GLP-1 and GLP-2, glucagon-like peptides 1 and 2; IP-1 and IP-2, intervening peptides 1 and 2; GLUTag, glucagon-SV40 T antigen; BrDU, bromodeoxyuridine; PCNA, Proliferating Cell Nuclear Antigen.

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experiment shown in Fig. 4) and 500 μl of peptide/gelatin or gelatin alone (or PBS) was injected subcutaneously every 12 hr for 10 days. Rat Glicentin, GLP-1, IP-2, and GLP-2 amino acid sequences were derived from the rat proglucagon sequence (20) and were synthesized by American Peptide Company (Sunnyvale, CA). Purity was assessed by amino acid analysis and HPLC, with final peptide purity ranging from 70% to 78%.

Analysis of Small Bowel Morphometry. Intestinal micrometry was performed using a Leitz microscope with a Mertz square-based micrometer. Tissue sections were cut longitudinally, fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. For comparative purposes, sections from each animal were obtained from the identical anatomical position (as measured in centimeters from the stomach and cecum) along the length of the small bowel. A minimum of 50 to a maximum of 150 longitudinally oriented villi were counted per section per animal in random order, and each section was counted twice. Small intestine epithelial height was expressed as crypt plus villus height in micrometers ± SEM, averaged for each intestinal segment for control or tumor-bearing nude mice.

Crypt Cell Proliferation Rate. Immunocytochemical staining with antisera directed against bromodeoxyuridine (BrDU; Boehringer Mannheim) was used to determine proliferation rates for age-matched control mice and mice carrying InR1G9, RIN1056A, and STC-1 tumors. Anti-BrDU shows no cross-reactivity with any endogenous cellular component (21). Two hours before being killed, the mice were weighed and then received an intraperitoneal injection of 50 mg/kg BrDU. For immunocytochemistry, the streptavidin-biotin-peroxidase complex technique was performed on 5-μm sections, and the reaction was visualized with 3,3′-diaminobenzidine. Two sets of negative controls were used: nude mice and CD1 mice that were not injected with BrDU, and intestinal sections from nude mice that were injected with BrDU but were not incubated with the primary antibody. Results are expressed as the percentage of positive-staining crypt cells. Crypt cell proliferation rates were assessed in nude mice and GLP-2-treated animals using immunocytochemistry and Proliferating Cell Nuclear Antigen (PCNA) as an indicator of cell proliferation (these measurements gave comparable results to the data obtained using BrDU).

RESULTS AND DISCUSSION

Previous experiments involving proglucagon-producing tumors propagated subcutaneously in mice or rats have demonstrated changes in pancreatic islet histology and islet gene expression, but intestinal morphology in these rodents was not reported (18, 22). Mice carrying three different subcutaneous proglucagon-expressing tumors for 30 days had 8- to 22-fold increases in the plasma levels of PGDPs (18) and increased small intestinal weights compared with control mice without tumors (P < 0.001; Fig. 2A). The gross and microscopic appearance of the small and large bowel (not shown) was normal in all experimental and control mice; however, the thickness of the small bowel wall was greater in the experimental animals. Histological sections from proximal jejunum, mid to distal jejunum, and distal ileum from all control and tumor-bearing mice were examined. A significant increase in thickness of the epithelial mucosa, measured as crypt plus villus height, was consistently observed in histological sections from all three lines of mice carrying subcutaneous proglucagon-producing tumors (P < 0.01; Fig. 2B). The increased mucosal thickness was observed along the entire length (proximal and distal jejunum and ileum) of the small bowel; the enlarged mucosal component of the bowel was the villus layer of the epithelium (P < 0.001), whereas crypt height and thickness of the muscle layers were comparable in control and experimental animals (data not shown).

Normal villus growth begins in the base of the crypt (23); as stem cells divide and differentiated cells migrate up the crypt axis and reach the villus, proliferation rates decline. Crypt cell proliferation rates in mice carrying the tumors (as assessed by BrDU labeling) were clearly increased compared with measurements obtained from control mice, and this increased proliferation rate was detected in sections from proximal and distal jejenum as well as ileum (P < 0.01–0.001; Fig. 2C). Taken together, these data demonstrate a consistent association between elevated plasma levels of the PGDPs and increased proliferation of the small bowel epithelium.

To identify a specific PGDP that functions as a small intestinal growth factor in vivo, we synthesized glicentin, GLP-1, IP-2, and GLP-2 (Fig. 1). As elevated plasma levels of glicentin have previously been associated with increased bowel growth (8, 9), we initially administered glicentin (or vehicle alone) subcutaneously to groups of 8-week-old female CD1 mice twice daily for 10 days. Glicentin produced a small but consistent increase in small bowel weight (P < 0.01; Fig. 3A). To ascertain whether additional PGDPs (Fig. 1) also displayed small bowel growth factor-like activity in vivo, we treated mice with GLP-1, IP-2, or GLP-2. Mice injected with GLP-2 (43.75 μg twice a day) for 10 days demonstrated a 50% increment in total small bowel weight (P < 0.001; Fig. 3A). In contrast, no increase in small bowel weight was observed in mice treated with GLP-1 or IP-2. The experiment was repeated on several different occasions with various concentrations of peptide, and even reduced amounts of GLP-2 (6.25 μg twice daily) consistently produced a 1.5- to 2-fold increase in small bowel weight (P < 0.01; Fig. 3B; unpublished data). GLP-2 (but not glicentin) was the only peptide to produce a statistically significant increase in mucosal thickness throughout the small intestine, as evidenced in histological sections prepared from both proximal and distal jejunum and ileum (P < 0.001; Fig. 3C). The increased mucosal thickness was attributable to an increase in villus height, whereas crypt depth and muscle thickness did not change (see Fig. 5).

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**FIG. 2.** (A) Small bowel weight in 60-day-old nude mice carrying transplantable subcutaneous glucagonomas for 30 days. For all data in Figs. 2–4, statistical significance between samples was assessed by ANOVA using a Statistical Analysis System package for IBM personal computers (SAS Institute, Cary, NC). All data are presented as the mean ± SD (n = 9–10 animals per group). ***, P < 0.001. (B) Small intestine crypt plus villus height in age-matched control and nude mice carrying InR1-G9, RIN1056A, and STC-1 subcutaneous tumors from A above (n = 8–9 animals per group). **, P < 0.01; ***, P < 0.001. (C) Crypt cell proliferation rates in the small intestine of nude mice carrying subcutaneous transplantable glucagonomas (from A above). A minimum of 15 to a maximum of 30 longitudinally oriented crypt/villus axes were counted per section per animal (n = 5–6 animals per group). ***, P < 0.001.

**FIG. 3.** (A) Small bowel weights after administration of synthetic glicentin, GLP-1[7–36]amide, IP-2, and GLP-2 (43.75 μg of peptide per injection in 16% gelatin, twice daily for 10 days) to 8-week-old CD1 female mice (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001. (B) Induction of small bowel growth in groups of 6-week-old female mice treated with vehicle alone (control) or GLP-2 (6.25 μg s.c. twice a day), for 10 days (n = 3). **, P < 0.01. (C) Small intestine crypt plus villus height, expressed as a percentage of control, in proximal jejunum, distal jejunum, and ileum, from vehicle and peptide-treated mice (n = 3). ***, P < .001.

In time course experiments, the growth-promoting properties of GLP-2 were clearly evident after only 4 days of peptide administration, and the changes in both bowel weight and villus...
proportions of stromal components (connective tissue and lymphocytes) so that the morphological appearance of the villi was identical (in control and GLP-2-treated mice) apart from the overall villus size (Fig. 5). The lining epithelial cells were histologically normal; the proportions of absorptive cells with striated borders and interspersed goblet cells were similar in the two groups of mice. The populations of Paneth cells and endocrine cells were comparable in control and GLP-2-treated mice.

Analysis of crypt cell proliferation in the GLP-2-treated and control mice demonstrated clearly increased PCNA-immunopositive cells in the GLP-2-treated group (Fig. 5 C and D). The total number of positive nuclei was increased, and there was a striking increase in PCNA-positive cells outside the crypts in the lower portion of the villus surfaces in GLP-2-treated mice (Fig. 5 C and D).

The glucagon-like peptides were identified carboxy-terminal to the glucagon sequence in a single prohormone precursor following the isolation of the cDNAs and genes encoding proglucagon (20, 24–26). Subsequent experiments demonstrated that GLP-1 functions as a potent regulator of glucose-dependent insulin secretion both in vitro and in vivo (27, 28). The biological functions of the remaining PDGFs shown in Fig. 1 have yet to be identified. The data presented here now identify GLP-2 as a major determinant of epithelial proliferation in the small intestine.

Posttranslational processing of proglucagon is highly tissue-specific (Fig. 1; ref. 29); in the pancreas, the sequence of GLP-2 is contained within a larger, incompletely processed peptide, designated the major proglucagon fragment. In contrast, processing of proglucagon in the intestine liberates glucagon, oxyntomodulin, GLP-1, and GLP-2. Initial studies of proglucagon gene expression suggested that GLP-2 may not be biologically important as the GLP-2 sequence was not detected in anglerfish islet proglucagon cDNAs (30, 31). Recent studies have shown that fish proglucagon does encode a GLP-2 sequence that is contained within a differentially spliced intestinal proglucagon mRNA transcript (32), providing further evidence for the biological importance of this peptide. The high degree of conservation of GLP-2 sequences across various mammalian species is consistent with the important biological role defined here for this peptide.

Immunocytochemical analyses using a variety of antisera directed against the proglucagon-derived peptides have demonstrated that proglucagon-immunopositive L cells are distributed throughout the epithelial layer of the small and large intestine. In all species examined to date, a proportionately greater number of L cells have been localized to the ileum and colon (7, 33, 34). Characterization of immunoreactive GLP-2 produced by the L cell has demonstrated that the predominant molecular form is proglucagon126–158, or GLP-213–33 (35, 36).

Elevated levels of the PGDPs have been associated with intestinal regeneration (8) and bowel resection (3, 8, 11, 12). The increased plasma levels of PGDPs in these models reflect increased synthesis and secretion of peptides from existing L cells and not simply L cell hyperplasia (11, 37, 38). The observation that human patients with proglucagon-producing tumors exhibit small bowel epithelial growth (9, 10) and the high degree of conservation of rodent and human GLP-2 sequences (20, 25) strongly imply that GLP-2 functions as a small bowel growth factor in humans. As peptide growth factors such as epidermal growth factor and IGF-1 have also been associated with bowel growth, the relative efficacy and importance of various specific growth factors in the control of small bowel proliferation remains to be determined (39). Nevertheless, in contrast to epidermal growth factor and IGF-1, the growth-promoting properties of GLP-2 appears to be highly tissue-specific, with proliferation detected only in the intestine. These observations may potentially be explained by tissue-specific expression of the receptor for GLP-2, a hypoth-

Fig. 4. (A) Temporal increase in small bowel weights in groups of 6-week-old female CD1 mice treated with vehicle alone (control) or with synthetic GLP-2 (5 μg in PBS) twice a day, subcutaneously for 1, 2, 4, or 6 days (n = 5). **, P < 0.01. (B) Crypt plus villus heights in proximal and distal jejunum and ileum from A above, treated for 6 days (n = 3). *, P < 0.05.
esis that should be testable following isolation of the GLP-2 receptor. Future experiments should focus on whether GLP-2 acts directly or indirectly in the induction of intestinal epithelial proliferation and on the isolation of the putative receptor that transduces the growth-promoting properties of GLP-2 in vivo.

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