Glucagon-like peptide-2 increases dysplasia in rodent models of colon cancer

Shivangi Trivedi,1 Stuart C. Wiber,1 Hala M. El-Zimaity,2 and Patricia L. Brubaker3

Departments of 1Physiology, 2Pathology and 3Medicine University of Toronto, Toronto, Ontario, Canada

Submitted 2 December 2011; accepted in final form 2 February 2012

Trivedi S, Wiber SC, El-Zimaity HM, Brubaker PL. Glucagon-like peptide-2 increases dysplasia in rodent models of colon cancer. Am J Physiol Gastrointest Liver Physiol 302: G840–G849, 2012. First published February 9, 2011; doi:10.1152/ajpgi.00505.2011.—The intestinal hormone, glucagon-like peptide-2 (GLP-2), enhances intestinal growth and reduces inflammation in rodent models. Hence, a degradation-resistant GLP-2 analog is under investigation for treatment of Crohn’s disease. However, GLP-2 increases colonic dysplasia in murine azoxymethane (AOM)-induced colon cancer. Considering the increased colon cancer risk associated with chronic colitis, we have therefore examined the effects of long-acting hGly2GLP-2, as well as of endogenous GLP-2 using the antagonist hGLP-23–33 in two novel models of inflammation-associated colon cancer: rats fed the carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and a high-fat diet (HFD) for one or three cycles, and mice with chronic dextran sodium-sulfate (DSS)-induced colitis administered AOM. hGly2GLP-2 treatment of one-cycle PhIP/HFD rats increased the number of colonic aberrant crypt foci by 72 ± 11% (P < 0.01). Fifty-one weeks after three PhIP/HFD cycles, hGly2GLP-2-treated rats had a 22% incidence of colon cancer, compared with 0% in vehicle-treated rats. AOM-DSS mice treated with vehicle or hGly2GLP-2 had high-grade dysplasia/colon cancer incidences of 56 and 64%, respectively, compared with 46% in hGLP-23–33-treated AOM-DSS animals (P < 0.05). Unexpectedly, hGLP-23–33 also reduced the colitis damage score by 32.0 ± 8.4% (P < 0.05). All high-grade dysplastic/cancerous tumors had nuclear localization of β-catenin although β-catenin mRNA transcript and protein levels did not differ between treatment groups. GLP-2 receptor mRNA expression also was not different. However, hGly2GLP-23–33-treated mice had markedly reduced numbers of doublecortin-and-calmodulin-kinase-like-1-positive stem cells, by 73.7 ± 8.6% (P < 0.05). In conclusion, the results of this study indicate a role for hGly2GLP-2 and endogenous GLP-2 as potential cancer promoters in rodents.

aberrant crypt foci; adenocarcinoma; azoxymethane; colonic damage score; dextran sulfate sodium; intestine; 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

GLUCAGON-LIKE PEPTIDE-2 (GLP-2) is an intestinal growth factor secreted from the enteroendocrine L cell after nutrient ingestion (51). The half-life of endogenous bioactive GLP-2 (GLP-21–33) is very short because of the presence of an alanine residue at position 2, which renders it a substrate for degradation by the enzyme dipeptidylpeptidase-IV (DPP-IV) (25; 62). Hence, the long-acting, degradation (DPP-IV)-resistant GLP-2 analog, hGly2GLP-2, is often used to examine the pharmacological actions of GLP-2. Furthermore, although the product of GLP-2 cleavage by DPP-IV, GLP-23–33, is a partial agonist of the GLP-2 receptor (GLP-2R), administration of this peptide at relatively low doses effectively antagonizes the actions of endogenous GLP-2 (56, 64). Indeed, GLP-23–33 has been used to demonstrate that the physiological role of GLP-2 is to induce mucosal regrowth of the intestine following refeeding after a fast, findings that have recently been confirmed in the GLP-2R-null mouse (2, 56). At pharmacological doses, both native GLP-21–33 and hGly2GLP-2 have many additional beneficial actions in the normal gut, including increased mucosal growth, mediated through proproliferative and antiapoptotic signaling, as well as increased barrier function, blood flow, digestive function, and nutrient absorption (5, 9, 17, 24). Hence, teduglutide, a degradation-resistant analog of GLP-2 has recently completed phase 3 clinical trials for the treatment of short bowel syndrome (8, 29).

GLP-2 is also effective in reducing mucosal injury and disease activity in rodent models of acute ulcerative colitis and enteritis (7, 19, 28, 33, 57), and preliminary studies in patients with Crohn’s disease (CD) have shown that treatment with teduglutide reduces disease activity index (11). However, chronic colonic inflammation attributable to CD of the colon and ulcerative colitis markedly increases the risk for colorectal cancer (CRC), in correlation with both the disease duration and the extent of colonic inflammation (21, 52). One of the major signaling mediators implicated in human CRC is the canonical Wnt pathway (1, 27, 58), which is also known to be activated in intestinal crypt cells upon administration of GLP-2 (20). Hence, because GLP-2 modulates three factors linked to CRC risk—intestinal mucosal growth, colonic inflammation, and cWnt signaling—investigation into the potential effects of GLP-2 as a colonic carcinogen is warranted.

Previous studies have examined the effects of exogenous and endogenous GLP-2 on intestinal cancer in mice. Hence, treatment with exogenous GLP-2 increased the number of small colonic adenomas in normal mice treated with the colon-specific carcinogen, dimethylhydrazine, whereas a long-acting GLP-2 analog increased the number of both large and small adenomas in these mice. In contrast, GLP-2 administration failed to increase the numbers of intestinal tumors in Apcmin/+ mice (32, 63). Furthermore, both exogenous long-acting GLP-2 and the endogenous peptide have been shown to increase colonic dysplasia and, possibly, cancer in normal mice treated with the dimethylhydrazine metabolite, azoxymethane (AOM) (26). Both dimethylhydrazine and AOM induce activating mutations in the cWnt signaling molecule, β-catenin, whereas Apcmin/+ mice have an inactivating mutation in the adenomatous polyposis coli (APC) gene, which prevents degradation of β-catenin (6, 36, 40, 50, 55, 59). To gain further insight into the broader applicability of GLP-2 as a potential colon cancerpromoter, we have now examined the effect of hGly2GLP-2 on colonic dysplasia and cancer in two novel models of inflammation-associated colon cancer, including a second species, rats, administered another carcinogen, the dietary heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in association with a high-fat diet (HFD), as well as mice administered AOM in the setting of chronic dextran sulfate sodium (DSS)-induced colitis.
MATERIALS AND METHODS

Animals

Adult (5–6 wk), male Fischer 344 (F344) rats and adult (6–10 wk), male C57BL/6 mice were purchased from Charles River Canada (Charles River Canada, St. Constant, QC, Canada). All animals were housed in a facility with a 12-h:12-h light/dark cycle and given ad libitum access to water and food. All experimental protocols were approved by the Animal Care Committee of the University of Toronto.

Experimental Protocols

PhIP-ACF study. A rat model of dietary carcinogen-induced pre-neoplastic colonic lesions, detected as aberrant crypt foci (ACF), was developed based on the protocol in Ref. 45 (Fig. 1A). In brief, all rats were fed regular AIN93G powdered chow diet with 16.8% fat-derived calories from soybean oil for 2 wk (Dyets, Bethlehem, PA), either alone or mixed with 400 ppm of the common dietary carcinogen (34), PhIP (Toronto Research Chemicals, North York, ON, Canada). Food intake and body weight were measured on alternate days during this period. This was followed by feeding of all rats for 4 wk of HFD with 59.2% fat-derived calories, obtained by supplementing AIN-93G diet with Primex (hydrogenated vegetable oil, Dyets), such that 41.3% of calories were derived from Primex and 17.9% from soybean oil. Feeding of a HFD is known to be associated with induction of mild intestinal inflammation (16, 35). During weeks 3 and 4 of the study, the rats were injected with either 50 mM ammonium bicarbonate (Bicarb, 200 μl sc bid; vehicle control; n = 4 for rats without PhIP feeding; n = 8 for rats with PhIP feeding) or hGly2GLP-2 (40 μg, 200 μl sc bid; American Peptide, Sunnyvale, CA; n = 8). During weeks 5 and 6 of the study, the dose of hGly2GLP-2 was increased to 60 μg to account for the increase in body weight. The rats were weighed twice weekly during weeks 3–6. The use of hGly2GLP-2 in all of the studies presented in this study precludes differences in DPP-IV levels (Bicarb, 200 μl sc bid; American Peptide); peptide doses were selected based on a previous validation study in normal mice treated with AOM (26). On the day of death, the mice were administered with vehicle, hGly2GLP-2, or hGLP-2–33 according to their respective groups 3 h prior to death. Small intestinal weight, colon weight, and colon length were obtained after gentle cleaning. Sections (0.5–2 cm in length) from the jejunum (5–10 cm proximal from the mid-small intestine) were frozen on dry ice or fixed in 10% neutral buffered formalin. Cleared colons were opened longitudinally, cut in three segments (proximal, middle, and distal), and fixed flat on Whatman paper between microscope slides in 10% neutral buffered formalin for histological analysis.

PhIP-tumor study. A rat model of colon cancer was developed on the basis of the PhIP-ACF protocol above and modifications of Ref. 65 (Fig. 1B). Briefly, some rats from the PhIP-ACF study were subjected to two additional cycles of AIN-93G diet containing PhIP (2 wk) and HFD (4 wk), followed by 42 wk of HFD (n = 9 for vehicle- and n = 10 hGly2GLP-2-treated groups). Weekly body weights were recorded throughout the study. The animals were also monitored for signs of colon cancer development such as weight-loss and rectal bleeding and were killed if deemed necessary by veterinarians at the animal facility. All remaining animals were killed after week 61 of the experimental protocol. After death, the small intestine, cecum, and large intestine were removed and examined for signs of macroscopic lesions, which were photographed in situ and then dissected intact, fixed in formalin, sectioned, and stained with hematoxylin and eosin (H&E) for tumor classification.

AOM-DSS study. A murine model of CRC was developed on the basis of a modified protocol from Refs. 42 and 48. In brief, mice were injected with AOM (10 mg/kg ip; Sigma-Aldrich Canada, Oakville, ON, Canada) and allowed to recover for 1 wk (Fig. 1C). At the beginning of week 2, they were given 2.5% DSS in drinking water for 1 wk, followed by 2 wk of recovery. This cycle was performed three times. The dose of DSS was determined following a pilot study in which morbidity and colonic damage score (CDS) was assessed in mice treated with 1–3% DSS alone; an intermediate dose of DSS was selected based on the findings (data not shown). During the DSS cycles, mice were monitored daily for signs of morbidity, dehydration, and blood in stools. Rodent chow was mixed with regular water to create a mash in cases of severe dehydration. In contrast to the pilot study, the concentration of DSS administered had to be reduced or eliminated in some mice during the third cycle as a result of rectal bleeding, likely consequent to the prior administration of AOM; these mice were randomly distributed between the subsequent treatment groups, and the reduction in DSS did not affect the CDS in tissues collected upon completion of the study regardless of the treatment group. Mice were weighed weekly and were then randomized to one of three treatment groups after the last day of DSS treatment, with each treatment lasting for 2 wk: vehicle (50 mM Bicarb, 200 μl sc bid), hGly2GLP-2 (1.5 μg in 200 μl sc bid), or hGLP-2–33 (50 ng in 200 μl sc bid; American Peptide); peptide doses were selected based on a previous validation study in normal mice treated with AOM (26). On the day of death, the mice were administered with vehicle, hGly2GLP-2, or hGLP-2–33 according to their respective groups 3 h prior to death. Small intestinal weight, colon weight, and colon length were obtained after gentle cleaning. Sections (0.5–2 cm in length) from the jejunal (5–10 cm proximal from the mid-small intestine), ileum (5–cm proximal from the ileocecal valve), and colon were frozen on dry ice or fixed in 10% neutral buffered formalin. Any tumors found were photographed and dissected, and pieces were fixed in 10% neutral buffered formalin or frozen on dry ice for storage at −80°C. The total number and size of tumors could not be determined because the growths merged into large continuous masses in the majority of animals (not shown). Because of the large number of animals involved in this study, this experiment was performed using three separate cohorts: set 1 (all 3 treatment groups; n = 8–10 per group), set 2 (vehicle and hGly2GLP-2 treatments only; n = 18–19 per group), and set 3 (all 3 treatment groups; n = 18–19 per group).
set 3 (vehicle and hGLP-233 only; n = 9–17 per group), to make a total of n = 35 for vehicle, n = 28 for hGLy2GLP-2, and n = 26 for hGLP-233. No statistical differences were found between the sets for any experimental parameter, except for colon length, in which the Bicarb-mice from set 3 demonstrated a small but significant increase compared with the Bicarb-mice in set 2 (data not shown). Nonetheless, these findings did not alter the overall analysis, and all data were therefore combined for analysis and presentation.

Histopathology. To identify neoplastic lesions in the rat colon, longitudinally opened colons were stained with 0.05% (wt/vol) methylene blue for 1 min and rinsed in PBS. These colons were viewed under ×5 and ×10 objectives in a blinded manner to identify ACF, as described previously (10). To stain for mucin-depleted foci, the colons were then rinsed in 70% ethanol and treated with a high-iron diamine solution (2.4 g/l N-N-dimethyl-m-phenylene diamine, 0.4 g/l N-N-dimethyl-p-phenylene diamine, 1.68% ferric chloride) for 18 h followed by staining with Alcian blue (1% Alcian Blue in 3% acetic acid) for 1 min and rinsing in 80% ethanol (12). Colons were scored for mucin-depleted foci in a blinded fashion using a ×5 objective. All lesions were visualized using a Zeiss AxioPlan microscope with AxioPlan software (Carl Zeiss Canada, Don Mills, ON, Canada). The control group of animals, receiving no PhIP and vehicle only during the HFD period, was used to provide baseline values for both ACF and mucin-depleted foci.

Morphometry. Jejunal crypt depth and villus height were determined in a blinded fashion on digital images obtained with a Zeiss AxioPlan microscope, for an average of 44 well-oriented crypts and 39 villi from H&E-stained sections. As described previously (19, 33), colonic injury was quantified from digital images of H&E-stained sections by grading normal crypts as grade 0, loss of the bottom one-third of crypts as grade 1, loss of the bottom two-thirds as grade 2, and loss of the entire crypt structure as grade 3, in a blinded fashion. CDS was calculated as the extent of damage, where 1 = 10% up to 10 = 100% of mucosal area, multiplied by the respective grade of damage. Hence, tissue with 100% normal crypt architecture has a CDS score of 0, whereas complete destruction of the colonic mucosa would generate a CDS score of 30.

Immunohistochemistry. Immunohistochemistry for the proliferative marker Ki67 was performed using a rat anti-mouse Ki67 antibody (1:150 diluted by the Tech-Center, DakoCyntomation, Glostrup, Denmark), followed by visualization using a biotinylated mouse anti-rat secondary antibody (Vector Laboratories, Burlingame, CA) with horseradish peroxidase staining. Immunoreactivity was visualized using diaminobenzidine followed by counterstaining with hematoxylin. For each specimen, 20–40 well-oriented crypts from three cross sections of the jejunum or colon were subjected to positional analysis for Ki67, obtained by scoring cells from the crypt bottom (position 1) up to position 20 as positive or negative, in a blinded fashion.

Immunohistochemistry for the epithelial cytokeratins AE1/AE3 was performed using a monoclonal mouse anti-human cytokeratin AE1/AE3 antibody (1:150 dilution; Dako North America, Carpinteria, CA). A goat anti-mouse secondary antibody (Vector Laboratories), followed by horseradish peroxidase treatment, diaminobenzidine staining, and H&E counterstain, was used to visualize the immunoreactivity. Tissue sections were then scored as having high- or low-grade dysplasia or intramucosal cancer. A two-tiered grading system was used for assessing the degree of dysplasia. The diagnosis of high-grade dysplasia was based primarily on architectural features, supplemented by appropriate cytology. The normal architecture includes cribriform glandular arrangement with “back-to-back” glands, prominent glandular budding, and intraluminal papillary tufting. These architectural features are usually accompanied by cytological features, such as loss of cell polarity and nuclear stratification (49). Diagnosis of intramucosal cancer was based on observations of AE1/AE3-positive epithelial cell invasion within the muscularis.

Immunohistochemistry for the quiescent stem cell marker doublecortin-and-caldesmon-kinase-like-1 (DCAMKL-1) was performed using an anti-human DCAMKL-1 COOH-terminal purified rabbit polyclonal antibody (1:30 dilution; Abgent, San Diego, CA). Cells positive for DCAMKL-1 were counted and classified as belonging to normal, dysplastic, or tumor tissue, in a blinded fashion. The total area of the section, scored as normal mucosa, dysplastic tissue, or tumor, was quantified using AxioVision software, and the number of DCAMKL-1-positive cells per unit area was determined.

Immunohistochemistry for β-catenin was performed using a purified mouse anti-mouse β-catenin antibody (1:300 dilution; BD Transduction Laboratories, Mississauga, ON, Canada), followed by H&E staining as described previously (20).

Semi-quantitative real-time RT-PCR. Frozen normal colon and colonic tumor tissue sections were lysed, and the RNA was isolated using the RNeasy Plus Mini Kit per manufacturer’s instructions (Qiagen, Mississauga, ON, Canada). The extracted RNA was then treated with DNase, quantified, and subjected to treatment with Superscript II Reverse Transcriptase. The cDNA was analyzed by PCR using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) in a Chromo4 Continuous Fluorescence Detection unit with Opticon Monitor Analysis software (Bio-Rad Laboratories, Mississauga, ON, Canada). Amplification was determined to be linear over three orders of magnitude for the GLP-2R (Mm01329473_m1), β-catenin (Mm00483039_m1), and 18S (endogenous control, Hs99999901_s1) primers. The ΔΔCt(t) method was used to calculate relative mRNA expression (47).

Western blot. Tumors were sonicated in radioimmunoprecipitation assay buffer containing protease inhibitors, and equal amounts of protein were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated on 7.5% SDS-PAGE gel, transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA), and incubated overnight with a mouse β-catenin antiseraum (1:1,000; BD Biosciences, Mississauga, ON, Canada) and a rabbit anti-actin antiseraum (1:1,000; Sigma Chemical, St. Louis, MO). After addition of mouse and rabbit horseradish peroxidase-anti-IgG secondary antisera (1: 2,000; Cell Signaling, Danvers, MA), bands were visualized using an electrochemical luminescence detection system (Amersham Pharmacia Biotech, Baie d’Urfe, QC, Canada) and imaged on a Kodak Image Station 4000MM PRO system.

Statistical Analysis

All data are presented as means ± SE. Statistical significance was established by one-way ANOVA followed by Bonferroni correction, Tukey’s or n-1 custom hypotheses tests, as appropriate, or by chi-square test, using GraphPad (San Diego, CA) or Statistical Analysis System (Cary, NC) software. CDS data were normalized by log10 transformation to reduce variance. Statistical significance was defined as P < 0.05.

RESULTS

Rat PhIP-ACF Study

To determine the effects of GLP-2 on the development of ACF, in a novel species treated with a unique carcinogen, rats were acutely fed the colon cancer-inducing agent, PhIP, and then subjected to a HFD (Fig. 1A). Compared with vehicle-treated rats fed the control AIN-93G diet alone (i.e., NoPhIP + Bicarb group), rats fed with the PhIP diet had reduced gain in body weight over the 6-wk experiment (266 ± 6.9% vs. 214 ± 11.7%, P < 0.05). However, normalized small intestinal and colon weights, as well as crypt and villus lengths were similar between the NoPhIP + Bicarb and PhIP + Bicarb animals, indicating that PhIP feeding per se did not have any effect on parameters of intestinal growth (Fig. 2, A–C). In contrast, whereas administration of hGLy2GLP-2 did not alter
body weight gain among the PhIP-fed animals, exogenous hGly2GLP-2 led to a 67.4 ± 4.5% (P < 0.001) and 43.0 ± 6.3% (P < 0.001) increase in normalized small intestinal weight and villus height, respectively, without any changes in crypt depth (Fig. 2, A and B). There was also a trend toward increased colon weight with hGly2GLP-2 treatment although this did not reach significance (Fig. 2C). Together, these findings indicated that the hGly2GLP-2 utilized was biologically active in PhIP-fed rats.

Although a few ACF were detected in the NoPhIP/Bicarb rats, animals in the PhIP+Bicarb group had a 470 ± 106% increase (P < 0.01) in the number of ACF, compared with the NoPhIP+Bicarb animals (Fig. 2D). Moreover, PhIP-fed rats treated with hGly2GLP-2 demonstrated an even greater increase in the number of ACF, by 72 ± 11%, compared with rats in the PhIP+Bicarb group (P < 0.01). This increase was associated with enhanced numbers of one-crypt ACF (P < 0.05), but not of two- and three-crypt ACF (Fig. 2E). The distribution of ACF along the length of the colon was not different between the treatment groups, nor were there any differences in the number of mucin-depleted foci (data not shown).

**Rat PhIP-Tumor Study**

To evaluate the progression of ACF to cancer, rats were subjected to repeated cycles of PhIP and a HFD, followed by a HFD for an additional 42-wk period (Fig. 1B). By the 52nd wk of the study, three PhIP/HFD rats were found dead or were euthanized due to significant weight loss or other morbidity. Among these, at necropsy, one rat from the Bicarb-treated group was found with a noncancerous intestinal tumor, whereas a second died due to large-cell lymphocytic leukemia, both as assessed by the veterinarian of the animal facility. One hGly2GLP-2-treated rat died in association with excessive bleeding from the mouth and anus that was not further assessed. Analysis of tumor sections from the rats that survived to the end of study revealed that none of the PhIP+Bicarb-
treated rats had large intestinal cancer (i.e., 0 of 7 rats), whereas 22% of the rats given the hGly2GLP-2 treatment had cancerous tumors in the cecum or colon (i.e., 2 of 9 rats; Fig. 2F).

Mouse DSS-AOM Study

To determine whether the effects of AOM on colon cancer are modified in a model of increased risk, mice were treated with DSS for three cycles to mimic the setting of chronic colitis (Fig. 1C). A slight decrease in body weight was noted after each DSS cycle; however, body weights on the day of death were not different for the vehicle-, hGly2GLP-2-, and hGLP-23–33-treated groups (27.0 ± 0.3 g for Bicarb vs. 26.7 ± 0.3 g for hGly2GLP-2 and 27.8 ± 0.3 g for hGLP-23–33). Administration of hGly2GLP-2 significantly increased small intestinal weight by 48.8 ± 6.0% (P < 0.001; Fig. 3A), as well as jejunal villus height and crypt length of mice by 42.8 ± 3.5% and 10.1 ± 2.4%, respectively (P < 0.01–0.001; Fig. 3B). In contrast, blocking the actions of endogenous GLP-2 by administration of hGLP-23–33 did not alter normalized small intestinal weight, villus height, or crypt depth (Fig. 3, A and B). Finally, whereas administration of hGly2GLP-2 increased jejunal crypt cell proliferation at positions 2 and 20 (P < 0.05–0.01), administration of hGLP-23–33 reduced proliferation in positions 10, 14, 16, 17, and 19 (P < 0.05–0.01; Fig. 3C). Together, these results indicate that both the hGly2GLP-2 and hGLP-23–33 were biologically active in the murine DSS-AOM mouse model.

All colon tissue sections with suspected tumors were assessed for the presence or absence of low- or high-grade dysplasia and colon cancer, using both H&E- and AE1/AE3-stained sections. Whereas 56% of vehicle-treated (control) mice demonstrated high-grade dysplasia/cancer colon cancer, this was increased to 64% in the hGly2GLP-2-treated animals, and this trend was reversed (decreased to 46%) in mice administered hGLP-23–33 (Fig. 4). Thus the incidence of high-grade dysplasia and cancer was increased by 15% in mice administered hGly2GLP-2, whereas that in hGLP-23–33-treated animals was decreased by 17% (Fig. 4). When taken together, chi-square analysis for trends indicated a significant relationship between GLP-2 levels and dysplasia (P < 0.05, such that the lowest rate was observed with antagonist treatment (e.g., reduced endogenous GLP-2 levels), followed by an intermediate incidence with Bicarb treatment (e.g., with normal endogenous GLP-2 levels) and the highest rate with exogenous administration of hGly2GLP-2. To determine whether the treatments with hGly2GLP-2 or hGLP-23–33 altered parameters of colonic inflammation, the normalized colon weight, length, and CDS were quantified. There was no change in either normalized colon weight or length with hGly2GLP-2 or hGLP-23–33 administration (Fig. 5, A and B). However, quantification of colonic damage by measurement of CDS revealed that, whereas hGly2GLP-2 treatment did not alter the CDS, blocking endogenous GLP-2 action with hGLP-23–33 did reduce CDS by 32.0 ± 8.4% (P < 0.05; Fig. 5C). Collectively, these data indicate that, although the experimental treatments did not alter several parameters of colonic growth, blocking endogenous GLP-2 reduces colitis damage in the colons of AOM-treated mice with chronic DSS-associated colitis.

To further examine possible mechanisms underlying the changes in high-grade dysplasia/cancer incidence with GLP-2 treatment, qRT-PCR was performed on total RNA extracted from cancer tissues. No differences in expression of the GLP-2R (Fig. 5D) or β-catenin (Fig. 5E) were observed between the treatments although there was a trend toward decreased GLP-2R levels in the hGly2GLP-2 and hGLP-23–33 groups. β-Catenin protein levels were also not different between Bicarb-treated and either hGly2GLP-2- or hGLP-23–33-treated animals (Fig. 5F). However, immunohistochemistry for β-catenin revealed that, although this transcription factor was present mostly at the cellular membrane in normal tissue, it was localized throughout the cytosolic and nuclear fractions in many of the cells found in the tumor and dysplastic tissues (Fig. 5G). Finally, quantification of DCAMKL-1-positive cells per unit area of normal vs. dysplastic and cancer tumor tissue revealed markedly fewer DCAMKL-1-positive cells in the tumors compared with normal tissue (Fig. 5H). Moreover, within the tumors, there was a 73.7 ± 8.6% reduction in the number of these cells in mice administered hGLP-23–33 compared with the Bicarb-treated controls (P < 0.05).

**DISCUSSION**

GLP-2 is an intestinal hormone with a multitude of beneficial effects that lead to improved intestinal growth and function (51). Hence, long-acting analogs of GLP-2 are now being considered as treatment options for patients with intestinal insufficiency or inflammatory bowel disease (IBD) (8, 11, 29). The growth-promoting actions of both exogenous and endogenous GLP-2 in the intestinal mucosa are of particular concern in context of the finding that individuals with IBD are at increased risk of developing colon cancer (21, 52). Moreover,
it has also been shown that patients with active IBD have greater levels of endogenous bioactive GLP-2 (67) although this does not appear to be the case in patients with mild IBD (54). However, studies in the literature examining the effect of GLP-2 on colon cancer have used only mice to date, and only in models of sporadic colon cancer (26, 32, 63). Hence, to examine the broader applicability of GLP-2 as a potential cancer promoter, the effect of GLP-2 on dysplasia and cancer in the colon was studied herein using both a different species and a novel inducer, namely rats fed the carcinogen PhIP in association with a HFD, which not only induces mild intestinal inflammation, but also promotes the carcinogenic effect of PhIP (16, 34, 35, 41, 45). Of note, PhIP is a common dietary carcinogen found in cooked meat and fish that has been estimated to contribute to nearly 50% of the cancer risk associated with normal consumption of these foods (34). Moreover, to assess the effect of GLP-2 on colon cancer in association with more severe, chronic colonic inflammation, DSS-AOM was used to create a murine model of colon cancer-associated colitis. Together, these models cover two species with dietary and chemical carcinogenesis, in association with mild dietary- and more severe chemically-induced intestinal inflammation, respectively. When taken together with findings of a previous study in normal mice treated with AOM (26), the present results indicate that both exogenous and endogenous GLP-2 increase colonic dysplasia and, possibly, colon cancer in a variety of rodent models of this disease.

The major finding of this study was that, in rats fed PhIP, treatment with the long-acting GLP-2 analog hGly2GLP-2 enhanced dysplastic colonic growth. Although this change occurred predominantly at the level of one-crypt ACF, which are considered to be less dysplastic than larger ACF (60), it corresponded with the finding of colon cancer only in the PhIP-HFD rats treated with hGly2GLP-2. Similar findings were made in the AOM-DSS mice, such that the incidence of high-grade dysplasia and cancer was increased by exogenous GLP-2, whereas the development of these growths was decreased by GLP-2R antagonism. The changes in colon cancer incidence were relatively small in the present study, ranging from 22% in the rats to 15–17% (for exogenous and endogenous GLP-2, respectively) in the mice. Nonetheless, the findings are in accord with our previous observation of a 30–63% increase in ACF in AOM-mice treated with hGly2GLP-2 for 4 wk and a 22–39% decrease following administration of hGLP-23–33 with mucin-depleted foci and colon cancer found in the hGly2GLP-2-treated animals only (26). Similar effects have also been observed in mice administered the carcinogen dimethylhydrazine, wherein treatment with both GLP-2 and hGly2GLP-2 increased the number of colonic polyps (63). Although these findings add PhIP to AOM and dimethylhydrazine as dysplastic agents for which the effects are modifiable by GLP-2 treatment, the results are difficult to reconcile with the findings of another study using APCmin/H11001 mice in which GLP-2 was not found to alter the incidence of adenomas (32). The major difference between the PhIP, AOM, and dimethylhydrazine rodent models of colon cancer and the APCmin/H11001 mouse is the driving agent for the abnormal growth, such that PhIP, AOM, and dimethylhydrazine are all models of sporadic colon cancer compared with the genetically driven small and large intestinal tumorigenesis caused by the APCmin/+ mutation (14). When taken together, therefore, these findings suggest that, although both exogenous and endogenous GLP-2 can enhance dysplastic growth in the rodent colon, the effects are dependent on the model utilized. These findings may also have clinical implications for patients treated with long-acting analogs of GLP-2, such that more frequent screening for colonic dysplasia may be preemptive.

To examine possible mechanisms underlying the effects of exogenous and endogenous GLP-2 on dysplasia and cancer in the AOM-DSS mice, colonic damage score, GLP-2R expres-
Fig. 5. Effect of hGly2GLP-2 and hGLP-23–33 on colonic damage, GLP-2R expression, doublecortin-and-calmodulin-kinase-like-1 (DCAMKL-1)-positive stem cells and β-catenin levels in AOM-DSS mice. Normalized colonic weight (A; n = 25–31), colon length (B; n = 25–32), colonic damage score (CDS) (C; n = 26–34), GLP-2R mRNA transcript levels (D; n = 5–9), β-catenin mRNA transcript levels (E; n = 8–9 for normal tissue; n = 22 for high-grade dysplasia/cancer), β-catenin protein levels (F; n = 5 for normal tissue; n = 5 for high-grade dysplasia/cancer; a representative blot is shown) are shown. Representative β-catenin staining in normal tissue and high-grade dysplasia/cancer (G) and number of DCAMKL-1-positive stem cells (H; n = 10–14; a representative photomicrograph is shown) in mice treated with Bicarb (open bars), hGly2GLP-2 (shaded bars), or hGLP-23–33 (solid bars; *P < 0.05 as indicated) are shown.
GLP-2 ENHANCES COLONIC DYSPLASIA

sion, β-catenin accumulation, and stem cell growth were examined. Unexpectedly, although hGly2GLP-2 treatment has been shown to increase growth and reduce CDS in models of acute colitis, including that induced by DSS (19, 28, 33, 57), treatment with hGly2GLP-2 did not change these parameters in the setting of AOM-induced cancer with chronic colitis. Indeed, blocking endogenous GLP-2 action actually reduced CDS in this model. However, the model used in the present study was one of, not only repeated cycles of inflammation, but also cancer induction. It is therefore not clear whether the findings can be extended to humans with IBD, who have been shown in one trial to respond favorably to treatment with a long-acting GLP-2 analog (11). Nonetheless, the reduction in CDS corresponded with the finding of decreased carcinogenesis in the hGLP-23–33-treated mice and may have been a contributing factor, given the established link between inflammation and colon cancer risk (21, 52).

In contrast to the findings with CDS, no significant changes in GLP-2R mRNA transcript levels were found between the treatment groups. However, analysis of mRNA transcript levels may not reflect GLP-2R protein levels, and there are currently no commercially available antisera with which these can be ascertained. Therefore, β-catenin expression was analyzed as an indirect measure of GLP-2R signaling in the tumors. The cWnt pathway is known to be involved in tumorigenesis of human cancers and is a target for mutations in both tumors. The cWnt pathway is known to be involved in tumor progression, and both protein expression and localization. Although the mRNA and protein levels of β-catenin were found to be similar in all treatment groups, immunohistochemistry revealed that there were higher levels of nuclear β-catenin in the tumor tissues. This result is consistent with the finding that β-catenin accumulation in colonic tumor tissues is a result of mutations in the β-catenin (Ctnnb1) and APC genes that prevent the ubiquitination and degradation of β-catenin (46). Notwithstanding, none of these findings appear to account for the findings of altered dysplasia and cancer rates in mice consequent to either increased or decreased GLP-2 activity.

Previous studies have demonstrated that intestinal crypt stem cells, not only regulate normal intestinal growth and repair, but also can play a role in tumorigenesis (3, 39). The two main populations of stem cells are the rapidly cycling crypt-based columnar cells, expressing Lgr5, and the quiescent stem cells found at position 4, identified by staining for DCAMKL-1 (4, 38). It has previously been reported that the DCAMKL-1-positive stem cells can be modulated by intestinal growth factors, such as gastrin, as well as by the presence of carcinogenic mutations (31, 37). Our finding that neither hGly2GLP-2 nor GLP-23–33 altered the numbers of these cells in the normal colon indicates that the growth effects of GLP-2 in the normal gut may not be mediated by increasing the prevalence of this population of stem cells although alterations in the cell cycle length cannot be precluded (53). Nonetheless, in high-grade dysplasia and cancer tissues, a marked reduction in DCAMKL-1-positive stem cells was observed in response to blockade of endogenous GLP-2, in association with reduced cancer incidence, suggesting a possible role for these cells in the actions of endogenous GLP-2 as a promoter of colitis-associated cancer.

Finally, it remains unclear as to the exact mediator by which the cancer-promoting effects of both endogenous and exogenous GLP-2 are mediated. Previous studies have demonstrated that the intestinal growth effects of exogenous GLP-2 are exerted indirectly through ErbB ligands and receptors, insulin-like growth factor-1 and its receptor, and keratinocyte growth factor (51), of which both the ErbB and insulin-like growth factor pathways have been implicated in the development of colon cancer (13, 23). Whether this is also the case for endogenous GLP-2, the relative potencies of the two peptides in the promotion of colon cancer, and the direct effects of these mediators of GLP-2 action on intestinal stem cells remains to be determined.

Collectively, the results of these studies demonstrate that hGly2GLP-2 promotes the development of preneoplastic lesions in rats fed with the dietary carcinogen PhIP, in support of previous findings made with a different carcinogen (i.e., AOM) in another species (i.e., murine; Ref. 26). Although only a small role for GLP-2 as a cancer promotor was identified in PhIP-fed rats and mice with AOM-induced colitis-associated cancer, blocking endogenous GLP-2 action was found to correspondingly reduce cancer, in association with factors that are known to drive dysplastic growth, such as colitis-associated colonic damage (a measure of inflammation) and stem cell numbers (3, 22, 44, 66). Although there have been no reports to date of intestinal dysplasia in patients with short bowel treated for up to 2 yr with long-acting analogs of GLP-2, or in those with Crohn’s disease treated for 8 wk, it has been suggested that such individuals may benefit from preventative surveillance (11, 30, 43). The results of the present study are clearly consistent with this recommendation, particularly for those with an intact colon who are treated chronically and/or have increased cancer risk in association with IBD. Furthermore, because blocking endogenous GLP-2 reduced colitis damage score as well as DCAMKL-1-positive cancer stem cells, these findings also suggest that hGLP-23–33 may be useful as a potential cancer-preventing biological agent in patients at increased risk for colon cancer.

ACKNOWLEDGMENTS

The authors are grateful to Dr. K. Banks, University of Toronto, for veterinary assistance and to Mr. A. Izzo for technical assistance.

GRANTS

S. Trivedi was supported by a graduate studentships from the Canadian Institutes of Health Research (CIHR; Frederick Banting and Charles Best Canada Graduate Scholarship) and the Department of Physiology, University of Toronto. S. Wiber was supported by summer studentships from the Canadian Association of Gastroenterology/CIHR and the Department of Physiology/University of Toronto Research Opportunity Program. P. Brubaker was supported by the Canada Research Chairs program. This work was supported by operating grants from the Canadian Institutes of Health Research (MOP-9940 and NMD-94732).

DISCLOSURES

P. Brubaker has received consulting fees from NPS Pharmaceuticals.

AUTHOR CONTRIBUTIONS

Author contributions: S.T. and P.L.B. conception and design of research; S.T. and S.C.W. performed experiments; S.T., S.C.W., H.M.E.-Z., and P.L.B. analyzed data; S.T., S.C.W., and P.L.B. interpreted results of experiments; S.T.
GLP-2 ENHANCES COLONIC DYSPLASIA


REFERENCES


