Increased Colorectal Epithelial Cell Proliferation and Crypt Fission Associated with Obesity and Roux-en-Y Gastric Bypass

Anita Sainsbury,1 Robert A. Goodlad,3 Sarah L. Perry,1 Stephen G. Pollard,2 Gerard G. Robins,1 and Mark A. Hull1

1Section of Molecular Gastroenterology, Leeds Institute of Molecular Medicine, University of Leeds, St. James’s University Hospital; 2Department of Obesity Surgery, St. James’s University Hospital, Leeds, United Kingdom; and 3Histopathology Unit, London Research Institute, Cancer Research UK, London, United Kingdom

Abstract

Background and Aims: The relationship between obesity, weight reduction, and future risk of colorectal cancer is not well understood. Therefore, we compared mucosal biomarkers in normal weight individuals [body mass index (BMI), 18.5-24.9 kg/m²] with those in morbidly obese patients (BMI >40 kg/m²) before and 6 months after Roux-en-Y gastric bypass (RYGB).

Methods: Rectal epithelial cell mitosis, crypt area, and crypt branching were measured following whole crypt microdissection. Apoptosis was measured by immunohistochemistry for neo-cytokeratin 18 on fixed tissue sections. Serum levels of C-reactive protein and cytokines were assayed in combination with quantification of mucosal proinflammatory gene expression by real-time RT-PCR.

Results: Twenty-six morbidly obese patients (mean BMI, 54.4 kg/m²) had significantly increased mitosis, crypt area, and crypt branching (all P < 0.01) compared with 21 age- and sex-matched normal weight individuals (mean BMI, 22.5 kg/m²). Morbidly obese patients underwent a mean excess weight loss of 41.7% at a mean of 26 weeks after RYGB. Surprisingly, this was associated with a further increase in mitosis and decreased apoptosis of epithelial cells. At the same time, lower levels of serum C-reactive protein and interleukin-6 following RYGB were accompanied by a reduction in mucosal IL-6 protein content but elevated mucosal expression of other proinflammatory genes such as cyclooxygenase-1 and cyclooxygenase-2.

Conclusions: Mucosal biomarkers, accepted as indicators of future colorectal cancer risk, are increased in morbidly obese patients compared with normal weight controls. The hyperproliferative state that exists 6 months after RYGB may have important implications for long-term colorectal cancer risk in bariatric surgery patients. (Cancer Epidemiol Biomarkers Prev 2008;17(6):1401–10)

Introduction

Multiple epidemiologic studies have described excess body weight as an independent risk factor for development of colorectal cancer (1, 2). In general, the relative risk of colorectal cancer increases with excess body weight from overweight [defined as body mass index (BMI; weight/height²) 25-29.9 kg/m²] to obesity (BMI >30 kg/m²), compared with normal weight individuals (BMI, 18.5-24.9 kg/m²). The relative risk of colorectal cancer in subjects in the highest BMI group (usually stratified as BMI >28-30 kg/m²) has been reported to vary between 1.5 and 2.0 compared with normal weight controls, with the association being stronger for colonic than rectal cancer, and for men rather than women (1, 2). It has been estimated that 69,000 colorectal cancer–related deaths per year (based on WHO mortality rates for the year 2001) worldwide can be attributed to being overweight and obesity (3). The burden of excess body weight–related colorectal cancer is likely to increase significantly in the future with the continuing “obesity epidemic” (4).

Elevated BMI is also associated with increased risk of colorectal adenoma (1, 2), which suggests that body size influences the premalignant stages of colorectal carcinogenesis during tumor initiation and adenoma growth. A relationship between excess body weight and early stages of colorectal carcinogenesis implies that weight loss in overweight/obese individuals could lead to reduced future risk of colorectal cancer. However, the hypothesis that weight loss reduces subsequent colorectal cancer risk has not been addressed directly by any long-term cohort epidemiologic study, apart from the Iowa Women’s Health Study, which showed a nonsignificant 18% decrease in colorectal cancer incidence in those postmenopausal women who had at least one previous episode of intentional weight loss of >20 pounds (5).

An alternative experimental strategy is to quantify colorectal epithelial cell and crypt biomarkers as surrogate measures of future neoplastic risk (6).
Epithelial cell proliferation and expansion of the crypt proliferative compartment have been associated with factors known to increase colorectal cancer susceptibility (e.g., advanced age, ulcerative colitis, and familial adenomatous polyposis) and carcinogen exposure in rodent colorectal cancer models (6). Therefore, we compared mucosal biomarkers of proliferation (7), apoptosis (8), and crypt branching (or fission; ref. 9) in the rectum of morbidly obese patients (defined as BMI >40 kg/m² or BMI >35 kg/m² in the presence of significant comorbidity) and normal weight individuals. We then studied the effect of marked weight loss in the same morbidly obese patients undergoing Roux-en-Y gastric bypass (RYGB) to test the hypothesis that weight loss in obese individuals leads to a change in rectal mucosal biomarkers. We chose morbidly obese patients undergoing bariatric surgery as model subjects for our study, rather than overweight or obese subjects enrolled in calorie restriction and/or exercise programs, to ensure significant weight loss in a translational research setting. RYGB consistently induces marked weight loss over a short period of time (~30% weight loss or 60% of excess body weight at 12 months; ref. 10), which enabled us to investigate whether weight loss is associated with short-term changes in mucosal biomarkers in a relatively small, homogeneous population of obese subjects, each acting as their own control.

The mechanistic basis of the link between excess body weight and increased colorectal cancer risk has not yet been elucidated. Obesity-related insulin resistance, leading to increased colonic insulin and insulin-like growth factor-1 exposure, increased adipokine levels, and elevated colonocyte oxidative stress and energy substrate availability have been proposed to explain this relationship (1, 2). An alternative hypothesis stating that chronic inflammation links both obesity and colorectal carcinogenesis reconciles independent observations that obesity is a state of chronic systemic inflammation (11) and that chronic mucosal inflammation promotes colorectal carcinogenesis (12). Therefore, we also measured levels of serum markers of inflammation [C-reactive protein (CRP), interleukin (IL)-6, tumor necrosis factor α (TNFα), and macrophage migration inhibitory factor (MIF)] and mucosal proinflammatory mediators [including mRNA levels of cytokines such as IL-1β, IL-6, TNFα, the cyclooxygenase (COX) enzymes, etc.] in morbidly obese patients. Because weight loss induced by RYGB is associated with decreased levels of CRP and IL-6 (13), we also investigated whether weight loss induced by RYGB was associated with a reduction in biomarkers of systemic and mucosal inflammation.

**Materials and Methods**

**Ethical Approval.** All aspects of the study were approved by the Leeds (East) Research Ethics Committee. Written consent was obtained from all patients before recruitment.
Human Subjects and Study Design

**RYGB Patients.** Consecutive patients undergoing RYGB for morbid obesity at St. James’s University Hospital, Leeds between February and December 2005 inclusive were recruited to the study on the day before elective surgery. Inclusion and exclusion criteria for study entry are described in Fig. 1.

A detailed drug history was obtained, including specific questions about nonsteroidal anti-inflammatory drug use (including low-dose aspirin), proton-pump inhibitor therapy, and oral calcium supplementation in the previous 3 months. Symptoms or antibiotic treatment compatible with a recent or current acute illness or inflammatory condition were noted. The height and weight of each subject were measured. Finally, blood was taken for serum collection. The next day, six mucosal biopsies were obtained from the posterior wall of the unprepared rectum, between 8 and 10 cm from the dentate line, using a rigid sigmoidoscope and 2.2-mm jaw, single-use biopsy forceps (Boston Scientific), while the patient was under general anesthesia, just before open RYGB.

In all cases, RYGB was done by one surgeon (S.G.P.) and involved stapling the stomach to produce a 20-mL capacity gastric pouch. The jejunum was then divided 150 cm distal to the ligament of Treitz and the distal end of the divided bowel was anastomosed to the gastric pouch. The Roux-en-Y construction was produced by side anastomosis of the proximal end of the divided bowel to the gastric limb, 150 cm downstream of the gastroenterostomy. Patients who had gallstones shown by preoperative abdominal ultrasonography also underwent cholecystectomy. Postoperatively, patients received 400 mg ferrous sulfate, 1 g elemental calcium, a multivitamin preparation (including 300 units of vitamin D), and 20 mg rabeprazole on a daily basis.

Patients then underwent identical clinical assessment and blood/rectal mucosal sampling at the first routine outpatient clinic visit ~6 months following RYGB.

**Normal Weight Individuals.** Patients with a normal BMI, who had a normal, diagnostic flexible sigmoidoscopy (no macroscopic evidence of mucosal inflammation, diverticulosis, or polyps to the splenic flexure) for investigation of bright-red rectal bleeding or abdominal pain, were sex and age matched (within 4 y) to RYGB patients. All patients received one Fleet Phosphate Enema within 60 min of the procedure. Clinical information and mucosal biopsies were obtained in an identical manner to RYGB patients.

Additionally, two mucosal biopsies were obtained from an area of active mild/moderate proctitis in two patients with ulcerative colitis and two patients with Crohn’s disease. These samples acted as positive controls for the reverse transcription-PCR analysis of mucosal proinflammatory gene expression only.

**Measurement of Serum Inflammatory Markers.** Blood was allowed to clot for 30 min at 20°C and then centrifuged at 3,000 rpm for 10 min at 20°C. Serum was aspirated and stored aliquoted at −80°C. Serum CRP was measured using a high-sensitivity immunoassay (Advia 1650/2400, Bayer HealthCare Diagnostics). Serum cytokine levels were determined using ELISAs from R&D Systems (Europe).

**Whole Crypt Microdissection.** Two rectal biopsies were fixed in Carnoy’s solution for 2 h at 20°C and then transferred to 70% (v/v) ethanol for storage at 20°C. Crypt microdissection of Schiff’s reagent–stained mucosa was done as described (7) using a Lynx stereodissecting microscope.

**Measurement of Crypt Mitosis, Crypt Area, and Crypt Branching.** All analyses were done by one observer blind to the identity of each sample. Twenty randomly chosen, nonbranching whole crypts were analyzed from each biopsy. The total number of mitotic figures in each crypt was counted using a Leica MZ APO stereomicroscope (magnification, ×400 to ×600). The microscope was fitted with an eye-piece graticule to analyze the zonal distribution of mitoses [1 (crypt −5 (lumen)]. Crypt area was measured using Lucia G software (version 4.6, Nikon UK Ltd.). Subsequently, all microdissected crypts from each biopsy were examined for evidence of branching (7). Data from both biopsies were combined to provide individual patient values.

**Immunohistochemistry.** Two biopsies were fixed in 10% (v/v) formalin overnight at 20°C. Biopsies were orientated for embedding in paraffin to produce longitudinal (3-μm thickness) sections.

Immunohistochemistry for neo-cytokeratin 18 (CK18) using M30 antibody was done as described (14). The number of epithelial cells with cytoplasmic brown staining was counted only in complete U-shaped crypts cut in midaxial, longitudinal section from the muscularis mucosa to the lumen. Data are presented as the mean number of apoptotic epithelial cells per whole crypt counted from two biopsies.

Immunohistochemistry for IL-6 on formalin-fixed, paraffin-embedded mucosal sections was carried out with rabbit polyclonal anti-human IL-6 antibody [1:1,600 dilution in 5% (v/v) swine serum in PBS for 60 min at 20°C; Abcam]. Immunoreactive IL-6 was visualized with the ABC/horseradish peroxidase technique (DakoCytomation). Omission of the primary antibody or incubation with an equivalent concentration of nonimmune rabbit IgG (DakoCytomation) was used as a negative control. Human colorectal cancer liver metastasis tissue was used as a positive control (15).

Immunoreactivity for IL-6 in epithelial cells, basement membrane, lamina propria cells, and lamina propria extracellular matrix was scored separately from 0 to 4 based on the distribution and intensity of staining [0, no staining; 1, patchy (<30%) and weak staining; 2, 30-70% cells or stroma stained at moderate intensity; 3, widespread (>70%) staining of moderate intensity; 4, diffuse, strong intensity staining]. A total IL-6 protein score was then assigned to each section (possible range, 0-16).

All immunohistochemical analyses were done by one observer blind to the identity of each section.

**RNA Isolation and Real-time Reverse Transcription-PCR.** These methods are described in Supplementary Methods.

**Statistical Analysis.** Normally distributed biomarker data were analyzed using Student’s unpaired or paired t test. Crypt apoptosis data were compared using either a Mann-Whitney U test or Wilcoxon signed rank test. The percentage of rectal biopsies, which contained no
branching crypts, was compared using χ² or Fisher’s exact test. Categorical patient data were analyzed using χ² or Fisher’s exact test. Mucosal IL-6 protein scores before and after RYGB were compared using the Wilcoxon signed rank test. The correlation between the change in IL-6 mRNA and protein levels following RYGB was tested using Spearman’s correlation coefficient. Statistical significance was assumed if \( P \leq 0.05 \).

**Table 1. Characteristics of morbidly obese RYGB patients and normal weight individuals**

<table>
<thead>
<tr>
<th></th>
<th>Normal weight</th>
<th>Morbid obesity</th>
<th>( P )</th>
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<tbody>
<tr>
<td>( n )</td>
<td>21</td>
<td>26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (mean ( \pm SE; \text{kg/m}^2 ))</td>
<td>22.5 ( \pm ) 0.5</td>
<td>54.4 ( \pm ) 2.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age [mean (range); y]</td>
<td>41 (21–59)</td>
<td>44 (31–60)</td>
<td>0.21</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>15.6</td>
<td>17.9</td>
<td>0.66</td>
</tr>
<tr>
<td>Smoker [n (%)]</td>
<td>8 (38)</td>
<td>1 (4)</td>
<td>0.01</td>
</tr>
<tr>
<td>NSAID use* [n (%)]</td>
<td>3 (14)</td>
<td>6 (23)</td>
<td>0.71</td>
</tr>
<tr>
<td>Previous cholecystectomy [n (%)]</td>
<td>0 (0)</td>
<td>2 (8)</td>
<td>0.50</td>
</tr>
<tr>
<td>Type II diabetes melitus [n (%)]</td>
<td>0 (0)</td>
<td>7 (27)</td>
<td>0.01</td>
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<tr>
<td>Serum level of inflammatory mediators (mean ( \pm SE ))</td>
<td></td>
<td></td>
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<tr>
<td>CRP (mg/L)</td>
<td>0.78 ( \pm ) 0.34</td>
<td>8.65 ( \pm ) 1.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.83 ( \pm ) 0.64</td>
<td>5.16 ( \pm ) 0.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>1.15 ( \pm ) 0.07</td>
<td>1.70 ( \pm ) 0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MIF (ng/mL)</td>
<td>6.95 ( \pm ) 0.67</td>
<td>8.61 ( \pm ) 0.72</td>
<td>0.10</td>
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</table>

Abbreviation: NSAID, nonsteroidal anti-inflammatory drug.

* NSAID use was defined as any use in the previous 3 mo including low-dose aspirin.

† Twelve patients received a cholecystectomy at the same time as RYGB (paired preoperative and postoperative mucosal biomarker data available in 11 cases).

Results

**Patient Characteristics.** Thirty-three consecutive morbidly obese patients were invited to participate in the study. Figure 1 describes patient flow through the study. Morbidly obese patients and normal weight individuals were well matched on age, sex, and nonsteroidal anti-inflammatory drug use (Table 1). No study participant received calcium supplementation before rectal biopsy.

**Increased Rectal Epithelial Cell Mitosis, Crypt Area, and Crypt Branching in Morbidly Obese Patients Compared with Normal Weight Individuals.** The mean number of mitoses per crypt was significantly higher in the rectum of morbidly obese patients before RYGB compared with normal weight individuals (Fig. 2A and B). Zonal analysis of mitotic cells indicated an increase in the number of mitotic cells in zones 4 and 5, along with simultaneous shrinkage of the proportion of mitotic cells in zone 1, in morbidly obese patients compared with normal weight individuals (Fig. 2C). The mean percentage of mitotic epithelial cells in superficial zones 4 to 5 of rectal crypts from morbidly obese patients before RYGB was significantly higher than the mean value for normal weight individuals \( (P = 0.04, \text{Student’s unpaired } t \text{ test}) \).

In keeping with increased epithelial cell proliferation, crypt area was also significantly larger in morbidly obese patients before RYGB than in normal weight individuals (Fig. 2D). The mean number of mitoses per crypt and crypt area remained significantly higher in morbidly obese patients compared with normal weight individuals after exclusion of smokers (data not shown).

We also counted the number of branching crypts as a measure of crypt fission (Fig. 3A and B). Six of 21 (28.6%) individuals with a normal BMI had at least one branching crypt visible in two separate rectal biopsies (Fig. 3C). There was a significant increase in the proportion of morbidly obese patients who had at least one detectable branching crypt (70.8%; \( P = 0.005, \chi^2 \) test) and a significant increase in branched crypt multiplicity in “positive” patients \( (P = 0.04, \text{Student’s unpaired } t \text{ test}; \text{Fig. 3C}) \).

Apoptotic cells were detected infrequently in rectal mucosa from individuals with a normal BMI (Fig. 4A and B). Only 6 of 21 (28.6%) cases had neo-CK18–positive cells evident in complete longitudinal crypts (mean crypt number, 9; range, 2-23). Eleven of 24 (45.8%) morbidly obese patients had crypts that contained neo-CK18–positive cells (mean crypt number, 8; range, 2-19). Neither the percentage of neo-CK18–positive patients nor the overall number of apoptotic cells per crypt was significantly higher in morbidly obese patients compared with normal BMI individuals \( (P = 0.23, \chi^2 \) test) and \( P = 0.16 \) (Mann-Whitney \( U \) test, respectively).

Taken together, these mucosal biomarker data indicate that morbidly obese patients have increased rectal epithelial cell proliferation, compared with individuals with a normal BMI, which is not balanced by an increased level of epithelial cell apoptosis, thus leading to increased crypt size. Having shown significant differences in mucosal biomarkers of future colorectal cancer risk in morbidly obese patients, we next investigated the effect of surgically induced weight loss on elevated mucosal biomarkers in morbidly obese patients.

**RYGB Is Associated with a Further Increase in Rectal Epithelial Cell Mitosis but Decreased Apoptosis.** Postoperative follow-up of patients who underwent RYGB occurred at a mean of 26 weeks (range, 22-34 weeks) after surgery. As expected, there was significant, uniform weight loss (Table 2) with a mean (± SE) excess weight loss of 41.7 ± 2.5% (16).

Surprisingly, there was a highly significant 2-fold increase in the number of mitoses per crypt in rectal mucosa obtained from patients after RYGB compared with paired mucosa from the same individuals before surgery (mean fold increase in mitoses per crypt, 2.1
(95% confidence interval, 1.5-3.7); \( P < 0.001 \), Student’s paired \( t \) test; Fig. 2A]. This was associated with a change in distribution of mitotic epithelial cells in crypts in postoperative biopsies so that there were fewer mitoses in zone 1 and increased numbers of mitotic epithelial cells in zones 4 to 5 compared with pre-RYGB samples (\( P = 0.016 \), Student’s paired \( t \) test; Fig. 2C). There was no significant difference in the increase in crypt mitosis following RYGB in those patients who received either a simultaneous (\( n = 11 \)) or prior cholecystectomy (\( n = 2 \)) compared with individuals who did not have a cholecystectomy (\( n = 11 \)) during RYGB (data not shown).

Despite changes in epithelial cell mitosis following RYGB, there was no significant increase in mean crypt area in paired rectal mucosa after RYGB (Fig. 2D). Similarly, there was no increase in the number of branching crypts in rectal mucosa from morbidly obese patients after RYGB (Fig. 3C). Although the percentage of patients who had at least one branching crypt present increased to 87.5% (Fig. 3C), this was not significantly different from the corresponding value in morbidly obese patients before surgery (70.8%; \( P = 0.29 \), Fisher’s exact test).

The increase in epithelial cell mitosis was accompanied by an overall decrease in the number of apoptotic cells per crypt post-RYGB (Fig. 4B; \( P = 0.033 \), Wilcoxon signed rank test).

**Changes in Serum Inflammatory Markers following RYGB.** As expected (13), our cohort of morbidly obese patients had significantly higher levels of serum CRP and IL-6 than normal weight controls (Table 1). Moreover, RYGB-induced weight loss was associated with a significant reduction in circulating levels of CRP and IL-6 (Table 2). In keeping with the persistent excess body weight of patients ~6 months after RYGB, levels did not fall into the range of values associated with normal body weight (Table 1).

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**Figure 2.** Comparison of mucosal biomarkers in individuals with a normal BMI and morbidly obese patients before and after RYGB. 
**A.** Mean number of mitoses per crypt. Forty whole crypts were evaluated per patient. For **A** and **C**, each data point represents a subject with a normal BMI (\( n = 21 \); open circle) or a patient before (pre) and 6 mo after (post) RYGB (\( n = 24 \); filled circles joined by a line). Bars represent the mean value for each group, with the value noted above. *, \( P < 0.01 \); ***, \( P = 0.001 \). **B.** Counting of mitoses in Schiff’s reagent–stained microdissected rectal mucosa. The microscopic focal plane was moved through the three-dimensional crypt structure to count all mitoses (detected as darker, condensed nuclear material compared with background epithelial cells; arrowheads show examples) in each examined crypt. **C.** Mean percentage number of mitoses in crypt zones 1 to 5. **D.** Mean crypt area. *, \( P < 0.01 \).
Because TNFα and MIF have been implicated in colorectal mucosal inflammation and carcinogenesis (17), we also measured serum levels of these cytokines. Consistent with the idea that obesity is a state of systemic chronic inflammation, serum levels of TNFα and MIF were higher in morbidly obese patients before RYGB compared with individuals with a normal BMI (Table 1), although the difference in serum MIF levels failed to reach a prespecified level of statistical significance. However, in contrast to the reduction in CRP and IL-6 levels associated with RYGB-induced weight loss, serum TNFα and MIF levels were both increased significantly following RYGB (Table 2).

Figure 3. Crypt branching in rectal mucosa from individuals with a normal BMI and morbidly obese patients before and after RYGB. Low-power (A) and higher-power (B) photomicrographs of branching crypts (arrowheads) in microdissected, Schiff’s reagent–stained rectal biopsies. C. Number of branching crypts per patient. Each data point represents the mean number of branching crypts in two biopsies from each individual. Normal BMI individuals with no branching crypts are not represented. The percentage of patients in each group, in whom no branching crypts were observed, is noted below. Lines join paired data from individual morbidly obese patients before (pre) and 6 mo after (post) RYGB. For RYGB patients, paired data including zero scores are included in the figure. Bars represent the mean number of branching crypts per biopsy with at least one branching crypt. *, P < 0.05.

The Reduction in Serum IL-6 Is Associated with a Decrease in Mucosal IL-6 Immunoreactivity after RYGB. Next, we tested whether RYGB-associated changes in serum IL-6 were mirrored by changes at the mucosal level by immunohistochemistry for IL-6 protein on formalin-fixed, paraffin-embedded rectal mucosal sections (Fig. 5). Immunoreactive IL-6 protein was present in surface epithelial cells, but not crypt epithelium, in rectal mucosa (Fig. 5C). There was also prominent, diffuse staining of the lamina propria (Fig. 5D), particularly the basement membrane (Fig. 5E), consistent with known binding of IL-6 to extracellular matrix glycoproteins (18). IL-6 protein was also localized to a small number of lamina propria mononuclear cells (Fig. 5F). Consistent with the reduction in serum IL-6 levels post-RYGB, there was a significant decrease in IL-6 protein immunoreactivity in mucosa collected 6 months after RYGB compared with paired preoperative mucosa [n = 25; median IL-6
protein score, 8 (interquartile range, 6.5-11) versus 5 (interquartile range, 4-6.5), pre-RYGB versus post-RYGB, respectively; \( P < 0.001 \), Wilcoxon signed rank test.

The Effect of RYGB on Mucosal Proinflammatory Gene Expression. We also examined mRNA levels of several proinflammatory genes relevant to colorectal carcinogenesis (\( IL-6, \) \( IL-1\beta, \) \( TNF\alpha, \) \( MIF, \) \( COX-1, \) and \( COX-2 \)), which related, in part, to the serum inflammatory markers that we measured (Supplementary Fig. S1). There was a significant increase in mRNA levels for \( COX-1 \) (\( P = 0.016 \)) and \( COX-2 \) (\( P = 0.006 \)) following RYGB, with a mean fold increase in mRNA levels (calculated as \( 2^{\Delta \Delta Ct} \)) of 7.6 and 4.1, respectively, compared with pre-RYGB values (Supplementary Fig. S1B; Supplementary Table S2).

Interestingly, there was an overall significant increase in IL-6 mRNA levels following RYGB \( (2^{\Delta \Delta Ct} 3.4; P = 0.019; \) Supplementary Fig. S1B) despite the reduction in IL-6 protein levels. Indeed, there was a significant negative correlation (Spearman \( r = -0.41, \) \( P = 0.044 \)) between the change in IL-6 protein and mRNA levels in individual patients (Supplementary Fig. S2).

Mucosal \( IL-6, \) \( COX-1, \) and \( COX-2 \) transcript levels after RYGB were still significantly lower than those associated with active inflammatory bowel disease (Supplementary Table S2). Consistent with the finding that mRNA levels of these proinflammatory genes did not reach levels observed in macroscopically inflammed mucosa from patients with inflammatory bowel disease, all rectal mucosal samples obtained from morbidly obese patients before and after RYGB were macroscopically and microscopically normal with no evidence of increased lamina propria inflammatory cell infiltration.

Figure 4. Apoptotic epithelial cell counting by immunohistochemistry for neo-CK18. A. Rectal mucosa from a morbidly obese patient before RYGB showing apoptotic, neo-CK18–positive cells (arrows). Bar, 20 \( \mu m \). B. Number of apoptotic neo-CK18–positive epithelial cells per crypt in 21 individuals with a normal BMI and in 24 morbidly obese patients before (pre) and 6 mo after (post) RYGB (no complete longitudinal crypts were visible in two cases). Each data point represents an individual patient value. Lines connect paired data. In 10 paired biopsies from RYGB patients, both values were zero (\( \times 10 \)). In two cases, a pre-RYGB value of 0.25 dropped to zero after surgery (\( \times 2 \)). \( *, P = 0.033 \).
This is the first report of an association between excess body weight and elevated mucosal biomarkers of epithelial cell proliferation and crypt fission, which are considered to predict future colorectal cancer risk based on their association with other colorectal cancer risk factors (6). Morbid obesity can now be added to the list of conditions (familial adenomatous polyposis, ulcerative colitis) and patient characteristics (old age, first-degree relative with colorectal cancer, presence of a sporadic adenomatous polyp(s) or colorectal cancer; refs. 6, 19) in which increased epithelial cell mitosis and/or luminal extension of the crypt proliferation zone in nonneoplastic mucosa is associated with an increased risk of future colorectal neoplasia. The increase in crypt area shown in morbidly obese patients compared with individuals with a normal BMI is consistent with data from Goodlad et al. (20), who have previously shown a good correlation between mitosis frequency and crypt area in human colorectal crypts. Crypt branching or fission occurs in "normal" colorectal mucosa (9) and is believed to be responsible for the spread of genetic mutations in the human colon (21). Previously, increased crypt branching has been shown in colorectal mucosa from patients with familial adenomatous polyposis, ulcerative colitis, and Crohn's disease (9). We now report that the degree of crypt branching is also elevated in mucosa from morbidly obese patients compared with normal weight individuals.

An important aspect of our study was paired analysis of unprepared rectal mucosa from patients before and after RYGB. However, we were only able to obtain mucosa from individuals with a normal BMI by biopsy during elective diagnostic flexible sigmoidoscopy, which

### Table 2. Changes in serum inflammatory markers associated with RYGB-induced weight loss

<table>
<thead>
<tr>
<th></th>
<th>Pre-RYGB*</th>
<th>Post-RYGB*</th>
<th>P</th>
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<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>54.4 ± 2.0</td>
<td>41.8 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>151.8 ± 6.3</td>
<td>116.6 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>Serum inflammatory markers</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CRP (mg/L)</td>
<td>8.65 ± 1.60</td>
<td>3.83 ± 0.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>5.16 ± 0.54</td>
<td>3.15 ± 0.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>1.70 ± 0.08</td>
<td>1.93 ± 0.07</td>
<td>0.002</td>
</tr>
<tr>
<td>MIF (ng/mL)</td>
<td>8.61 ± 0.72</td>
<td>11.69 ± 1.12</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data for 26 RYGB patients.

* Student's paired t test.

** Mean ± SE.

### Discussion

This is the first report of an association between excess body weight and elevated mucosal biomarkers of epithelial cell proliferation and crypt fission, which are considered to predict future colorectal cancer risk based on their association with other colorectal cancer risk factors (6). Morbid obesity can now be added to the list of conditions (familial adenomatous polyposis, ulcerative colitis) and patient characteristics (old age, first-degree relative with colorectal cancer, presence of a sporadic adenomatous polyp(s) or colorectal cancer; refs. 6, 19) in which increased epithelial cell mitosis and/or luminal extension of the crypt proliferation zone in nonneoplastic mucosa is associated with an increased risk of future colorectal neoplasia. The increase in crypt area shown in morbidly obese patients compared with individuals with a normal BMI is consistent with data from Goodlad et al. (20), who have previously shown a good correlation between mitosis frequency and crypt area in human colorectal crypts. Crypt branching or fission occurs in "normal" colorectal mucosa (9) and is believed to be responsible for the spread of genetic mutations in the human colon (21). Previously, increased crypt branching has been shown in colorectal mucosa from patients with familial adenomatous polyposis, ulcerative colitis, and Crohn's disease (9). We now report that the degree of crypt branching is also elevated in mucosa from morbidly obese patients compared with normal weight individuals.

An important aspect of our study was paired analysis of unprepared rectal mucosa from patients before and after RYGB. However, we were only able to obtain mucosa from individuals with a normal BMI by biopsy during elective diagnostic flexible sigmoidoscopy, which
entailed a preparatory phosphate enema. Phosphate enema treatment has been associated with an increase in \textit{ex vivo} \[^{3}H\]thymidine incorporation in rectal mucosa (22). If bowel preparation did alter mitosis frequency in our study, it would be likely only to minimize the apparent difference between the normal weight and morbidly obese patient groups. In our study, we would argue that enema preparation is unlikely to have affected the observed mitosis frequency given the short period of time between administration of the enema and mucosal biopsy, as well as the similarity between the mitosis scores in the normal weight individuals that we studied and previously published data (20). However, phosphate enema preparation has been reported to increase ornithine decarboxylase activity in rectal mucosa (23), and profound changes in inducible gene expression could realistically occur in the 30- to 60-minute period between enema administration and sigmoidoscoposcopic biopsy. Therefore, we did not measure mucosal cytokine mRNA levels in normal BMI individuals.

We deliberately studied a cohort of morbidly obese patients to gain “proof-of-principle” on a link between excess body weight and elevated mucosal colorectal cancer biomarkers in humans. Similar studies of less obese patients are now required to distinguish between the possibilities that there is either a linear relationship between excess body weight and changes in mucosal biomarker values or a threshold level at which excess body weight begins to be associated with elevation of mucosal biomarkers.

An unexpected finding was that significant weight loss (~40% excess weight loss) 6 months after RYGB was associated with increased crypt epithelial mitosis and luminal migration of the crypt proliferation zone, rather than a reduction toward values observed in normal weight individuals. We believe that 6 months is sufficient time to exclude a nonspecific mucosal response to abdominal surgery. However, we still do not know whether the hyperproliferative state apparent at 6 months is a transient phenomenon or will persist during longer periods of observation. The duration of a hyperproliferative response is of crucial clinical importance because persistent elevation of this biomarker of colorectal cancer risk would imply that obese patients, who have undergone RYGB, are at long-term increased risk of colorectal neoplasia. Previously, there have only been two anecdotal reports of colorectal cancer in a short, retrospective follow-up study (mean duration, 18.5 months) of RYGB patients (24).

Increased proliferation in post-RYGB patients was not mirrored by further crypt expansion or more frequent crypt branching. It is possible that a longer duration of the hyperproliferative state is necessary for development of increased crypt size and/or increased crypt branching. Consistent with this concept, the crypt fission cycle time in human colon has been estimated to be 27 months (21).

Jejuno-ileal bypass for treatment of obesity is associated with a persistent (at least 2 years after surgery) increase in \textit{ex vivo} \[^{3}H\]thymidine incorporation in human rectal mucosa compared with non-bypass controls with a similar BMI (25). It has been proposed that mucosal hyperproliferation after jejuno-ileal bypass may due to increased exposure of colonocytes to luminal secondary bile acids (25). RYGB also involves proximal small intestinal bypass. Therefore, one hypothesis is that hyperproliferation following RYGB is explained by alterations in colorectal luminal content, including secondary bile salt concentrations.

An alternative hypothesis to explain the hyperproliferative state following RYGB is that profound weight loss (on a background of morbid obesity) per se drives mucosal proliferation. However, data from the only other study of the effect of weight loss in obese individuals (mean 8.6% decrease in weight over 12 weeks by calorie restriction) on a human rectal mucosal colorectal cancer biomarker (which showed a mean 34% decrease in \textit{ex vivo} \[^{3}H\]thymidine incorporation) would not support this theory (26). Taken together, the data from our study and that of Steinbach et al. (26) suggest that there are differential effects on colorectal epithelial cells related to calorie restriction versus surgical weight loss strategies.

Patients received calcium supplementation following RYGB. In the \[^{3}H\]thymidine incorporation study of jejuno-ileal bypass patients (25), supplementation with a similar amount of elemental calcium per day to that which our RYGB patients received reduced mucosal \[^{3}H\]thymidine incorporation down to levels observed in obese non-bypass controls. Therefore, it is possible that the mitosis frequency values observed in post-RYGB patients could be even higher in the absence of calcium supplementation.

Approximately half of the patients who underwent RYGB had a previous cholecystectomy or a cholecystectomy done at the same time as RYGB. It has been shown that the mitotic index (measured by mitotic figure counting in mucosal sections) of colonic mucosa is increased following cholecystectomy (27). However, in our study, we did not detect any significant difference in crypt epithelial mitosis between RYGB patients who did or did not have a concurrent cholecystectomy (data not shown).

Alternative confounders of the postoperative data from the morbidly obese cohort include administration of a proton pump inhibitor and/or iron supplementation. Long-term (>3 years) follow-up and biomarker assessment in RYGB patients is planned and should determine whether changes in epithelial cell biomarkers persist despite cessation of short-term drug therapy.

Elevated serum CRP and IL-6 levels in morbidly obese patients compared with normal BMI individuals and a decrease in serum levels following RYGB-induced weight loss are consistent with previously published data (13). Serum TNFα levels have also been shown to be elevated in obese individuals compared with nonobese controls (28). We report, for the first time, that there is a small but significant increase in serum TNFα levels in obese patients at 6 months post-RYGB. This phenomenon was not shown in a previous study of weight loss secondary to gastric banding (28). Serum MIF levels were also increased at 6 months following RYGB in morbidly obese patients, unlike patients who had similar degrees of excess weight loss secondary to gastric banding (29).

An important observation was that changes in mucosal IL-6 mRNA levels related to RYG did not reflect alterations in mucosal or serum IL-6 protein levels. The negative correlation between changes in mucosal IL-6 transcript and IL-6 protein levels in individual patients following RYG suggests that this...
cytokine may regulate its own gene expression, as has been described in murine cardiac myocytes (30). In light of the IL-6 data, it is now clear that measurement of mucosal cytokine protein and activity levels will be mandatory for further investigation of a link between excess body weight, weight loss, and mucosal inflammation, rather than reliance on measurement of serum cytokine levels and mucosal gene transcript analysis.

IL-6 has been shown to drive human colorectal cell proliferation and invasiveness (31). Therefore, decreased mucosal IL-6 content following RYGB-induced weight loss might be expected to reduce mucosal carcinogenic potential. On the other hand, mRNA levels of both the COX isoforms were increased after RYGB, consistent with increased protumorigenic potential (32). Levels of other proinflammatory cytokine mRNAs, such as IL-1β, TNFα, and MIF, did not change significantly. Therefore, we did not obtain any definitive evidence for lower inflammatory potential in colorectal mucosa following RYGB-induced weight loss.

In summary, this translational biomarker study has provided the first evidence that a mucosal hyperproliferative state exists in excess body weight and elevated colorectal cancer risk, using the clinical model of morbidly obese patients undergoing bariatric surgery. Our data should stimulate further translational and clinical studies of the colorectum of obese individuals, which will lead to greater understanding of the link between obesity and increased colorectal cancer risk, in turn leading to enhanced health education strategies targeting obesity, as well as the development of novel chemoprevention therapy for obesity-related colorectal cancer. The unexpected finding that RYGB-induced weight loss is associated with biomarker changes compatible with further increased risk of colorectal neoplasia has potentially important clinical ramifications for obese patients undergoing bariatric surgery.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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