# Regional Factors Affecting Proliferation in the Large Intestine of the Rat (43405)

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*Abstract.* **Colonic neoplasia is more frequent in the distal colon than in the proximal colon in spontaneous human disease and in carcinogen-induced tumors in rodents. The possibility that this may reflect regional differences in morphology and in proliferative responses to fasting and refeeding was explored in this study in rats. Scanning electron microscopy revealed that the density of colonic crypts was 36% higher in the distal than in the proximal colon, while light microscopy revealed that distal crypts had 70% more colonocytes than proximal crypts. Thus, the number of colonocytes per unit area in the distal colon is approximately twice that in the proximal colon. Proliferation was assessed by the uptake of bromodeoxyuridine *in vivo* and showed that regions of the distal colon had greater suppression of proliferation during fasting than the cecum, and greater enhancement of proliferation during refeeding than that observed in the cecum or the proximal colon. Changes in proliferation associated with fasting and refeeding were accompanied by changes in the concentrations of short chain fatty acids, but the data did not support the hypothesis of a direct relationship between increasing concentrations of short chain fatty acids and enhanced proliferation. Regional differences in morphology and proliferation could be relevant to the greater susceptibility of the distal colon to neoplasia.** [P.S.E.B.M. 1992, Vol 200]

Colorectal neoplasms are not uniformly distributed throughout the large bowel. The majority of neoplasms are located in the left colon and there is clustering of hyperplastic, adenomatous, and malignant polyps in individual patients (1). Furthermore, adenomas in the distal colon tend to be larger, with a higher frequency of villous change and high-grade dysplasia (2, 3). The reasons for these anatomic patterns of occurrence and progression are unknown, although epidemiologic studies have identified associations with age, sex, diet, occupation, and physical activity (4, 5). The density of cells may also be relevant to the location of colon cancer (6), although there are little data on regional variation in crypt density or numbers of cells per crypt.

The factors that control the normal growth and differentiation of colonocytes and the events leading to proliferative abnormalities continue to be unclear. Epidemiologic studies have shown an association between colon cancer and diets high in fat and calories and low in fiber (7). These associations could be mediated by a variety of factors, including luminal concentrations of bile salts and short chain fatty acids (SCFA) and other effects of diet on microflora and large bowel motility. One SCFA, butyrate, is of particular interest because it is a preferred fuel for colonocytes and has additional effects on proliferation and differentiation (8, 9). Furthermore, regional differences in the luminal concentrations of butyrate have been shown with lower concentrations in the distal colon than in the proximal colon in humans and experimental animals (10, 11).

Persuasive data exist to support a relationship between enhanced rates of cell proliferation and increased susceptibility to colon cancer (12). For example, higher rates of proliferation in uninvolved colonic and rectal mucosa have been demonstrated in patients with colon cancer using a variety of techniques, including uptake of tritiated thymidine (13) and bromodeoxyuridine (14) and cell cycle analysis using flow cytometry (15). These enhanced rates of cell proliferation could increase the formation of DNA adducts, decrease the time for DNA repair and may be a prerequisite for oncogene activation and gene deletions that are associated with cancer formation (16, 17). The purpose of this study was to

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examine morphological characteristics and proliferative kinetics in different regions of the rat colon showing differences in susceptibility to carcinogen-induced neoplasia. Proliferation was suppressed by fasting and enhanced by refeeding after fasting, and changes were related to luminal concentrations of SCFA.

## Materials and Methods

The studies were performed in female Sprague-Dawley rats, 250–350 g body weight, maintained on a standard pellet diet (18). The animals were housed in groups of two in wire-bottom cages in a room at constant temperature and with a 12-hr light-dark cycle (0600–1800 hr). Eighteen rats were divided into three equal groups. One group was fed *ad libitum*, a second was fasted for 56 hr and then sacrificed, while a third group was fasted for 56 hr but allowed to refeed for 15 hr prior to sacrifice. Animals were killed between 0900 hr and 1000 hr to avoid any circadian effects on cell proliferation.

At 30 min prior to sacrifice, animals were injected intraperitoneally with bromodeoxyuridine (80 mg/kg). After sacrifice, the abdomen was opened and segments were taken from the duodenum, midjejunum, ileum, cecum, proximal colon, transverse colon, distal colon, and rectum (Fig. 1). The digesta from the cecum and proximal and distal colon were gently expelled and the lumen rinsed with a measured volume of water. The rinsings and the digesta were pooled and weighed, the pH was measured, and an aliquot was reserved for later determination of the concentrations of SCFA by gas liquid chromatography. Mucus was removed by incubation of everted, inflated sacs of intestine with 1.6 m*M* dithiothreitol in Dulbecco's phosphate buffer for 15 min. Thereafter, segments of intestine were opened and pinned on dental wax to maintain a similar degree of tension. They were then fixed with either glutaraldehyde for scanning electron microscopy or with Bouin's solution for assessment of the labeling index (19). Using scanning electron microscopy, the density of crypts in the proximal and distal colon was determined in specimens from 10 animals and at least 10 areas per region were examined. Total cell numbers were determined by counting the numbers of epithelial cells on one side of each of 10–20 well-oriented crypts. The number of cells per crypt was calculated from the product of the height of the crypt (cells) × the number of cells in the crypt circumference. For determination of the labeling index, paraffin sections (5 microns) were incubated with a monoclonal antibody to bromodeoxyuridine (Bioclone, Sydney, Australia), and cell nuclei in S phase were identified by the immunoperoxidase technique of De Fazio *et al.* (20). The labeling index was calculated as the ratio of labeled cells to total cells per crypt and expressed as a percentage.

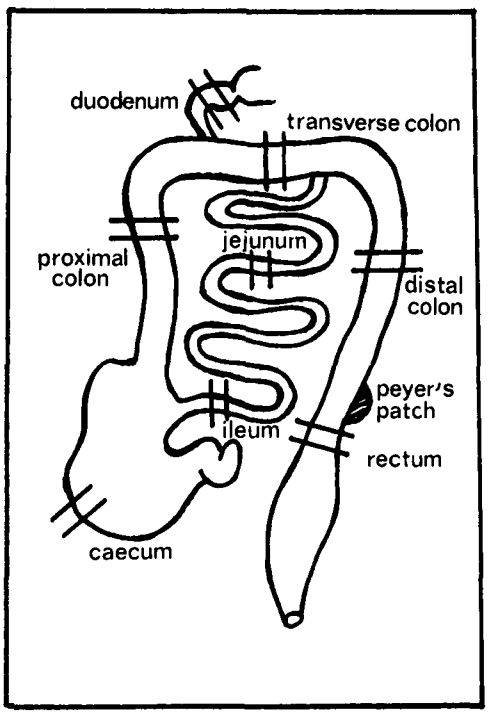


**Figure 1.** A schematic diagram of the rat colon showing the regions from which samples were taken for measurement of proliferative activity.

For determination of SCFA, an aliquot of intestinal contents (0.5–2.5 ml) was dispensed into a disposable glass test tube containing an equal volume of 10% sulfosalicylic acid. The acid precipitate was centrifuged at 2000*g* for 10 min, the supernatant was decanted, and a 100-μl aliquot was mixed with 500 μl of internal standard solution (1 m*M* caproic acid) in a 5-ml flask and shell-frozen in alcohol at −80°C. The flask was attached to a Y piece on the vacuum manifold and further cooled before reducing the pressure. The receiver was cooled and the sample carefully distilled by warming in a waterbath at 50°C. When distillation was complete, the vacuum was released and the distillate thawed and made alkaline with 0.1 *M* NaOH to a pH of >10. The distillate procedure was repeated to yield a dry residue of sodium salts. These were dissolved in 100 μl of 0.1 *M* phosphoric acid for analysis by gas liquid chromatography. One microliter of the sample was injected into the gas liquid chromatograph using TENAX GC/1% phosphoric acid with a column temperature programmed at 130–160°C at 4°C/min. Quantitation was by computing integrator with an internal standard set at 50 mg/ml and a multiplier set to allow for the original solid content of the sample.

## Statistics

Differences between groups were analyzed using the Wilcoxon rank sum test and the Wilcoxon signed rank test. Differences were regarded as significant when $P < 0.05$ using a two-tailed test.

## Results

**Regional Differences in Crypt Density and Cell Number.** A representative scanning electron microscopic image (Fig. 2) shows the surface of the colon and the area defined by the square. The mean number of crypts per unit area (± SE) was calculated to be 526 ± 39 crypts/mm$^2$ ($n$ = 10) in the proximal colon and 714 ± 68 crypts/mm$^2$ ($n$ = 10) in the distal colon ($P <$ 0.05). Thus, crypts were more densely packed (36%) in the distal than in the proximal colon. In the proximal colon, the mean crypt height (± SE) was 21 ± 0.5 cells and the mean circumference was 16 ± 0.7 cells, resulting in a mean cell number per crypt of 336 ± 12 ($n$ = 8). For the distal colon, the mean height was 29 ± 0.5 and the mean circumference was 20 ± 0.8, for a mean cell number per crypt of 580 ± 18 ($n$ = 8). Thus, there were approximately 70% more cells per crypt in the distal than in the proximal colon ($P < 0.05$). Because the distal colon has 36% more crypts per unit area, this region has approximately twice as many cells per unit area as the proximal colon.

**Regional Differences in Proliferative Responses to Fasting/Refeeding.** The labeling indices in the three groups in various regions of the small and large bowel are shown in Figure 3. Fasting resulted in lower mean values in all regions, but the degree of suppression was more prominent in the large bowel than in the small bowel. Furthermore, in the large bowel, the labeling indices in the cecum were higher ($P < 0.01$) than in other regions. On refeeding after a fast, the mean labeling index was higher in all regions, but the increase in proliferation (fasting versus refeeding) was greater in the large bowel than in the small bowel and greater in the distal colon and rectum than in the cecum and proximal and transverse colon. When compared with fasting values, the labeling index after refeeding was significantly higher in all regions of the small and large bowel except the jejunum. Furthermore, after refeeding, the labeling indices of the distal colon and rectum were significantly higher ($P < 0.01$) than those of the cecum and proximal and transverse colon. Rats fed *ad libitum* had proliferative indices intermediate between those of fasting and refeeding and differences between groups reached statistical significance only in the proximal colon, distal colon, and rectum.

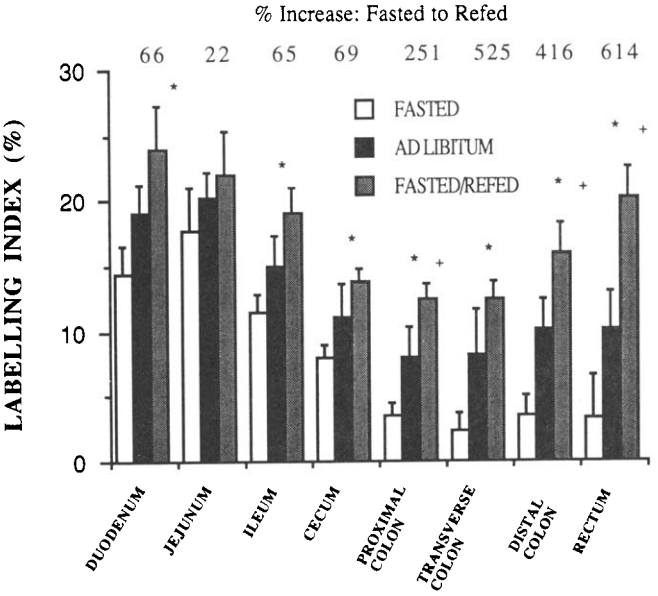


**Figure 3.** The labeling index (%) in biopsies from the duodenum to rectum in rats fed *ad libitum* and in rats after fasting and fasting-refeeding. A comparison of the labeling index after refeeding with that after fasting showed significant differences ($P < 0.01$) (*) in all regions except the jejunum. Differences in the labeling index between refed and *ad libitum* fed rats reached statistical significance ($P <$ 0.05) (+) only in the proximal colon, the distal colon, and the rectum.

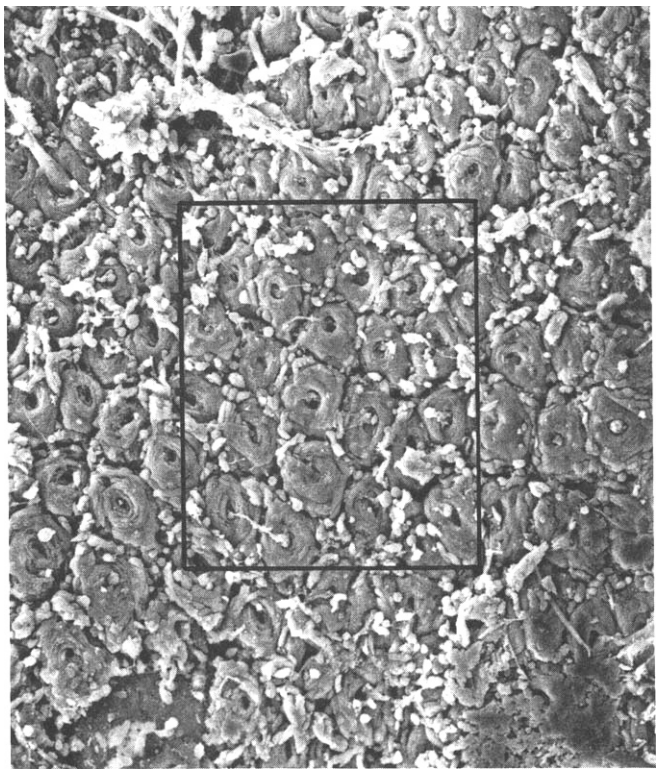

**Figure 2.** A surface scanning electron microscopic view of the rat colon after removal of mucus using 1.6 m*M* dithiothreitol. The boxed area shows a region assessed for the density of crypts.

**Regional Differences in Luminal SCFA Concentrations after Fasting/Refeeding.** After 56 hr of fasting, the rats lost up to 20% of their initial body weight. When refed for 15 hr, the pattern of food intake at five hourly intervals, expressed as a percentage of the total, was 42%, 42% and 16%. The effects of fasting and refeeding on luminal pH and on the mass of digesta in various regions of the large bowel were similar to those we have described previously (11). Concentrations of total and individual SCFA in segments of the large bowel are shown in Figure 4. During feeding *ad libitum*, mean concentrations of total and individual SCFA were

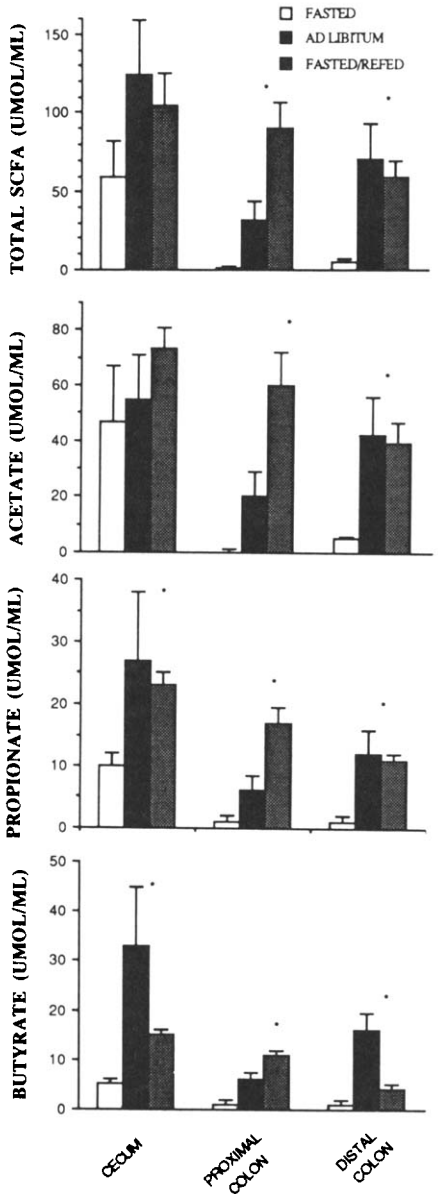


**Figure 4.** Concentrations of individual SCFA and total SCFA in the cecum, proximal colon, and distal colon in rats fed *ad libitum* and in rats after fasting and fasting-refeeding. After fasting, concentrations of total and individual SCFA were significantly lower in all regions ($P < 0.05$) (*) except concentrations of total SCFA and acetate in the cecum.

lower in the proximal and distal colon than in the cecum. After fasting, concentrations of total and individual SCFA declined in all regions, particularly in the proximal and distal colon. After refeeding, concentrations of total SCFA were restored in the cecum and distal colon, but higher concentrations ($P < 0.05$) were observed in the proximal colon. Similar patterns were seen for acetate and propionate. For butyrate, however, concentrations after refeeding remained low in the cecum and distal colon. The highest concentrations of SCFA were observed in the cecum, which showed the smallest change in the labeling index after fasting and refeeding. Indeed, after refeeding, there was a significant negative correlation between the labeling index in the cecum, and proximal and distal colon and concentrations of butyrate ($r = -0.89$, $P < 0.001$), propionate ($r = -0.59$, $P < 0.05$), acetate ($r = -0.55$, $P < 0.05$), and total SCFA ($r = -0.68$, $P < 0.01$).

## Discussion

Results from this study provide two possible explanations for the higher frequency of colonic neoplasms in the left colon in experimental animals. In morphological studies, the crypt density and the number of cells per crypt were significantly higher in the distal colon than in the proximal colon. This 2-fold increase in cell density could increase the likelihood of mutations that lead to the formation of adenomas and cancer. However, care should be taken with such an assumption, since susceptibility to mutational change may be more likely in proliferating crypt cells than in surface mucosal cells. An additional consideration is variation in the exposure of crypt and surface cells to carcinogens and other damaging agents in the gut lumen. These possibilities will only be clarified by the identification of those cells susceptible to malignant transformation and the extent to which such cells are influenced by the luminal environment in the colon.

Greater changes in cellular proliferation with fasting and refeeding were noted in the large bowel than in the small bowel. Furthermore, the labeling index in the small bowel was somewhat higher than in the large bowel, indicating that factors other than proliferation account for the low frequency of neoplasms in the former region. In the large bowel, the degree of change induced by fasting and refeeding was lowest in the cecum and highest in the rectum. Conceivably, the overall cell turnover may be higher in the distal than in the proximal colon, although this has not been clearly established, as yet, either in humans or experimental animals (21). Such an effect, however, could increase the risk for distal neoplasms, perhaps because of an increase in the risk for DNA damage or a decrease in the time for DNA repair. Another possibility is that the frequency of mutations is influenced by fluctuations in proliferation as well as by overall rates of cell division.

Changes in the luminal concentration of SCFA are one possible explanation for greater fluctuations in proliferation in the distal colon. In this study, fasting resulted in a marked decrease in luminal concentrations of SCFA, particularly in the proximal and distal colon. After refeeding for 15 hr, luminal concentrations of SCFA largely returned to levels seen in rats fed *ad libitum*. Although previous studies have provided persuasive data on a role for butyrate in colonic proliferation (9, 22), the present study indicates that the relationship between luminal concentrations of butyrate and proliferative indices is likely to be complex. For

example, in the current study, there was an inverse correlation between luminal concentrations of butyrate and the labeling index. One possibility is that effects on proliferation are dependent upon the concentration of butyrate and the degree of change in the butyrate concentration induced by perturbations such as fasting and refeeding. This would be consistent with studies in the sheep rumen, in which proliferation was enhanced by the acute administration of butyrate and suppressed by prolonged infusions (23). If these observations are relevant to rats, the greater stability of the cecal mucosa in regard to proliferation could be due to maintenance of luminal concentrations of butyrate. In contrast, in the distal colon, concentrations of butyrate fall to extremely low levels during fasting, such that the reappearance of butyrate after refeeding could result in enhanced proliferation.

The above observations raise the possibility that the predilection of neoplasms for the distal colon is related to an increase in cell numbers in the distal colon or to changes in proliferative kinetics that increase the risk of cell mutation. These changes in proliferation are associated with changes in the luminal concentrations of SCFA, but the data do not support the hypothesis of a direct relationship between increasing concentrations of SCFA and enhanced proliferation.


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