Proliferative Activity of Colonic Mucosa at Different Distances from Primary Adenocarcinoma as Determined by the Presence of Statin: A Nonproliferation-Specific Nuclear Protein

Shlomo Kyzer, M.D.,* Benjamin Mitmaker, M.D., Ph.D., F.R.C.S.(C),*
Philip H. Gordon, M.D., F.R.C.S.(C), F.A.C.S.,*
Hyman Schipper, M.D., Ph.D., F.R.C.P.,‡ Eugenia Wang, Ph.D.†

*From the *Department of Surgery, †Departments of Medicine and Anatomy, and ‡Department of Neurology, Sir Mortimer B. Davis-Jewish General Hospital, Lady Davis Institute for Medical Research, and McGill University, Montreal, Quebec, Canada*

The field change is one hypothesis concerning the development of colorectal carcinoma. Removal of a carcinoma without its entire surrounding altered mucosa may result in the development of a recurrence. S44, a monoclonal antibody directed against statin, a nuclear protein expressed in nonproliferating cells in either a quiescent or senescent state, was used to determine the rate of cell growth in colorectal mucosa at different distances from carcinomas. The specimens of 18 patients undergoing resection of a colorectal carcinoma were immediately opened after operation, and strips of mucosa were taken at distances of 1 cm, 5 cm, and 10 cm from the carcinoma. For each location, 10 longitudinally oriented crypts were evaluated for statin-positive cells identified by the presence of a dark brown peroxidase-conjugated antibody reaction product. The average percentage of statin-positive cells per crypt was significantly lower at a 1-cm distance from the carcinoma compared with the mucosa located 5 and 10 cm from the carcinoma (20.89 ± 4.33 at 1 cm, 32.41 ± 5.27 at 5 cm, and 34.23 ± 6.45 at 10 cm). None of the calculated parameters showed any significant difference between the 5-cm and 10-cm locations. The fact that the proliferation rate of the mucosal cells returns to the normal level at 5 cm from the margin of the carcinoma suggests that cells located within this distance still retain proliferative potential even though they are morphologically indistinguishable from their normal counterparts. We conclude that failure to remove this transitional, potentially proliferative mucosa may result in subsequent development of anastomotic or perianastomotic recurrences. [Key words: Proliferative adjacent mucosa; Colon carcinoma; Statin]

Kyzer S, Mitmaker B, Gordon PH, Schipper H, Wang E. Proliferative activity of colonic mucosa at different distances from primary adenocarcinoma as determined by the presence of statin: a nonproliferation-specific nuclear protein. Dis Colon Rectum 1992;35:879–883.

The field change hypothesis is one of the theories proposed for the development of colorectal carcinoma. According to this hypothesis, a field of growth is initiated in a part of the colonic mucosa, and some of the altered colonic cells may be promoted to undergo complete malignant change. Removal of the carcinoma without its entire surrounding changed mucosa may result in the development of local recurrence.

The high iron diamine Alcian blue (HID-AB) stain, which differentiates patterns of mucosubstances in the goblet cells of the colonic mucosa, can define a transitional zone composed mainly of sialomucins proximally and distally to colorectal malignancies.[1–6] This zone extends an average of 3.4 cm from the carcinoma.[5] In addition, the presence of sialomucin at the resection margin was found to be an independent prognostic variable for the development of local recurrence of carcinoma and of subsequent survival for patients with colorectal carcinoma.[7–9] In *in vitro* ^{3}H-thymidine, microautography performed by Ponz de Leon *et al.*[10] failed to demonstrate any appreciable differences

This study was conducted with support from the Sir Mortimer B. Davis-Jewish General Hospital Foundation and the American Physician Fellowship and with grants to Eugenia Wang from the Medical Research Council of Canada and from the National Institute on Aging of the National Institutes of Health of the U.S.A.

Address reprint requests to Dr. Gordon: Sir Mortimer B. Davis-Jewish General Hospital, 3755 Cote Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada.

in cell proliferation between colonic mucosa taken from close proximity (2 cm) of a colorectal carcinoma and mucosa taken from various other distances (4, 8, >10 cm) from the margin of the carcinoma.

S44 is a monoclonal antibody directed against statin, a 57-kilodalton nuclear protein that was first detected in cultures of nonproliferating senescent human fibroblasts and in young fibroblasts where growth was inhibited by serum starvation.[11] Subsequent work has demonstrated statin expression in most, if not all, nonproliferating mammalian and avian tissues.[12, 13] S44, a marker for nongrowing cells, is then a powerful tool for the determination of an altered proliferation rate in normal and neoplastic tissue because any change in the number of the actively dividing cells results in a proportional change in the number of cells that present in the nonproliferative phase. The aim of the present study was to determine S44 immunoreactivity of colorectal mucosa at different distances from carcinomas of the large bowel and thus reveal the indices of growth quantitatively as judged by their respective proximity to the carcinoma.

METHODS

Patients

A group of 18 patients who underwent resection of colorectal adenocarcinomas between November 1989 and March 1990 were entered into the study. Immediately after removal of the specimen, the colon was opened, and strips of mucosa, measuring 0.75 × 1.0 cm and denuded from the muscular layer, were excised 1 cm, 5 cm, and 10 cm proximal to the carcinoma. In cases of carcinomas located in the cecum and proximal ascending colon, mucosal specimens were taken at the same distances distal to the lesion. Specimens were embedded in O-chlorotoluene compound (Miles Labs., Inc., Elkhart, IN), frozen in liquid nitrogen, and stored at −84°C until ready for tissue processing.

Immunohistochemistry

Seven-microns-thick, longitudinally cut colonic sections were mounted on poly-L-lysine (Sigma Chemical Co., St. Louis, MO)-coated slides. These slides were then air-dried for 20 minutes and fixed in methanol/acetone (1:1 v/v) for 10 minutes at −20°C. After fixation, the slides were washed twice in phosphate-buffered saline (PBS), preincubated with 3 percent hydrogen peroxide for 30 minutes, washed in PBS for another 20 minutes, exposed to a diluted normal (blocking) serum for 20 minutes, and incubated in either the antistatin monoclonal antibody (S44)[14] at dilutions of 1:300 or control (PAI ascites) hybridoma fluid overnight in a humidified slide chamber at room temperature. Immunohistochemistry was performed using the vectastain avidin-biotin-complex technique.[15] The revelation medium consisted of diaminobenzidine-H_2O_2 with cobalt chloride ($CoCl_2$) intensification: preincubation with 50 mg of diaminobenzidine plus 1 percent $CoCl_2$ in 100 ml of PBS for five minutes followed by incubation with diaminobenzidine plus $CoCl_2$ plus 0.3 percent H_2O_2 for an additional five minutes. Sections were counterstained with Kernechtrot,[16] dehydrated in graded ethanol, cleared in toluene, and mounted in Permount.

Statin immunoreactivity was determined in the crypt of the colonic mucosa at the different distances from the carcinoma. Statin-positive cells were identified by the presence of a dark brown reaction product diffusely distributed over the entire nucleus or limited in the form of a ring or crescent reaction at the nuclear membrane.

Quantitation of Statin Immunoreactivity

For each mucosal location, 10 longitudinally oriented crypts were evaluated for statin immunoreactivity. Cells from one side of each crypt (column) were counted from the surface epithelium to the base, noting the total number of cells and the number of statin-positive and statin-negative cells. From the ratio of the average number of statin-positive cells per crypt to the average total number of cells per crypt, the percentage of statin-positive cells was calculated. Differences in statin immunoreactivity at the various locations were assessed using chi-squared analysis.

RESULTS

Qualitative Observations

Statin-positive epithelial cell nuclei were found to be located particularly at the upper one-quarter to one-third of the colonic crypt (Fig. 1). Epithelial cells in the rest of the crypt were found to be statin negative, although some scattered statin-positive nuclei were encountered in the lower two-thirds of the crypt, especially at the crypt base. The non-

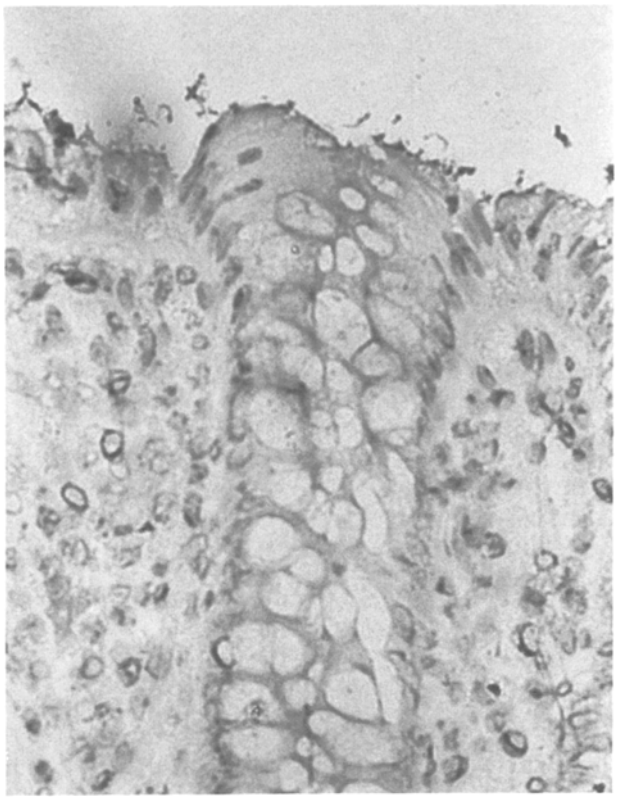

Figure 1. Statin-positive nuclei in upper one-quarter to one-third of colonic crypt.

proliferating pericryptal cells and smooth muscle nuclei of the muscularis mucosa were mostly statin positive, and this phenomenon acts as an internal control (Fig. 2).

Quantitative Results

The average number of statin-positive, statin-negative, and total crypt epithelial cell counts and average percentage of statin-positive cells per crypt are summarized in Table 1. Although the average total crypt cell number does not differ significantly at the different locations (*i.e.*, 1 cm, 5 cm, and 10 cm), the average number of statin-positive cells was found to be significantly lower at the 1-cm location, with a proportionally significantly higher number of statin-negative cells, compared with the mucosa 5 and 10 cm from the carcinoma (Table 1; Fig. 3). The average percentages of statin-positive cells per crypt were significantly lower at the 1-cm distance from the carcinoma compared with the mucosa located 5 and 10 cm from the carcinoma (Table 1). None of the calculated parameters showed any significant difference between the 5-cm and 10-cm locations (Table 1).

DISCUSSION

Recurrence after resection for colorectal carcinoma, although a disappointing event for the surgeon, becomes a calamitous event for the patient. The reported local recurrence rates vary between 2 percent and 20 percent,[17–22] and in many cases, especially those developing after resection of a rectal carcinoma, the recurrence is unresectable and unresponsive to radiotherapy or chemotherapy.

The exact etiology of recurrence of carcinoma at or around anastomoses has been controversial. Explanations such as inadequate primary resection,[23] the presence of residual occult metastases in the lymphatics,[24–27] implantation of viable malignant cells,[28–31] and metachronous carcinogenesis[4, 32–36] all have their advocates but are not totally satisfactory. According to the field change hypothesis, a colorectal carcinoma recurrence may develop because of inadequate resection margins that fail to remove altered mucosa at or near the anastomosis.

Our present study demonstrates a quantitative change in the proliferative state of the normal-appearing mucosa in close proximity to the primary colorectal carcinoma. The increased mucosal pro-

Figure 2. Statin-positive nuclei of pericryptal cells.

Table 1.
Average Number of Statin-Positive Cells at Different Distances from the Carcinoma

	1 cm	5 cm	10 cm
Statin$^+$ cells	16.16 ± 4.31	23.21 ± 4.00	24.17 ± 6.38
Statin$^-$ cells	60.70 ± 10.67	47.75 ± 9.75	45.82 ± 7.67
Total no. of cells	76.54 ± 12.42	71.18 ± 11.25	69.87 ± 9.62
Percent statin$^+$	20.89 ± 4.33	32.41 ± 5.27	34.23 ± 6.45

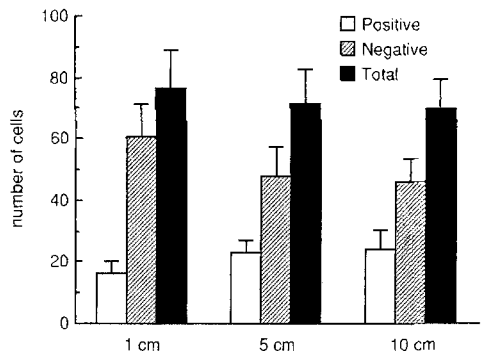

Figure 3. Quantitation of statin positivity at 1 cm, 5 cm, and 10 cm from the carcinoma.

liferation rate disappeared somewhere between and 5 cm from the margin of the carcinoma. This finding supports the presence of mucosa with transitional proliferative activity adjacent to colorectal malignancies. The presence of "transitional" mucosa is also supported by the HID-AB stain, which demonstrates a high content of sialomucin at an average of up to 3.6 cm from the margin of the carcinoma.[5] According to Mori *et al.*,[37] this "transitional" change cannot be classified as a premalignant phenomenon, but rather a nonspecific secondary effect in the mucosa adjacent to the carcinoma. In a recent study using S44 immunoreactivity in our laboratory, we were unable to determine any altered mucosal proliferative activity at colonic anastomoses (unpublished data). The study included a group of patients undergoing resection for colorectal carcinoma 1 to 14 years earlier. Adequate resection margins may explain this finding.

^{3}H-Thymidine labels cells during DNA synthesis (the S phase) of the cell cycle. It thus remains uncertain whether radioactive, negatively labeled cells are still undergoing cell cycle traverse but are in the G_1 or G_2 phase. Our ability to detect statin, a specific G_0 phase marker, overcomes this disadvantage by means of separating the entire population into subfractions of noncycling *vs.* cycling cells. This may explain the fact that ^{3}H-thymidine labeling could not determine the presence of quantitative differences in labeling index among mucosa taken from different distances from colorectal carcinomas.[10]

CONCLUSION

Our study supports the presence of "transitional" mucosa adjacent to colorectal carcinomas. Failure to remove this highly proliferative mucosa may result in subsequent development of anastomotic or perianastomotic recurrences.

REFERENCES

1. Filipe MI. Value of histochemical reactions for mucosubstances in the diagnosis of certain pathological conditions of the colon and rectum. Gut 1969;10: 577–86.
2. Filipe MI. The value of a study of the mucosubstances in rectal biopsies from patients with carcinoma of the rectum and lower sigmoid in the diagnosis of premalignant mucosa. J Clin Pathol 1972; 25:123–8.
3. Rosenberg IL. The etiology of colonic suture line recurrence. Ann R Coll Surg Engl 1979;61:251–7.
4. Habib NA, Dawson PM, Blount MA, Cox S, Kravitz I, Wood CB. Study of the histochemical changes in mucus from normal and tumor-bearing mucosa in patients with colorectal cancer. Eur J Surg Oncol 1985;11:243–5.
5. Greaves P, Filipe MI, Branfoot AC. Transitional mucosa and survival in human colorectal cancer. Cancer 1980;46:764–70.
6. Robey-Cafferty SS, Ro JY, Ordonez G, Cleary KR. Transitional mucosa of colon. A morphological, histochemical and immunohistochemical study. Arch Pathol Lab Med 1990;114:72–5.
7. Dawson PM, Habib NA, Rees HC, Williamson RC, Wood CB. Influence of sialomucin at the resection margin on local tumor recurrence and survival of patients with colorectal cancer. A multivariate analysis. Br J Surg 1987;74:366–9.
8. Habib NA, Salem R, Luck RJ, Blount MA, Rifoot MA, Wood CB. A histochemical method that predicts local recurrence after curative resection in carcinoma of the colon and rectum. Surg Gynecol Obstet 1984;159:436–8.

9. Wood CB, Dawson PM, Habib NA. The sialomucin content of colonic resection margins. Dis Colon Rectum 1985;28:260–1.
10. Ponz de Leon M, Roncucci L, Di Donato P, *et al.* Pattern of epithelial cell proliferation in colorectal mucosa of normal subjects and of patients with adenomatous polyp or cancer of the large bowel. Cancer Res 1988;48:4121–6.
11. Wang E. A 57 kDa molecular weight protein uniquely present in non-proliferating cell and senescent human fibroblasts. J Cell Biol 1985;100:545–51.
12. Wang E. Statin, a non-proliferation specific protein, is associated with the nuclear envelope and is heterogeneously distributed in cells bearing quiescent state. J Cell Physiol 1989;140:418–26.
13. Wang E, Krueger JG. Application of a unique monoclonal antibody as a marker for proliferating subpopulations of cells of same tissue. J Histochem Cytochem 1985;33:582–94.
14. Sester U, Moutsatsos IK, Wang E. A rat liver 57-kDa protein is identified to share antigenic determinants with statin, a marker for non-proliferating cells. Exp Cell Res 1989;182:550–8.
15. Hsu SM, Raine L, Fanger H. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J Histochem Cytochem 1981;29:577–80.
16. Bogden A, Gaboreanu M. Techniques of normal and pathological histochemistry. Bucharest: Ceres Publishing, 1976:78–9.
17. Gill PG, Morris PJ, The survival of patients with colorectal cancer treated in a regional hospital. Br J Surg 1978;65:17–20.
18. Hickey RL, Romsdah MM, Johnson DE, *et al.* Recurrent cancer and metastases. World J Surg 1982;6: 585–95.
19. Labow SB, Salvati EP, Rubin RJ. Suture line recurrence in carcinoma of colon and rectum. Dis Colon Rectum 1975;18:123–5.
20. Long RT, Edwards PH. Implantation metastasis as a cause of local recurrence of colorectal carcinoma. Am J Surg 1989;157:194–201.
21. Pihl E, Hughes ES, McDermott FT, Price AB. Recurrence of carcinoma of the colon and rectum at the anastomotic suture line. Surg Gynecol Obstet 1981; 153:495–6.
22. Rich T, Gunderson L, Lew R, Galdibini J, Cohen A, Donaldson G. Patterns of recurrence of rectal cancer after potentially curative surgery. Cancer 1983;52: 1317–28.
23. Morson BC, Vaughan EG, Bussey HJ. Pelvic recurrence after excision of the rectum for carcinoma. BMJ 1963;2:13–8.
24. Olson RM, Perencevich NP, Malcolm AW, Chaffey JT, Wilson RE. Patterns of recurrence following curative resection of adenocarcinoma of the colon and rectum. Cancer 1980;45:2969–74.
25. Rao AR, Kagan AR, Chan PM, Gilbert HA, Nussbaum H, Hintz BL. Patterns of recurrence following curative resection alone for adenocarcinoma of the rectum and sigmoid colon. Cancer 1981;48:1492–5.
26. Rosenberg IL. The aetiology of colonic suture line recurrence. Ann R Coll Surg Engl 1979;61:251–7.
27. Schackert HK, Fidler IJ. Development of an animal model to study the biology of recurrent colorectal cancer originating from mesenteric lymph system metastases. Int J Cancer 1989;44:177–81.
28. Fermor B, Umpleby HC, Lever JV, Williamson RC. Proliferative and metastatic potential of exfoliated colorectal cancer cells. JNCI 1986;76:347–9.
29. Symes MO, Fermor B, Umpleby HC, Williamson RC. Cells exfoliated from colorectal cancers can proliferate in immune deprived mice. Br J Cancer 1984; 50:423–5.
30. Umpleby HC, Fermor B, Symes MO, Williamson RC. Viability of exfoliated colorectal carcinoma cells. Br J Surg 1984;71:659–63.
31. Vink M. Local recurrence of cancer in the large bowel. The role of implantation, metastases and bowel disinfection. Br J Surg 1954;41:431–3.
32. Lipkin M, Newmark H. Effect of added dietary calcium on colonic epithelial-cell proliferation in subjects at high risk for familial colonic cancer. N Engl J Med 1985;313:1381–4.
33. Shields HM. Occurrence of an adenocarcinoma at the choledochoenteric anastomosis 14 years after pancreatoduodenectomy for benign disease. Gastroenterology 1977;72:322–4.
34. Sooriyaarachhi GS, Johnson RO, Carbone PP. Neoplasms of the large bowel following ureterosigmoidostomy. Arch Surg 1977;112:1174–7.
35. Terpstra OT, Peterson Dahl E, Williamson RC, Ross JS, Malt RA. Colostomy closure promotes cell proliferation and dimethylhydrazine-induced carcinogenesis in rat distal colon. Gastroenterology 1981;81: 475–80.
36. Williamson RC, Bauer FL, Oscarson JE, *et al.* Promotion of azoxymethane-induced colonic neoplasia by resection of the proximal small bowel. Cancer Res 1978;38:3212–7.
37. Mori M, Shimono R, Adachi Y, *et al.* Transitional mucosa in human colorectal lesions. Dis Colon Rectum 1990;33:498–501.