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Immunohistochemical analysis of epithelial cell proliferation in normal-appearing rectal mucosa of patients with colorectal adenoma and cancer using an in vitro labeling method with bromodeoxyuridine.

Nobumasa Ikeda* Motowo Mizuno† Hiroyuki Okada‡

Jun Tomoda** Takao Tsuji††

*Okayama University,
†Okayama University,
‡Okayama Univeristy,
**Okayama University,
††Okayama University,

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Nobumasa Ikeda, Motowo Mizuno, Hiroyuki Okada, Jun Tomoda, and Takao Tsuji

Abstract

To identify diffuse mucosal changes which may precede the development of colorectal cancer and a possible indicator for detecting high-risk populations, we immunohistochemically studied cell-cycle events in crypts of normal-appearing rectal mucosa of patients with colorectal adenoma and cancer using an in vitro labeling method with bromodeoxyuridine (BrdU). Biopsy specimens of endoscopically normal-appearing rectal mucosa were obtained during colonoscopy from 20 patients with colorectal adenocarcinoma, 20 with adenoma, and 15 without apparent colorectal diseases. The specimens were incubated with BrdU in vitro, and labeled S-phase cells were identified immunohistochemically using a monoclonal antibody to BrdU. Modification of the BrdU-labeling pattern in the normal appearing rectal mucosa, such as the presence of BrdU-labeled cells at the mucosal surface or in the upper one-fifth of the crypt column, was observed in 15 of the 20 patients with adenocarcinoma, 17 of the 20 patients with adenoma and 6 of the 15 controls. This upward shift in the frequency of proliferating cells in the crypt was significantly higher in the patients with colorectal adenoma and cancer than in the controls, and may be used to identify subjects at high risk for colorectal cancer.

KEYWORDS: colon cancer, bromodeoxyuridine

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Immunohistochemical Analysis of Epithelial Cell Proliferation in Normal-Appearing Rectal Mucosa of Patients with Colorectal Adenoma and Cancer Using an *In Vitro* Labeling Method with Bromodeoxyuridine

NOBUMASA IKEDA, MOTOWO MIZUNO*, HIROYUKI OKADA, JUN TOMODA AND TAKAO TSUJI

First Department of Internal Medicine, Okayama University Medical School, Okayama 700, Japan

To identify diffuse mucosal changes which may precede the development of colorectal cancer and a possible indicator for detecting high-risk populations, we immunohistochemically studied cell-cycle events in crypts of normal-appearing rectal mucosa of patients with colorectal adenoma and cancer using an *in vitro* labeling method with bromodeoxyuridine (BrdU). Biopsy specimens of endoscopically normal-appearing rectal mucosa were obtained during colonoscopy from 20 patients with colorectal adenocarcinoma, 20 with adenoma, and 15 without apparent colorectal diseases. The specimens were incubated with BrdU *in vitro*, and labeled S-phase cells were identified immunohistochemically using a monoclonal antibody to BrdU. Modification of the BrdU-labeling pattern in the normal appearing rectal mucosa, such as the presence of BrdU-labeled cells at the mucosal surface or in the upper one-fifth of the crypt column, was observed in 15 of the 20 patients with adenocarcinoma, 17 of the 20 patients with adenoma and 6 of the 15 controls. This upward shift in the frequency of proliferating cells in the crypt was significantly higher in the patients with colorectal adenoma and cancer than in the controls, and may be used to identify subjects at high risk for colorectal cancer.

Key words: colon cancer, bromodeoxyuridine

Studying patients with familial adenomatous polyposis, Vogelstein *et al.* (1) suggested that malignant transformation of colorectal mucosa to adenocarcinoma was a multi-step process and that several genetic alterations had already occurred before the development of colorectal cancer (2). Alterations in DNA synthesis activity have also been shown to accompany normal-appearing mucosa that may be an intermediate stage in the development of colorectal cancer (3-6). Studies on cell-cycle events using tritiated thymidine (^{3}H-TdR) revealed an abnormal pattern of ^{3}H-TdR incorporation in normal-appearing colon mucosa among polyps of patients with familial polyposis (3) and in normal-looking colorectal mucosa of patients treated for colorectal cancer (4) or with adenomatous colorectal polyps (5, 6). However, the disadvantages, including the time required to use radioactive isotope labeling have limited the clinical applicability as a means of identifying populations at high risk for colorectal cancer.

In 1984, Raza *et al.* described a simple method of identifying S-phase cell using a thymidine analog, bromodeoxyuridine (BrdU), instead of ^{3}H-TdR (7). The BrdU containing S-phase cells could be detected immunohistochemically using a monoclonal antibody to BrdU. In this study, we examined the cell-cycle events in crypts of normal-appearing rectal mucosa of patients with colorectal cancer using an *in vitro* labeling method with BrdU to identify diffuse mucosal changes which may precede the development of colorectal cancer to determine whether or not this parameter can be used to detect high-risk populations.

Subjects and Methods

Patients. Endoscopic biopsies were obtained during colonoscopic examination from 20 patients with colorectal cancer, 20 with colorectal adenoma, and 15 without apparent colorectal disease. The specimens were obtained from the lesions and from colonoscopically normal-appearing rectal mucosa at least 5 cm from the

* To whom correspondence should be addressed.

lesions. Informed consent was obtained from each patient. The 20 patients with colorectal cancer included 15 men and 5 women with a mean age of 63 years (from 50 to 75). Nine patients had advanced cancer, and 11 patients had early stage cancer. Five cancer lesions were located on the right side of the colon, 10 on the left side, and 5 in the rectum. The 20 patients with adenoma included 15 men and 5 women with a mean age of 54 years (from 42 to 74). The size of the polyps ranged from 2 to 20 mm in diameter. Eight patients had a single polyp, and 12 had multiple polyps. In the patients with multiple polyps, specimens obtained from the largest polyp were examined. Fourteen polyps were histologically tubular, and 6 were tubulovillous adenoma. The 15 control patients included 6 men and 9 women with a mean age of 49 years (from 26 to 79).

Incubation and immunohistochemical detection of BrdU. The biopsy specimens were incubated for 1h in 95 % O_2 and 5 % CO_2 at 37 °C in 100 ml of FC43 solution (Green Cross, Tokyo, Japan) (8) containing 320 μM 5-bromo-2-deoxyuridine (Sigma Chemical Co., St. Louis, MO, USA). The tissues were then fixed in 70 % ethanol overnight, embedded in paraffin, and sectioned. The immunohistochemical detection of incorporated BrdU was performed by the avidin-biotin-peroxidase complex method using the Vectastain ABC Kit PK4002 (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidase activity was blocked by immersing the sections in a 3 % H_2O_2 solution for 5 min at room temperature, with subsequent washing in water. DNA was denatured by treating the sections with 4N HCl for 20 min at room temperature to expose the incorporated BrdU in the DNA chain. Then, the slides were incubated with normal horse serum for 30 min and with anti-BrdU mouse monoclonal antibody (IgG1) (Becton-Dickinson, Mountain View, CA, USA) for 30 min at room temperature. After washing, biotinylated horse anti-mouse immunoglobulin G antibody was applied for 30 min, and the sections were incubated with avidin-biotinylated horseradish peroxidase complex for 45 min at room temperature. The sections were then submerged in 0.06 % 3, 3'-diaminobenzidine tetrahydrochloride (Sigma) solution containing 0.03 % H_2O_2 for 3 min at room temperature to show the immunologic reaction. The reaction products were amplified using a DAB enhancement kit (Amersham, Buckinghamshire, UK), and nuclei were weakly counterstained with hematoxylin.

Immunohistologic analysis. Two sections were examined for each sample. In the normal- appearing rectal mucosa, crypts longitudinally sectioned from the bottom to the top were evaluated. A mean number of 19 well-oriented crypts were examined for each section. The labeling index (LI) was determined by counting the number of BrdU-labeled cells in the total number of epithelial cells in a crypt column of the normal mucosa or in the total number of tumor cells of adenoma and cancer tissues. A minimum of 1,000 nuclei were scored to determine LI. Changes in the distribution of the BrdU-labeled cells in the crypt of the normal-appearing rectal mucosa was evaluated by detecting upward shift of the BrdU-labeled cell in the crypt. The upward shift was arbitrarily defined as the presence of the BrdU-labeled epithelial cell in the mucosal surface or in the upper one-fifth of the crypt column. Differences in LI among groups were analyzed using Student's *t*-test to determine statistical significance, and the tabular data of the distribution of the labeled nuclei in the rectal mucosa was analyzed using the chi-square test.

Results

LI of BrdU in the colorectal cancer specimens ranged from 8 % to 51 % (mean $\pm$ SD, 28.1 % $\pm$ 11.3 %), in the adenoma from 5.4 % to 19 % (12 % $\pm$ 4.1 %) and in the normal rectal mucosa of the control patients from 1.2 % to 8.3 % (4.3 % $\pm$ 2 %). The LI of the cancer was significantly higher than those of the adenoma ($P < 0.001$) and the normal mucosa ($P < 0.001$), and the LI of the adenoma was significantly higher than that of the normal mucosa ($P < 0.001$) (Fig. 1).

LI in the normal-appearing rectal mucosa ranged from 1 % to 6.8 % (4.3 % $\pm$ 1.6 %) in the patients with the colorectal cancer, from 1 % to 8.4 % (4.9 % $\pm$ 2 %) in those with adenoma and from 1.2 % to 8.3 % (4.3 % $\pm$ 2 %) in those of the control patients. There was no significant difference between the LIs in the normal-appearing rectal mucosa of the patients with colorectal cancer or adenoma and that of normal controls (Fig. 2).

BrdU-labeled nuclei were found primarily in the lower portion of the crypt in the normal rectal mucosa (Fig. 3), and were present at the mucosal surface or in the upper one-fifth of the crypt column in 6 of the 15 normal controls. In contrast, in 15 of the 20 patients with the colorectal cancer and in 17 of the 20 with adenoma, BrdU-labeled epithelial cells were observed at the surface or in the upper one-fifth of the crypt in the normal-

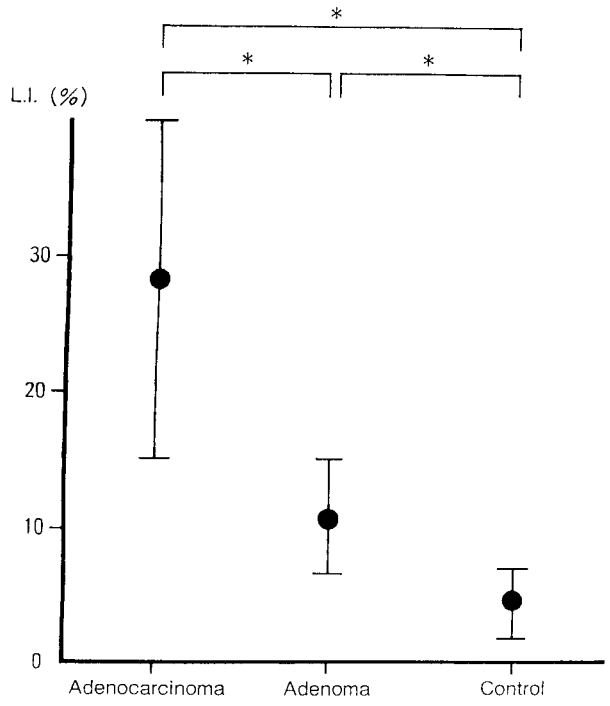

Fig. 1 Bromodeoxyuridine (BrdU)-labeling index (LI) of adenocarcinomas, adenomas, and rectal mucosa of normal control subjects. * $P < 0.001$, Mean ± SD

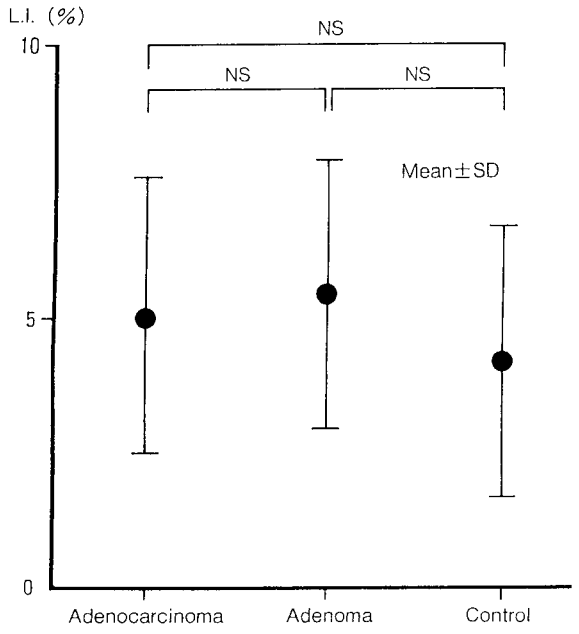

Fig. 2 BrdU-labeling index (LI) of the normal-appearing rectal mucosa in the patients with adenocarcinoma and adenoma and the normal control subjects. NS = not significant. BrdU: See Fig. 1.

appearing rectal mucosa (Fig. 4). The frequency of BrdU-labeled cells at the surface or in the upper one-fifth

Table 1 Incidence of the upward shift of the BrdU-labeled epithelial cells in the normal-appearing rectal crypt in the patients with adenocarcinoma and adenoma and the normal control subjects

Groups	Number	BrdU-labeling of cells in upper one-fifth of crypts
Adenocarcinoma	20	15(75 %) *
Adenoma	20	17(85 %) **
Control	15	6(40 %)

*: $P < 0.05$ **: $P < 0.01$ by chi-square test.
BrdU: Bromodehydroxyuridine

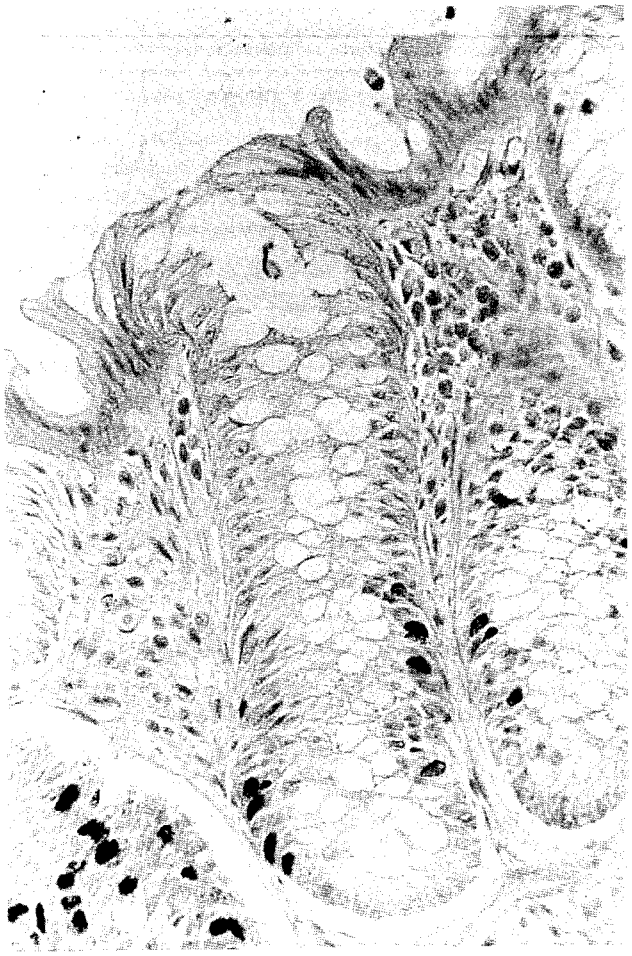

Fig. 3 Immunohistochemical detection of BrdU-labeled cells in the normal colonic mucosa. The labeled cells are observed in the lower portion of the crypt. BrdU: See Fig. 1.

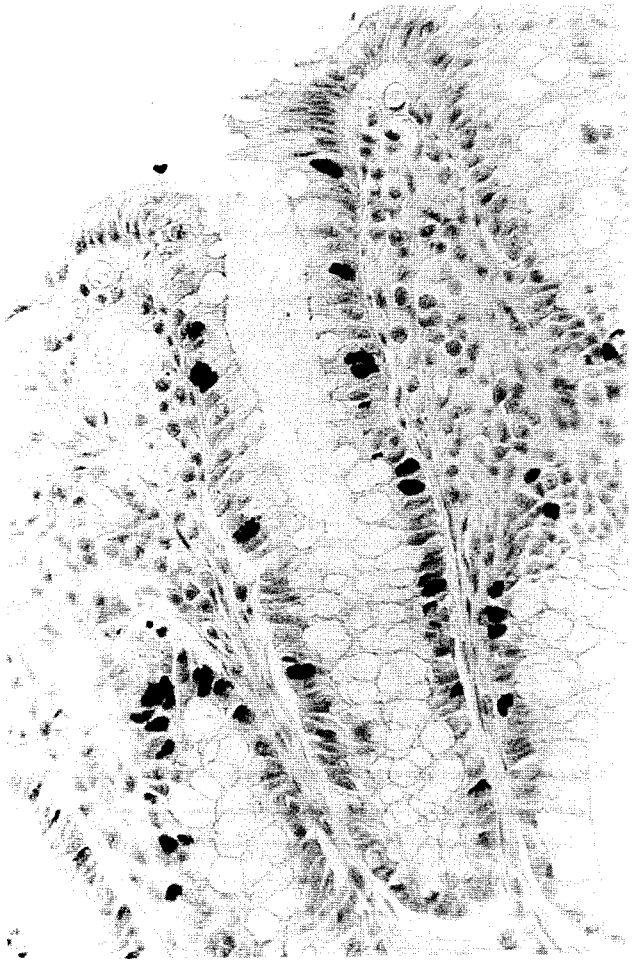

Fig. 4 Immunohistochemical detection of BrdU-labeled cells in the normal appearing rectal mucosa of the patient with adenocarcinoma. The labeled cells are present in the upper one-fifth of the crypt column. BrdU: See Fig. 1.

of the crypt, indicating the increased proliferative zone to the surface of the rectal crypt, was significantly greater in the patients with cancer ($P < 0.05$) or adenoma ($P < 0.01$) than in the control subjects (Table 1).

Discussion

This study demonstrated that the BrdU-labeled epithelial cells were shifted upward in the crypt of the normal-appearing rectal mucosa of the patients with colorectal adenoma and cancer using the *in vitro* labeling method with BrdU. Our findings are compatible with those reported previously in studies using ^{3}H-TdR autoradiography (3, 4, 5, 9). Deschner and Lipkin (3, 10), who studied the mucosa of patients with familial polyposis, demonstrated ^{3}H-TdR incorporation into the surface epithelial cells of the intervening flat mucosa. The ^{3}H-TdR incorporation into the surface epithelial cells was also shown in the mucosa adjacent to the polyp in the patients with sporadic polyps (5, 9). Maskens and Deschner (4) reported a significant upward shift of the proliferating cell compartment in the histologically normal colorectal mucosa of patients treated for rectal or sigmoid cancer. We extended these observations and showed here that the upward shift of the proliferating epithelial cells was detected in the rectal mucosa far from the lesions, which may suggest that modification of the distribution of S-phase cells is not limited in the mucosa adjacent to the lesions but rather detected diffusely in the mucosa with colorectal adenoma and cancer.

We used the *in vitro* BrdU-labeling method to identify S-phase cells instead of ^{3}H-TdR. The labeled cells could be easily identified immunohistochemically, as we had previously described (11), and the method is more rapid and less cumbersome, since it does not involve the use of radioisotopes, than the ^{3}H-TdR autoradiography. The values of LI obtained for the normal-appearing colorectal mucosa were similar to those reported using ^{3}H-TdR (9) and BrdU labeling (12), but were lower than other reports (5, 13, 14). The differences may be due to variations in the handling of biopsy material, especially the culture conditions and the amount of ^{3}H-TdR or BrdU used. Although the method employed here yielded lower LI values than those reported after intravenous infusion of BrdU (15, 16), this *in vitro* technique for labeling the rectal biopsy with BrdU was sensitive enough to disclose changes in the cell-cycle events during colorectal carcinogenesis and may prove useful in the screening of high risk populations for colorectal cancer.

In the carcinogenesis of the colorectal cancer, a multi-step process has been suggested (1). Genetic alterations including abnormal expression of several oncogenes (17, 18) as well as alterations in DNA synthesis (19, 20) have been shown to precede the development of the cancer. Another important hypothesis in colorectal carcinogenesis is the so-called adenoma-carcinoma sequence, where a malignant lesion develops in a precursor adenoma (21-23). In this study, changes in the BrdU-labeling pattern were observed even in the normal-appearing rectal mucosa in the patients with adenoma, suggesting that

changes in the cell proliferation may be an early event during colorectal carcinogenesis which may precede the development of the adenoma itself. Identification of changes at the genetic level involving deregulation of DNA synthesis which resulted in the appearance of the cells with persistent DNA synthesis at the mucosal surface requires further investigation.

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