Bromodeoxyuridine Uptake and Proliferating Cell Nuclear Antigen Expression throughout the Colorectal Tumor Sequence

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Abstract

In vitro uptake of bromodeoxyuridine and expression of proliferating cell nuclear antigen (PCNA) were evaluated histochemically in rectal mucosa of control subjects and subjects with colorectal neoplasia in large intestine adenomas and adenocarcinomas. Both labeling indices progressively increased along the path of tumor progression, as did the difference between them (PCNA labeling indices were always greater than those of bromodeoxyuridine). The correlation between them was fairly close in the controls and in adenomas with low-grade dysplasia, whereas no significant linear relations were noted in adenomas with high-grade dysplasia or in adenocarcinomas. The progressive increase in PCNA would thus seem to be related to both hyperproliferation and neoplastic deregulation of PCNA synthesis. In the mucosa of subjects with colorectal neoplasia, PCNA labeling revealed hyperproliferation but not the surface-wards shift of the proliferative compartment detected by bromodeoxyuridine. PCNA expression, therefore, is not a sufficiently sensitive marker of the risk of tumor transformation in the intestinal mucosa.

Introduction

Cell proliferation is a fundamental biological characteristic of tumor progression in various organs (1). Since changes can be detected even in the premorphological stage of carcinogenesis, they are currently the most exhaustive biomarker available for the detection of groups at risk. This is particularly true of gastrointestinal cancer (2, 3). There is thus a particular need for methods permitting accurate evaluation of this parameter in situ. The benchmark method uses the uptake of labeled DNA precursors to identify cells in S phase. Initially precursors readily identifiable by autoradiography were used for this purpose (4), whereas more modern techniques have resulted in the widespread use of 5-bromo- and 5-iododeoxyuridine, two derivatives of the methyl group of the pyrimidine ring obtained by substitutive halogenation. These compounds are then detected immunohistochemically with specific anti-halopyrimidine MAb (5). The LI of the two techniques are comparable (6). Precursor diffusion/uptake in vitro is greatly influenced by the structural features of the tissue concerned and critically limited by the volume of the sample (7), although ways of getting over these difficulties exist (8). Similar limitations do not apply to in vivo BrdU labeling. This method, however, is suitable for small, highly selected series only (9) and impracticable for the study of populations or groups.

Immunohistochemical identification of endogenous antigens expressed by a cell in one or more phases of its cycle is an alternative approach to the investigation of cellular proliferation. The markers thus obtained include the antigen recognized by the Ki67 MAb, PCNA, DNA polymerase [alpha], P 125, nucleolar and mitotic antigens, and the transferrin receptor (10, 11). They differ in their specificity for the phases of the cell cycle and in the conditions required for their use. PCNA is of particular interest. By contrast with other antigens associated with proliferation, it can be identified in fixed, paraffin-embedded tissues and can thus be used on routinely treated specimens from series held on file. It is an auxiliary DNA polymerase [delta] protein and plays a critical role in the initiation of cell proliferation (12). It is not expressed in significant amounts by cells in G0 and G1, whereas there is a steady increase through the advanced G1 phase and even more in the S phase, followed by intermediate values in G2 and M (13). PCNA is a stable protein and is not degraded during the passage into the noncycling compartment: 40% is still present 48 h after the entry into G0 (14). A distinction can be drawn between two PCNA pools: 30% is tightly associated with DNA replication sites, its antigen determinants are preserved by fixation in formalin or methanol, and it is specifically correlated with the S phase; the second pool is diffused in the nucleoplasm, present in short-term G0 cells, and only preserved by fixing with formalin (14, 15).

This article describes cell proliferation levels and distribution in bowel tumor progression as evaluated by immunohistochemical detection of in vitro BrdU uptake and PCNA expression. In the majority of cases, this well-known model reflects the morphological and biological stages of the adenoma-carcinoma sequence. The diagnostic categories considered in this survey, therefore, run from normal mucosa and histologically normal mucosa

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The abbreviations used are: MAb, monoclonal antibody; BrdU, bromodeoxyuridine; C, compartment; PCNA, proliferating cell nuclear antigen; LI, labeling index; LI, %, percentage of compartments containing at least one labeled cell; PCNA, proliferating cell nuclear antigen; LI, crypt LI.
with cell proliferation abnormalities to colorectal adenoma and adenocarcinoma.

Materials and Methods

Tissue Specimens. The study was conducted on endoscopically normal rectal mucosa from 50 patients (29 men; 21 women), aged 17-68 years (median, 42 years) with no personal or familial history of colorectal neoplasia, and 30 patients (18 men; 12 women), aged 31-74 years (median, 56 years) with single adenomas (14 cases), multiple adenomas (6 cases), and colorectal adenocarcinomas (10 cases). Three adjoining biopsies were taken from each patient at distances which varied from 3 to 22 cm from the tumor lesions (median, 7 cm). Two were fixed for 24 h in 10% buffered formalin and methacarn, respectively, and embedded in paraffin for PCNA detection. The third was incubated for 90 min at 37°C with RPMI (GIBCO, Paisley, Scotland) containing 300 μM BrdU (Sigma Chemical Co., St. Louis, MO), fixed overnight in 70% ethanol, and embedded in paraffin.

Eighty endoscopically resected polyps (diameter, 0.4-2.8 cm) with a histological diagnosis of tubular adenoma (60 cases), tubulovillous adenoma (12 cases), and villous adenoma (8 cases) with a low grade (59 cases) and a high grade (21 cases) of dysplasia and 20 colorectal adenocarcinomas (15 moderately differentiated; 4 poorly differentiated; 1 well differentiated; 11 pT3N1, 4 pT3N0, 5 pT2N0) were studied. Histological diagnosis and grading were according to the WHO criteria (16), staging in accordance with the TNM system. Five random specimens were taken by hand from each polyp and carcinoma with a scalpel blade, cut into fragments with a maximum diameter of 3 mm, and incubated with BrdU in accordance with the procedure used for the mucosa. Other specimens taken according to customary and histopathological diagnosis protocols were fixed in 10% buffered formalin for 24 h and embedded in paraffin.

Immunohistochemistry. Four-μm sections were mounted on slides pretreated with Vectabond (Vector Laboratories, Burlingame, CA), air-dried overnight at room temperature, dewaxed, and rehydrated. Detection of BrdU and PCNA was done by means of the avidin-biotin-peroxidase complex method with the Vectastain ABC PK-4002 kit (Vector Laboratories). The anti-BrdU (mouse IgG₁) MAb (Becton-Dickinson, Mountain View, CA) was diluted 1:50 in phosphate-buffered saline after endogenous peroxidase blockade with 3% H₂O₂ and used after DNA denaturation with HCl 2 N at 37°C for 30 min. Immunostaining for PCNA was done with PC 10 (mouse IgG₂b) MAb (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) diluted 1:200 in phosphate-buffered saline after endogenous peroxidase blockade with 3% H₂O₂. At the end of the procedures, the slides were weakly counterstained with hematoxylin, dehydrated, clarified, mounted in Entellan (Merck, Darmstadt, Germany), and examined under a standard light microscope.

Immunohistochemical Analysis. Immunostaining for BrdU was uniformly intense and the labeled nuclei were readily identified without a background. Since BrdU diffusion and uptake are critically limited by the distance from the tissue/medium interface, attention was confined to areas with unmistakable evidence of exposure to BrdU, namely clusters of labeled epithelial cells and/or labeled lymphocytes in the stroma (1). PCNA immunostaining, on the other hand, was very different from one case to another and even within the same case. Fields with both high and low nucleus labeling percentages were therefore examined. Subjective scoring was minimized by regarding all labeled nuclei as positive irrespective of staining intensity (17-19).

The LI was obtained by dividing the number of labeled cells by the total number of cells (at least 2000). In the mucosa, the number and distribution of labeled nuclei were determined by dividing each crypt into 5 equal longitudinal compartments (1 to 5 from the base to the mouth). The following values were calculated: TLI; the LI in the pT, and the C, as described elsewhere (20).

Statistics. Results were expressed as means ± SEM. Student's t test was used to assess the significance of the differences between the means for adenomas and adenocarcinomas and in the base and intermediate crypt compartments. The Mann-Whitney nonparametric test was used for the surface compartment data. Linear regression and Pearson's coefficient were used to evaluate the correlation between the BrdU and PCNA LI. P < 0.05 was chosen as the significance threshold.

Results

Highly significant (P < 0.001) intergroup PCNA LI differences were observed (Fig. 1): normal mucosa fixed in formalin 10.4 ± 0.7 (mean ± SEM); adenoma with low-grade dysplasia 18.9 ± 1.70; adenoma with high-grade dysplasia 29.9 ± 2.1; adenocarcinoma 41.5 ± 2.3. A similar pattern was observed for BrdU LI (mucosa, 9.7 ± 0.2; adenoma with low-grade dysplasia, 16.31 ± 2.1; adenoma with high-grade dysplasia, 22.02 ± 1.28). The adenocarcinoma BrdU LI (23.5 ± 1.9) was not significantly different from that in the high-grade dysplasia group. In each group, the PCNA LI was higher than the BrdU LI (P < 0.001; except mucosa, P = 0.08), and the percentage of difference was higher in adenocarcinoma (43.37%) and high-grade dysplasia (26.35%) than in low-grade dysplasia (13.7%) and normal mucosa (6.7%). Fig. 2 shows that the slope of the regression equation and the correlation coefficient diminish as a tumor progresses, with close correlation between the two LIs in normal mucosa (P < 0.001) and low-grade dysplasia (P < 0.05) but not in high-grade dysplasia and adenocarcinoma.

Table 1 shows the distribution of BrdU and PCNA-labeled cells in normal mucosa and that from patients with neoplasia, whose TLI, L1, , P1, , and C1, values for BrdU are significantly increased and those for C, are significantly decreased, reflecting contemporary hyperproliferation and a shift of the area of maximum proliferation from the bottom to the middle and surface crypt compartments. PCNA labeling of both formalin- and methacarn-fixed biopsies disclosed the increase in TLI, L1, , P1, , and C, but no significant change occurred in C, . Hyperproliferation, in other words, was not accompanied by any significant shift in PCNA positive cell distribution. The two labeling profiles were similar in the control mucosa: proliferation was confined to compart-
Correlations between the BrdU and PCNA LI in the rectal mucosa and in colorectal adenomas and adenocarcinomas (mucosa: $y = 3.07 + 0.84x$, $r = 0.7$, $P < 0.001$; low-grade dysplasia adenoma: $y = 9.9 + 0.65x$, $r = 0.61$, $P < 0.05$; high-grade dysplasia adenoma: $y = 27.06 + 0.38x$, $r = 0.23$, $P = $ not significant; adenocarcinoma: $y = 38.8 + 0.1x$, $r = 0.15$, $P = $ not significant).

Fig. 2. Correlations between the BrdU and PCNA LI in the rectal mucosa and in colorectal adenomas and adenocarcinomas (mucosa: $y = 3.07 + 0.84x$, $r = 0.7$, $P < 0.001$; low-grade dysplasia adenoma: $y = 9.9 + 0.65x$, $r = 0.61$, $P < 0.05$; high-grade dysplasia adenoma: $y = 27.06 + 0.38x$, $r = 0.23$, $P = $ not significant; adenocarcinoma: $y = 38.8 + 0.1x$, $r = 0.15$, $P = $ not significant).

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Discussion

It has been established that, in normal intestinal mucosa, the accuracy of BrdU (6) and PCNA labelings (15) coincides with the $[^{3}H]$thymidine autoradiographic determination of S phase cells. Conversely, the results obtained with the two methods are contradictory when compared with those of other immunohistochemical markers of cell proliferation in preneoplastic or neoplastic lesions. Ki67 immunoreactivity is only roughly descriptive of proliferative activity in ulcerative colitis (21) when compared to BrdU uptake (22). Mean values of $[^{3}H]$thymidine and BrdU LI are similar in colorectal adenomas and adenocarcinomas (1, 23), and the correlation between BrdU LI and Ki67 index is good in malignant tumors (24), but no correlations have been found between Ki67 and PCNA in advanced gastric carcinoma (25) or in breast cancer (19). However, the two antigens are expressed by the same cell population in malignant lymphomas (18).

PCNA expression, as displayed by the PC 10 MAb, runs parallel to changes in the synthetic DNA cell population in the colorectal tumor sequence, increasing from normal mucosa through adenomas to adenocarcinoma. In adenomas with low-grade dysplasia, too, the percentage of PCNA-positive cells is significantly lower than in high-grade forms. Furthermore, the absolute PCNA index values in all groups in this study were higher than the corresponding BrdU values as reported by other workers (26–28), since immunohistochemical evaluation in formalin-fixed tissues reveals PCNA diffused in the nucleoplasm as well as S phase-specific PCNA bound to DNA replication sites (14, 15, 29). In addition, the specificity of the MAb for the PCNA associated with these sites is not related to its functional activity, thus they identify specific PCNA epitopes in a replication machinery that has ceased to function even though its structure is still intact (28). The discrepancy vis-à-vis the S phase markers is also due to the stability of PCNA. Its half-life is about 20 h and it can thus be identified in the nucleus of cells in $G_{0}$ for not more than 24 h (14). Even so, these mechanisms do not explain the two distinctive findings in this study, i.e., a gradual increase in the difference between the PCNA and BrdU LI and their reduced degree of correlation along the tumor sequence. This suggests that there may be a PCNA pool that is not correlated with the DNA replication mechanisms and is progressively expressed during tumorigenesis. It has been shown that the PCNA concentration in the nucleus is greater than that necessary for replication alone (30) and that PCNA itself has several functions that are associated with differences in its antigen structure and not necessarily linked to DNA replication (31). Its repair function (32–34), for example, could explain the discrepancies between its LI and that of other proliferation indices (19). Tumor deregulation above all brings about the involvement of PCNA in the unscheduled synthesis of DNA (35), and this mechanism is probably responsible for the gradual separation for the PCNA and BrdU LI in the large bowel tumor sequence. In other words, the disproportionate increase in the PCNA along the path from normal mucosa to adenocarcinoma expresses an increase in both PCNA associated with DNA replication in proliferating cells and PCNA engaged in unscheduled/deregulated DNA synthesis.

During the premorphological stages of carcinogenesis, two kinetics abnormalities are noted in intestinal...
mucosa cell proliferation: (a) hyperproliferation (increased LI; expansion of the proliferation zone along the crypt); and (b) a shift of the proliferative compartment to the surface of the crypt (20). Both changes have been identified with S phase markers, i.e., BrdU (20) and [3H]-thymidine (36). The present study has shown that PCNA (b) crypt); and increased LI; expansion of the proliferation zone along the shift has been shown experimentally in vivo. Aspects of the molecular biology of PCNA could explain compartment shift, even in methanol-fixed biopsies. It is mucosa cell proliferation: (a) hyperproliferation (increased LI; expansion of the proliferation zone along the crypt); and (b) a shift of the proliferative compartment to the surface of the crypt (20). Both changes have been identified with S phase markers, i.e., BrdU (20) and [3H]-thymidine (36). The present study has shown that PCNA detection clearly reveals hyperproliferation but not the compartment shift, even in methanol-fixed biopsies. It is unlikely that this shift is due to poor or irregular in vitro diffusion of DNA precursors into the basal areas of the mucosa, since ways of checking the uniformity of uptake have been devised and, above all, because the same shift has been shown experimentally in vivo (37). Some aspects of the molecular biology of PCNA could explain its ineffectiveness as an immunohistochemical marker of some early changes in intestinal mucosa cell kinetics. PCNA expression is regulated at both the transcriptional and the posttranscriptional level (30, 38); the gene is effectively transcribed in both quiescent and proliferating cells, but accumulation of the specific mRNA takes place in the latter only. Several factors, including the growth factors, regulate and modulate the stability of PCNA mRNA (35, 39). Our preliminary data (40) point to increased expression of epidermal growth factor receptors in crypts with proliferation abnormalities. One can reasonably suppose that these receptors take part in the altered PCNA expression in the lower part of the crypt and thus mask the surface-wards shift of the proliferative compartment.

Further research into other less frequent intestinal carcinogenesis sequences will be necessary to gain knowledge of BrdU and PCNA labeling patterns and of the mechanisms governing them. One such sequence is ulcerative colitis, which shows quantitative and distributive changes of S phase cells in the mucosa (41) and in which the tumor progression is associated with genetic pathways different from those of the adenoma-carcinoma sequence (42). Since the correspondence of PCNA expression and in vivo BrdU uptake has recently been demonstrated in normal rat colon (43), the two markers should also be studied during the morphogenetic sequences induced by carcinogens in animal models.

In conclusion, changes in PCNA expression along the colorectal tumor sequence are related to both proliferative activity and progressive neoplastic deregulation of PCNA synthesis. Immunohistochemical detection of PCNA, too, is not sensitive enough to reveal all intestinal mucosa cell proliferation abnormalities in neoplasia. Such abnormalities are independent and express different aspects of carcinogenesis and levels of risk of colorectal cancer. PCNA expression, therefore, is not a suitable marker of tumor transformation in the intestinal mucosa.

References

Table 1 Cytokinetic values in rectal mucosa. Controls versus colorectal neoplasia

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* Results are expressed as mean ± SEM.
* M. methacarn-fixed specimens.
* P. Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas, and adenocarcinomas of the large bowel.
* C. Controls versus colorectal neoplasia.
* P < 0.05 versus controls.
* P < 0.001 versus controls.

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