The Effect of Incubation of Rectal Biopsies on Measures of Proliferation Using Proliferating Cell Nuclear Antigen in Comparison with 5-Bromo-2-deoxyuridine

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Abstract

Rectal epithelial cell kinetics are used as intermediate markers for colorectal cancer and relate to risk. In this study, measures of proliferation using direct immunohistochemistry for proliferating cell nuclear antigen (PCNA) were compared to in vitro labeling by bromodeoxyuridine (BrdUrd) and incubated biopsies that were later stained for PCNA (PCNA-I) in human rectal biopsies. The study group consisted of 20 sets of biopsies from 12 subjects participating in an intervention trial. Fresh nonincubated biopsies were fixed in methacarn and stained immunohistochemically for PCNA (clone 19A2). In parallel biopsies, BrdUrd was incorporated into the DNA of S-phase cells during a 2-h incubation at 37°C under hyperbaric conditions and localized by immunohistochemistry. Additionally, biopsies were incubated under hyperbaric conditions for 2 h at 37°C, fixed in methacarn, and stained for PCNA (PCNA-I). There was a highly significant difference in the labeling index between the three methods (P < 0.01), but there was no significant difference between subjects (P = 0.439). The mean labeling index was 2.3 ± 0.1% for PCNA, 2.9 ± 0.1% for PCNA-I, and 4.1 ± 0.1% for BrdUrd. The proportion of labeled cells in the top two-fifths was significantly higher (P = 0.01) for BrdUrd (5.5 ± 0.8%) and PCNA-I (6.4 ± 1.1%) compared to PCNA (3.1 ± 0.6%), and a significant difference was seen between subjects (P = 0.038). PCNA-I and BrdUrd methods had similar crypt heights with 73.5 ± 1.8 and 71.2 ± 1.3 cells/crypt column, respectively, but were significantly shorter (P < 0.001) than PCNA with 83.4 ± 1.5 cells/crypt column, indicating a loss of cells during organ culture. The simplicity of the PCNA technique, which avoids potential perturbations occurring during organ culture, has considerable appeal as a marker for colorectal cancer risk, but additional studies are needed to correlate PCNA with neoplastic risk.

Introduction

An increased rate of proliferation of rectal epithelial cells and an extension of the major zone of proliferation toward the lumen are risk markers for colorectal neoplasia (1). LI and the proportion of labeled cells in the top two-fifths have been used as predictors of risk, although they may be independent of one another (1, 2). A number of techniques are available for studying cell kinetics, including tritiated thymidine incorporation (3-5), BrdUrd incorporation (6-8), flow cytometry (9), and direct immunohistochemistry using monoclonal antibodies specific for cell cycle antigens such as Ki67 (10) and PCNA (11, 12).

Tritiated thymidine has been the standard method used for measuring cell kinetics. It involves the incorporation of the label into the nuclei of cells in the S phase of the cell cycle and detection by autoradiography. An exposure time of 2–3 weeks is necessary before the results are obtained. The technique is time-consuming, and the results are not rapidly available. In addition, the use of radioactive material is expensive and potentially hazardous. An alternative method to [3H]thymidine is BrdUrd, an analogue of thymidine that becomes incorporated into DNA-synthesizing cells during organ culture and is detected immunohistochemically using a monoclonal antibody to BrdUrd. The technique is relatively simple, with the results available in 2–3 days. However, uneven diffusion of BrdUrd occurs in organ culture, giving rise to patchy staining (13). This can be minimized by cutting the biopsies into smaller fragments (1) and incubating under increased atmospheric pressure, ensuring uniform diffusion into the tissue (8). For flow cytometry, single-cell suspensions are required, which results in the loss of the information available from crypt architecture.

Direct immunohistochemical methods using monoclonal antibodies are of considerable appeal because of their simplicity and the preservation of tissue morphology without the need for organ culture or in vivo injections. Proliferation antigens such as Ki67 and PCNA are expressed over a wider range of the cell cycle. Ki67 is a nuclear antigen expressed in all cycling cells (14-16) of paraffin sections and staining with anti-MIB-1 monoclonal antibody may overcome this, but it is

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1 The abbreviations used are: LI, labeling index; PCNA, proliferating cell nuclear antigen; BrdUrd, 5-bromo-2-deoxyuridine; df, degrees of freedom.
not clear whether this technique is analogous to staining frozen sections.

PCNA is a 36-kDa auxiliary protein associated with DNA polymerase-δ at the sites of DNA synthesis during the S phase of the cell cycle. The synthesis of PCNA increases during the G1-S phase of the cell cycle, peaking during the S phase and rapidly declining in the S-G2 phase (17, 18). It has been reported that two forms of PCNA are present in cell nuclei. One form is located in the nucleoplasm of quiescent cells and is readily extracted with detergent or high salt concentrations, whereas the second is bound at the sites of DNA synthesis and cannot be released by either detergent or high salt concentrations (19). Both forms of PCNA are detected after formaldehyde fixation, whereas only the PCNA associated with DNA replication is detected after methanol or methacarn fixation. PCNA antibodies can be directly applied to paraffin-embedded sections, selectively staining proliferating cells. PCNA may serve as a useful marker for measuring cell proliferation.

In this study, our aim was to correlate PCNA immunoreactivity with the in vitro labeling of BrdUrd in human rectal biopsies. We also stained incubated biopsy specimens for PCNA to determine whether part of the difference in results between the PCNA and BrdUrd techniques may be due to the incubation process and to see whether PCNA staining changed during incubation.

Materials and Methods

Subjects. The study group consisted of 12 subjects (5 women and 7 men; 40–68 years of age; mean, 53 years) with a history of colorectal adenomas. Some subjects were on a normal diet, whereas others participated in a dietary intervention trial consuming a high fiber and low fat diet. For four subjects, multiple sets of biopsies were taken before and after interventions in a trial testing the effect of different fibers on cell proliferation, accounting for the total sample size of 20. Six rectal biopsies (two for each method) of macroscopically normal mucosa were taken 10–15 cm from the anal verge using colonoscopic biopsy forceps, mostly without any bowel preparation, for each of the 20 comparative studies.

Biopsies. Upon removal, the biopsies were immersed in cold RPMI 1640 containing 50 μg/ml penicillin, 2 mm glutamine and 30 milliliters/ml insulin and transported to the laboratory on ice. The biopsies were distributed equally between the PCNA, BrdUrd, and PCNA-I methods. Biopsies for PCNA were fixed immediately in 5 ml of methacarn (methanol:chloroform:acetic acid, 6:3:1) for 1 h. For BrdUrd, the biopsies were cut into two to three fragments using a scalpel blade and placed in a Petri dish with complete media consisting of the basic medium plus 10% FCS, 500 μM BrdUrd, and 500 μM deoxyctydine. They were incubated at 37°C for 2 h with hyperbaric carbon gas at a pressure of 0.6 bar. At the end of the incubation, the biopsy specimens were fixed in Carnoy’s fluid (ethanol:chloroform:acetic acid, 6:3:1) for 1 h. Incubated PCNA involved incubation of the biopsies in complete media without the nucleotides under the same hyperbaric conditions as the BrdUrd biopsies and fixing in methacarn for 1 h. After fixation, the biopsies were dehydrated in three changes of absolute ethanol over 3 h, cleared in three changes of chloroform over 3 h, and vacuum-embedded in three or four changes of paraffin wax over a 1-h period. Serial sections were cut at 2 μm and mounted in sequence on numbered slides.

Immunohistochemistry. Paraffin sections for PCNA and PCNA-I biopsies were stained immunohistochemically for PCNA using a mouse monoclonal antibody (clone 19A2; Coulter Immunology, Hialeah, FL), as reported previously (20). A slightly modified method was used for BrdUrd immunohistochemistry. Briefly, sections were dewaxed and brought down to distilled water through 2× 100 and 75% ethanol. The sections were hydrolyzed in 5n HCl for 30 min, washed in distilled water, and placed in Tris buffer for 3 min. Nonspecific binding sites were blocked by incubating the sections in Tris buffer containing 10% FCS for 10 min. After washing in Tris buffer, the sections were incubated with a 1:200 dilution of a mouse monoclonal antibody to BrdUrd (Biocline) for 90 min at room temperature, washed in three changes of Tris buffer over 10 min, and incubated with a 1:100 dilution of a rabbit antimouse immunoglobulin for 30 min. Once washed, the sections were treated with 0.9% hydrogen peroxide for 15 min, washed in Tris buffer, and then incubated with a 1:40 dilution of a swine antirabbit immunoglobulin for 30 min, followed by incubation with a 1:40 dilution of rabbit horseradish peroxidase antiperoxidase for 30 min. The 3,3’-diaminobenzidine reaction and counterstaining were conducted as described in the PCNA protocol.

Analysis of Crypts. Crypts were counted only if sectioned longitudinally from surface to base, with a single layer of epithelial cells and with the lumen identifiable at least at the base. Each crypt column (hemicrypt) was counted from the center of the base of the crypt up to the level of the collagen table beneath the surface epithelium. Both crypt columns were counted for each crypt. For BrdUrd, a cell was considered labeled if there was any nuclear staining, regardless of the intensity of the stain. For PCNA, only the cells with uniform dark brown staining of the nucleus were considered labeled (17, 21). By counting only the intensely dark nuclei, cell kinetics of cells in the S phase of the cycle are measured, potentially correlating with BrdUrd, which is S-phase specific. The total number of cells/crypt column, the number of labeled cells/crypt column, the LI, which is the ratio of labeled cells to the total number of cells expressed as a percentage, and the numerical position of the labeled cells/crypt column were recorded for each sample and for each method. The number of labeled cells within five crypt compartments, each containing equal numbers of cells, was used to compare the distribution of the proliferative zone between methods and subjects.

Statistical Analysis. Comparisons between methods with respect to the LI and the percentage of labeled cells in the top two-fifths were made using ANOVA and Friedman’s rank-based nonparametric test. In addition, log-linear models were used with χ² tests of the likelihood ratio statistic (22) to compare the distribution of labeled cells in the four compartments, considering each of the bottom three-fifths and the top two-fifths combined. The 20 samples from the 12 subjects were treated independently. In the four subjects from whom multiple biopsies were taken, significant dietary changes were made over the course of the sampling as part of a low fat/high fiber intervention trial.

Results

The measures of proliferation are shown in Table 1. An average of 27 ± 0.6 crypts/sample were counted for PCNA, 22 ± 0.9 crypts/sample were counted for PCNA-I, and 21 ± 2.0 crypts/sample were counted for BrdUrd. There was a highly significant difference in the LI between the three methods (F = 91.2 on 2 and 38 df; P < 0.001; Fig. 1), but there was no significant difference between subjects (F = 1.04 on 19 and 38 df; P = 0.439). The mean LI for BrdUrd (4.1 ± 0.1%) was significantly higher than that for PCNA-I (2.9 ± 0.1%), both of which were
Table 1  Measures of proliferation

<table>
<thead>
<tr>
<th>Method</th>
<th>PCNA</th>
<th>PCNA-I</th>
<th>BrdUrd</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>No. of crypts counted [mean (SD)]</td>
<td>27 (2.7)</td>
<td>22 (4.2)</td>
<td>21 (8.7)</td>
</tr>
<tr>
<td>No. of labeled cells/crypt [mean (SD)]</td>
<td>3.7 (0.6)</td>
<td>4.3 (1.0)</td>
<td>5.8 (0.9)</td>
</tr>
<tr>
<td>No. of cells/crypt column [mean (SD)]</td>
<td>83.4 (6.5)</td>
<td>73.8 (4.0)</td>
<td>71.2 (6.0)</td>
</tr>
<tr>
<td>Total LI [mean (SD)]</td>
<td>2.3 (0.4)</td>
<td>2.9 (0.5)</td>
<td>4.1 (0.5)</td>
</tr>
<tr>
<td>% of labeled cells in compartments [mean (SD)]</td>
<td>33.6 (8.0)</td>
<td>30.6 (11.0)</td>
<td>29.4 (8.3)</td>
</tr>
<tr>
<td>% Compartment 1</td>
<td>45.9 (7.4)</td>
<td>41.5 (8.1)</td>
<td>42.7 (4.8)</td>
</tr>
<tr>
<td>% Compartment 2</td>
<td>17.4 (5.6)</td>
<td>21.3 (8.3)</td>
<td>22.4 (6.7)</td>
</tr>
<tr>
<td>% Components 3 and 5a</td>
<td>3.1 (2.8)</td>
<td>6.4 (5.0)</td>
<td>5.5 (3.7)</td>
</tr>
</tbody>
</table>

Fig. 1. The differences in LI between methods for each sample. The LI is the ratio of labeled cells to the total number of cells, expressed as a percentage. Using ANOVA, a significant difference was seen between methods, with PCNA-I and BrdUrd producing significantly higher LIs than the PCNA method (P < 0.001). PCNA; ○, PCNA-I; ◯, BrdUrd.

Fig. 2. The proportion of labeled cells (expressed as a percentage of all labeled cells) in the top two-fifths of crypts (C2 measure) is a measure of the expansion of the proliferative zone toward the lumen. For both PCNA-I and BrdUrd, there was a significant increase in the proportion of labeled cells in the top two-fifths of crypts when compared to PCNA (P = 0.01 using ANOVA). PCNA; ○, PCNA-I; ◯, BrdUrd.

Discussion

We found higher LI and percentages of labeled cells in the top two-fifths with incubated techniques, regardless of the staining technique (BrdUrd or PCNA), when compared with direct
Fig. 3. Each crypt was divided into five crypt compartments of equal size. The percentage of labeled cells in each of the five crypt compartments was used to determine the distribution of labeled cells along the crypt. Few cells are found in compartments 4 and 5, so they were combined to form four crypt compartments. The distribution of labeled cells along the crypt is shown for each sample and for each method. Individual subjects are identified by number, and multiple biopsies are identified by letter. However, only 5 of 20 samples had statistically significant distribution patterns between methods (P < 0.05). □, PCNA; ○, PCNA-I; ◊, BrdUrd.
immunohistochemistry for PCNA without incubation. Positive correlations between PCNA and \(^{3}H\)thymidine have been found in several studies using dual labeling in both human and rat colonic tissue after the incubation required for thymidine uptake (12, 23). Similarly, studies comparing PCNA and BrdUrd in human colonic tissue after incubation (for BrdUrd) on serial sections from the same set of biopsies have also found positive correlations (13). Risio et al. (2) and Weisgerber et al. (17) found positive correlations between PCNA and BrdUrd in rectal mucosal biopsies from humans (r = 0.7 and 0.6, respectively) using separate biopsies for each method, with direct staining for PCNA and immunohistochemical detection of BrdUrd after incubation. However, in Weisgerber’s study (17), similar numbers of labeled cells/crypt column and similar LIs were calculated despite differences in the total number of cells/crypt column, rendering his conclusion on LI difficult to interpret. In addition, Einspahr et al. (24) found highly significant correlations between PCNA, BrdUrd, and \(^{3}H\)thymidine in subjects with a history of colorectal cancer using single labeling on separate sets of biopsies for each method. All immunohistochemical staining is subject to discretionary determination of positive versus negative cells.

To maintain crypt architecture, cell proliferation, which occurs at the base of the crypt, must be balanced by an equal rate of cell loss (25). As cells mature, they migrate up the crypt, differentiate, and then shed into the lumen. Part of the explanation for our results of higher LI with the incubation techniques is that the number of cells (labeled and unlabeled)/crypt fell during culture, probably from the upper region, where unstained cells are more numerous. We hypothesize that this would result from shedding or apoptosis of cells during the incubation phase, thus introducing an artificial increase in the LI. Apoptosis has been shown to occur in the nonproliferative compartments of the gut, the top of villi in the small intestine, the top part of the crypts, and the surface epithelium of the large intestine. Hall et al. (26) have shown that delayed fixation increases the number of apoptotic cells in the top part of the villus. Incubation of colonic mucosa from guinea pigs for 150 min with butyrate has shown an increase in the number of apoptotic cells and apoptotic bodies on the mucosal surface (27). Similarly, preliminary studies have shown that incubation of human colonic biopsies under conditions identical to the present study induces apoptosis, as shown by the terminal deoxynucleotidyl transferase-mediated nick end labeling staining technique, in the surface epithelium.4 By incubating and delaying fixation by 2 h, the number of apoptotic cells may have increased and may have been phagocytosed by macrophages or adjacent epithelial cells, thus explaining the shorter crypts seen after incubation for the PCNA-I and BrdUrd techniques. Part of the difference can also be attributed to an increase in the number of labeled cells with each of the incubated techniques (significantly for BrdUrd, and almost significant for PCNA-I). This implies recruitment of cells into cycling during the 2-h incubation. This process inherently may be more subject to environmental conditions during incubation than biological risk influences in the subjects from which the biopsies were taken.

The significant difference in absolute values of LI between methods is not of major concern, providing that ranking orders are maintained. However, ranking orders for LIs between methods were not correlated, nor were they correlated for the percentage of labeled cells in the top two-fifths (2\(q_0\)), implying either that the methods are not equivalent measures of proliferation and each needs to be assessed as to its reliability as a surrogate end point for neoplasia or that there is little difference between subjects due to their common history of adenomas (and thus risk status), and that four subjects were restudied. A good measure of proliferation will be highly reproducible in that the variation between biopsies taken from the same individual a few days apart will be smaller than the variation between subjects, especially subjects with different risks of neoplasia. Thus, if the different staining methods were comparable, it would be expected that there would be no interaction between subjects and methods, but a significant difference between subjects where they are at risk. A significant difference between methods would not by itself be of much concern, because the ranking order of measures does not change.

Distribution of labeled cells should be a more robust measure for comparison, independent of absolute labeling levels. We have documented this in previous studies of quality control (20, 28). Even using this end point for analysis, significant differences were detectable between methods for 5 of 20 samples in the current study, indicating the possibility that each of the 3 methods is measuring a different biological parameter. Because there was no significant difference in distributions of labeled cells between the two incubation techniques, this measure of proliferation seems to be more consistent for incubation techniques. Optimal correlations with neoplastic risk between the three measures have yet to be determined.

With the LI, there was either a significant interaction between subject and method or there was a large residual error. A subject-method interaction could conceivably occur if the result from one method or the other was determined to some extent by a subject-related variable. For example, a genetically determined proliferation response might be more evident from the standard PCNA technique, whereas such a genetic effect may be overridden by perturbations occurring during incubation and thus would not be subject-dependent. Comparing the way the values change between the 20 samples in Fig. 1, it can be seen that there is either a significant interaction between sample and method or a large residual SD. A residual SD that is large compared with the difference between subjects implies the method is unlikely to be of much use for determining the effect of intervention and possibly even a subject’s risk of neoplasia.

As stated, the differences in the methodologies are important because the ranking order of results for the different methodologies is not consistent. Thus, if subjects are to be compared using different methodologies, different results can be anticipated. It is still uncertain as to which of these or other measures of proliferation correlates best with neoplastic risk or which correlates best ultimately to a favorable (antineoplastic) outcome from an intervention. Biologically, it is not clear whether the tighter cell cycle ($S$ phase) measurement achieved with BrdUrd or the broader cell cycle staining of PCNA is likely to more closely relate to risk (29). Quality control and colorectal cancer risk assessment studies using Ki67 with direct immunohistochemistry have not been reported to date, nor have studies with whole crypt mitotic counts.

The robustness of the measures of proliferation (subject-related, time-related, and technique-related measures) has to be carefully defined in quality control of the laboratories engaged in proliferation studies. Minimizing the number of variables is important, given the error variance inherent at each point in the assessment of proliferation (18).

Our preference for methodology remains with the PCNA

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4 D. H. Barkla and P. R. Gibson, personal communication.
direct immunohistochemical staining technique, but with carefully defined quality control systems in place. Results are best when performed in a single laboratory, with a single experienced observer counting crypts carefully selected for validity and using well-defined criteria for positivity.

Data significantly relating proliferation with risk are mostly reported using the incubation ([3H]thymidine and BrdUrd) techniques. Thus, despite the inherent technique limitations of uneven nucleotide uptake and potential for perturbations in vitro, correlations with neoplastic risk can be identified in clinical studies using these incubation techniques. There is still a paucity of studies relating direct PCNA immunohistochemistry with either neoplastic risk or interventional effects. More studies of this are needed to substantiate that the PCNA technique without incubation is capable of detecting risk-related differences in proliferation and provides an even better risk separation than BrdUrd with incubation.

Acknowledgments

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References

The effect of incubation of rectal biopsies on measures of proliferation using proliferating cell nuclear antigen in comparison with 5-bromo-2-deoxyuridine.

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