Epidemiological Use of Rectal Proliferation Measures

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Abstract

Measures of rectal mucosal proliferation have been developed and used in research clinical settings, but their utility for larger-scale epidemiological studies remains uncertain. We assessed the suitability of bromodeoxyuridine (BrdUrd) and proliferating cell nuclear antigen (PCNA)-labeling indices (LIs) in the setting of a multicenter clinical trial of adenoma recurrence. Subjects at participating practices were asked to permit biopsy of normal rectal mucosa during a colonoscopy scheduled for other reasons. PCNA and BrdUrd labeling was performed, and corresponding LIs were computed.

In general, subjects were willing to undergo biopsy during their scheduled procedures; less than 10% refused. Preparation for PCNA was acceptable; the mean number of scorable crypts (± SE) was 12.99 ± 0.37. Preparation for BrdUrd labeling was less successful, with a higher proportion of unscorable specimens and a lower mean number of scorable crypts.

Among the 54 specimens with both LIs computed, the LI for PCNA was modestly higher than that for BrdUrd LI (4.1 ± 0.2 and 3.7 ± 0.2 respectively; P = 0.03). The rank order correlation between the two indices was 0.38. There was variation across centers in the PCNA LIs but few differences according to number of crypts scored.

Measurement of rectal mucosal proliferation is feasible among endoscopy patients in large studies if PCNA is used; BrdUrd seems more difficult. The relationship between these two labels requires further study.

Introduction

Cell proliferation is an important factor in carcinogenesis (1), and its measurement in rectal mucosa has been proposed as a tool for the understanding of colorectal carcinogenesis (2). The techniques commonly used to measure proliferation in the rectal mucosa all involve the manipulation of biopsy specimens, with subsequent labeling of proliferating cells. The labeling is done by either incorporating exogenous labels into the cells or identifying natural proteins associated with proliferation. Tritiated thymidine and BrdUrd are the most commonly used exogenous labels, and PCNA and Ki 67 the most frequently utilized natural proteins. On the basis of the numbers of labeled and unlabeled cells, LIs are computed as the proportion of all cells in a crypt that are labeled (3, 4).

To date, these techniques have been used in relatively small studies closely associated with experienced laboratories; their suitability for larger studies in less tightly controlled settings is not clear. All of the methods noted above require specimen preparation after biopsy to preserve mucosal crypt architecture and permit computation of the LIs. This involves manipulation of the specimens under magnification (typically with a dissecting microscope) to ensure that the mucosa is not folded or twisted. In addition, tritiated thymidine and BrdUrd labeling require simple organ culture, a technique which could pose difficulties in some circumstances. In contrast, PCNA requires only proper specimen preparation immediately after the biopsy is taken. Ease of preparation might be expected to make PCNA the preferred technique for epidemiological use, but a comparison of PCNA and BrdUrd has not been performed in these settings.

In this report, we describe our experience using rectal proliferation markers in a large-scale clinical trial and compare BrdUrd- and PCNA-labeling indices.

Subjects and Methods

Rectal mucosal specimens were obtained as part of the Calcium Polyp Prevention Study, a multicenter randomized double blind trial of the efficacy of calcium carbonate as a preventive intervention against the recurrence of large bowel adenomas. There are six clinical centers: The Cleveland Clinic; Dartmouth Medical School; The University of California at Los Angeles/Kaiser Hospital; the University of Iowa; The University of Minnesota; and the University of North Carolina. Dartmouth Medical School is the coordinating center, and the M. D. Anderson Cancer Center is the laboratory center for proliferation measures.

Subjects were recruited at the clinical centers within 3 months of the removal of at least 1 large bowel adenoma and the clearing from the bowel of all identified polyps. After confirmation of eligibility and the signing of an informed consent document, subjects entered a 3-month placebo run-in period, during which likely compliance with the study was assessed. If subjects were considered suitable, they were randomized to receive either 3000 mg calcium carbonate (containing 1200 mg elemental calcium) or an identical placebo. Information regarding medical history,
diet, and personal habits was collected by questionnaire prior to randomization. Follow-up colonoscopy occurs 1 and 4 years after the qualifying endoscopy. The follow-up period for the study is the interval between the year-1 and the year-4 exams. This allows for a second colonoscopy to detect and remove polyps missed on the qualifying examination, and also permits a latent period of effect to be present without distorting study findings. Recruitment has been completed, and 930 subjects randomized.

Our goal was to obtain rectal mucosal specimens from 300 subjects during the year-1 colonoscopy. Biopsies were also obtained near baseline in a few subjects, but these sporadic specimens are not included in this report. Recruitment was conducted at all centers, although many associated private practice sites were not included because of the anticipated difficulties in obtaining and processing specimens there. Preparation for PCNA labeling was conducted at all centers, but the more demanding BrdUrd labeling was started at only three institutions.

Specimens were obtained and prepared according to a standard protocol: (a) subjects received bowel preparation with p.o. polyethylene glycol lavage and/or saline or tap water enemas [phosphate enemas were not allowed because their tendency to irritate the rectal mucosa (5)]; (b) at the beginning of the endoscopy, at least 4 and preferably 6 mucosal biopsies were taken approximately 8–10 cm from the anus using standard biopsy forceps. If only PCNA specimens were obtained then at least 2 and preferably 3 biopsies were requested; (c) specimens were immediately removed from the forceps using a strip of bibulous paper and placed in warmed MEM solution for transport to a work area in no more than 15 min. There, the specimens were flattened (and for BrdUrd labeling oriented mucosal side up); (d) PCNA specimens were immediately fixed in 70% ethanol and stored until shipment; (e) BrdUrd specimens were subjected to crypt organ culture. Briefly, the biopsy specimens were immersed in MEM solution containing 50 μM BrdUrd (Sigma Chemical Co.) and incubated at 37°C for 1 h in a shaking water bath without oxygen enrichment or hyperbaric pressure. After incubation, specimens were fixed in 70% ethanol and stored until shipment at scheduled intervals to the laboratory center at M. D. Anderson Cancer Center; and (f) study staff recorded the bowel preparation used, the time between the first biopsy taken, and the placement of the fixation of the PCNA specimens or placement of the BrdUrd specimens into the incubating bath (if those specimens were obtained).

Study coordinators were first instructed in specimen handling through printed materials and a video. Subsequently, a training session was held at the laboratory center. Five of 6 study coordinators attended and received direct instruction in preparation of the specimens.

Further manipulation of the specimens proceeded as follows: (a) upon receipt, specimens were checked for damage in transit and logged into a tracking database. Batched samples were sent for histological preparation. Paraffin-embedded 4-μm histological sections were cut from each biopsy. Steps were taken to maximize antigenicity of the samples, including preparing the section at 50–54°C and fixing serial sections to poly-L-lysine-coated slides prior to immunocytochemistry; and (b) anti-BrdUrd (Becton-Dickinson) or anti-PCNA (PC-10 clone, Signet) mAbs were used for detection of proliferating cells. A dilution series, as recommended by the manufacturers, was used prior to each staining procedure. The staining for BrdUrd was standardized using a DNA hydrolysis step, followed by a series of steps using a kit (Signet) for visualization of proliferating cells using diaminobenzidine as the chromogen. Negative and positive controls were used during each staining batch. Staining was assisted semiautomatically using the Sequenza (Scimetrics, Inc.). A diluted hematoxylin (Fisher Scientific) was used as the counterstain. For PCNA, staining proceeded using the Vectastain ABC kit (Vector Laboratories) with the same counterstain.

Scoring of the crypt labeling was performed as below, by one reader blind to characteristics of the subjects: (a) well oriented U-shaped crypts were selected for counting, provided that they were open from the apical lumen to the base, and that the base actually contacted the muscularis mucosa. If a crypt was well shaped but the muscularis was missing, it was also accepted if its height was not markedly different from neighboring crypts for which the muscularis was present; (b) for BrdUrd, all labeled cells were uniformly stained within the nuclear area, and the intensity of label was invariant. For PCNA, a stringent gradient was used. Only the deepest staining or the next lighter shade of brown were taken as positive. The pattern of PCNA coloration was accepted as positive only if the stain was nuclear and homogeneous. Light staining cells, which would not fit into this description above, were not counted as positive; and (c) a LI was calculated for each scorable crypt, i.e. those satisfying criteria a above, and having at least one labeled cell (or surrounded by crypts with at least one labeled cell). The number of cells per U-shaped crypt column was enumerated, and the number and position of each labeled cell was recorded. The crypt LI was generated as the number of labeled cells divided by the total number of crypt cells in scorable crypts with at least one labeled cell. Crypt LIs were averaged to produce an average LI for each subject.

All scorable crypts identified were included in the analysis for each specimen. In a validation study, repeated scoring was performed for 20 subjects by the technician who scored all of the samples for the study. The intraclass correlation for the repeated LI measurements was 0.40. The LIs and the numbers of scorable crypts were compared using t tests or analysis of covariance. Spearman Rank correlation coefficients and t tests were used to compare BrdUrd and PCNA LIs (6).

**Results**

A total of 468 patients had year-1 colonoscopies at locations where biopsies could be processed, and of these 401...
were asked to participate in the rectal mucosal proliferation study (Table 1). Only 39 (10%) refused, but other problems prevented successful biopsy in 7 (2%). A total of 355 PCNA specimens were obtained, but difficulties with BrdUrd preparation led to the discontinuation of preparation for this label, so only 97 BrdUrd specimens were submitted.

Tables 2 and 3 summarize the quality of the specimen preparation.

**Table 2** Number of scorable crypts in specimens submitted for PCNA labeling index

<table>
<thead>
<tr>
<th>No. of scorable crypts</th>
<th>Before training session (%)</th>
<th>After training session (%)</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5 scorable crypts</td>
<td>4 (48)</td>
<td>18 (66)</td>
<td>22 (62)</td>
</tr>
<tr>
<td>6-10 scorable crypts</td>
<td>12 (14.5)</td>
<td>42 (15.4)</td>
<td>54 (15.2)</td>
</tr>
<tr>
<td>11-15 scorable crypts</td>
<td>20 (24.1)</td>
<td>59 (21.7)</td>
<td>79 (22.2)</td>
</tr>
<tr>
<td>Total</td>
<td>83 (100)</td>
<td>272 (100)</td>
<td>355 (100)</td>
</tr>
</tbody>
</table>

**Table 3** Number of scorable crypts in specimens submitted for BrdUrd labeling index

<table>
<thead>
<tr>
<th>No. of scorable crypts</th>
<th>Before training session (%)</th>
<th>After training session (%)</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5 scorable crypts</td>
<td>16 (41.0)</td>
<td>23 (39.7)</td>
<td>39 (40.2)</td>
</tr>
<tr>
<td>6-10 scorable crypts</td>
<td>12 (30.8)</td>
<td>11 (19.0)</td>
<td>23 (23.7)</td>
</tr>
<tr>
<td>11-15 scorable crypts</td>
<td>6 (15.4)</td>
<td>10 (17.2)</td>
<td>16 (16.5)</td>
</tr>
<tr>
<td>Total</td>
<td>39 (100)</td>
<td>58 (100)</td>
<td>97 (100)</td>
</tr>
</tbody>
</table>

**Table 4** Mean number of PCNA scorable crypts and labeling indices, and preparation time, by study center

<table>
<thead>
<tr>
<th>Center</th>
<th>No.</th>
<th>No. of scorable crypts</th>
<th>Labeling index</th>
<th>Preparation time</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47</td>
<td>13.4 ± 0.87</td>
<td>3.5 ± 0.2</td>
<td>14.7 ± 0.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>9.9 ± 0.77</td>
<td>4.1 ± 0.2</td>
<td>15.3 ± 1.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>11.2 ± 0.88</td>
<td>3.9 ± 0.2</td>
<td>55.9 ± 7.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>15.4 ± 0.77</td>
<td>4.1 ± 0.1</td>
<td>16.5 ± 1.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>14.6 ± 0.86</td>
<td>3.7 ± 0.1</td>
<td>16.6 ± 1.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
<td>10.8 ± 0.92</td>
<td>3.9 ± 0.1</td>
<td>51.9 ± 12.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total</td>
<td>333</td>
<td>13.0 ± 0.37</td>
<td>3.9 ± 0.1</td>
<td>23.6 ± 1.7</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* Percent of specimens.
* Percent of specimens with at least one scorable crypt (crypt architecture preserved and at least one labeled cell, or surrounded by such labeled, preserved crypts; n = 333).
* Percent of specimens with at least one scorable crypt (crypt architecture preserved and at least one labeled cell, or surrounded by such labeled, preserved crypts; n = 58).
* P = 0.57 for before-after training difference.

A total of 54 subjects had both PCNA and BrdUrd specimens with at least one countable crypt. PCNA labeling yielded a slightly higher mean LI, 4.14 ± 0.16, versus 3.69 ± 0.19 for BrdUrd (P = 0.03). The correlation between the two LIs was 0.38 (P = 0.005).
Among subjects with at least one scorable crypt, techniques could be managed by our study personnel. Our experience with the epidemiological use of rectal mucosal proliferation measures is in many ways encouraging; the SDs for the number of crypts scored (Table 5), but there were some differences according to category of number of scorable crypts in the specimens. These findings remained after inclusion of age, sex, and lifetime number of previous adenomas in the analysis.

### Discussion

Our experience with the epidemiological use of rectal mucosal proliferation measures is in many ways encouraging; it was quite feasible to obtain biopsy specimens among subjects already having an endoscopy, and the PCNA techniques could be managed by our study personnel. However, only a small proportion of BrdUrd specimens submitted were optimally prepared for analysis, as reflected in the number of scorable crypts. While the manner of preparation was presumably a factor in specimen quality, there was little evidence that the time involved had a direct impact on the LIs obtained. In any case, BrdUrd seems much less suitable for studies such as ours than PCNA.

Since PCNA is expressed in a wider range of the cell cycle than is reflected in BrdUrd incorporation (7), it is not surprising that the PCNA LI was somewhat higher than that for BrdUrd. The correlation between the two measurements, although statistically significant, was also modest. The lack of perfect correlation may be due to at least two possibilities. On the one hand, the two LIs may be measuring somewhat different aspects of rectal mucosal proliferation. The second possibility is that the observed modest correlation may be due to the inherent variability of the two measures. A lack of repeatability will result in the observed correlations being low, even if the correlation between the "true" PCNA LI and the "true" BrdUrd LI is higher (8).

### References


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