Proliferation cell nuclear antigen (clone 19A2) correlates with 5-bromo-2-deoxyuridine labelling in human colonic epithelium

U M Weisgerber, H Boeing, R Nemitz, R Raedsch, R Waldherr

Abstract

Measurements of cell proliferation can be used as biomarkers of preneoplastic change. In this study, two immunocytochemical methods that measure different components of the cell cycle were compared to assess cell proliferation on biopsy samples from human colonic mucosa. These methods are based on a monoclonal antibody against 5-bromo-2-deoxyuridine (BrdU), which is confined to S phase cells, and a more broad assessment of proliferation based on an antibody against proliferating cell nuclear antigen (PCNA, clone 19A2). In the PCNA assay, only strongly immunostained nuclei were included. The proliferation index was assessed in colonic mucosa from patients with no colonic disorders. Correlation between individual total proliferation indices determined by either method was significant with r=0.6 (p<0.05). The mean proliferation index in the study group was 7-79% using BrdU and 7-64% using PCNA immunocytochemistry. Distribution of BrdU-labelled PCNA nuclei was similar with respect to the two methods with a peak at the 18th and the 24th percentile in the case of BrdU and at the 23rd percentile for PCNA. Variance component analysis showed that at least two biopsy specimens should be evaluated per subject to allow a precise individual characterisation. It is concluded that PCNA (19A2) immunocytochemistry may be used as an operational marker of cell proliferation in normal colonic mucosa. A significant correlation and an agreement in the mean proliferation index between PCNA (19A2) and BrdU can only be achieved by a strictly standardised enumeration of labelled cells limited to strongly stained nuclei in the PCNA evaluation.

Biological markers are becoming increasingly important for early detection of an increased cancer risk and as intermediate end points for chemoprevention trials. Many studies have shown the importance of cell kinetic parameters in the progression of colorectal cancer. Methods that detect the incorporation of a DNA precursor such as [3H]-thymidine labelling or immunocytochemical detection of 5-bromo-2-deoxyuridine (BrdU) are direct and confine measurements of DNA synthesis in active S phase proliferating cells. They are labour intensive, however, and complex to perform on a large scale, for example, in clinical regimens. Proliferation markers based on the immunocytochemical finding of endogenous antigens such as Ki67 or proliferating cell nuclear antigen (PCNA) generally express a wider range of the cell cycle, but their application in clinical practice is considerably less complex. The simplicity is particularly true for PCNA, because the antibody can be applied in paraffin embedded tissue.

PCNA is a cell cycle related protein, which is expressed increasingly through G1 phase, reaching its maximum in early S phase, and persisting with decreasing detectability in G2 phase. Bravo and MacDonald-Bragov described a soluble form of PCNA, which is lost on fixation in organic solvents and does not participate in DNA replications, and an insoluble form, which is associated with the sites of ongoing DNA synthesis and is expressed particularly during S phase and is detectable by immunofluorescence. Bravo and MacDonald-Bragov report that the soluble form is not detected by immunofluorescence because methanol is used for fixation. Moreover, various PCNA antibodies define different epitopes and fixation conditions may affect epitope location within cells. Therefore, PCNA may serve as a useful marker for proliferation if the conditions of fixation are held constant and the same antibody is used throughout the experiment.

Use of a monoclonal antibody to PCNA in investigative studies requires standardisation of the method in relation to established methods for measuring cell proliferation. Comparative studies have shown that PCNA provides a strong correlation with [3H]-thymidine labelling and flow cytometric assessment of S phase fraction in rat colon. PCNA and immunocytochemical labelling in human tissue.

The determination of proliferation indices in colonic epithelium by BrdU incorporation in colonic biopsies and subsequent immunocytochemical detection has been found to be equivalent to the standard autoradiographic method by our group (unpublished data).

In this study, we describe use of a monoclonal antibody (19A2) against PCNA on methanol fixed, paraffin embedded human colonic biopsy specimens using a routine biotin streptavidin immunocytochemical system. Measures of proliferative activity were compared with results obtained by BrdU incorporation and subsequent ABC immunocytochemistry in the same group of patients. We decided to use the term 'proliferation index' to describe the results of the PCNA assay because the antibody to PCNA does not label only S phase cells; we use the same term for the results of the BrdU assay, although the term 'labelling index' is frequently used by other groups. The aim of this study was to evaluate...
how the proliferation index obtained by PCNA (19A2) correlates with that determined by conventional BrdU immunocytochemistry.

Subjects and methods

SUBJECTS
The study group consisted of 17 subjects (10 women, 7 men) with a mean age of 47 years (range, 19–75). All subjects had a normal diet and were free of inflammatory bowel disease and of benign and malignant tumours at the time of complete colonoscopy. Personal and family anamneses for colorectal cancer (first degree relatives) were negative in all patients. Histological evaluation showed normal mucosa. Bowel preparation was performed by oral administration of two litres of a PEG electrolyte solution. This preparation has been shown not to affect epithelial cell proliferation. The study was approved by the ethical committee of the University of Heidelberg.

A minimum of four biopsy samples were obtained from each patient from macroscopically normal mucosa at the rectosigmoidal junction (about 20 cm from the anal verge), using standard forceps. The samples were distributed equally to the BrdU and PCNA protocols.

BrdU protocol

The biopsy specimens were immersed immediately in Dulbecco’s modified Eagle’s medium (Serva, Heidelberg), prewarmed to 37°C, with the mucosal side upwards and placed on a filter paper under a dissecting microscope. Incubation was conducted in small Petri dishes containing 10 ml of Eagle’s medium containing 10% fetal calf serum and 160 μM BrdU (Amersham, Braunschweig, Germany) for two hours at 37°C. The biopsy specimens were then washed with phosphate buffered saline (PBS) and fixed overnight (at least 18 hours) in absolute methanol at 4°C. After dehydration and removal of the filter paper, the flat, fixed biopsy specimens were oriented in a vertical position in one paraffin block. Specimens were embedded in paraplast with a melting temperature of 54–56°C (Klinpath, Zevenaar, Netherlands).

Between 80 and 100 serial sections (3 μm thick) were cut on a rotary microtome and mounted on glass slides. Because the lumen of the same crypt is visible within a maximal range of 50 μm, every 15th section was stained and counted to ensure that the same crypt was not counted twice. The slides were dried overnight at 37°C for better adherence of the sections. Sections were dewaxed in xylene and rehydrated through graded ethanol to 0:01 M PBS, pH 7:4. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 0.1% sodium azide for 30 minutes at room temperature. After rinsing in PBS, acid hydrolysis was performed by incubating the sections in 2 N HCl for 30 minutes at room temperature followed by washing in two successive baths (three minutes each) of 0.1 M borax (Na2B4O7) at pH 8.5 to neutralise the acid.

After being washed in PBS (twice for five minutes), the slides were incubated overnight with mouse monoclonal antibody against BrdU (Amersham, Braunschweig, Germany) at 4°C in a humid chamber. The sections were then incubated with biotinylated anti-mouse immunoglobulins from sheep (1:50 for 30 minutes; Amersham), followed by incubation with streptavidin biotinylated peroxidase complex (1:100 for 30 minutes; Amersham). Biotinylated anti-mouse immunoglobulins and streptavidin biotinylated peroxidase complex were diluted in PBS containing 1% human IgG. After each step, the sections were rinsed in PBS (3 × 5 minutes).

The reaction product was visualised using a substrate solution containing 0.05% 3,3-diaminobenzidinemetahydrochloride (DAB) (Serva, Heidelberg, Germany), 0.01% hydrogen peroxide, 0.01% nickel chloride, and 0.01% cobalt chloride in TRIS buffered saline (pH 7.6) with a usual reaction time of 10 minutes under microscopic examination. After being washed in distilled water, the slides were counterstained with 0.1% nuclear fast red in 5% aluminium sulphate for five minutes, dehydrated, and mounted using a synthetic mounting medium (Vitro-Clud, Langenbrink, Emmendingen, Germany).

PCNA protocol

For the PCNA immunoreaction, biopsy specimens were washed in PBS, flattened on a filter paper for better orientation after fixation, and fixed overnight in absolute methanol at 4°C. Before establishing the final protocol, various tissue fixation methods were compared to investigate their influence on staining intensity for both the PCNA and the BrdU protocol. As an alternative to absolute methanol, biopsy samples were subdivided and fixed for 18–24 hours in 10% buffered formalin, 70% ethanol, 70% methanol, and a commercially available alcoholic fixative called ‘Primafix’. Subsequent tissue handling was the same as that in the BrdU protocol.

In the immunocytochemical staining procedure, sections are digested gently by incubation in 0.02% Saponin for 30 minutes at room temperature (Wargovich, personal communication). This step replaces DNA denaturation by acid hydrolysis with subsequent neutralisation in the BrdU protocol.

After the sections had been washed in PBS, a mouse monoclonal antibody against PCNA (IgM isotype; clone 19A2; Camon, Wiesbaden, Germany) was applied and incubated overnight at 4°C in a humid chamber. Biotinylated anti-mouse immunoglobulins, streptavidin biotinylated peroxidase complex, DAB reaction, and the final counterstaining were conducted as described for the BrdU protocol.

Determination of proliferation index

All sections were examined by one observer (RN) who had no knowledge of the clinical features or the histological diagnosis (magnification ×1000). At least 15 crypts were evaluated per subject and per method in sections from one
or two, and rarely three different biopsies. A crypt was analysed if the entire length of the crypt was visible in the longitudinal section and the base of the crypt contacted the muscularis mucosa.

In each crypt column – that is the single column of epithelial cells lining one side of a sectioned crypt meeting the criteria – the total number of cells and the number of labelled cells and their position in the crypt column relative to the base of the crypt were noted. In the BrdU assay, a cell was considered positive if there was any nuclear staining present. For the PCNA assay faint staining was seen frequently, probably reflecting the relatively long half life of immunoreactive PCNA and the fact that PCNA is expressed in phases of the cell cycle other than S phase. For comparison with BrdU, therefore, only the strongly stained nuclei, which are assumed to be in S phase, were counted.

The total number of cells, the number of labelled cells, and the proliferation index, which is the ratio of labelled cells to the total number of cells, were determined for each crypt column. The proliferation index was also calculated separately for each of five compartments of equal size into which each crypt column was divided. The same procedure was repeated for percentiles of the crypt column, and a running mean over five percentiles was calculated to smooth the distribution.

To estimate intraobserver variation, four randomly selected cases were counted on two separate occasions by the same observer.

**Statistical analysis**

Non-parametric statistical methods were used whenever possible, because neither the basic data nor the log transformed data were normally distributed. Correlation coefficients were calculated as Spearman rank correlations and comparison between the two methods by the sign test.

Variance component analysis was used to estimate the number of crypts and the number of biopsy samples that must be evaluated for each method to classify an individual correctly. A nested variance component model with random effects was calculated with the patient as unit and crypt and biopsy specimens as classification variable. The coefficient of variation (SD/mean) was calculated from this variance component model for each variable of the model. The variance within a patient was calculated by:

$$S_p^2 = \frac{S_c^2 + n S_b^2}{nb}$$

where: $S_p^2$=variance of the sample mean per determinand; $S_c^2$=variance of crypts (from the variance component analysis); $S_b^2$=variance of biopsies (from the variance component analysis); n=number of crypts; b=number of biopsy specimens. The 95% confidence intervals (CI) was estimated by: 95% CI=1.96$\sqrt{S_p^2}$ (see reference 20).

**Results**

**Proliferation index**

About 37,000 colonic epithelial cells were evaluated per method and more than 15 crypts were evaluated per subject. When scoring only the strongly reactive nuclei in the PCNA assay, which were assumed to represent the S phase cells, the individual proliferation indices between the two methods showed a Spearman correlation coefficient of $r_s=0.6$ ($p=0.011$) (Fig. 1). The regression equation was estimated as PCNA=0.0459+0.3912 BrdU ($p=0.015$). The mean proliferation index in the study group obtained by the BrdU technique (7.79%) was virtually equivalent to that obtained by the PCNA technique (7.64%) ($p=0.8$). Inclusion of the minor reactivity in the PCNA assay gave a mean proliferation index of 9.1% and the correlation failed to be significant. A statistically significant difference between the two methods was found in the total number of cells per crypt column, the crypts appearing longer in the PCNA assay (Table I). The mean proliferation indices within compartments were nearly identical for both assays. Slightly higher proliferation indices were found for the 4th and 5th compartments with the BrdU than with the PCNA technique.

Figure 2 shows the percentile proliferation indices obtained with the two methods. The proliferation index peaked at the 23rd percentile with the PCNA method. BrdU showed two peaks at the 18th and the 24th percentile. The proliferation index was slightly lower with BrdU

<table>
<thead>
<tr>
<th>Compartment</th>
<th>PCNA (mean (SD))</th>
<th>BrdU (mean (SD))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component 1</td>
<td>13-33 (6-52)</td>
<td>13-43 (9-46)</td>
</tr>
<tr>
<td>Component 2</td>
<td>15-22 (9-29)</td>
<td>16-73 (12-67)</td>
</tr>
<tr>
<td>Component 3</td>
<td>7-49 (6-52)</td>
<td>7-54 (9-99)</td>
</tr>
<tr>
<td>Component 4</td>
<td>2-52 (2-11)</td>
<td>1-25 (3-52)</td>
</tr>
<tr>
<td>Component 5</td>
<td>0-11 (0-19)</td>
<td>0-05 (0-77)</td>
</tr>
</tbody>
</table>

*Difference statistically significant, two sided p value: <0.05; **PPI, proliferation index in percent; percentage of labelled cells calculated for each patient separately; percentage of labelled cells calculated for each compartment and each patient separately.

**Figure 1:** Correlation of individual proliferation indices (PI) measured by the BrdU and by the PCNA method. ($r_s=0.6$, $p<0.05$; $b=0.39$.)
than with PCNA between the 20th and 42nd percentiles and higher between the 60th and the 80th percentile.

**INTROOBSERVER VARIATION**

Re-evaluation of four randomly selected subjects showed a 94.9% agreement on proliferation index in the case of BrdU and a 90.3% agreement in the case of PCNA. The percentage value of agreement on the total number of cells per crypt column, was 99.1% for BrdU and 97.3% for PCNA.

**INDIVIDUAL VARIATION**

Variance component analysis was used to calculate the contribution of the variations within subjects, biopsy samples, and crypts to the total variation of the proliferation index per method. The variance within subjects and within biopsy samples was found to be higher for the BrdU method than the PCNA method, but the variance within crypts was equal (Table II).

Variance component analysis was also used to calculate the required number of crypts (equation [1] resolved for n) to estimate the proliferation index for a subject within a 95% confidence interval with one, two, or three biopsy samples separately for each method (Table III). The gain in precision by taking two biopsy samples compared with one is striking for both methods; this effect is because of variation between biopsy specimens within subjects. The difference between the two methods is most obvious, when the precision of the point estimate is allowed to include a 95% confidence interval of PI±2.5%. Such a precision cannot be achieved by using one biopsy sample with the BrdU method, whereas PCNA can provide a point estimate within PI±2.5% by using one biopsy sample with 30 evaluated crypts. Estimation of a subject's proliferation index with the same precision using two biopsy samples requires 10 crypts in total for PCNA and 14 for BrdU. Evaluating more than two biopsy samples leads to an alignment in the number of crypts required to achieve a certain precision.

**METHODS**

Differences in time and type of fixation affect the intracellular staining pattern—that is, the strength of the staining and therefore the number of labelled cells. Various fixatives were tested (Table IV). The best results were obtained using both methods with absolute methanol fixation at +4°C for 18–24 hours. Staining intensity in the PCNA assay also introduced the Saponin digestion step. Staining was optimal after 18–24 hours of fixation, but less intense after more than 48 hours of fixation. This effect was no longer seen when the DNA denaturation step in the PCNA protocol was replaced by the Saponin digestion step. Saponin pretreatment also resulted in an augmentation of PCNA staining and a decrease in background staining.

**Discussion**

In this study on biopsy samples from colonic mucosa of patients with no colonic disorders, the comparison between two methods of assessing cell proliferation showed a significant correlation (r = 0.6, p < 0.05). Mean proliferation index (7·79, 7·64 respectively) as well as its distribution within crypts was similar with the two methods in the study group. The BrdU index obtained in this material was higher than that reported by Risio et al. and lower than that reported in other studies. Different laboratory practices do not permit direct comparison of values; for example, Wilson et al. used hyperbaric gas phase during incubation but a lower BrdU concentration in the medium, Risio et al. did not add fluorodeoxyuridine to the medium to enhance BrdU uptake. Our results agree in the distribution of proliferative activity.
tion indices by compartments, in that mostly the highest proliferative activity was found in the second compartment when five compartments were calculated. In this comparative study PCNA data resembled BrdU data when scoring was restricted to the more strongly reactive PCNA nuclei. Inclusion of all reactive nuclei gave PCNA values, which were on average about 20% higher than BrdU labelling. This variability in strength of nuclear reactivity has also been briefly reported by Battersby et al. On the other hand, Gawand and others showed in their comparison with [3H]-thymidine labelling that BrdU resulted in S phase cells becoming stained, when methanol fixation was used. But, they did not report a great variability in the intensity of nuclear staining.

The variance component analysis shows that the variation between biopsy specimens of one subject is larger for the BrdU method compared with the PCNA method. Basically, the intra-individual variability can be caused either by real variability from site to site within the circle of the rectosigmoidal junction or by variability in the sample preparation. The higher variability between biopsy samples in the BrdU method is likely to be because of the second reason - that is, this method is more affected by the conditions of incubation and subsequent processing. Therefore, in terms of precision for a given combination of crypts and biopsy specimens from a subject, the PCNA method seems preferable. Because of the variability between biopsy samples, the gain in precision of defining a subject’s proliferation index by taking two biopsy samples compared with one is remarkable for both methods. This finding implies that not only is the total number of evaluated crypts a crucial criterion for a precise estimation of a proliferation index, but also the number of biopsy samples per subject being used for the evaluation. It seems worthwhile to include at least two biopsy samples in the evaluation, for the additional gain in precision is the highest when increasing the number of biopsy samples from one to two samples.

Type of fixation may influence the accessibility of the PCNA protein to antibody detection although results from other studies do not support this conclusion. The use of absolute methanol in the PCNA protocol for fixation of the tissue yielded the most consistent staining results in our study. This finding is in line with the hypothesis that with this fixation the insoluble form of PCNA, which is associated with the DNA replication sites, can be detected. Galand and others also found that PCNA shows best agreement with [3H]-thymidine labelling when using methanol fixation. Our finding concerning dependency of staining intensity on duration of fixation has also been made by Battersby et al. The introduction of Saponin in the PCNA protocol before the application of the PCNA antibody was helpful in that PCNA could be identified even after more than two weeks of fixation. This step prolonged the localisation of PCNA immunoreactivity in time, which is especially useful in multicentre studies, where biopsy specimens have to be sent from several endoscopy units to a central laboratory.

The higher total number of cells per crypt column in the PCNA assay compared with the BrdU assay was suspected to be as a result of an observer effect during the counting process. The slightly stronger staining of labelled cells in the case of BrdU compared with PCNA may facilitate the identification of labelled nuclei. The faster counting could then lead to fewer actually seen nuclei in the BrdU assay compared with PCNA. Re-examination of four randomly selected patients for both methods showed a high agreement on the average total number of cells for each subject. Thus, the observer effect is not a plausible explanation of the phenomenon. A difference because of the handling of the specimens can also be excluded, because specimens for both BrdU and PCNA assay were equally flattened on a filter paper before fixation. An increased cell loss during incubation with BrdU as another possible explanation is not supported by published works. The significant difference in the number of cells per crypt column needs further investigation with regard to its cause and its possible confusing effects on the analysis of labelled cell distribution.

It is concluded that staining nuclear non-S phase specimens is less reliable compared to staining of replicating cells, PCNA antibodies may be more reliable markers to detect in situ cells with S phase contents. To detect S phase cells by means of PCNA assay, scoring procedures have to be strictly standardised to consistently score only strongly stained nuclei, which may be achieved best by using image analysis. If these conditions are not met, we assume that it might be more reliable to regard PCNA as a marker with its own evidence to characterise proliferative activity by scoring all PCNA reactive nuclei as positive regardless of staining intensity.

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