Proliferating cell nuclear antigen (PCNA): a new marker to study human colonic cell proliferation

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Abstract

Immunohistochemistry of the S phase related proliferating cell nuclear antigen (PCNA) was studied as an alternative to ex-vivo bromo-deoxyuridine (BrdU) immunohistochemistry for assessment of human colonic cell proliferation. From 16 subjects without colonic disease biopsy specimens were collected from five different sites along the colorectum and processed for BrdU and PCNA immunohistochemistry. The mean proliferation index of PCNA was significantly higher at 133% of the value obtained with BrdU. There was, however, a good correlation between the results from both techniques ($r=0.6275$; $p<0.05$). Decrease in proliferation index along the colorectum was seen with both staining methods but was clearer with PCNA immunohistochemistry (caecum/ascending colon v rectum: 12.0 v 7.2; $p<0.004$). The total number of crypt cells also decreased from proximal to distal (134 to 128; $p<0.06$) but at no site correlated significantly with the proliferation index. It is concluded that in clinical cell kinetic studies staining for PCNA may serve as an attractive alternative to the BrdU incorporation assay.

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In experimental carcinogenesis, changes in the proliferative characteristics of the colonic mucosa have been found to come before the development of frank neoplasia.[1-3] In subjects at risk for the development of colorectal carcinoma similar changes have been found including increased proliferation and an abnormal distribution of proliferating cells along the colonic crypts. Therefore, study of the proliferative phenotype may become an important clinical marker to identify subjects at risk to develop neoplasia and to determine early effects of treatment aimed at reducing this risk.[4-6]

For many years ex vivo labelling with tritiated thymidine followed by autoradiography has been used to determine the percentage of S phase cells or labelling index. This technique, however, is labour intensive and requires facilities to handle radioactivity. An improvement was achieved by ex vivo labelling with bromo-deoxyuridine (BrdU), a thymidine analogue and its visualisation with a monoclonal antibody using routine immunohistochemical methods.[7-9] Together with the difficulties in fixation and processing, partly adjustable by enzymatic pretreatment, the ex vivo labelling step remains a potential source for differences in labelling efficacy.

Proliferating cell nuclear antigen (PCNA) is an intranuclear 36 kD polypeptide[10 11] whose expression[12] and synthesis[13] is linked with cell proliferation. Flow cytometry on the human lymphoblastoid cell line Molt 4 using anti-PCNA monoclonal antibody and propidiumiodide showed predominant expression of PCNA during the S phase of the cell cycle but also during the late G1 and early G2 phase.[14]

In this study human colorectal mucosa was ex vivo labelled with BrdU to investigate the correlation between BrdU and PCNA immunoreactivity and the relation between these parameters and crypt height. Biopsy specimens were obtained in a programmed way from various sites over the entire colorectum to establish proliferative parameters in relation to site.

Materials and methods

BIOPSY SPECIMENS

Biopsy specimens of endoscopically normal colonic mucosa were taken at five different sites from proximal to distal in the colorectum (ascending, transverse, and descending colon, sigmoid, and rectum) of 16 subjects who had had diagnostic colonoscopy because of abdominal complaints and who were found not to have any organic colonic disorders (seven men, nine women, mean age 49 years, range 17 to 76). All specimens were taken by one endoscopist (LGJBE) and completely processed by the same technician (AP-H). Specimens were immediately placed in RPMI 1640 tissue culture medium containing 10% fetal calf serum, 20 μM 5-fluoro-uracil, 20 μM bromodeoxyuridine (Serva, Heidelberg, Germany), and 0·9% hydrogenperoxide during one hour at 37°C.

The composition of the tissue culture medium was based on a 3×3 experiment in which optimal conditions for ex vivo labelling of mouse and human tissue with BrdU were determined. Intestinal specimens were incubated in RPMI 1640 medium containing 10% fetal calf serum and concentrations of 1, 2 or 3×10⁻⁵) M BrdU and 0·5, 1 or 2 10⁻⁵) M 5-fluorouracil. We found that the addition of hydrogen peroxide seemed to improve the label uptake, therefore in this experiment hydrogen peroxide 30% was added to final concentrations of 0·3, 0·9, and 1·8% respectively. Specimens were immersion fixed in 70% ethanol for at least 24 hours. Best results with regard to immunoreactivity and morphology, were obtained by incubation with RPMI 1640 medium containing 10% fetal calf serum, 2×10⁻⁵) M 5-fluoro-uracil, and 0·9% hydrogen peroxide.

As maximum incorporation of BrdU can be expected after depletion of the endogenous pool

of thymidine monophosphate by inhibition of its de novo synthesis with 5-fluorodeoxyuridine,[15][16] addition of 5-fluorouracil, which is intracellularly converted to 5-fluorodeoxyuridine, resulted in slightly better immunostaining without differences in proliferation index.

Comparison of in vivo labelling with tritiated BrdU with 5 and 50 mg BrdU/kg in mice showed a sensitivity of 94·8 and 98·4% and a specificity of 99·6 and 99·4% respectively.[9] Comparison of ex and in vivo labelling with BrdU in mice showed a very good correlation between both techniques. From five hours of ex vivo labelling onwards, the proliferation index decreased in comparison with the proliferation index obtained by in vivo labelling.

Thereafter the specimens were immersion fixed in ethanol 70% at 4°C, and stored at the same temperature. Fixation and processing for anti-BrdU immunohistochemistry are potential sources of variability in proliferation index. It was previously shown that tissues fixed in denaturing fixatives such as ethanol or Carnoy's fluid invariably showed immunoreactivity, while cross linking agents, for example, formalin and glutaraldehyde showed weak or no reaction at all. By pretreatment of tissue sections with pepsin, the staining intensity of crypt cells was increased after formalin fixation. The effect of pepsin digestion can be explained by a facilitation of DNA denaturation as a result of digestion of chromatin associated proteins.[17] Finally, specimens were embedded in paraplast and cut at 2 μM on a Reichert-Jung microtome (type 2540, variation according to manufacturer's specifications 10%).

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After removal of paraffin and rehydration, endogenous peroxidase was blocked in 0·3% hydrogenperoxide in methanol for 20 minutes. In the slides to be processed for anti-BrdU immunohistochemistry, DNA was hydrolysed in 2 N HCl for 30 minutes at 37°C and sections were subsequently rinsed in 0·1 M sodium tetraborate (pH 8·5). After incubation with a monoclonal anti-BrdU antibody[9] or an IgM anti-PCNA antibody (clone 19A2, Coulter Electronics, Mijdrecht, The Netherlands[11]) for one hour at 37°C, sections were incubated with rabbit anti-mouse IgG-HRP (one hour, room temperature). As peroxidase substrate 3,3' diaminobenzidine-tetrahydrochloride (Sigma, St Louis, USA) was used according to Graham and Karnovsky.[18] Nuclei were counterstained with haematoxylin.

Positive controls included in vivo labelled specimen of mice or rat and ex vivo labelled human biopsy specimens known to be positive. Negative controls included human and rat specimen with and without incorporated BrdU, leaving out the anti-BrdU or anti-PCNA antibody or the conjugated rabbit anti-mouse polyclonal antibody. Only slides of batches with correct controls were evaluated.

SCORING AND METHOD OF COUNTING OF STAINED SECTIONS

Only U shaped longitudinally cut crypts with open lumina along the crypt axis, with the base of the crypt touching the muscularis mucosae, were evaluated. The total number of immunoreactive nuclei/crypt was counted as well as the total number of crypt nuclei. To avoid selection bias, all crypts showing at least one BrdU immunoreactive nucleus were included and compared with the appropriate PCNA immunoreactive nuclei. The proliferation index of that crypt was defined as the percentage immunoreactive crypt cells of the total number of crypt cells. In addition, the distribution of immunoreactive cells along the crypt lining was studied by dividing the crypt into three parts (lower, middle, and upper) and by determining in what part the labelled cells were localised predominantly. Slides were analysed by one investigator (FJGMK) with a blinded method. The repeatability coefficient[19] was calculated at 1·08% (n=10), which means that 95% of differences in repeated measurements (intraobserver variability) are expected to be less than 1·08%.[20] Data were analysed as immunoreactive nuclei/crypt, not per number of crypt cells. Based on the 'running average'[21] data in three patients, we could show that six well oriented and well stained crypts were sufficient to obtain a reliable proliferation index (Fig 1). Therefore the analysis was restricted to biopsy specimens that fulfilled this criterium. Paired and unpaired t tests were used to compare BrdU/PCNA staining and staining at different colonic sites respectively.

Results

BrdU staining was successful in only 44% of well oriented crypts compared with 86% with PCNA (Table I). In some mucosal sections good BrdU staining was seen in some parts whereas in other parts crypts were clearly negative. This shows that labelling inefficiency rather than immunohistochemistry problems contributed to the comparatively low success rate. This is substantiated by the high success rate of PCNA staining,

Figure 1: The running average of proliferation index in normal looking colonic mucosa of one subject.
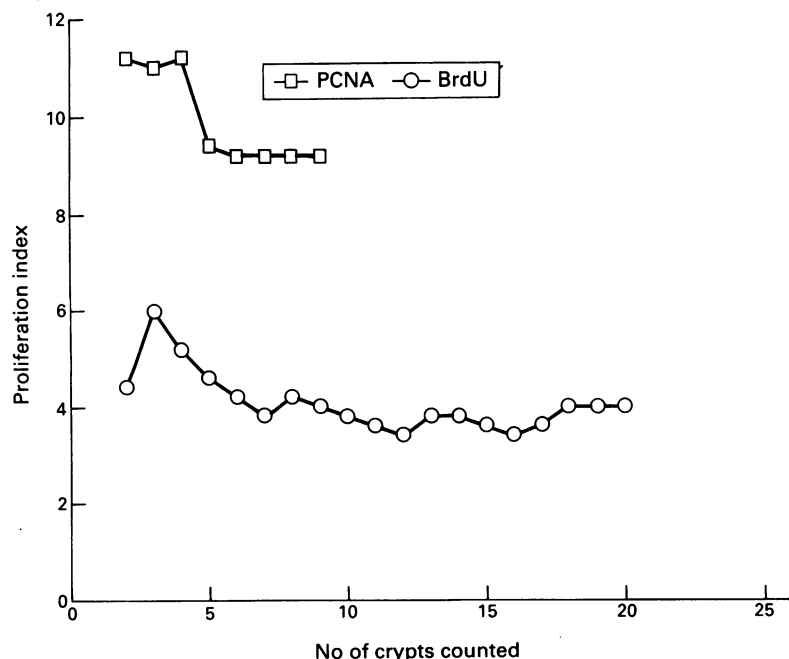
TABLE I   *Success rate of BrdU and PCNA immunohistochemistry*

	Staining	
	BrdU	PCNA
No of crypts examined	2035	761
No of positive crypts	901 (44%)	651 (86%)

BrdU = bromodeoxyuridine.
PCNA = proliferating cell nuclear antigen.

TABLE II   *Comparison of BrdU and PCNA immunohistochemistry of the human colorectal mucosa*

Staining	Mean profliferation index	SD	Range
BrdU	6·9	2·4	2·1–12·6
PCNA	9·2*	3·6	4·0–16·1

Sixteen specimens with at least six evaluable crypts for each staining. *p = 0·0004 (paired t test); abbreviations as in Table I.

which is not dependent on a previous ex vivo labelling step.

BrdU and PCNA staining were then compared on the single crypt level. For this we selected 16 specimens for which at least six crypts with BrdU were available, thereby making a direct comparison with PCNA possible (Table II). The mean

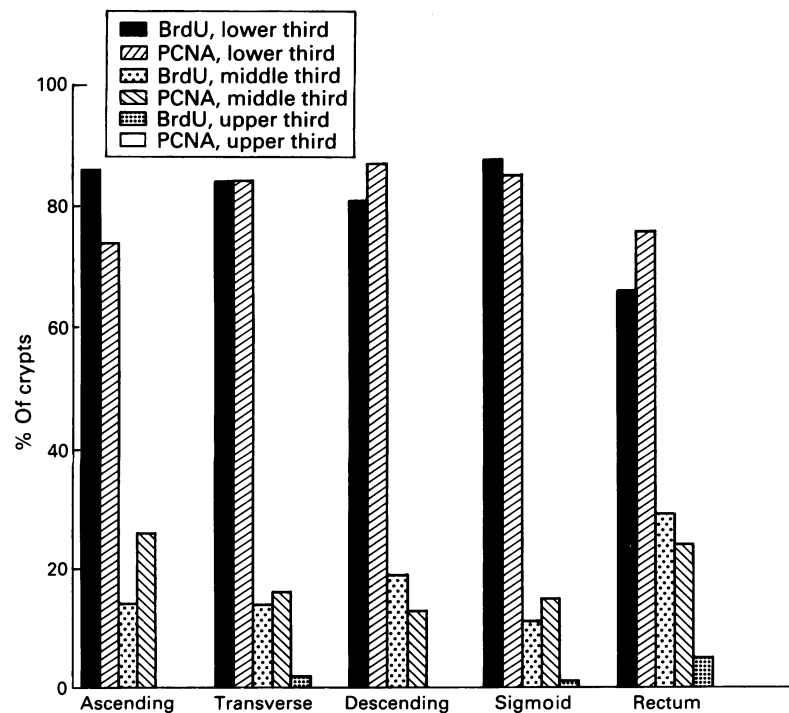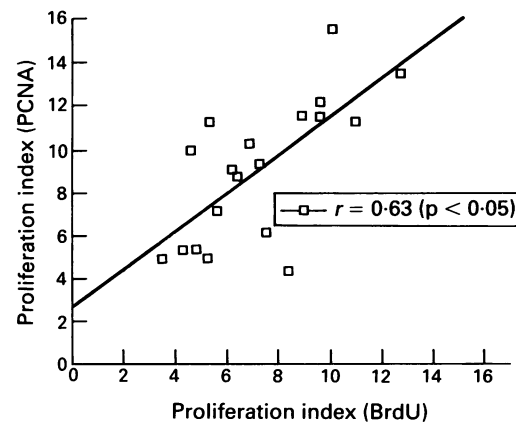*Figure 2: Correlation BrdU/PCNA immunohistochemistry.*

—□— $r = 0.63$ (p < 0·05)

PCNA proliferation index was significantly higher at 133% of the BrdU proliferation index. This difference seems to be systematic as the correlation matrix is showing significant correlation ($r = 0·63$; p<0·05) (Fig 2). Furthermore, the distribution of BrdU and PCNA immunoreactive cells along the colonic crypt was similar. In Figure 3, this distribution over the lower, middle, and upper compartments of the colonic crypt is depicted, showing in both cases that 80% of these cells are located on the lower third and virtually none in the upper third. This is illustrated (Fig 4) in two serial sections of the same colonic crypt.

BrdU and PCNA immunoreactivity were determined in relation to the site along the human colorectum (Fig 5). Both proliferation indices decreased from proximal to distal; this was most pronounced for the PCNA staining index with a decrease of 40% between the right sided colon and the rectum. For the PCNA staining index, the differences between rectum and sigmoid were not significant; differences with and between the descending, transverse, and ascending colon were, however, statistically significant (p = 0·0004 and p = 0·02 respectively).

Crypt height defined as the number of mucosal cells present in one column of a longitudinally cut crypt, was also determined along the entire colorectum (see Fig 5). Crypt height was the lowest in the rectum, but the differences were not statistically significant compared with descending, transverse, and ascending colon (p = 0·06). BrdU immunoreactivity (correlation coefficient 0·0008, p value not significant) and PCNA immunoreactivity (correlation coefficient 0·1616, p value not significant) were not significantly correlated with the crypt height.

Discussion

Changes in proliferative characteristics of colorectal mucosa are important markers for an increased risk of developing neoplasia. In addition to the problems in fixation, the ex vivo labelling step with BrdU is an important source of problems in the implementation of anti-BrdU immunohistochemistry for assessment of proliferation characteristics in routine endoscopy laboratories. More importantly, poor penetration of the DNA precursors or the finish of DNA synthesis in the ex vivo situation may cause the labelling to be heterogenous and of low efficacy. For instance in this study less than 50% of evaluable crypts was adequately labelled with BrdU despite measures to facilitate labelling by performing incubation next to the endoscopy room, by using a rich incubation medium, and by adding hydrogen peroxide to improve oxygen tension. This clearly illustrates the need for the development of logistically more easy and technically more reliable ways to determine proliferative characteristics. Our experiments were carried out to validate the use of PCNA immunohistochemistry as an alternative proliferative assay for human colorectal mucosa.

In Table III, some ex vivo cell kinetic studies on endoscopically and histologically normal human colonic tissue in normal subjects are summarised, showing a proliferation index
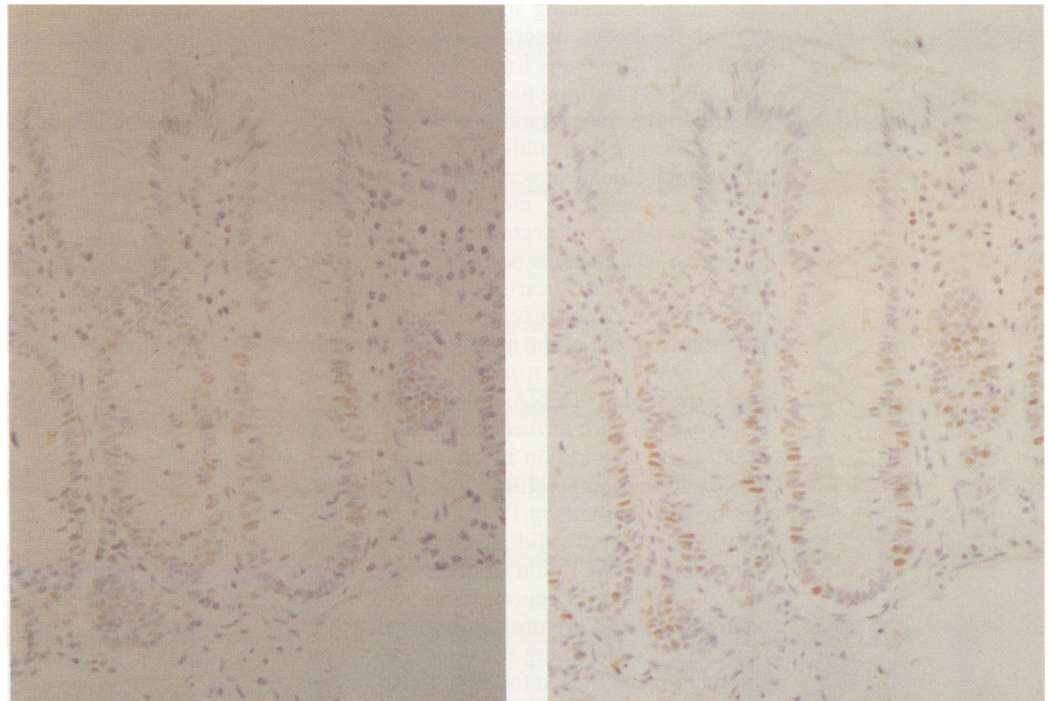

Legend:
■ BrdU, lower third
▨ PCNA, lower third
▩ BrdU, middle third
▨ PCNA, middle third
▦ BrdU, upper third
□ PCNA, upper third

Figure 3: Predominant site of BrdU or PCNA staining along colorectal crypts.

Figure 4: BrdU (left) and PCNA (right) immunoreactivity in two serial sections of the same colonic crypt.

ranging from 4·9–11·3% for tritiated thymidine labelling and from 5·9–6·9 for BrdU. The proliferation index with BrdU was slightly higher than reported in published works (showing that the simple ex vivo labelling procedure used is at least as efficient as more sophisticated alternatives commonly used[21][27] but still in the range of values obtained using tritiated thymidine).

By comparing PCNA and BrdU immunoreactivity in a systematic way differences as well as similarities were found. The main difference is the frequency of immunoreactive cells that was almost always higher in PCNA compared with BrdU stained crypts. The two proliferation indices significantly correlated, however, showing that the enhanced detection of PCNA immunoreactivity is a systematic rather than a random phenomenon. Immunohistochemical studies using IgG type anti-PCNA antibodies and BrdU incorporation showed a good correlation in stimulated peripheral blood mononuclear cells.[28] In tissues fixed in methanol the PCNA proliferation index did not differ by more than 6% from the tritiated thymidine labelling index.[29] In an animal study, using normal rat colon after in vivo labelling with BrdU and tritiated thymidine no significant differences between the labelling indices with these two DNA precursors and PCNA immunoreactivity was found.[30] Another animal study showed a strong correlation between in vivo labelling with tritiated thymidine and PCNA ($r=0·982$; p= 0·018) and between location of maximal staining for PCNA and tritiated thymidine ($r=0·997$; $p<0·05$).[31] The conclusion from these studies, that PCNA immunostaining may be a good alternative for BrdU or tritiated thymidine labelling is supported by our findings, showing a good correlation between BrdU and PCNA proliferation index along the entire colorectum. The systematic difference of around 2% may be the consequence of poor penetration of the label, early cessation of DNA synthesis or the immunoreactive PCNA present in late G1 or G2 cells. The fact that with in vivo labelling the BrdU and PCNA proliferation index are equal, at least in rats, suggests that the first explanation is more probable. It has to be emphasised, however, that with different fixatives, for example, formalin, with differences in immunocytochemical procedures, and with different cell sources, for example, cell lines, discordances between PCNA

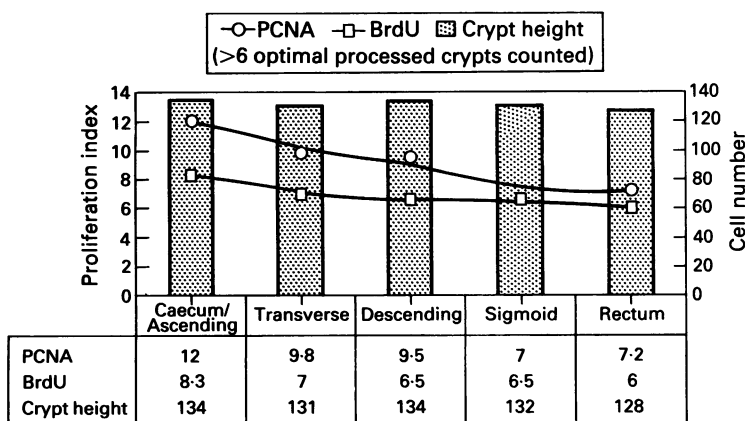*Figure 5: Proliferation index and crypt height along the normal human colon.*

-O- PCNA   -□- BrdU   ▨ Crypt height
(>6 optimal processed crypts counted)

	Caecum/Ascending	Transverse	Descending	Sigmoid	Rectum
PCNA	12	9·8	9·5	7	7·2
BrdU	8·3	7	6·5	6·5	6
Crypt height	134	131	134	132	128

TABLE III   *Proliferation index of endoscopically and histologically normal human colonic mucosa in normal subjects*

Reference	No of subjects	Tritiated thymidine	BrdU	PCNA
22	24	6·7 (0·9 SEM)		
6	16	4·9 (0·2 SEM)		
23	33	6·5 (2·5 SD)		
24	10	9·3 (0·3 SEM)		
	10	9·5 (0·3 SEM)		
25	19	11·3 (0·3 SEM)		
26	14	5·8 (1·6 SD)		
27	10	5·7 (0·7 SEM)		
21	40		5·9 (0·4 SEM)	
This study	16		6·9 (2·4 SD)	9·2 (3·6 SD)

Abbreviations as in Table I.

staining and labelling indices with DNA precursors have been described by others. For the time being it remains important to validate the use of PCNA staining for the tissue type and the species under investigation.

Extension of the proliferative compartment to the luminal side of the crypt has been described as being a sensitive measure of proliferative abnormalities in colorectal mucosa. It is also one of the earliest changes occurring during induction of colonic adenocardinoma in mice by 1,2-dimethylhydrazine. It is therefore important to note that staining of the middle and upper thirds of the crypt was not appreciably different with anti-BrdU or anti-PCNA antibodies.

Terpstra *et al* described a decrease in tritiated thymidine proliferation index along the normal looking human colorectum.[6] For BrdU, the same has been described by Potten *et al*.[32] If PCNA staining is to be a valid alternative to BrdU or thymidine incorporation studies, it is mandatory that this finding is reproducible with the new technique. We therefore compared BrdU labelling and PCNA staining at various sites along the colorectum and indeed found both parameters of proliferation to decrease from the ascending colon to the rectum. This decrease was more prominent with PCNA staining, which underscores the validity of this technique to measure differences in proliferation.

As Figure 2 shows, small percentages of crypts with BrdU but without PCNA in the upper third of the crypts were seen, but only in the rectosigmoid region. As the PCNA proliferation index is generally higher then that with BrdU, this seems surprising. Figure 5 shows, however, a more prominent decrease in PCNA proliferation index compared with BrdU, suggesting that the number of cells in G1 or G2M phase of the cell cyclus decreases from proximal to distal in the human colon. This would explain why, in the distal colon, BrdU immunoreactive nuclei are seen without simultaneous PCNA immunoreactive nuclei.

The crypts of mice with a genetic susceptibility to develop colorectal cancer are not only characterised by an increased proliferation but also by increased crypt size.[33] This suggests that in these animals the increased proliferation is not the result of an increased cell loss, but rather a reflection of an inherently increased proliferative activity. We examined whether the crypt height in the human colorectal mucosa is related to proliferative activity as reflected in the BrdU labelling index or PCNA staining index. It was found that the crypt height, expressed as the number of cells/colonic crypt was constant over the colorectum and in individual cases did not correlate with the markers of proliferation. It remains to be investigated if the crypt height is an alternative or additional biomarker for the risk of developing colorectal adenoma or carcinoma.

In summary, in the human colorectum the rate and site of cell proliferation determined by immunostaining for PCNA reflects the proliferative activity determined by labelling with the DNA precursor BrdU. The technique avoids the use of an ex vivo labelling step and is therefore technically more easy and less amenable to

disturbances because of inadequate penetration of the label or early cessation of DNA synthesis. It remains to be seen if PCNA immunostaining may indeed substitute for DNA precursor labelling in studies aimed at identifying patients at risk of develping colorectal neoplasia.

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