

# Immunohistochemical detection of abnormal cell proliferation in colonic mucosa of subjects with polyps

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## Abstract

Previous studies have shown the presence of increased proliferation in the large bowel epithelium of those at high risk of developing colon cancer. An *in vitro* technique for labelling large bowel mucosa with the thymidine analogue bromodeoxyuridine (BrdU) was therefore developed and its ability to distinguish differences in mucosal proliferation between subjects with colorectal adenomas and normal controls was assessed.

Sigmoid biopsy specimens from 15 subjects with polyps and 15 age and sex matched controls were labelled and the incorporated BrdU visualised with an immunohistochemical technique. Mean labelling index (LI) was significantly higher in those with polyps than in controls. Differences in the pattern of labelling in colonic crypts were compared by the generation of cumulative labelling distributions. Analysis showed a significant expansion of the proliferative compartment in the colon crypts of those with polyps.

It is concluded that *in vitro* labelling with BrdU provides a useful method for the assessment of mucosal proliferation in subjects at high risk of developing colon cancer.

Many studies have shown abnormalities of mucosal epithelial proliferation in diseases which predispose to gastrointestinal cancer. Autoradiography following  $^3\text{H}$ -Thymidine ( $^3\text{H}$ -T) incorporation into mucosal fragments *in vitro* has been the most widely used technique for studying cell proliferation in subjects at increased risk for colonic neoplasia.<sup>1</sup> Using this technique, significant differences in the labelling index (LI) and distribution of proliferative cells within large bowel crypts have been described in hereditary non-polyposis colon cancer,<sup>2</sup> familial polyposis coli,<sup>3,4</sup> and in the presence of sporadic polyps or carcinoma.<sup>5,6</sup> *In vitro*  $^3\text{H}$ -T labelling has therefore been proposed as a potential method for identifying high risk subjects,<sup>7,5</sup> and has also been used as an "intermediate biomarker" of colon cancer risk in assessing the short term effects of dietary intervention in man.<sup>8,9</sup> It is an exacting technique, however, with the disadvantage of requiring the use of radioactive isotopes and

considerable delays (often several weeks) while autoradiographs are developed.

The recent development of a monoclonal antibody against the thymidine analogue bromodeoxyuridine (BrdU) now permits much more rapid identification of S-phase cells (and thus the LI) by immunohistochemical techniques.<sup>10</sup> Measurement of BrdU incorporation is comparable in sensitivity to that of  $^3\text{H}$ -T *in vivo*.<sup>11</sup> We have therefore explored its use for *in vitro* labelling of human colorectal mucosa, and assessed its use as a means of discriminating those at increased risk of colon neoplasia by comparing its incorporation in the sigmoid mucosa from subjects with large or multiple colonic polyps and normal controls.

## Methods

### LABELLING TECHNIQUE

Biopsy specimens of sigmoid mucosa taken through a colonoscope were obtained from patients with one or more large adenomas (> 1 cm) and age and sex matched non-polyp bearing controls. All biopsy specimens from the patients were taken a minimum of 5 cm away from any macroscopic adenomas. Control subjects were selected on the basis of negative colonoscopy and barium enema in addition to having no family history of large bowel neoplasia. Biopsy specimens were taken between 9 and 11 am to exclude any theoretical effects of diurnal variation.<sup>12</sup> Immediately after removal specimens were transported to the laboratory in complete medium, pre-gassed with 95% oxygen and 5% carbon dioxide at 37°C, and established in organ culture. The organ culture technique is essentially that described by Pritchett *et al.*<sup>13</sup> Whole biopsy specimens were orientated, luminal surface

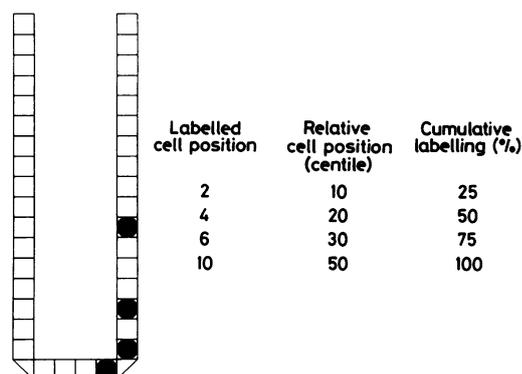


Figure 1 Stylised crypt column showing scoring of labelled cell positions and calculation of cumulative labelling distribution in a crypt containing 20 cells.

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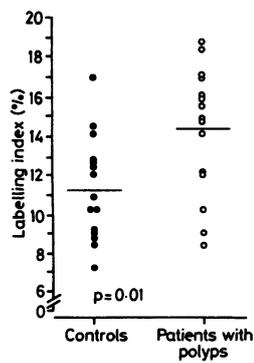


Figure 2 Labelling indices of controls and patients. (— = mean LI).

up, on steel grids and placed in organ culture dishes. The culture medium used comprised Weymouth's MB752 medium, supplemented with 10% fetal calf serum, L-glutamine 1.5 mM, ascorbic acid 300  $\mu\text{g}/\text{ml}$ , hydrocortisone 3.0  $\mu\text{g}/\text{ml}$ , ferrous sulphate 0.45  $\mu\text{g}/\text{ml}$  and gentamycin 50  $\mu\text{g}/\text{ml}$ ,<sup>14</sup> to which Brdu was added to a final concentration of 100  $\mu\text{mol}$ , and fluorodeoxyuridine at a concentration of 10  $\mu\text{mol}$ .<sup>15</sup> Tissue explants were maintained at the gas fluid interface in a sealed modular incubator containing 95% oxygen and 5% carbon dioxide at a pressure of two atmospheres. Explants were labelled at 37°C for one hour, rinsed and fixed by submerging the grids in Carnoy's fixative for 12–16 hours.

The biopsy specimens were then processed to paraffin wax and carefully embedded on edge to ensure that subsequent sections were taken through the vertical crypt axis. Sections 3  $\mu\text{m}$  thick were cut at intervals of 100  $\mu\text{m}$  through each tissue block and plated onto poly L-lysine coated slides for subsequent immunostaining.

#### IMMUNOSTAINING TECHNIQUE

Sections were dewaxed and placed in 1% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in methanol for 20 minutes to block endogenous peroxidase. After washing in TRIS buffered saline (TBS), pH 7.6, DNA was denatured by immersion of the slides in 1 N HCl for eight minutes at 60°C<sup>16</sup> to expose bound Brdu for immunostaining with an avidin-biotin peroxidase technique using a standard ABC kit (Dako). Tissue sections were incubated successively in 20% rabbit serum in TBS, 1/500 mouse anti-Brdu (Becton-Dickinson) for 30 minutes at room temperature, 1/400 biotinylated rabbit anti-mouse for 30 minutes, and finally in peroxidase avidin-biotin

complex for 30 minutes. After each step sections were rinsed in TBS. The antibody-peroxidase complex was visualised by incubation in 0.02M TRIS/HCl buffer (pH 7.6) containing 0.05% diaminobenzidine and 0.1%  $\text{H}_2\text{O}_2$  for five minutes. Sections were counterstained with Meyer's haematoxylin, dehydrated through graded alcohol, cleared in HistoClear, and mounted in synthetic resin.

#### SCORING OF BRDU LABELLING

Stained sections were scored for Brdu labelling under a light microscope at a magnification of  $\times 400$ . Forty longitudinally sectioned hemi-crypts (crypt-columns) were analysed from each patient.<sup>5</sup> For each crypt column the total number of cells and the number and position of Brdu positive cells were directly keyed into a microcomputer for further analysis. The LI (percentage of cells in S-phase) for each patient was determined from the percentage of Brdu positive cells in each of the 40 crypt columns.

To discern differences in the distribution of labelled cells within crypts cumulative labelling distributions of Brdu positive cells were generated. This method entails normalising each crypt column to a notional 100 cell positions, with cell position 0 at the crypt base and cell position 100 at the luminal surface. The distribution of positive cells is then expressed as a cumulative percentage for each crypt cell position centile. For example, if a crypt column contains 20 cells and the second, fourth, sixth and tenth cells from the bottom are labelled then these cells are assigned to the 10th, 20th, 30th, and 50th centiles of that crypt. The cumulative labelling corresponding to these centiles would then be 25, 50, 75 and 100% (fig 1). The results from all crypt columns in a sample can be combined to generate a cumulative labelling distribution for that sample.

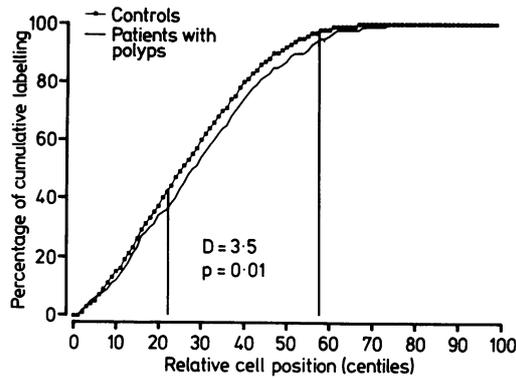


Figure 3 Photomicrograph of Brdu labelling from a control showing labelled cells confined to the basal region of the crypts.



Figure 4 Photomicrograph of Brdu labelling from patient with expansion of the proliferative compartment towards the luminal surface of the colonic crypt.

Figure 5 Cumulative labelling distributions in sigmoid mucosa of patients and controls. The curve is significantly shifted towards the right (the luminal surface of the crypts) in polyp bearing mucosa.



Comparison of LI and crypt column height were performed using Student's *t* test. To test for significant differences between the cumulative labelling distribution of patients and control subjects a two sample two-tailed Kolmogorov-Smirnov analysis was used. This non-parametric test defines a critical value *D* for any chosen probability at which the differences between cumulative distributions become significant.<sup>17</sup>

### Results

Biopsy specimens were obtained from eight male and seven female patients with colonic polyps with a median age of 60 years (range 41–79) and from 15 age and sex matched controls with a median age of 61 years (range 42–76).

Mean LI was significantly higher in the patients than in the control group (14.3 (SEM) 0.85 *v* 11.27 (0.7), *t* = 2.76, *p* = 0.01), although there was considerable overlap in the range of values within the two groups (fig 2).

Mean crypt column size was similar in the controls and patients (56.6 (SD) 8.5 *v* 57.2 (8.4), *p* = ns). In addition to differences in the LI, extension of the proliferative compartment was found in the mucosa from the patients (figs 3–4). Analysis of the cumulative labelling distributions showed an upward shift of the proliferative compartment in the colonic crypts of patients, with 100% labelling being present by the 64th centile in control crypts compared with the 75th centile in the patients. This shift in the cumulative labelling distribution curve was significant between the 22nd and 57th centile (*D* = 3.5, *p* = 0.01; fig 5). Colonic mucosa from patients therefore showed a significant expansion of the proliferative compartment towards the luminal surface throughout the middle third of the colonic crypt.

### Discussion

This study shows that subjects with colonic adenomas have an increased LI in the large intestinal mucosa, as has been reported previously in studies using <sup>3</sup>H-T autoradiography.<sup>15</sup> Brdu labelling is analogous to <sup>3</sup>H-T labelling *in vivo*<sup>11,18</sup> and *in vitro* using organ culture of fetal mouse colon.<sup>19</sup> The values obtained for human colonic mucosa are similar to those reported using <sup>3</sup>H-T labelling in several studies,<sup>6,20,21</sup> but are higher than in some others.<sup>3,9</sup> These differences may be explained

by variations in the handling of biopsy material, the available amount of <sup>3</sup>H-T in the culture medium, and the criteria used when scoring autoradiographs for labelled cells.

Risio *et al* reported a mean LI of 5.7% using *in vitro* Brdu in 10 samples of normal descending colon,<sup>22</sup> which contrasts with the mean value of 11.5% in this study. These authors counted fewer crypt columns, however, and experienced uneven or patchy uptake of Brdu. Our methods also differ from theirs in the use of an organ culture technique, the addition of fluorodeoxyuridine to enhance uptake of Brdu,<sup>15</sup> and the presence of a hyperbaric gas phase during labelling. The presence of a hyperbaric gas phase produces better correlation between *in vitro* labelling immediately after biopsy and *in vivo* labelling with <sup>3</sup>H-T<sup>23</sup> and Brdu.<sup>11</sup> Moreover, the LI values obtained with Brdu in this study are similar to those reported after intravenous infusion of Brdu in man.<sup>24</sup>

Traditionally, the distribution of the proliferative compartment within the colonic crypt has been analysed by dividing the crypt column into four, five, or even 10 separate compartments, with subsequent comparison of the LI within individual compartments.<sup>2,4–6,25</sup> Analysis of autoradiographs (after <sup>3</sup>H-T labelling) have shown an increase in proliferative activity in the upper portions of the colonic crypts in familial polyposis coli,<sup>27</sup> hereditary non-polyposis colon cancer,<sup>2</sup> and sporadic adenomas and carcinomas,<sup>5,6</sup> compared with controls. The generation of cumulative labelling distributions shows a significant shift in the zone of major proliferative activity towards the luminal surface in the presence of sporadic adenomas in a manner analogous to the “stage 2” proliferative defect described in previous studies.<sup>25,26</sup>

We conclude that *in vitro* labelling with Brdu is a rapid and sensitive method that is capable of showing the presence of abnormal crypt cell kinetics in the large intestinal mucosa of subjects at increased risk of developing colon cancer. Moreover, determination of the LI in conjunction with the cumulative labelling distribution may be applicable as a screening tool for subjects at risk of colon cancer, and it provides a useful “biomarker” for determining the short term effects of dietary intervention in such subjects.

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