Study of the proliferation in human gastric mucosa after in vivo bromodeoxyuridine labelling

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Abstract

Studies to measure human gastric crypt or gland cell proliferation may have a number of practical clinical applications in relation to both benign and malignant gastric conditions. Bromodeoxyuridine (BrdUrd) labels human gastric mucosal cells in the S phase. Computer aided data analysis of labelled mucosa allows static proliferative indices to be estimated, including the crypt labelling index (LI), the peak labelling position, the distribution of labelled cells and indirectly the crypt growth fraction. Multiparameter flow cytometric analysis of labelled nuclei allows the S phase duration (Ts) of mucosal cells to be estimated. Specimens of histologically normal gastric body (GB, n=16) and antral mucosa (GA, n=10) were obtained from 25 patients with gastric carcinomas who received a bolus dose of 250 mg BrdUrd between 3·0 and 15·7 hours before surgery. Tissue sections were stained by an immunohistochemical method and subjected to detailed counting of up to 50 longitudinal crypts per specimen. The total crypt labelling index was calculated by a grid counting method. A significant difference existed between the proliferative compartments of gastric antral and body mucosa measured by a number of criteria. The median lengths of the crypts were 137 cells (GB) and 188 cells (GA). The median peak labelling positions were cell 26 (GB) and cell 61 (GA) from the crypt orifice. The mean crypt labelling indices were 2·8% (GB) and 4·8% (GA). The mean Ts of GA cells was 7·7 hours and of GB cells was 10·8 hours.

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Gastric mucosal proliferation has been characterised in animals using tritiated thymidine, including the mouse[1 2] and dog.[3] Some aspects of the sequence of migration and differentiation of cells in mammalian gastric crypts have been reported. Limited studies have been published on human gastric mucosal cell proliferation[4–6] and a detailed overview of such work has been published.[7] In vivo studies in man have been limited by ethical and technical constraints.

The clinical study of human tissue proliferation has been advanced by the use of the halogenated pyrimidine S phase markers bromo- and iodo-deoxyuridine (BrdUrd and IdUrd),[8–10] which share S phase labelling characteristics with tritiated thymidine.[11 12] In vivo BrdUrd labelling is suitable both for histochemical and multiparameter flow cytometric analysis. We have adapted a proprietary computer based data analysis programme for intestinal mucosa to study and assess the distribution of S phase cells and to estimate the crypt labelling index in surgical gastric mucosal biopsy specimens from various anatomical sites. Multiparametric flow cytometric analysis allows dynamic data on cell kinetics to be obtained from a single injection and biopsy specimen.[13–16] In particular, for the purposes of this study, it can measure the S phase duration (in hours) of crypt cells in gastric mucosa. From these data, the crypt growth fraction, the cell cycle time, and the crypt turnover time can also be estimated.

The relative complexity of gastric crypt anatomy compared with colorectal mucosa has discouraged its use as an experimental model in proliferation research. Gastric mucosa is nevertheless a structured, spatially organised tissue of considerable clinical and biological importance. The study of the proliferation of human gastric mucosa may provide clues to the development of invasive adenocarcinoma, ulcers, mucosal erosions, and to the actions of putative mutagenic drugs in the stomach.

Methods

DRUG ADMINISTRATION

The study was made on full thickness tissues excised from surgical resection specimens in conjunction with an in vivo labelling study of primary gastric or oesophageal carcinomas. Each patient gave consent to have a single intravenous dose of 250 mg 5-bromo 2-deoxyuridine (BrdUrd) (Takeda, Japan) given over 30 seconds in 10 ml saline, between 3·0 and 15·75 hours before surgery. No detectable side effects were noted with this procedure. Hospital Ethical Committee approval was obtained.

IMMUNOHISTOCHEMICAL LOCALISATION OF BRDURD

Histologically normal mucosal strips were excised at least 5 cm from the tumour and stored in 70% ethanol. Nine 3 micron sections (every fourth section) were cut from each formalin fixed, wax embedded block and placed on gelatinised slides. Dewaxed slides were air dried and the endogenous peroxidase activity was blocked in 1% H_2O_2 in methanol for 30 minutes. Slides were hydrolysed in 1 N HCl at 60°C for eight minutes and then neutralised in boric acid buffer. After washing in phosphate buffered saline the sections were covered with normal rabbit serum diluted 1:20 in phosphate buffered saline for 30 minutes and kept moist. The primary antibody (rat anti BrdUrd, Sera Labs, Crawley Down, Sussex) was then added (1 in 5 in phosphate buffered saline) for one hour at 20°C. This was followed by washing with phosphate buffered saline and the addition of the secondary

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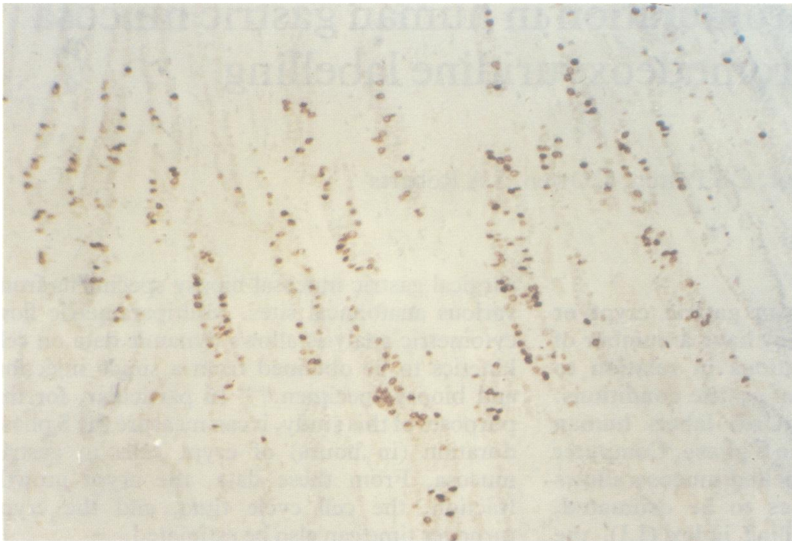

Figure 1: A photomicrograph of gastric body mucosa showing the clarity of peroxidase staining of bromodeoxyuridine labelled cells. (Original magnification ×16.)

antibody (rabbit anti rat peroxidase, Dako, High Wycombe, Bucks) 1 in 100 in 10% normal mouse serum for 1 hour at 20°C. The slides were then rewashed in phosphate buffered saline and treated with diamino-benzidine tetrahydrochloride (Sigma, Poole) for 15 minutes in the dark. Finally, the slides were weakly stained with haematoxylin for one minute before dehydrating and mounting in Xam (BDH, Poole).

SCORING AND METHOD OF COUNTING OF STAINED SECTIONS

The anatomy of gastric crypts varies through the stomach. Gastric pits become progressively deeper and more convoluted from the proximal cardia to the distal pyloric region. Each crypt consists of a superficial, longitudinal segment and a convoluted deep segment, the second usually appearing in transverse or oblique view on cut sections. Crypts were categorised by site of origin into proximal, or gastric body (n=16) and distal, or gastric antrum (n=10).

The study of labelled crypt cells is helped by computerised data analysis, as described for colorectal crypts.[9] A trained observer selected longitudinal crypts and counted labelled and unlabelled cells in the proliferative compartment, entering data directly into a BBC Microcomputer.

BrdUrd labelling was confined to the superficial zone of human gastric crypts (Fig 1). Up to 50 histologically normal crypts were analysed from each specimen where the entire proliferating compartment was contained within the longitudinal fragment of the crypt selected for counting. Position one was chosen as the first cell in the vertical column at the crypt orifice (Fig 2) as a reference point for the computer program. This was in contrast with other scoring studies, where the reference point was fixed at the crypt base. Fifty half crypt sections were scored for each sample. Scoring was performed for each cell along the crypt column to a point immediately beyond the proliferative zone where crypt continuity was lost. Slides were scored under a ×40 oil objective. The abscissa scale has been reversed to aid comparisons with colorectal data.[9,10,12]

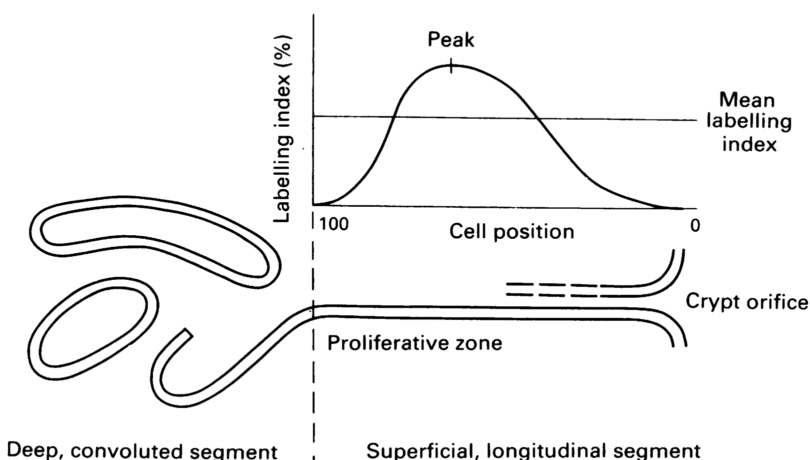

Figure 2: Diagram of the method of obtaining data in this study. The computer assisted counting was confined to the longitudinal portions of crypts in which the entire proliferative compartment was contained. Total crypt length and labelling index were estimated indirectly (see text).

INDIRECT ESTIMATION OF THE TOTAL CRYPT SIZE

Entire crypts are rarely displayed on single sections. Indirect estimates of total crypt length were therefore made. The total number of labelled and unlabelled cells was counted under an optical grid over the full thickness of the mucosa at a number of sites chosen at random on each specimen (approximately 1000 cells per site). The average number of labelled cells (x) per 50 hemicrypts in the proliferation zone was used to extrapolate to the total crypt size. For example, if x=4, and there were 20 labelled cells in a population of 1000 cells, it could be assumed that the cell population represented 20/4, or five hemicrypts. Hence the mean hemicrypt length was 1000/5, or 200 cells. Factors such as oblique and transverse cuts and cell overlap would affect the accuracy of this estimation.[7]

CALCULATION OF THE FLOW CYTOMETRY S PHASE DURATION

Suspensions of mucosal cells were prepared from fragments of mucosa by a pepsin extration technique. The method of preparation and of the analysis used to calculate the S phase duration, Ts, was identical to that reported elsewhere.[9,13,16]

DYNAMIC INDICES OF MUCOSAL PROLIFERATION

The crypt turnover time (Tt) can be estimated from the crypt labelling index (LI) and the S phase duration as follows: LI=Ts/Tt; assuming that the growth fraction (GF) at the position of the peak LI is unity, the cell cycle time (Tc) can be calculated: Tc=(Ts/peak LI)×GF. The overall crypt growth fraction is the ratio of Tc/Tt.

PARAMETERS MEASURED

Data which were derived from this analysis included: (a) the size and location of the proliferative zone; (b) the cell position of the peak labelling index (peak LI), and the upper and

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Results for pooled analyses of gastric body (16 patients, 780 hemicrypts) and gastric antral (10 patients, 500 hemicrypts)

Parameter	*Gastric body 780 hemicrypts (SD)*	*Antral mucosa 500 hemicrypts (SD)*
Static indices		
Total mean crypt labelling index %*	2·8 (2·4)	4·8 (3·1)
Proliferative range (no of cell positions)	60	90
Peak labelling position†	26 (6·1)	61 (10·6)
Calculated crypt length (cells)†	137 (20)	188 (28)
Peak labelling index %	11·9 (11·6)	13·9 (10·6)
Cell position of median labelling index†	28·8 (6·7)	52·0 (8·2)
Cell position of 95%ile†	17·0 (4·2)	46·9 (8·9)
Cell position of 05%ile†	26·2 (15·0)	74·1 (6·7)
Overall crypt growth fraction	0·11 (0·05)	0·19 (0·05)
Dynamic indices		
S phase duration (h)	10·8 (4·1)	7·7 (3·1)
Crypt turnover time (Tt) (days)	28·1	11·5
Cell cycle time (Tc) (h)	83 (29)	62 (28)

*This is the upper estimate as an unknown number of unlabelled cells occur at the lower cell positions.
†Counting from the orifice of the crypt.

lower limits of proliferation, the 95%ile and 05%ile; (c) the mean, median, and peak labelling indices; (d) the crypt growth fraction; (e) the cell cycle time; (f) the crypt turnover time.

STATISTICAL METHODS
Comparisons between the various subgroups of patients were performed using the Mann-Whitney U test. A Wilcoxson signed rank test was used for the paired test of samples. A significance value of p=<0·05 was used.

Results

PATIENT DETAILS
Twenty six mucosal specimens were obtained from 25 patients who had palliative or radical surgery for gastric adenocarcinoma (n=19), pancreatic adenocarcinoma (n=2), or oesophageal squamous carcinoma (n=4). There were 17 male and eight female patients, with ages between 43 and 82 years (median 66). Three underwent gastrotomy and intubation or gastroenterostomy and biopsy only, four underwent partial oesophagectomy for squamous tumours, 10 underwent total gastrectomies, and seven had partial gastrectomies. There was one modified Whipple's procedure.

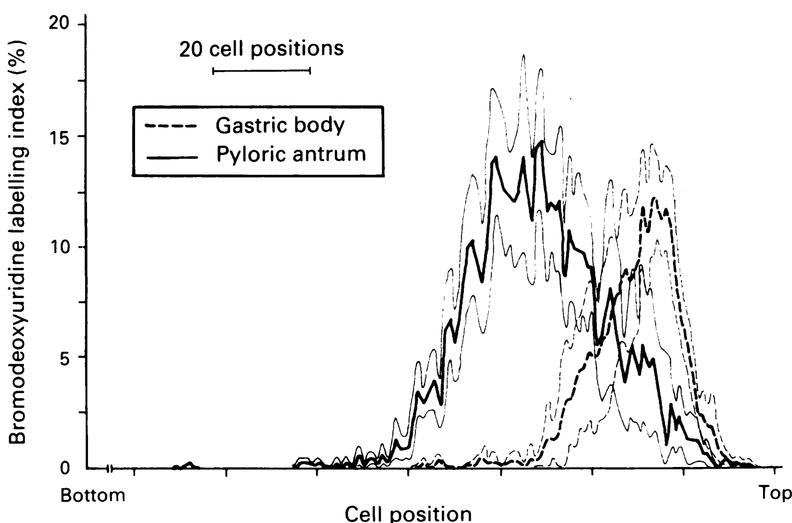

Figure 3: The distribution of labelled cells in gastric body and antral mucosa, emphasising the regional variation in crypt anatomy. The crypt length has been normalised and cell positions are expressed from the crypt orifice, labelled 'top' in this figure. The solid line represents the mean values, and the error limits are shown by the faint lines (see text).

CELL COUNTING
There were 780 pooled hemicrypts of gastric body mucosa (n=16) and 500 pooled hemicrypts of antral mucosa (n=10). The Table and Figure 3 shows the results.

STATIC INDICES
The total mean crypt labelling index was 2·8% in gastric body mucosa compared with 4·8% in antral mucosa, with a significantly larger proliferative range of cell positions in the gastric antrum (90 *v* 60 cells). The position of peak labelling was deeper in the antral crypts at position 61 compared with position 26 in body mucosa. Antral crypts were estimated to be longer than gastric body crypts. Figure 3 shows the distribution of the crypts described by the median value, 5th and 95th percentiles.

Significant differences (p=0·001) were found between these two gastric regions with regard to crypt length, size of the proliferating compartment, and the position of peak labelling. The overall growth fraction (<20%) was similar in both antral and body mucosa.

DYNAMIC AND FLOW CYTOMETRY DATA
The flow cytometrically derived S phase duration was similar in gastric body and antral cells. (All normal mucosal specimens had diploid DNA profiles.) The median Ts of gastric body mucosa was estimated to be 10·4 hours (range 3·8–19·7 hours) and of antral mucosa to be 7·3 hours (4·1–12·8 hours). The cell cycle time was estimated to be similar in both regions of mucosa, but the crypt turnover time seemed to be significantly longer (28·1 days) in gastric body than in antral (11·5 days) mucosa.

Discussion

Human gastric mucosa is a complex proliferation model when compared with colorectal mucosa, because of the convolution of the crypts and because the proliferation zone is located in the middle or upper part of the crypt, possibly producing bidirectional movement of maturing cells. An opportunity arose from a primary study of proliferation in gastrointestinal malignancy to investigate BrdUrd labelled gastric mucosa. There were no controls from non-malignant cases, but all specimens were well separated from the tumour, were normal on microscopy, and by all histological criteria were representative of normal gastric mucosa. We have previously reported[10] how mucosal proliferation may be influenced by proximity to a tumour. This would be difficult to test in gastric mucosa, because crypt anatomy varies throughout the stomach, and normal anatomical variation might mask any tumour associated changes.

There is no simple method to assess proliferation in single gastric crypts. We have adopted a pragmatic method to estimate the crypt size and

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the total crypt labelling index. We acknowledge the limitations imposed by the small size of this pilot study, and the arbitrary grouping of specimens into proximal and distal mucosa. In practice the changes in crypt structure along the stomach are almost a continuum.

Multiparameter flow cytometry adds the dimension of time to static analyses, where cells have been pulse labelled in vivo and where adequate time has elapsed between labelling and biopsy.[13 16] The technique does not distinguish between crypt and stromal cell nuclei in suspension, but S phase estimations are valid because most S phase cells are demonstrably cryptal rather than stromal in stained sections. Because of the variable time between pulse labelling and biopsy, there will be a trend to higher crypt labelling subsequent on cell division over the longer time periods. We were unable to show a statistically significant effect, however, of this time on the labelling index in a large and similarly prepared series of colorectal mucosa.[9]

Valid comparisons are difficult to draw from existing publications, particularly between species. Similar labelling index distributions have been published for the dog[3] and in the rat,[17] as reviewed.[7] With regard to man, Lipkin *et al*[4] studied tritiated thymidine labelling in the gastric mucosa of a patient with oesophageal malignancy and a gastrostomy in serial biopsies. In the gastric body crypts, intense labelling occurred in the proliferative zone, with cell migration up the crypts taking place over 96 hours. The S phase duration was calculated to be 10 hours. There were a mean 58 cells per column, of which 6·8 were labelled within 60 minutes. In the proliferation compartment, the labelling fraction was 30%, and the crypt cell birth rate was estimated to be 3 cells per 100 cells per hour.

Our data describe a technique which has allowed detailed study of the proliferation compartment of human gastric mucosa, about which little has been published. The study of gastric mucosal proliferation may have important clinical applications. The stomach is exposed to dietary hazards and potential carcinogens throughout life, but the origins of gastric carcinoma within normal mucosa remain unknown. Other fields for study include the proliferative response in healing gastric ulcers and the changes that may contribute to the clinicopathological diagnosis of Barrett's oesophagus.

We suggest that in vivo labelling of gastric mucosa offers considerable physiological and technical advantages over in vitro and crypt squash techniques, because the delivery of a thymidine analogue throughout the three dimensional structure of a tissue (or tumour) is assured in the presence of normal vascularisation. S phase gastric crypt cells display BrdUrd consistently (Fig 1). In vivo BrdUrd based studies, however, have serious practical limitations. Although BrdUrd appears to be safe for in vivo pulse labelling studies in low dose by the intravenous route, its use in vivo is confined to the study of malignancy.[18] In vitro incubations of fresh gastric mucosal biopsy specimens with BrdUrd or tritiated thymidine overcome the constraints of in vivo labelling at the cost of the loss of the dynamic function of pulse labelling. Studies of crypts labelled in vivo with BrdUrd may provide a reference against which other putative intrinsic proliferation markers such as Ki-67 and proliferating cell nuclear antigen may be assessed. By these means it is hoped that further understanding of gastric mucosal proliferation and its controls in normal conditions and in disease may be achieved.

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