Comparison of labelling by bromodeoxyuridine, MIB-1, and proliferating cell nuclear antigen in gastric mucosal biopsy specimens

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Abstract

***Aims*—To compare proliferating cell nuclear antigen (PCNA) and MIB-1 with bromodeoxyuridine (BrdU) pulse labelling, a specific marker of cell proliferation, in endoscopic gastric biopsy specimens.**

***Methods*—Twenty four biopsy specimens were obtained from 12 patients: 10 antral and eight body specimens were suitable. Each specimen was routinely processed and stained with haematoxylin and eosin. A modified Giemsa stain was used to detect the presence of *Helicobacter pylori*. Sections of the specimens were labelled with BrdU, MIB-1, and PC10. Gastric mucosa specimens were divided into three zones. The numbers of positively staining nuclei for 500 epithelial cell nuclei were counted in each zone and expressed as a percentage.**

***Results*—The proportion of PCNA positive cells (range 0–90%) was much greater in all specimens (10 antrum, eight body). BrdU positive cells were virtually all confined to zone 2 (0–17% cells in this zone were positive) (zone 1 = surface and gastric pit, zone 2 = isthmus, zone 3 = gland base), while PCNA positive cells were present in all three zones (1 = 23–90%, 2 = 43–90%, 3 = 0–74%). Spearman's rank coefficient correlation of 0·57 confirmed that the percentage of positively staining cells varied in the same direction for both PCNA and BrdU ($p < 0\cdot001$). PCNA, however, was overexpressed in all zones of the gastric epithelium compared with BrdU. In 38 biopsy specimens from 19 patients, of which 14 antrum and 11 body were suitable, the proportion of MIB-1 positive cells (0-59%) was greater than BrdU in most. As with BrdU labelling, the MIB-1 positive cells were confined to zone 2 (zone 1 = 1–11%); zone 2 = 21–59%; zone 3 = 0–13%) and the coefficient correlation for MIB-1 and BrdU was 0·63 ($p < 0\cdot001$).**

***Conclusions*—MIB-1 accurately reflects the S-phase fraction in gastric mucosa, determined by BrdU labelling in conventionally processed gastric biopsy material. Caution is needed in the interpretation of PCNA labelling detected by PC10, which should not be accepted uncritically as a marker of cell proliferation in paraffin wax embedded material.**

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Gastric epithelium exists in a state of equilibrium between cell proliferation in the gastric pits and cell loss into the lumen. This state is influenced by both physiological and pathological processes. Gastric mucosal biopsy specimens provide "snapshots" of the mucosa, and labelling with a marker of cell proliferation allows the proliferative activity of the epithelium in various disease states to be compared. The use of flexible endoscopy has produced a large archive of biopsy material which could be studied with a cell proliferation marker designed for use on routinely processed, paraffin wax embedded material.

Proliferating cell nuclear antigen (PCNA) is a 36 kilodalton DNA polymerase δ auxiliary protein essential for DNA synthesis. It is associated with the cell cycle.[1 2] PC10 is a monoclonal antibody directed to genetically engineered PCNA and has been reported as a marker of cell proliferation suitable for use on conventionally fixed and processed normal human tissue.[3]

MIB-1 is one of three recombinant monoclonal antibodies reactive to parts of the Ki-67 antigen.[4] The monoclonal antibody Ki67 reacts with a human nuclear cell proliferation associated antigen that is expressed in all active parts of the cell cycle and is well established as a marker of cell proliferation.[5 6] The main disadvantage of the Ki-67 method is that fresh frozen material is required. Recently MIB-1 has been reported to stain a true Ki-67 equivalent using immunostaining techniques on frozen sections. Poor results were obtained initially on conventionally fixed and processed biopsy material, but heating the dewaxed sections in a microwave oven prior to immunostaining produced strong nuclear staining of proliferating cells.[4]

It is important to compare a new technique with a recognised standard to assess its suitability as a measurement of cell proliferation. Bromodeoxyuridine (BrdU), a thymidine analogue, can be incorporated into the cell cycle during S phase (DNA synthesis). Immunohistochemical techniques using an antibody to BrdU on tissues exposed to it either in vivo or in vitro, produce a marker of cell proliferation which is regarded as a "gold standard".

Methods

Patients undergoing routine diagnostic endoscopy were recruited after informed consent had been obtained. Using standard forceps two adjacent biopsy specimens were taken at endoscopy from the gastric antrum and body

Table 1 Labelling indices in each zone (1–3) of gastric antral and body mucosa using BrdU and PCNA labelling

Case No	Diagnosis	Antibody	Antrum 1	Antrum 2	Antrum 3	Body 1	Body 2	Body 3
1	Normal	BrdU	0·2	2·0	0			
		PC10	45	43	61			
2	Normal	BrdU	0	6	0	0	6	0
		PC10	67	83	67	23	51	0
3*	Normal	BrdU	3	17	0	1	5	0
		PC10	74	85	74	90	83	56
4*	Normal	BrdU	2	8	0	1	6	0
		PC10	70	79	66	77	81	36
5*	Normal	BrdU	2	5	0	3	9	0
		PC10	79	68	49	69	70	59
6	Chronic gastritis	BrdU	0·4	11	0	2	0	0
		PC10	75	80	38	62	63	42
7	Chronic gastritis	BrdU	2	15	0			
		PC10	81	90	64			
8	Chronic gastritis	BrdU	3	16	0	3	9	0
		PC10	78	69	56	52	61	51
9	Chronic gastritis	BrdU	0	8	0			
		PC10	66	69	53			
10	Chronic gastritis	BrdU				0·2	0·4	0
		PC10				69	86	63
11	Chronic gastritis	BrdU				0	6	0
		PC10				76	89	54
12	Chronic gastritis	BrdU	2	6	0			
		PC10	83	86	56			

*Sections labelled with three immunostains.

and placed on filter paper. One biopsy specimen from each site was placed in 10% buffered formalin, routinely processed, and stained with haematoxylin and eosin. A modified Giemsa stain was used to detect *Helicobacter pylori*. The other specimen was taken for immunohistochemical study and was placed in RPMI 1640 Medium without L-Glutamine (Gibco) containing BrdU (5 mg/10 ml) for 60 minutes at 37°C in a waterbath, then fixed in formalin, and routinely processed to paraffin wax. Before immunostaining, the sections were cut and dried at room temperature. Sections for labelling with BrdU were placed in hydrochloric acid (2M). Sections for labelling with MIB-1 were placed in citrate buffer and incubated twice for 5 minutes (650 Watts) in a microwave oven (Miele M696). The sections were stained with BrdU (1 in 20 dilution; Dakopatts) and PC10 (1 in 50; Dakopatts), or MIB-1 (1 in 50; Binding Site, Birmingham) antibody for 60 minutes using a three step avidin-biotin complex immunoperoxidase technique. Only sections with a full thickness of mucosa (epithelium to muscularis mucosae) and orientated perpendicular to the epithelial surface were counted. For the purposes of this study, the gastric mucosa was divided into three zones: zone 1 = surface and gastric pit; zone 2 = isthmus; zone 3 = gland base. The number of positively staining nuclei per 500 epithelial cell nuclei (or whole section when less than 500 cells were present) was counted in each zone and expressed as a percentage. This number was determined by counting consecutive high power fields until the continuous mean varied by less than 5%. Only unequivocally stained cells were counted as positive. Some biopsy specimens were too small to provide sections for all three immunostains. To provide an adequate number of sections for comparison, the PC10 and MIB-1 labelling was performed on different sections in most cases (the BrdU labelling was performed on both sets of sections).

Table 2 Labelling indices in each zone (1–3) of antral and body gastric mucosa using BrdU and MIB-1 labelling

Case No	Diagnosis	Antibody	Antrum 1	Antrum 2	Antrum 3	Body 1	Body 2	Body 3
1*	Normal	BrdU	3	17	0			
		MIB-1	11	48	4			
2*	Normal	BrdU				1	6	0
		MIB-1				3	24	1
3*	Normal	BrdU	2	5	0	3	9	0
		MIB-1	2	26	2	3	37	3
4	Chronic gastritis	BrdU	3	13	7			
		MIB-1	1	38	2			
5	Normal	BrdU				3	11	4
		MIB-1				0	21	0
6	Chronic gastritis	BrdU				9	8	9
		MIB-1				9	59	4
7	Chronic gastritis	BrdU	5	11	0			
		MIB-1	2	39	4			
8	Normal	BrdU	2	18	2			
		MIB-1	2	25	4			
9	Normal	BrdU				1	11	1
		MIB-1				6	35	5
10	Chronic gastritis	BrdU	4	14	2	6	15	10
		MIB-1	8	39	13	2	47	3
11	Chronic gastritis	BrdU				3	17	7
		MIB-1				8	48	8
12	Chronic gastritis	BrdU	5	6	0			
		MIB-1	1	43	2			
13	Chronic gastritis	BrdU	1	9	1	3	12	1
		MIB-1	2	35	2	4	33	2
14	Chronic gastritis	BrdU	2	11	2			
		MIB-1	4	37	1			
15	Chronic gastritis	BrdU	2	15	0	5	16	3
		MIB-1	5	46	1	2	42	6
16	Chronic gastritis	BrdU	3	8	3	5	17	10
		MIB-1	6	57	3	5	46	6
17	Chronic gastritis	BrdU	4	17	10			
		MIB-1	5	57	2			
18	Chronic gastritis	BrdU	5	7	2	8	11	5
		MIB-1	3	31	2	2	31	5
19	Chronic gastritis	BrdU	2	7	1			
		MIB-1	3	48	2			

Results

PCNA COMPARED WITH BrdU

Twenty four biopsy specimens for immunostaining were obtained from 12 patients: 10 antral and eight body specimens were suitable for counting. Five patients had normal gastric histological results, and seven had chronic *H pylori* associated gastritis. The proportion of PCNA positive cells was much greater than BrdU positive cells in all biopsy specimens (range 0–90%). BrdU positive cells were virtually all confined to zone 2 (0·4–17%), while in zones 1 and 3 0–3% and 0%, respectively, were positive. PCNA positive cells were present in all three zones (zone 1, 23–90%; zone 2, 43–90%; zone 3, 0–74%) (table 1). Figure 1 illustrates the distribution of BrdU compared with PCNA percentage positivity. When this was analysed using the Spearman rank correlation coefficient test, a correlation coefficient of 0·57 ($p < 0·001$) was found. This confirms that the percentage of positively staining cells varies in the same direction for both PCNA and BrdU.

MIB-1 COMPARED WITH BrdU

Thirty eight biopsy specimens were obtained from 19 patients: 14 antral and 11 body biopsy specimens were suitable for immunostaining. Five patients had normal gastric histology, nine had chronic *H pylori* associated gastritis, and five had *H pylori* negative gastritis. The proportion of MIB-1 positive cells (range 0–59%) was greater in most biopsy specimens than BrdU positive cells (range

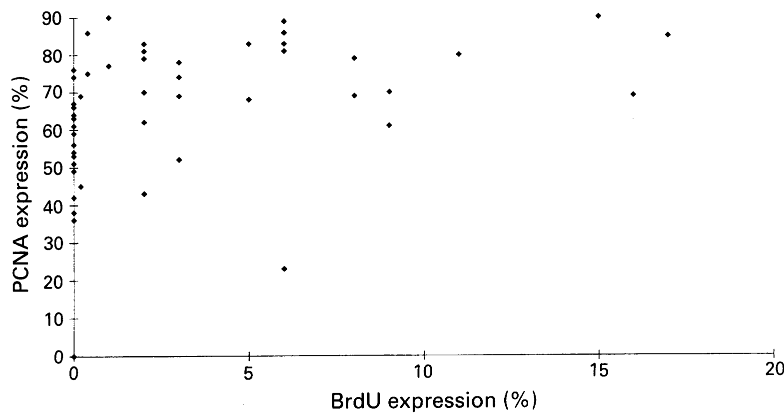

Figure 1 Plot of BrdU and PCNA percentage positivity in all three zones of gastric antral and body biopsy specimens showing a correlation coefficient of 0·57.

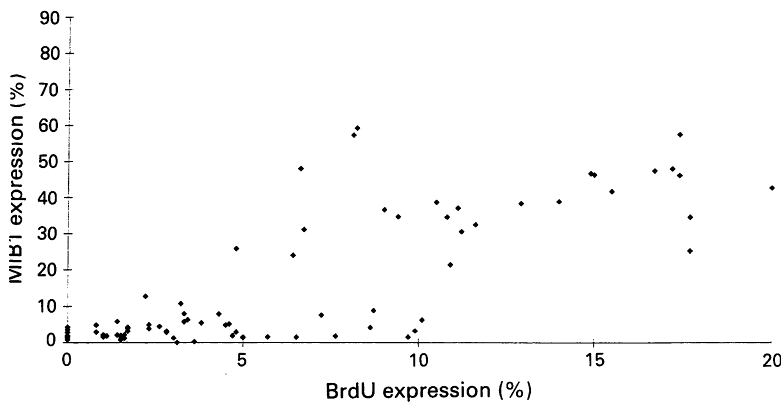

Figure 2 Plot of BrdU and MIB-1 percentage positivity in all three zones of gastric antral and body mucosa showing a correlation coefficient of 0·63.

0–18%). The MIB-1 positive cells were mainly confined to zone 2 (zone 1 = 1–11%; zone 2 = 21–59%; zone 3 = 0–13%) as were those staining positively for BrdU; zone 1 = 0·8–9%; zone 2 = 5–18%; zone 3 = 0–10% (table 2). The correlation coefficient for MIB-1 and BrdU was 0·63 ($p < 0·001$; Spearman's rank correlation coefficient) (fig 2).

Discussion

Thymidine is taken up by cells during DNA synthesis, or S phase, of the cell cycle. The development of monoclonal antibodies to BrdU, a thymidine analogue, has allowed the "S-phase labelling index" to be measured, using an immunoperoxidase technique, on in vivo and in vitro labelled material. The "S-phase labelling index" is regarded as an accurate estimate of the proliferating cell population, but the in vitro method of BrdU labelling, though not invasive, requires incubation of fresh tissue and thus precludes the study of biopsy material not already treated with the reagent.[7–9] There is a need for an accurate measurement of cell proliferation which can be performed on routinely processed material, which is neither time consuming nor labour intensive. Identification of PCNA by the PC10 antibody and Ki-67 antigen by MIB-1 have been put forward as possible contenders.[2,4] PC10 immunoreactivity has been shown to correlate with flow cytometry in gastrointestinal lymphomas,[10] and with Ki-67 staining in nodal lymphomas.[11] It also correlates with BrdU labelling in phytohaemagglutinin-stimulated human peripheral blood mononuclear cells.[3] Ki-67 is well established as an accurate marker of cell proliferation on fresh frozen tissue,[5,6] and MIB-1 has been shown to stain accurately for the Ki-67 antigen. A study of gastric carcinoma, however, has shown that Ki-67 labelling does not correlate with PC10.[12] This observation raises concern about the applicability of identification of PCNA by PC10 in gastric mucosa. To determine whether PC10 and MIB-1 labelling can be used as markers of cell proliferation in gastric mucosa we have compared them with in vitro pulse labelling with BrdU. The latter method has been shown to correlate well with in vivo staining in normal and neoplastic gastric mucosa.[13] Our results show a much greater expression of PC10 than BrdU in all zones of the gastric epithelium. Though there is a correlation which confirms that positivity with the two antibodies varies in the same direction, the discrepancy between the two indices is very pronounced. In contrast, MIB-1 expression, though greater than that of BrdU, is much less than that of PCNA and correlates more closely with BrdU labelling.

Ohyama *et al* have shown that BrdU labelling index was 6·9% (SEM 1·8%) in 42 normal gastric mucosae and 14·5% (11·5%) in 56 gastric cancers.[13] Jain *et al* have shown that the mean PC10 labelling index in 93 gastric cancers was 40·7%, range of 0–90%.[12] These data are in broad agreement with our results. A recent study of rat intestine and rat mammary tumours has also shown a similar discrepancy between PCNA and BrdU based labelling indices.[14]

The substantial overexpression of PCNA recognised by PC10 can be explained by its presence in all phases of the cell cycle with a half-life of about 20 hours.[15,16] This relatively long half-life leads to its expression in cells which have left the cell cycle and are therefore not synthesising DNA. This is in contrast to Ki-67 which is expressed in the active parts of the cell cycle—G1, S, G2 and mitosis, but not G0. The overexpression of PC10 is said to be particularly prominent in formalin fixed tissue, but in the study of PCNA in rat gastric mucosa and rat mammary tumours methacarn was used as the fixative and, in spite of this precaution, similar high concentrations of the protein were found.[14] In neoplastic lesions of the stomach overexpression may be due to deregulation of PCNA or stimulation by tumour associated growth factors.[3] Recently, EGF and TGF α have been shown to increase expression of PCNA in the mouse pancreas.[16] In the present study PCNA overexpression is present in histologically normal gastric mucosa as well as in chronic gastritis and growth factors may contribute to this process. A noticeable difference between PCNA and BrdU labelling has not been observed in the colon where the labelling indices are relatively close.[17] Factors which

promote PCNA expression may be present in the stomach but absent in the colon. Whatever the reason for the high PC10 expression in the stomach, our finding of up to 90% positivity in normal gastric epithelium leaves very little room for the detection of increased cell turnover in disease states.

In conclusion, MIB-1 immunostaining of conventionally fixed and processed gastric mucosal biopsy specimens, following incubation in a microwave oven, correlates closely with that of BrdU labelling, and may be used as a method of determining cell proliferation in these. In contrast, PCNA, as detected by PC10, does not accurately reflect the S-phase fraction in gastric mucosa, as determined by BrdU labelling. In view of all the factors already known to influence PC10 labelling, extreme caution is needed in its interpretation, and it should not be accepted uncritically as a marker of cell proliferation in paraffin wax embedded material.

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