Accelerated gastric epithelial proliferation

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Abstract

Gastric body mucosal proliferation was quantified and localised under conditions of increased gastrin drive using a variety of techniques. Rats were given omeprazole 400 μ mol/kg/day by gavage and after 30 days mean serum gastrin rose 11-fold ($p < 0.001$). Total mucosal polyamines rose 220% from 15.9 to 50.9 nmol/mg protein ($p < 0.001$). This was associated with a 238% increase in crypt cell production rate from 0.541 to 1.83 crypt cells/h by vincristine metaphase arrest ($p < 0.02$). Using computer aided counting of proliferating cell nuclear antigen (PCNA) immunostained nuclei to assess epithelial proliferation in hypergastrinaemia rat stomach: mucus neck cell PCNA labelling was increased by 41% ($p < 0.001$) and gland cell PCNA labelling was increased by 222% ($p < 0.001$). PCNA/AgNOR (argyrophilic nuclear organiser regions) co-stained sections were used to assess proliferative activity in cycling and non-cycling cell populations. Data from these experiments suggest that, in addition to increasing the number of mucosal cells in cycle, cell life and cell cycle duration may be reduced in hypergastrinaemia.

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Gastrin stimulates acid secretion in the gastric body and is trophic to both epithelial and enterochromaffin like cells of the gastric body mucosa.^{1,2} Administration of omeprazole, a substituted benzimidazole, causes achlorhydria by inhibition of the proton pump in gastric parietal cells.² In animals and humans with an intact gastric antrum, this results in a sustained, progressive but reversible increase in serum gastrin.^{3,4}

The proliferative compartment of the gastric oxyntic mucosa is thought to be confined to the mucus neck cells. Expression of proliferation associated antigens is uncommon in foveolar, surface, and gland cells, which are fed by migration of cells from the neck cell compartment.⁵ The experiments described give new insight into this process.

Different techniques for measuring cellular proliferation give different types of information. In recognition of this we have performed studies in parallel relating to proliferation associated biochemical events, the number of cells in cycle, the site of cellular proliferation, the duration of the cell cycle, and the number of new cell births. The techniques used include analysis of mucosal polyamine content, prolifer-

ating cell nuclear antigen (PCNA) immunostaining, counting of nuclear organiser region numbers, and measurement of crypt cell production rate. All were performed in the same rat model of accelerated gastric epithelial proliferation.

Polyamine biosynthesis is closely linked to epithelial proliferation. The polyamines spermidine, spermine, and their precursor diamine putrescine are present in all cell types.⁶ They are synthesised from the amino acid ornithine, in a reaction catalysed by the enzyme ornithine decarboxylase (ODC). While the precise function of polyamines is still uncertain, one of the important interactions of the polyamines in eukaryotic cells is their ionic interaction with DNA, which has implications for both DNA structure and function.⁷ Proliferative responses are often associated with a rise in ODC activity and a rise in cellular polyamine concentrations, which are permissive in many cases.^{8,9} Proliferative modulators such as gastrin¹⁰⁻¹² and epidermal growth factor^{13,14} cause a rise in ODC activity. ODC mediated polyamine biosynthesis is consequently a secondary messenger for proliferative modulators.¹⁴ We have included measurements of mucosal polyamines to provide a link with existing studies.

Vincristine metaphase arrest is a technique for measuring the number of new cells produced within an intestinal crypt or gastric pit over a period of time: the crypt cell production rate.¹⁵ The technique is dependent upon the uniformity of the number of cells within the individual crypt and has the advantage over mitosis counting of identifying all the arrested metaphases within microdissected crypt squashes rather than within a section through the crypts or in the case of stomach gastric glands. The technique gives important information about the rate of cell birth rather than the state of cells in cycle at any one time.¹⁵

The PCNA is a 36 kD nuclear protein, which accumulates progressively through G₁-M of the cell cycle and disappears at the end of mitosis. It has been identified as an auxiliary protein to DNA polymerase delta and is postulated to be a cell cycle regulator protein.¹⁶ The PC10 antibody is a mouse monoclonal raised against recombinant rat PCNA. PC10 is of the IgG2a isotype and has been extensively characterised by western blotting and ELISA.¹⁶ This antibody recognises PCNA in formalin fixed, wax embedded tissues and can be used as an operational marker of cell proliferation.¹⁷

Nucleolar organiser regions (NORs) are segments of DNA encoding for ribosomal RNA, which are of importance during the transcription of nucleic acid to protein.¹⁸ Proteins, associated with NORs can be shown by a simple silver staining technique and are then

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Figure 1: (Left) Microdissected gastric pit squashes prepared for counting of vincristine metaphases. Stained with Feulgen's reagent. (Right) Vincristine metaphase stained with Feulgen's reagent within a gastric pit squash.

Biology, Molndal, Sweden. Controls received vehicle.

Radioimmunoassay of serum gastrin

Serum samples were obtained by cardiac puncture under general anaesthesia, immediately centrifuged, and snap frozen in liquid nitrogen. Duplicate samples were assayed in batches. Radioimmunoassay was performed as previously described, using an antibody L2 against the biologically active forms of gastrin G-17 and G-14.²¹

Mucosal thickness

Specimens of stomach were processed routinely for histological examination. Sections were examined under a $\times 40$ objective and well orientated areas selected for measurement. Using a calibrated graticule, five measurements were made of the mucosal thickness and the mean calculated for each stomach.

Mucosal polyamines

The stomach was removed, opened, and washed in ice cold Hartmann's solution. Mucosal scrapings were taken using two microscope slides over glass on ice. Mucosal scrapings were placed in pre-labelled cryotubes and snap frozen in liquid nitrogen. The acid soluble polyamines were separated from cellular protein by perchloric acid extraction. Polyamines were quantified separately by high pressure liquid chromatography after the method of Wallace *et al*^{22,23} and protein was quantified by the Coomassie blue technique all as previously described.²⁴

Crypt cell production rate

Rats received an intraperitoneal injection at time zero of vincristine 1 mg/kg to bring about metaphase arrest. Individual animals were killed at 15 minute intervals from 30 minutes after injection. Samples of oxyntic mucosa taken 0.25 cm from the squamocolumnar junction were processed and microdissected. Crypt squashes were prepared and stained with Feulgen's reagent as previously described¹⁵ (Fig 1 left panel). Using a conventional microscope the pits were examined and arrested

visualised as brown/black dots called argyrophil NORs (AgNORs). Numbers of AgNORs in interphase nuclei are related to cellular protein transcription and proliferation levels and have been used to distinguish normal from reactive and neoplastic human tissues. Counts in thin sections are only representative of the true total nuclear AgNOR number, but a standardised method permits comparisons to be made between sectioned nuclei.^{18,19}

The aim of this study is to quantify and localise the degree of proliferation in the different functional compartments of the mucosa, under conditions of accelerated gastric epithelial proliferation. The numbers of cells in cycle and cell cycle kinetics are investigated.

Methods

Achlorhydria/hypergastrinaemia

Male Sprague-Dawley rats were acquired with a mean body weight of 100 grams. Two groups of 10 rats were used for each experiment; mucosal thickness, mucosal polyamines, and vincristine metaphase arrest were performed on the same animals. PCNA immunocytochemistry and AgNOR counts were performed on two separate groups of 10 animals. Omeprazole powder was suspended as a 1% solution in 0.25% methyl cellulose solution, buffered by the addition of sodium hydrogen carbonate 2 mg/ml, and adjusted to pH 8 with sodium hydroxide. A fresh suspension was prepared each day and given once daily by gavage for 30 days. A dose of 400 $\mu\text{mol/kg/day}$ was used, which has previously been shown to cause achlorhydria.²⁰ Omeprazole was the generous gift of Dr H Mattsson, AB Hassle, Gastrointestinal Research, Department of

Figure 2: (Left) PCNA immunostaining of oxyntic mucosa in a hypergastrinaemic animal. The chromagen is DAB and no counter stain has been used. This upper band is the mucus neck cell compartment. (Right) A PCNA positive nucleus in the gland compartment of the oxyntic mucosa of a hypergastrinaemic rat.

metaphases were counted (Fig 1 right panel). Forty pits per biopsy specimen were examined. The mean metaphase count in 40 crypts was plotted against time and a best fit line calculated by linear regression. The slope of the line is the crypt cell production rate. The gradients (metaphase accumulation/min) are compared for significance using a *t* test that takes into account scatter around the best fit line. The term crypt cell production rate is used here when referring to cell births in the gastric pits/glands.

PCNA immunocytochemistry (Fig 2)

Fresh rat stomachs were fixed in Carnoy's solution for four hours and stored in 70% alcohol. From wax embedded blocks three micron sections were cut, mounted on glass slides, and air dried at room temperature overnight. Sections were dewaxed, taken through alcohol, and then immersed for 10 minutes in 25% phosphate buffered saline in methanol with 0.5% hydrogen peroxide to block endogenous peroxidase activity. Sections were subsequently taken to water and immunostaining was performed using the ABC method (Dakopatts, High Wycombe, England), with primary incubation of PC10 antibody overnight at a dilution of 1:200 in triethanolamine buffered saline as previously described.²⁵ Diaminobenzidine was used as a chromagen. No counterstain was used to maximise the differential between nuclear staining and background. Tonsillar

germinal centres were used as positive controls and were uniformly positive. Omission of the primary antibody and the application of irrelevant antibodies of the same isotype acted as negative controls and excluded non-specific binding in all cases.

Assessment of proliferation by computer aided image analysis (Figs 3 and 4)

PCNA stained sections were examined using a Carl Zeiss Jenaval microscope with a Hitachi KP140 solid state camera attached. The video interface with a Joyce Loebble Mini Magiscan was amplified and converted into a digital signal. The system uses GENIAS (General Purpose Image Analysis Software) to analyse the image. This adjusts for staining intensity and after subtraction of background and artefact, separates overlapping features and counts stained nuclei as previously described.²⁶

Sequential PCNA and AgNOR staining (Fig 5)

Separate 3 micron sections were cut and incubated overnight with PC10 at a dilution of 1 in 200. Immunostaining was carried out with the APAAP method using the streptavidin ABC kit (Dakopatts, UK). No counterstain was used. The PC10 stained slides were then used for AgNOR staining by the Rowland's modification of the Ploton method.²⁷ Counting of the AgNORs was carried out by one investigator (SJD) by a standard cumulative mean technique as previously described.²⁸ Well orientated sections were examined using a green filter to increase definition. At least 100 PCNA positive and 100 PCNA negative cells were counted in both the neck and gland areas. The Student's *t* test on paired and unpaired data was applied as appropriate (all groups had a normal distribution) at 95% confidence intervals.

Results

Serum gastrin

In vehicle control rats the mean serum gastrin was 24.8 fmol/ml (median 25.1; range 11.9–37.4 fmol/ml). After 30 days of omeprazole administration 400 µmol/kg/day, this rose 11-fold to a mean serum gastrin of 270.8 fmol/ml (median 245; range 173–445 fmol/ml) ($p < 0.01$ Mann-Whitney U test).

Figure 3: Digitised image of the PCNA immunostained section of stomach produced by the GENIAS system.

Figure 4: Bar chart generated by GENIAS showing PCNA positive cell counts in hypergastrinaemia and control. The counts are plotted from 0–312 μm from the gastric lumen (neck cell compartment) and from 312–624 μm (gland cell compartment).

Mucosal thickness

Oxyntic mucosal thickness was increased 16% from 4.9 mm (4.42–5.11 mm) to 5.69 mm (5.4–7.05 mm) in the hypergastrinaemic rats ($p < 0.001$).

Mucosal polyamine content

In omeprazole treated rats mucosal putrescine content rose from a median of 0.2 (0.08–0.31) to 0.33 nmol/mg (0.16–0.76) ($p < 0.04$).

Spermidine content rose from a median of 7.2 (3.62–19.24) to 19.6 nmol/mg (12.5–33.67) ($p < 0.001$). Spermine rose from 7.02 (5.18–13.11) to 32.4 nmol/mg (12.96–52.56) ($p < 0.001$) and total polyamines rose 220% from 15.9 (9.45–26.38) to 50.9 nmol/mg (29.04–80.41) ($p < 0.005$).

Spermine to spermidine ratio changed from 1.1 to 1.5, but this did not reach statistical significance ($p = 0.07$).

Crypt cell production rate (Fig 6)

Gastric body crypt cell production rate measured by vincristine metaphase arrest was 0.541 crypt cells per hour in vehicle controls. This was increased by 238% to 1.83 crypt cells per hour in omeprazole treated rats ($p < 0.02$ Student's *t* test comparison of linear regression gradients).

Figure 5: PCNA/AgNOR sequentially stained mucus neck cells from a normogastrinaemic stomach. (Photographed with a red filter). Darker PCNA positive nuclei contain up to four AgNORs. PCNA immunocytochemistry using the APAAP method.

PCNA immunocytochemistry

Mucus neck cell PCNA labelling increased 41% from 460/mm² (209–616/mm²) in normogastrinaemic rats to 649.9/mm² (493–819/mm²) ($p < 0.001$) in the hypergastrinaemic animals.

Gland cell PCNA labelling increased 222% from 63.9/mm² (35–88/mm²) in normogastrinaemic rats to 220/mm² (144–377/mm²) ($p < 0.001$). Repeated examination of the same section showed a standard deviation of 2.7%.

Sequential PCNA and AgNOR staining

The method resulted in brown/black silver stained AgNOR dots against a red background in the nuclei of PCNA positive cells and against a golden background in the nuclei of PCNA negative cells. The dots were easily enumerated. PCNA negative cells had 1–2, or rarely 3, AgNORs per nucleus. PCNA positive cells had generally 1–3, and occasionally 4, AgNORs per nucleus.

In the hypergastrinaemic rats the AgNOR counts per PCNA positive neck cell nucleus (2.29 (0.11), mean (SD)) were similar to AgNOR counts in PCNA positive gland cell nuclei (2.26 (0.14)).

These values exceeded AgNOR counts in the normogastrinaemic rats for both PCNA positive neck (1.71 (0.07)) and PCNA positive gland cells (1.65 (0.1)) ($p < 0.001$). In normogastrinaemic rats, neck and gland PCNA positive cells had similar AgNOR counts.

The AgNOR counts per PCNA negative nucleus of hypergastrinaemic rats were similar in both the neck cells (1.31 (0.1)) and the gland cells (1.33 (0.09)). These values exceeded AgNOR counts in the normogastrinaemic rats PCNA negative nuclei of neck (1.23 (0.05)) and gland cells (1.25 (0.1)) ($p < 0.001$). In normogastrinaemic rats PCNA negative neck and gland cells had similar AgNOR counts.

Discussion

Our experiments have quantified accelerated gastric epithelial proliferation in the stomach of hypergastrinaemic rats using a number of different methods. Gastrin is known to stimulate cell proliferation in the stomach and removal of antral gastrin secretion leads to gastric mucosal atrophy. The novel aspects of our studies relate to the nature and relation between different cellular events, the site of these events, and the relevance to therapeutic acid suppression in humans.

Our findings suggest that the greatest increment in cell proliferation in response to increased gastrin drive occurs in the gland component of the gastric mucosa. Conventionally the mucus neck cell compartment has been viewed as the sole site of cell proliferation in the stomach. We have shown that the greatest density of PCNA positive cells is found in the neck cell compartment. After induction of hypergastrinaemia, however, the greater increase in PCNA positive cells occurred in the gland cell compartment; 238% in the gland cell

Figure 6: Gastric body crypt cell production rate.

compartment compared with 41% in the neck cell compartment. The neck cell compartment contributes an extra 170 cells/mm² compared with the 148 cells/mm² in the gland cell compartment but because the gland cell compartment has a greater area, it contributes the greatest total number of new PCNA positive cells. The presence of mitoses in the gland cell compartment confirms the presence of active cell division in this area. This suggests that recruitment of terminally differentiated cells into cycle may play a part in increased cell production under conditions of increased trophic drive to the mucosa. Although it is possible that PCNA positive cells migrated from the neck cell compartment into the gland cell compartment it seems unlikely, as the half life of PCNA is about 20 hours and this is much shorter than the migration time measured in previous experiments.⁵

Cell life may be reduced in accelerated gastric proliferation. The evidence for this comes indirectly from a comparison of the increase in proliferative activity when compared with mucosal hyperplasia. The pit length was increased by 16% whereas cell proliferation as assessed by polyamines content, crypt cell production rate, and PCNA positive cell numbers were increased by more than 200%. Under steady state conditions seen by 30 days, the mucosa is not undergoing further hyperplasia,²⁰ hence cells must be being born and shed at an increased rate. The implication of these findings is that individual cell lifespan is reduced.

Examining the PCNA/AgNOR data may give an insight into cell cycle duration. Whereas PCNA immunoreactivity is an all or nothing phenomenon (the image analysis system counts positive nuclei above background and takes no account of staining intensity after this) AgNOR counts give a graded score. Non-proliferating cells will have small numbers of AgNORs. AgNOR numbers increase with the level of proliferative activity. PCNA positive cells in the neck cell compartment have more AgNORs than their PCNA negative counterparts in each group as expected. PCNA positive cells have similar AgNOR scores in the neck cell compartment and gland cell compartment in each group suggesting that the level of proliferative activity, possibly reflecting cell cycle duration, is the

same in cycling cells in the gland cell compartment as in the neck cell compartment. Of particular interest is that the PCNA positive cell AgNOR count in the hypergastrinaemic group, exceed those of the PCNA positive cells of the normogastrinaemic group. This may suggest that cycling cells in the hypergastrinaemic animal are in a higher state of proliferative activity than cycling cells unstimulated by hypergastrinaemia. Similarly an excess of AgNORs in PCNA negative cells in the hypergastrinaemic rats over those of the normogastrinaemic rats suggests, that the increased proliferative activity is reflected in the non-cycling cell population. Increased AgNOR counts in PCNA negative cells may also imply a longer half life for NORs than PCNA protein.²⁹ A similar unexpectedly high count in PCNA negative cells has been seen in a study of high grade non-Hodgkin's lymphoma.³⁰ Thus in the rapidly proliferating mucosa reduced cell cycle duration is linked to reduced cell life.

The rise in serum gastrin seen in this model is in excess of that seen in humans in the clinical situation.^{3,4} The dose of omeprazole used in the rats rendered them achlorhydric.²⁰ Although the powder preparation of omeprazole used in the rat differs from the form used in humans, the effective dose used is likely to be at least 10 times that used in humans. Hypergastrinaemia induced by proton pump inhibitor treatment, is reversible in response to withdrawal of the drug.² In humans, gastric biopsy data in longterm studies with omeprazole have not shown treatment related changes in the gastric mucosa.³¹ No dysplasia has been seen after 84 months of follow up in patients receiving omeprazole and although increased enterochromaffin like cell numbers are seen, this is correlated with the degree of corpus gastritis and is more disease than drug related.^{31,32} Carcinoids have not been seen in patients receiving longterm acid suppression for reflux oesophagitis, although they are occasionally seen in Zollinger-Ellison syndrome with both H₂ and proton pump inhibitor treatment.³³

PCNA immunostaining data give results of the same order of magnitude as polyamine and crypt cell production rate data. The simplicity and reproducibility of the computer aided counting technique of PCNA immunostaining makes this a useful new research tool. Our data validate PCNA immunostaining by comparison with the crypt cell production rate, an established standard for proliferation measurement. PCNA immunostaining has the added advantage of giving spatial information. Anomalous PCNA expression discordant with cell cycle events has been reported in association with neoplasia.³⁴

In conclusion, hypergastrinaemia induces a state of accelerated gastric epithelial proliferation. There is a proportionally greater increase in proliferation in the gland compartment compared with that in the mucous neck cell compartment, although the neck cell compartment remains the main source of new cell births. AgNOR counts complement PCNA immunostaining. Together they suggest that

the level of proliferative activity in cycling cells of hypergastrinaemic rats is greater than that of cycling cells in normogastrinaemic rats, with increased cell turnover and shorter cell cycle duration.

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