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Cell kinetics of human gastric mucosa and gastric cancer in organ culture

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Summary. The cell kinetics of human gastric epithelium in organ culture have been measured using flash labelling with tritiated thymidine and the metaphase arrest technique to estimate cell birth rates. Normal gastric antral and body mucosa have been compared with mucosa showing gastritis and gastric carcinoma. Labelling indices with tritiated thymidine in normal gastric mucosa declined over a 48-h period suggesting that essential growth factors were lacking. Labelling indices and cell birth rates were higher in gastritis than in normal mucosa and highest in gastric carcinoma. Labelling indices were higher in intestinal-type gastric carcinoma than diffuse carcinoma. In metaphase arrest experiments carcinomas showed on average an eightfold increase in resistance to the metaphase-arresting properties of vincristine when compared with normal mucosa. The validity of using the metaphase arrest technique to measure cell birth rate in gastric cancers in view of this vincristine resistance is discussed.

Key words: Gastric mucosa – Gastrointestinal neoplasms – Cell cycle – Organ culture

Introduction

Organ culture is a useful method for studying functional properties of normal and pathologically altered human tissue under controlled conditions which preserve tissue architecture. In the gastro-intestinal tract it has been successfully applied to small and large intestinal mucosa and colorectal cancer (Browning and Trier 1969; Autrup et al. 1978; Pritchett et al. 1982), but by comparison gastric mucosa has proved relatively difficult to culture (Donaldson and Kapadia 1980). We have previously described an organ culture method applicable to human gastric mucosa which in animal studies preserved cell kinetic alterations observed in vivo following carcinogen treatment (Wilson and Macartney 1986, 1988). Here we report measurements of human gastric epithelial cell kinetics in organ culture. The main aim was to compare proliferative activity in normal gastric epithelium, gastritis and in gastric cancers using tritiated thymidine labelling indices and cell birth rates.

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Materials and methods

The proliferative activity of human gastric antral and body mucosal epithelium and gastric carcinoma was measured in an organ culture system (Wilson and Macartney 1986). Two methods, the flash labelling index with tritiated thymidine (LI) and the cell birth rate by metaphase arrest, were used. In addition dose-response curves for vincristine were measured in order to establish the optimum concentration of the drug for induction of metaphase arrest.

Tissue

Three endoscopic biopsies were taken from the gastric antrum and/or body from 39 patients; one biopsy was used for histological examination and the remainder transferred to ice-cold Hanks balanced salt solution for subsequent use in organ culture. None of these patients had a diagnosis of gastric carcinoma and all were undergoing investigation for non-specific upper gastrointestinal symptoms or unexplained iron-deficiency anaemia. Ethical permission for this part of the study was obtained from the Ethical Committee, St. Thomas's Hospital.

Human gastrectomy specimens for either peptic ulceration ($n = 27$) or gastric cancer ($n = 20$) were collected fresh from the operating theatre within 5 min of removal. Each specimen was opened longitudinally along the greater curvature and rinsed with cold 0.9% saline. Antral and/or body mucosa was freed from underlying submucosal connective tissue and removed in strips. Tumours were bisected and a solid piece ($1.5 \times 0.5 \times 0.5$ cm) resected out avoiding superficial or necrotic areas. Tissue for organ culture was then transported in Hanks balanced salt solution as for endoscopic biopsies. Tissue blocks for routine histology were also taken from adjacent sites. Carcinomas were classified into intestinal and diffuse subtypes (Lauren 1965).

Organ culture

Specimens obtained at endoscopy or from gastrectomies were transported in ice-cold Hanks balanced salt solution containing penicillin (500 IU/ml) and streptomycin (500 µg/ml). Endoscopic

biopsies were not further dissected. Tissue from gastrectomy specimens were cut into 1- to 2-mm squares and placed in organ culture.

The explants were grown as previously described (Wilson and Macartney 1986). Between 2 and 4 explants were grown in each culture dish on sterile stainless steel grids. The total number of explants set up from each case varied depending on whether endoscopic biopsies were being used (2 explants from each patient) or surgical resection specimens. Using surgical resection material between 40 and 50 explants were used per gastrectomy specimen for experiments on cell birth rates, vincristine dose response curves and LI studies. Culture conditions prior to the cell kinetic measurements were as previously described (Wilson and Macartney 1986) and consisted of supplemented Waymouth's MB 752/1 medium as described by Defries and Franks (1977) modified from Hodges and Melcher (1976). Culture dishes were sealed in an air-tight perspex box in an atmosphere of 95% O_2/5% CO_2 and incubated at 35° C.

Cell kinetic studies

Labelling index. After periods varying from 4 to 48 h in culture, the medium was changed to one containing 6-[^{3}H]-labelled thymidine (79 Ci/mmol, 20 μCi/ml medium, Amersham, UK). After a further 60 min incubation, tissue was rinsed, fixed in Carnoy's fluid histologically processed and autoradiographs prepared as previously described (Wilson and Macartney 1988). Briefly, histological sections are dipped in K-2 nuclear emulsion (Ilford, UK) exposed for 5 weeks, developed, fixed and counterstained with haematoxylin. The LI is estimated by counting the proportion of labelled and unlabelled gastric epithelial cell nuclei. By implication the LI represents the proportion of cells which are synthesizing DNA at a given time point. Nuclei were considered to be labelled if 5 or more silver grains lay over the nucleus. Counts were made at a magnification of × 500 and 3000 epithelial nuclei were counted in optimally orientated gastric pits away from the edges of the explant and avoiding necrotic areas. All nuclei from the openings of the pits to the base were counted. LI in endoscopic biopsies ($n=39$) and gastrectomies ($n=5$) were compared. Variation in LI over a 48-h period of culture were measured in normal gastric antral and body mucosa from 5 gastrectomies. In addition the LI in mucosa showing gastritis and metaplasia ($n=17$) and gastric cancer ($n=11$) specimens were compared.

Vincristine dose-response experiments. The optimum concentration of vincristine producing metaphase arrest in gastric epithelium in vitro was determined in a series of dose-response experiments. This was a pre-requisite for the metaphase arrest experiments where the cell birth rates were measured (see below). If too low a dose of vincristine is used incomplete arrest occurs, whereas with too high a dose, mitotic figures degenerate. Both these situations lead to under-estimates of the cell birth rate (Wright and Appleton 1980). Accordingly vincristine at doses of 0–10 μg/ml culture medium was added to organ cultures of normal gastric antral mucosa specimens ($n=11$) or gastric carcinoma specimens ($n=5$) after 24 h prior culture. Forty explants were used from each specimen with 4 explants per culture dish at each concentration of vincristine. After a further 150 min the explants were fixed in Carnoy's fluid and processed histologically. Histological sections stained with haematoxylin and eosin were used for counts of arrested metaphases in 3000 epithelial nuclei at a magnifications of × 500. Areas for counting were selected as for LI counts.

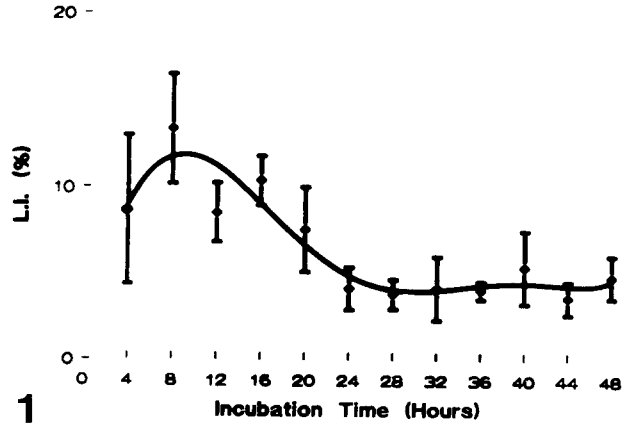
Cell birth rate studies. The cell birth rate in gastric mucosal epithelium and gastric cancers was measured by the technique of metaphase arrest and expressed as the number of new gastric cells produced per 1000 gastric epithelial cells per hour (Wright and Appleton 1980). The proportion of cells arrested in metaphase is calculated in histological sections of tissue taken at different time intervals after in vitro administration of the optimum dose of vincristine to explants. The rate of increase in the metaphase index with time equals the rate at which cells enter mitosis. The rate is calculated by measuring the slope of a line plotting the metaphase index against time after vincristine administration. In these experiments gastrectomy specimens of normal gastric antral mucosa ($n=6$), atrophic gastritis ($n=3$) and intestinal-type gastric cancer ($n=4$) were used. For each specimen 7 dishes each containing 4 explants were cultured for 24 h. Vincristine was then added at a concentration of 1 μg/ml medium for non-malignant specimens or 8 μg/ml for gastric cancers. These concentrations were chosen on the basis of the results of dose-response experiments. One dish containing 4 explants was fixed at 30-min intervals over a period of 3 h. Explants were then histologically processed. Counting procedures to determine metaphase indices were as described above for dose-response curves.

Results

Morphological preservation of both normal mucosa, atrophic gastritis with metaplasia and gastric cancers was similar to that previously reported by us (Wilson and Macartney 1986, 1988). After 48 h in culture 58% of normal antral explants and 52% of normal body mucosal explants were well preserved apart from simplification of the glandular architecture and oedema of the lamina propria. After the same period in culture 57% of all explants taken at endoscopy and 58% of all explants from gastrectomies were well preserved.

Figures 1 and 2 show the changes in the flash labelling indices in normal gastric antral and body mucosa measured at 4-h intervals over a period of 48 h culture. There is considerable variation and fluctuation which is most marked during the first 12 h. Thereafter the LI declines progressively to reach a basal level of about 5% by 24 h with probable further decline in body mucosa. Within explants zones of high and low LI could be identified. Uptake of tritiated thymidine was most marked at the edges of the explants where tissue architecture tends to breakdown in organ cultures (Fig. 3). The method of obtaining the explant, whether endoscopic biopsy or gastrectomy, did not affect the LI (Table 1, $P=$NS). Although the LI was initially higher in antral mucosa than in body mucosa, after 24 h culture this difference was no longer apparent (Figs. 1, 2; Table 1). Higher LI were seen in explants showing gastritis (Table 1) particularly where there was intestinal metaplasia (Fig. 4; Table 1). High LI were also seen in the intestinal form of gastric adenocarcinoma, but by comparison the LI in the diffuse form of gastric carcinoma was quite low (Table 1). These differences were statistically significant ($P=0.05$) using the Kruskal-Wallis one-way analysis of variance.

The effects of different concentrations of vincristine on the metaphase arrest index for normal gastric antral mucosa and the gastric cancers is shown in Figs. 5 and 6. Each point on the curve represents the mean of experiments on 6 different specimens. There is a broad peak of arrested mitoses in normal mucosa at vincristine concentrations of 1–4 μg/ml culture medium and a concentration of 1 μg/ml was used in subsequent cell birth rate measurements in normal mucosa. In contrast the gastric cancers are resistant to metaphase arrest. A dose of at least 8 μg/ml is required to produce mitotic arrest and this was the concentration used in subsequent cell birth



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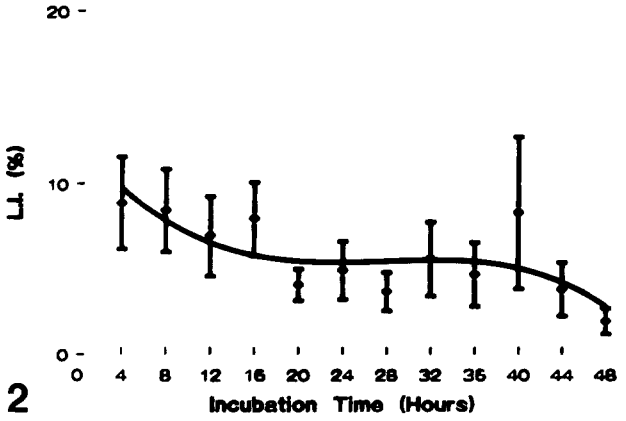

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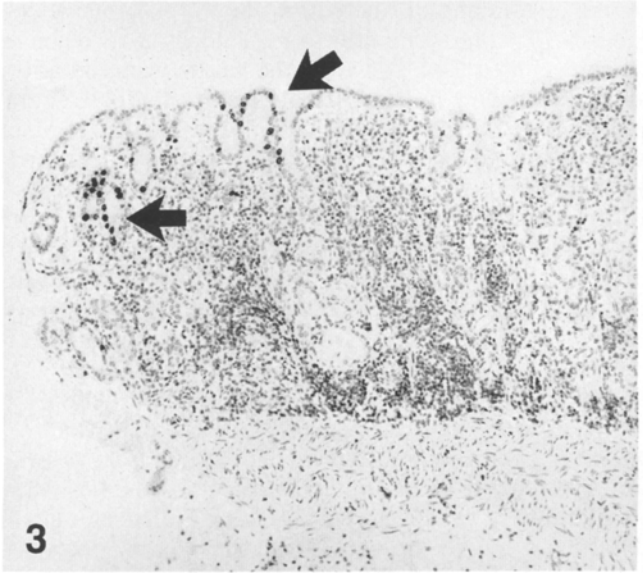

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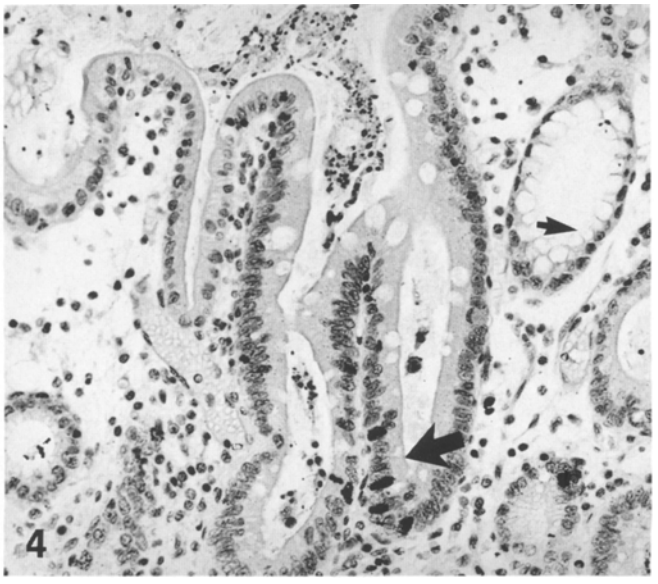

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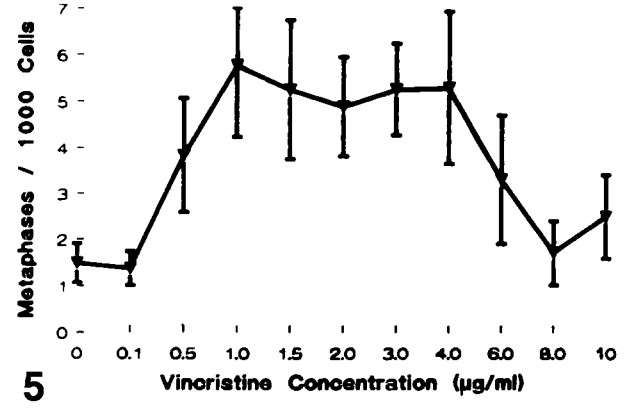

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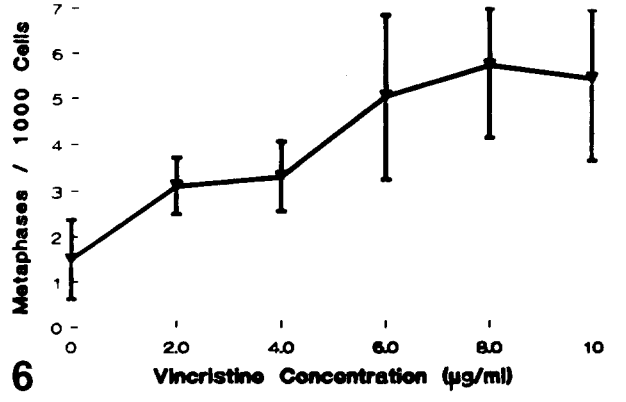

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Fig. 1. Mean flash labelling index (% ± SEM) in normal gastric antral mucosal epithelium at 4-h intervals up to 48 h

Fig. 2. Mean flash labelling index (% ± SEM) in normal gastric body mucosa

Fig. 3. The edge of an explant of gastric antral mucosa with gastritis. Epithelial cells near the edge are labelled with tritiated thymidine (*arrows*). × 80

Fig. 4. An area of intestinal metaplasia shows labelling (*large arrow*) which contrasts with the lack of labelling in an adjacent non-metaplastic gastric pit (*small arrow*). × 200

Fig. 5. Dose-response curve of normal gastric antral epithelium to the mitotic-arresting property of vincristine. Each point represents the mean and 95% confidence limits of six separate experiments

Fig. 6. Dose-response curve of gastric carcinoma to vincristine. Mean and 95% confidence limits are shown. The dose required to obtain mitotic arrest is much higher than in normal gastric antral mucosa

Table 1. Proliferative measurements in gastric mucosal epithelium

	Labelling index %	Confidence limits (95%)	CBR[a]	Confidence limits (95%)
Endoscopic biopsies	5.2	3.4– 7.0	ND	–
Gastrectomy specimens	4.2	1.4– 9.2	ND	–
Normal antral mucosa	5.2	2.3– 8.5	3.3	2.4–4.2
Normal body mucosa	5.0	1.8– 8.2	ND	–
Atrophic gastritis	5.5	1.2–12.8	4.6	2.9–6.3
Intestinal metaplasia	11.2	4.6–17.8	ND	–
"Intestinal" cancer	9.8	1.6–23.6	4.3	0.8–7.8
"Diffuse" cancer	3.3	1.3– 5.0	ND	–

[a] Cell birth rate expressed as epithelial cells produced/1000 epithelial cells per hour

rate experiments on cancers. However even this concentration may be too low since in two of the dose-response experiments evidence of metaphase arrest only occurred at the highest concentration of vincristine used (10 μg/ml).

The mean cell birth rate (expressed in epithelial cells produced/1000 epithelial cells/h) for normal antral mucosa ($n=6$) was 3.3 (SD 0.9). In atrophic gastritis ($n=3$) this rose to 4.6 (SD 1.7). In gastric cancers ($n=4$), which were all of intestinal subtype, the most striking finding was the wide variation (mean 4.3, SD 3.5). Although the trend was towards higher birth rates in gastritis and gastric cancer the differences were not statistically significant.

Discussion

Our results show increasing kinetic activity and heterogeneity when normal gastric mucosa, gastritis and gastric carcinoma are compared. This is in keeping with most results obtained using either conventional short-term flash-labelling with tritiated thymidine, DNA flow cytometry or in vivo stathmokinesis (Deschner et al. 1972; Wright et al. 1977; Hart-Hansen et al. 1979; Saski et al. 1984; Macartney and Camplejohn 1986). This sequence of increasing proliferative activity is held to support the concept of gastric cancer developing on a background of hyperproliferation and hyperplasia (Lipkin et al. 1985; Lehnert and Deschner 1986), which is known to occur in experimental gastric carcinogenesis. However, experimental carcinogenesis differs from human gastric cancer insofar as there is rarely evidence of significant intestinal metaplasia, nor is it clear whether the hyperplastic changes seen in experimental cancer are merely due to carcinogen-induced local toxicity and mucosal ulceraton (Matsukura et al. 1979). Secondly there are several lines of evidence to suggest that "diffuse" gastric cancer has rather low intrinsic proliferative activity when compared with the more common intestinal-type gastric carcinoma (Sasaki et al. 1984; Macartney et al. 1986). Whilst we do not dispute that the LI in intestinalised gastric mucosa is higher than normal gastric mucosa, the concept of hyperplasia appears an oversimplification applied to gastric mucosa showing variable degrees and types of intestinal metaplasia, glandular atrophy and inflammation.

Our results also highlight certain problems associated with the use of organ culture and raise questions about the validity of using stathmokinesis either in vivo or in vitro for measuring kinetic activity in gastrointestinal carcinomas.

In vivo measurements of LI show that tritiated thymidine uptake is higher in body mucosa than in the gastric antrum (Hart-Hansen et al. 1976). Certainly in the sequential study we obtained higher LI for body mucosa after 4 h culture in vitro, but this difference had disappeared after a 24-h culture period in vitro. More significant is our finding of fluctuations and a progressive decline in LI values as culture time increased to reach a basal level after 24 h. Thus all the values were obtained for flash labelling indices after 24 h culture (Table 1) are much lower than those obtained in other in vitro studies where measurements are made after only 1–2 h culture (reviewed in Lehnert and Deschner 1986). In contrast to organ culture studies of colonic mucosa (Senior et al. 1982), we have never observed a late resurgence of LI after several days in culture. This decline to basal levels in gastric mucosa after 24 h culture in vitro suggests that growth factors are missing from the culture medium and the system would be suitable for assaying growth factors as suggested by Finney et al. (1989) for colon.

The second question concerns the reliability of the stathmokinetic method applied to the study of gastric and possibly other gastrointestinal carcinomas. Both our own results for gastric cancer and those of Pritchett et al. (1982) for colorectal carcinoma show that there is marked resistance on the part of malignant cells to the mitosis-arresting properties of vincristine. Pritchett et al. (1982) report a sixfold increase in the concentration of vincristine required for complete metaphase arrest and we observed an eightfold increase in the dose required. However, although we never observed evidence of escape from metaphase in the subsequent birth rate experiments or tumours, nevertheless in at least one of the dose-response curves no definite evidence was obtained that the optimum metaphase arresting concentration of vincristine was reached. This raises the possibility that no concentration of vincristine can be chosen which will reliably induce metaphase arrest in all cancers unless dose-response curves are first plotted for each tumour followed by a stathmokinetic experiment on the same tumour. This is clearly impossible to achieve. There is also the further possibility that vincristine resistance varies in samples taken from different areas within the same tumour. We know of no data to refute this possibility.

In view of these difficulties the values for cell birth rates for in vivo studies (Wright et al. 1977; Tabuchi et al. 1980) must be viewed with caution, since dose-response curves to vincristine cannot be obtained for ethical reasons. Wright et al. (1977) obtained much higher estimates for cell birth rates than those reported here. They also observed higher values in diseased gastric mucosa and kinetic heterogeneity in gastric cancer. The

lower values in our study are explained by the suppression of kinetic activity observed in vitro compared with in vivo (Wilson and Macartney 1988). In contrast Tabuchi et al. (1980) in an in vivo study obtained cell production of the same order of magnitude as in our experiments. However, we consider that their experimental design is extremely suspect since the stathmokinetic study was conducted over a period of 24 h, which is well outside the normal duration for cell birth rate experiments (Wright and Appleton 1980). In a stathmokinetic experiment of that duration significant numbers of mitoses would be expected to degenerate, leading to underestimates of the cell birth rate. In addition nearly all the samples in their study were taken at a single time point (16 h which may have biased the results.

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