

Effects of gastrointestinal hormones on the growth of human intestinal epithelial cells *in vitro*

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SUMMARY The growth of cultured epithelium like cells from human normal embryonic intestine was studied in response to various hormones using a method that quantifies the number of cells by the amount of dye that they bind after fixation. Gastrin and neurotensin in the pg/ml range and higher caused small increases in cell growth. Glucagon and VIP were stimulatory in the low ng/ml range, whereas somatostatin and bombesin had no effect at the lower concentrations but were stimulatory at the highest concentration tested (10 and 100 ng/ml respectively). Secretin and pancreozymin (cholecystokinin) seemed to be ineffective.

Gastrointestinal hormones regulate the metabolism and growth of most gastrointestinal tissues.¹ In patients with the Zollinger-Ellison syndrome mucosal hyperplasia and hypergastrinaemia occur,^{2,3} whereas in antrectomised patients the oxyntic gland mucosa usually atrophies.⁴ These clinical observations provided initial evidence of a trophic role for gastrin. With gastrointestinal mucosae *in vitro* and *in vivo* gastrin and pentagastrin stimulate cell growth and the synthesis of protein, RNA, and DNA;^{5,6} pentagastrin increased the growth of human normal gastric mucosal cells and colonic malignant cells *in vitro*,⁷ and gastrin increased the proliferation and the rate of [³H]-thymidine incorporation into the DNA of the IEC-6 crypt cell line from rat small intestine.⁸

There is comparatively little work on the roles of various other gut hormones as growth regulators of gastrointestinal mucosae *in vivo* or *in vitro*.⁹⁻¹² A trophic effect of CCK on the digestive mucosa is disputed.¹²⁻¹⁵ Secretin *in vivo* is reported to have an antitrophic effect on rat jejunal and ileal mucosae,^{16,17} and it reduced the DNA and protein content in organ cultured rabbit ileum.¹² Secretin, however, had little or no effect on rabbit jejunum,¹² and it caused duodenal hyperplasia in the rat.¹⁸ Glucagon enhanced the growth of cultured human colorectal

cancer cells.¹⁹ Somatostatin 2-20 ng/ml increased the growth of human gastric cancer cells, but inhibited the growth of normal epithelial cells from human gastric mucosa at 20-100 ng/ml¹⁰ and had little or no effect on rat intestinal IEC-6 cells.⁸ Vasoactive intestinal peptide (VIP) and neurotensin do not appear to have been previously studied on any aspect of proliferation, and we have not found any reports on the effects of secretin, pancreozymin, or bombesin on the growth of isolated gut epithelial cells.

We now report studies of eight naturally occurring peptides on the growth in culture of epithelium like cells from human normal embryonic intestine. The peptides are gastrin (G-17), glucagon, pancreozymin, and secretin (which occur mainly in the gut), and somatostatin, neurotensin, bombesin, and VIP (which occur also in the central nervous system).

Methods

CELLS AND THEIR CULTURE

Epithelial-like cells from human normal embryonic intestine (Flow Laboratories, no 407) were grown as monolayers in Eagle's basal medium (BME) supplemented with 15% newborn bovine serum (NBS) and 50 IU/ml each of penicillin and streptomycin, in a humidified atmosphere of air/CO₂ (95:5) at 37°C. The medium was prepared from powder (Flow Laboratories), using water that was double distilled in glass; sterilisation was by filtration.

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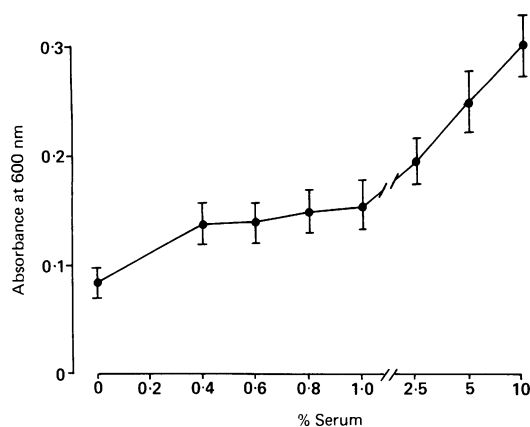


Fig. 1 Serum concentration effect curve for the growth of epithelium-like cells from human normal embryonic intestine, as measured spectrophotometrically by the amount of dye eluted. Results are expressed as the mean (SE) of the separate means from four experiments each with 16–24 replicates.

These cells grow rapidly in medium containing high concentrations of serum. In order to ensure that we could detect either stimulation or inhibition of growth by the peptides, we determined a lower serum concentration which gave moderate cell growth. From the curve (Fig. 1), constructed using 0–10% NBS by the method described below, 1% NBS seemed optimal.

Cell growth was assessed by a dye method.²⁰ The cells were detached by treatment with trypsin/EDTA (0.5%:0.02% w/v) to produce a single cell suspension, counted (Coulter counter, model DN), and diluted in BME+15% NBS so that 100 μ l contained 6000 cells. Aliquots of 100 μ l were added to each well of a 96-well microtest plate, except for one row of four which were used as medium blanks. The final volume in each well was made up to 200 μ l, and the cells were left to adhere to the microtest plates (five hours in air/CO₂ 95:5, 37°C). After removing the lid, each plate was inverted and the medium expelled by three firm downward shakes. The cells were then washed with 100 μ l serum free medium. Each peptide or its vehicle was added in 200 μ l BME containing 1% NBS. Two peptides and the vehicle(s) were studied simultaneously at all concentrations on the same plate. After three days' incubation, the medium was removed and the cells were fixed for 15 minutes by adding 100 μ l 10% formol saline to each well. After 15 minutes the fixative was replaced with stain (12.2 mM crystal violet in 154 mM NaCl, filtered) which was then removed 15 minutes later. The wells were rinsed twice with distilled water, dried in air for five to six hours, and the stain in the cells was

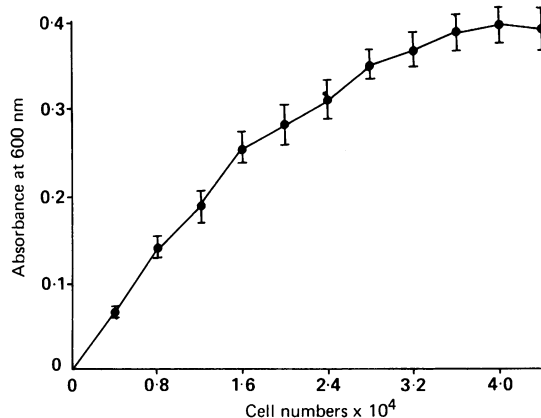


Fig. 2 Cell numbers showed a curvilinear relationship to the amount of dye eluted, as measured by spectrophotometry. Results are expressed as the mean (SE) of the separate means from five experiments each with eight replicates. All of our cell growth studies with hormones gave readings on the linear part of the curve.

eluted with 100 μ l acidified methanol (five drops 1 M HCl to 100 ml methanol). Light absorption through each well was read at 600 nm using a Dynatech microplate reader. Although the cells remained in each well, the absorption was almost entirely the result of the dye; the cells accounted for 0.5–1% of the readings, as shown by measurements before and after eluting the dye from them. Calibration curves were constructed to determine the relationship between cell numbers and dye concentration, using 4000 to 44 000 cells/well in increments of 4000 (eight wells/increment). When the cells had attached to the plate (at five hours) the medium was removed and the cells were fixed and stained as described above. Five separate experiments were done with each peptide (plus additional ones for gastrin, see later) giving a total of 40 replicates at each point. The results are expressed as the mean of the separate means (SE) from each experiment. Cell numbers showed a curvilinear relationship to the amount of dye eluted, as measured by spectrophotometry (Fig. 2).

Glucagon was dissolved in water adjusted to pH 8.8 using 0.1 M NaOH, and diluted in the same solvent. The other peptides were dissolved and diluted in sterile 154 mM NaCl. All the water was double distilled in glass, and sterilisation was by filtration. Log concentration effect curves were constructed for the eight gastrointestinal peptides, gastrin G-17, glucagon, pancreozymin (CCK), secretin, somatostatin, neurotensin, bombesin, and VIP (all from Sigma). They were used in five concentrations in the following 10-fold ranges: gastrin 2 to 20 000 pg/ml; pancreozymin 0.02–200

μ Crick units; glucagon, somatostatin, neurotensin, and VIP 1 to 10000 pg/ml; secretin 0.01 to 100 μ Crick units/ml; bombesin 10 to 100000 pg/ml.

In other experiments with gastrin, the cell diameters were measured using an eyepiece graticule calibrated with a microscope stage micrometer.

STATISTICAL ANALYSIS

In each experiment the mean reading in eight wells (12 for controls except in the cell size experiments) was calculated. The mean (SE) of the means was analysed by Student's *t* test for paired data (two-tailed) to determine the difference from vehicle controls.

Results

EFFECT OF GASTRIN ON CELL SIZE

The results for growth, expressed in absorbance units, are the mean of the four means from each experiment, with 40 replicates/experiment for controls, and eight replicates/experiment for gastrin. Cell diameter (μ m) is the mean of the means from 100 replicates/experiment for controls and 20 replicates/experiment for gastrin. The amount of dye bound to the fixed cells presumably depends on number and size. With gastrin, measurements of the amount of dye eluted from the cells probably represent cell numbers, because gastrin 20 and 200 pg/ml increased the amount of dye by 6 (2)% and 10 (3)% respectively ($p < 0.05$) but had little or no effect on cell size; the differences in mean cell diameters were -2.2% and 1.2% respectively ($p > 0.4$).

EFFECT ON CELL GROWTH, AS IMPLIED BY DYE BINDING

The effects of gastrin (G-17), neurotensin, glucagon, VIP, somatostatin, bombesin, secretin, and pancreozymin (CCK) on the growth of human intestinal epithelial cells (expressed as light absorbance at 600 nm \times 100) are shown in the Table. Each point is the mean (SE) of the separate means from six experiments each with eight replicates (eight wells) for peptides and 12 replicates for controls. The results with gastrin are separate from those above concerned with cell size.

Gastrin and neurotensin, in the pg/ml range and higher, caused small increases in cell growth, as indicated by the greater amount of dye (Table). Glucagon and VIP were stimulatory in the low ng/ml range, whereas somatostatin and bombesin had no effect at the lower concentrations but were stimulatory at the highest concentration tested (10 and 100 ng/ml respectively). Secretin and pancreozymin seemed to be ineffective at all concentrations tested (Table).

Table 1 Effect of various peptides on the growth of epithelium-like cells from human normal embryonic intestine

Peptide	1	10	100	1000	10000	100000	pg/ml
Gastrin*	12 (7)	19 (9)	18 (10)	21 (9)	18 (10)		
P	<0.02	<0.01	<0.02	<0.01	<0.05		
Neurotensin	6 (7)	8 (7)	10 (7)	10 (8)	15 (7)		
P	0.1	0.025	<0.05	<0.05	<0.02		
Glucagon	3 (9)	3 (9)	4 (7)	8 (6)	20 (10)		
P	<0.4	<0.4	<0.2	<0.05	<0.01		
VIP	1 (8)	4 (8)	4 (7)	15 (10)	23 (9)		
P	<0.9	<0.3	<0.4	<0.02	<0.005		
Somatostatin	-3 (7)	-1 (9)	9 (10)	10 (9)	26 (10)		
P	<0.7	<0.9	<0.3	<0.3	<0.05		
Bombesin		4 (4)	3 (5)	3 (6)	8 (8)	11 (8)	
P		<0.4	<0.3	<0.3	<0.1	<0.05	
	0.01	0.1	1	10	100		μ CU/ml
Secretin	6 (10)	-7 (6)	-6 (8)	0 (7)	15 (8)		
P	<0.9	<0.1	<0.2	<1.0	<0.2		
Pancreozymin*	11 (13)	2 (6)	2 (6)	6 (6)	7 (4)		
P	<0.2	<0.5	<0.3	<0.2	<0.2		

*Double the concentration shown - that is, 2-20000 pg/ml for gastrin and 0.02-200 μ CU/ml for pancreozymin. The results are % change in the amount of dye eluted, compared with vehicle controls. Increased cell growth occurred with gastrin (2-20000 pg/ml), neurotensin (10-10000 ng/ml), glucagon (1 ng/ml), VIP (10 ng/ml), somatostatin (10 ng/ml), and bombesin 100 ng/ml. Secretin and pancreozymin appeared to have no effect. P represents the probability of the difference compared with vehicle controls.

Discussion

As stated in the introduction, many studies *in vivo* and *in vitro* indicate that gastrin has a trophic effect on gastrointestinal tissues. Apart from clinical observations,²⁻⁴ most work has been in laboratory animals. Our studies show that gastrin has a trophic effect on cells from human embryonic intestinal epithelium. Although, we cannot exclude the possibility that gastrin or the other hormones altered the cell surface proteins or binding of dye by the cells, the effect of fixing by formaldehyde before adding the dye presumably far outweighs any small peptide mediated changes that might occur.

Glucagon also stimulated epithelial cell replication in our experiments, consistent with the finding that the hormone enhanced the growth of human colon cancer cells in culture.¹⁹ High doses of glucagon in suckling rats, however, did not induce the early adaptation of the small bowel mucosa which occurs at weaning,²¹ although in a patient with an endocrine tumour that secreted enteroglucagon (which is closely related to glucagon) the intestinal mucosa was hypertrophied;²² extracts of the tumour injected into mice also appeared to cause hypertrophy of the intestinal mucosa. Other extracts rich in enteroglucagon may directly stimulate crypt cell production.^{23,24}

Somatostatin stimulated intestinal cell growth at the highest concentration (10 ng/ml), but lower concentrations were ineffective. The growth of normal human gastric mucosal cells was enhanced by 1 ng/ml somatostatin, whereas inhibition occurred with 20–100 ng/ml.¹⁰ With rabbit cultured intestinal mucosa somatostatin reduced the DNA and protein content of the jejunum but not the ileum.¹¹ There was little or no effect of somatostatin 10–500 ng/ml on the numbers of rat IEC-6 cells or their incorporation of [³H]-thymidine into DNA.⁸

We found that bombesin stimulated the growth of human intestinal cells at the highest concentration tested. This peptide also stimulated the growth of the gastrointestinal tract and pancreas in suckling rats,²⁵ causing both hypertrophy and hyperplasia depending on the dose and the duration of the treatment.

Secretin was ineffective in our studies. In rats, secretin alone or with pentagastrin had little or no effect on the parietal cell mass,²⁶ but it inhibited gastrin stimulated DNA synthesis and accumulation in the gastric oxyntic region and duodenum.¹⁷

We obtained no effect of pancreozymin (CCK) on cell growth, consistent with the finding that CCK 0.1 nM–1 μM did not influence the DNA or protein content of rabbit small intestine in organ culture.¹³ In rats, however, acute and chronic administration of the CCK-related peptide caerulein induced duodenal hyperplasia, but caused only transient hypertrophy of the oxyntic gland area,¹⁸ and in another study chronic administration of caerulein to rats stimulated epithelial cell proliferation in the gastric antral mucosa.¹⁴

Our results with neurotensin and VIP appear to be the first concerning effects on cell growth. These findings, and those with the other peptides, represent the first direct evidence that some of them have a trophic action on epithelial cells from human embryonic intestine *in vitro*. The findings may be physiologically relevant, as the peptide concentrations were appropriately low,²⁷ and the results generally agree with *in vivo* studies.

The findings, however, must be treated with caution. As with all cell culture studies, it is not known to what extent cell changes during culture affect the responses, or how much resemblance there is to effects *in vivo*. The artificial conditions during culture unavoidably lead to great differences from those *in vivo*. We used newborn bovine serum which itself affects cell growth, and *in vivo* there may be simultaneous exposure to many hormones present in the bloodstream or reaching the cells by other means.

The 10000-fold range of concentrations that we used almost inevitably means that the higher amounts are physiologically excessive compared with normal blood concentrations. High concentrations

may occur in disease, however, and surges in the plasma concentrations of several gastrointestinal hormones, including enteroglucagon, gastrin, neurotensin, and pancreozymin occur after in full term²⁸ or preterm²⁹ enterally fed infants, but not in those deprived of enteral feeding.³⁰ These hormones may therefore have a role in the structural and functional changes that accompany postnatal adaptation to human extra-uterine nutrition.^{31,32} Our results showing that some of these hormones have a trophic effect on human embryonic intestinal cells *in vitro* support this hypothesis.

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