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ABSTRACT

Background Barrett's oesophagus is a common premalignant lesion caused partly by acid reflux. Although the requisite therapy, proton pump inhibitors (PPIs), have been implicated in the progression of Barrett's oesophagus in animal models, harmful effects of prolonged PPI therapy in Barrett's oesophagus is both inconclusive and controversial. We therefore aimed to test the role of PPI-induced hypergastrinaemia in vitro and see whether any biological parameters were useful surrogates of long-term therapy in man.

Methods We undertook detailed serological and tissue assessment of gastrin and CCK_2 receptors in 90 patients randomised to different doses of PPI therapy during a detailed 2-year follow-up. We also undertook a comprehensive study of cell models to study the consequential biological effects of gastrin on the mucosa.

Results Gastrin and its cognate receptor CCK_2R were expressed highest in the stomach, then less in Barrett's oesophagus and least in squamous oesophagus (SqE) ($n=20$ paired t-test, $p<0.01$). Analysis of the change in Barrett's oesophagus segment length change in 70 patients who were randomised to high or low PPI dose showed no difference over 2 years ($n=70$ t-test, $p=0.8$). Prolonged PPI use did, however, increase the serum gastrin, (36 pg/ml±57 pg/ml to 103 pg/ml±94 pg/ml (paired t test, $p<0.05$)). In vitro gastrin also induced changes in $OE33(E)_{cckr}$ Barrett's oesophagus cells, but not $OE21(E)_{cckr}$ squamous cells, transfected with CCK_2R; migration was induced by 1 ng/ml of gastrin but proliferation only increased with 100 ng/ml (paired t-test, $p<0.01$) and both were abolished by antagonists.

Conclusion While the short-term effects of gastrin enhance epithelial restitution in Barrett's oesophagus (but not squamous mucosa) there is no clinical evidence that Barrett's oesophagus length expands over time. This study, which is the largest and longest term randomised controlled trial of gastrin biology in Barrett's oesophagus, is further proof of the clinical safety of PPI therapy.

The number of incidences of oesophageal adenocarcinoma (OA) is increasing and has a poor prognosis.[1] The related premalignant lesion is Barrett's oesophagus.[2] The pathophysiology evolves from chronic gastro-oesophageal reflux disease (GORD).[2] Management of GORD involves the use of proton pump inhibitors (PPIs), yet these therapies do not fully reverse Barrett's oesophagus.[3] There is some literature indicating that PPI therapy may prevent acid damage in short- and medium-term studies, decrease proliferation in Barrett's oesophagus, and promote healing of the ulceration of the adjacent oesophagitis, thereby abrogating the 'growth and expansion of Barrett's oesophagus'.[4–6] Moreover, there is a suggestion that PPI therapy may actually increase the generation of Barrett's oesophagus in a proportion of patients.[7–9] This latter theory is further supported by animal studies which, despite their flaws, have been consistent in pointing to an increased propensity of oesophageal adenocarcinoma (EAC) with PPI use.[10 11] In this regard, gastrin, in its various forms (preprogastrin, progastrin and the main mature forms gastrin 34, gastrin 17 and gastrin 14) all have biological activity in the gastrointestinal (GI) tract. It is, however, primarily amidated gastrin-17 which has been shown to regulate normal gastrointestinal cells.[3] There is evidence that PPIs, perhaps by gastrin and its CCK_2 receptor (CCK_2R) interaction, are also involved in the development of pre-malignant gastrointestinal lesions and cancers.[5 8 12 13] There are also data showing that gastrin can influence the biology of gastric cell lines,[7–13] elevate the colon tumour burden in the *min* mouse[10] and induce proliferation in Barrett's oesophagus, and all through activation of the CCK_2R.[11 12] The data are contradictory, though short-term experiments in man suggest a beneficial effect of PPIs. Long-term follow-up in detailed epidemiological studies of continuous PPI therapy have also failed to show an increase in either adenocarcinoma rates of the colon or oesophagus.[14 15]

A real conundrum arises; how can these disparate in vitro and in vivo effects of PPI hypergastrinaemia be reconciled? While PPI alterations in growth are minimal, migration may be important in causing rapid selection for clones during acute repair.[16 17] Migration would allow clones with upregulated CCK_2R to rapidly cover ulcerated mucosa,[17] prior to even enhanced crypt bifurcation.[18 19] There is also compelling work that gastrin may induce migration by inducing integrins and mucosal regeneration in vitro, and even control stem cell fate in models in vivo, but these have never been tested in humans.[18–20]

We wished to test the theory that gastrin stimulation by long-term PPI therapy induces aberrant epithelial mucosal biology, leading to the spread of clones expressing the cognate CCK_2R. Our specific aims therefore were: (1) to examine the differential expression of gastrin and its cognate receptors in

benign Barrett's oesophagus, compared with adjacent tissues; (2) to assess long-term changes in Barrett's oesophagus length as well as any changes in gastrin and CCK_2R expression following PPI use in patients randomised to low- and high-dose PPI therapy; and (3) to assess the functional effects of exogenous gastrin and/or CCK_2R on a number of important cellular outcomes in vitro.

MATERIALS AND METHODS

Patients and tissue collection

Samples were collected from endoscopies by one trained upper GI endoscopist (JJ). Two groups of patients with Barrett's oesophagus and normal renal function were studied: 20 patients with Barrett's oesophagus on 'routine surveillance' and another 70 who were recruited into the AspECT trial. The former group was studied as approved according to the Leicestershire ethics committee guidelines No. UHL 6885. They were all also *Helicobacter pylori* negative, with an age range 34–77 years, and 13 patients (10 men, three women) were on PPI therapy (omeprazole 20 mg/day or equivalent) and another seven patients (five men, two women) were PPI naïve at the start of the study but they were commenced on esomeprazole 20 mg/day for 2 months. The 70 'AspECT trial' patients were studied as approved according to North East London ethics committee guidelines No. 04/Q0603. Their ages ranged from 34 to 79 years and there were 55 men and 15 women. The two arms of this trial randomised patients to two PPI-only regimens: high-dose esomeprazole 80 mg/day and low-dose esomeprazole 20 mg/day. Samples were collected using strict endoscopic and histological criteria by two experienced GI pathologists (RH and KW), as described previously.[21–23] The endoscopic length was measured using the previously validated C and M criteria.[24]

Cell culture

The OE33(E) cell line was derived from an oesophageal adenocarcinoma.[9 19 25] The $OE33(E)_{GR}$ cell line was a kind gift from Dr Varro (Liverpool University), and was established following stable transfection with human CCK_2R. The OE21(E) cell line was derived from an oesophageal squamous carcinoma. The $OE21(E)_{GR,}$ and $OE21(E)_{IV}$ were transfected with human CCK_2 receptor (pClneo $hCCK_2SR$), with the empty vector (pClneo plasmid), or the intron-IV human splice variant of CCK_2R (pClneo hCCK2i4R) respectively, using lipofectamine (Invitrogen, UK).[19] TE4 and TE7 cells were used as controls for OE21(E); the columnar cell lines SEG1 (lung cancer), BxPC3 (pancreatic adenocarcinoma), Panc1 (pancreatic adenocarcinoma), AGS (gastric cancer) were used as OE33(E) controls as they are known to respond to gastrin when transfected with CCK_2R and/or the variant intron 4 CCK_2R.[19]

Extraction of RNA for reverse-transcriptase and real-time PCR

RNA was extracted using TRIzol reagent (Total RNA Isolation Reagent; Gibco BRL, Invitrogen, Carlsbad, California, USA). Primers were CCK_2 receptor sense: 5′ TTCTTCATCCCAGGT GTGGTTATG 3′, and antisense; 5′ TTTGGCTGTCGCTGTC ACTGTC 3′ and for gastrin 17: sense; 5′ GCCCAGCCTCT CATCATCG 3′ and antisense, 5′ GCCGAAGTCCATCCATCCA 3′ (103 bp and 128 bp amplicons respectively). PCR was done in triplicate and normalised for epithelial/stromal content by expression of *Hprt1*. PCR products were sequenced. $CCK_{2iv4}R$ receptor was only found in the control cell lines.

Measurement of the cell cycle

Cells were exposed to gastrin at concentrations between 0.01 and 100 ng/ml (1–10 000 μmol/l) and time points between 24 and 96 h as described before.[21] Each experiment was performed on four occasions. DNA was stained with propidium iodide and was evaluated using a Beckman Coulter FACScan (Beckman Coulter, High Wycombe, Buckinghamshire, UK), using an XL System II software programme.

Measurement of cellular migration/motility

Cells were seeded at a density of between 5×10^5 and 1×10^6 in six-well plates and left to reach 95% confluency. The 'scratch wound' was made in triplicate as described[20 22] with either [Leu^{15}]-gastrin with or without the CCK_2R antagonists JB93182 or compound A (both competitive inhibitors of CCK_2R).[19 20 22] The distance that cells migrated was calculated from 10 random but marked positions on a grid. Hepatocyte growth factor was used as a positive control, as this is currently the most motogenic substance known in Barrett's cells.[22]

The filter migration assays were performed in duplicate using Nunc 10 mm cell culture inserts with 0.45 μm polycarbonate membranes (Becton Dickinson, Franklin Lakes, New Jersey, USA) as described previously[22] and were counted in ten randomly defined fields.

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Antibodies used were: (anti-human vimentin, 1 μg/ml (IgG1; Dako); anti-Ki67 (IgG1; Dako, Glostrup, Denmark), 0.1 μg/ml; anti-CCK_2 receptor 0.2 μg/ml (IgG1; Sigma, Poole, UK); and anti-gastrin-17 antibody, 0.1 μg/ml (IgG1; Sigma), ki67 0.01 μg/ml (IgG1; Sigma) and annexin 5 0.3 μg/ml (IgG1; Santa Cruz, California, USA). The sections were scored blind by two independent observers with regard to intensity: 0, −/+, + and ++ as described.[21]

MEASUREMENT OF HUMAN GASTRIN-17 PEPTIDE IN VIVO AND EX VIVO

Human gastrin-17 was measured using a competitive ELISA kit (R&D Systems, Minneapolis, USA) with a sensitivity of 1 pg/ml.

Statistical analysis

All statistical analyses were performed using Minitab, GraphPad Prism 5 or R statistical software. Distribution plots were made to establish whether the data were parametric or non-parametric. Significance was assessed by performing a parametric Student t test or a non-parametric Mann–Whitney U test or the Wilcoxon test. Paired tests were used where appropriate. Differences in proportions were assessed using Fisher's exact test.

RESULTS

In vivo expression of CCK_2R and gastrin mRNA in gastric epithelium, Barrett's oesophagus and normal (squamous) oesophageal epithelium

In order to assess objectively the differential expression of CCK_2R and gastrin-17 in vivo in the SqE and Barrett's oesophagus tissues as well as the stomach, we assessed matched biopsies from 13 patients (all *H pylori*-negative and taking PPI therapy omeprazole 20 mg/day or equivalent). Comparing the mean mRNA expression between the normal gastric tissue (fundus) and the SqE, there was 2.2-fold more CCK_2R in the former tissue (37.2±31 vs 16.4±29) ($p=0.05$, one tailed, Student t test) (figure 1).

Gastrin 17 was also expressed most in the gastric antrum compared with Barrett's oesophagus and SqE (52.0 ng±15.1, 0.22 ng±0.15 and 0.14 ng±0.07 respectively) (paired t-test, $p<0.01$).

In order to determine whether there was any differential expression of either CCK_2R or gastrin between phenotypically normal stomach or adjacent fundic cystic polyps (associated with chronic PPI use) we examined their in vivo mRNA levels. There was no significant difference in the mRNA expression of

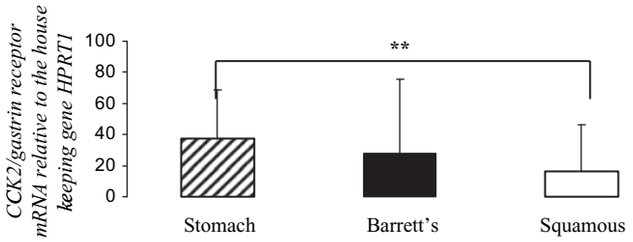

Figure 1 Ex vivo mRNA levels of CCK_2 receptor in stomach, benign Barrett's and normal squamous oesophagus (expressed in arbitary units). The levels of CCK_2 receptor mRNA were analysed using quantitative real-time PCR. The data are the means (with standard deviations) of three replicates, performed on 13 matched oesophageal biopsy tissues, which were normalised to the *Hrpt* housekeeping gene. **Signifies a significant difference ($p<0.05$) from the gastric samples.

CCK_2R or gastrin between the gastric and fundic cystic epithelium (table 1) (paired t-test $p>0.1$).

In vivo expression of CCK_2 receptor protein expression in gastric epithelium, Barrett's oesophagus and normal (squamous) oesophageal epithelium

In order to clarify whether the relative in vivo protein levels of CCK_2 receptor was also differentially expressed we used immunohistochemistry in matched gastric (antrum or fundus), Barrett's oesophagus and SqE as before.[20] The images contained in figure 2 were representative of six individual patients (one patient did not have a full set of paired biopsies) and confirmed that most CCK_2 receptor was expressed in the stomach (figure 2c,d), less in the Barrett's oesophagus being very patchy (arrowed) (figure 2b) and least in the normal oesophagus (figure 2a). The cellular localisation of the staining was typically localised in the cytoplasm with minimal involvement of the cell membrane. The parietal cells/epithelial cells lining the glandular pits of the gastric mucosa and both the columnar and goblet cells in Barrett's oesophagus expressed most CCK_2 receptor. The negative controls, in which either the primary antibody was omitted, or isotype matched control, were uniformly negative in the gastric tissue (figure 2e,f).

In order to assess the long term outcomes of hypergastrinaemia in two different doses of PPI we utilised data on file in the endoscopy records of patients in the AspECT trial. The trial randomises patients to two PPI arms: low dose esomeprazole PPI therapy (20 mg/day (figure 3)), and high dose PPI therapy (80 mg/day (figure 3)). Thirty-six patients were on high dose PPI therapy alone and 34 were on low dose PPI therapy alone. The change in length of Barrett's oesophagus, from baseline compared to 2 years of PPI therapy, was not significantly different in the low dose PPI group compared with the high dose group (figure 3) (t-test $p=0.38$), and, in fact, the change in Barrett's oesophagus length after 2 years of PPI treatment did not differ significantly from the baseline in either group. In addition there was also no difference in the changes of proliferation by flow cytometry or apoptosis as assessed by annexin 5 between low dose and high dose therapy arms after 2 years (data not shown).

Table 1 A summary of the expression profile of gastrin and CCK_2 receptor in vivo

		Normal gastric tissue	Normal oesophagus	Barrett's epithelium	Fundic cystic polyps
mRNA	Gastrin	(antrum) +++ (normalised ratio=2.3)	+ (normalised ratio=1)	+ (normalised ratio=1.7)	+++
	CCK_2 receptor	+++ (body)	+	++	+++
Protein	Gastrin	(antrum) +++	+	++	++
	CCK_2 receptor	+++ (body)	+	+	++

ELISA LEVELS OF GASTRIN PEPTIDE IN EX VIVO BIOPSY TISSUE

In order to assess the endogenous concentration of human gastrin-17 protein in SqE and gastric tissues we examined 10 patients undergoing endoscopy, using an ELISA. Gastric (antrum) tissue predictably expressed significantly more gastrin-17 ELISA (52 ng/ml±15.1) ($p<0.05$, paired t-test) compared with both Barrett's oesophagus (0.22 ng/ml±0.15) and SqE (0.144 ng/ml±0.07) (figure 4) ($p<0.01$, paired t-test). Although the difference between Barrett's oesophagus and SqE was not significant in eight of the 10 samples Barrett's oesophagus expressed over twice as much gastrin 17 than matched SqE ($p=0.06$ paired t-test).

In vivo serum levels of gastrin peptide

In order to assess the contribution of prolonged PPI therapy to circulating serum gastrin-17 tissue levels we measured samples in seven patients before and after the commencement of PPI therapy (2 months average). There was an increase in serum gastrin levels from 36 (±53) pg/ml at baseline, compared with 103 (±94) pg/ml ($p<0.05$, paired t-test) following PPI therapy (table 2).

In vitro expression of gastrin-17 and CCK_2/gastrin receptor mRNA

In order to validate the relative in vitro mRNA levels of gastrin-17 and its receptors in cell models we used quantitative real-time PCR in a panel of oesophageal and gastric cancer cell lines (table 3). Real-time PCR showed that the cell lines transfected with CCK_2 (wt) receptor mRNA, including $OE33(E)_{GR}$ (×1000 expression compared with parental), AGS_{GR} (×10 000 expression compared with parental), and $OE21(E)_{GR}$ (×100 expression compared with parental), expressed significantly more CCK_2 receptor than untransfected parental cells ($p<0.05$ paired t-test) (table 3).

Gastrin induces an increase in cellular proliferation in vitro

To determine the proliferative effect of $[Leu^{15}]$-gastrin treatment on cell cycle, analysis was carried out using flow cytometry. Subconfluent cells were treated with physiological concentrations of $[Leu^{15}]$-gastrin (0.01 ng/ml up to 100 ng/ml) in serum-free medium for 24 and 48 h treatment (only 24-h data shown).

Stimulation of the parental squamous OE21(E) cell line, $OE21(E)_{GR}$, or the cell line transfected with the empty vector $OE21(E)_{EV}$ with $[Leu^{15}]$-gastrin had no obvious effect on any of the phases of the cell cycle (data not shown). However, the growth inhibitor aphidocolin increased the G_0 fraction and decreased the S phase while 10% FCS decreased G_0 arrest in OE33 cells (figure 5a). Similarly stimulation of the parental OE33(E) cell line with $[Leu^{15}]$-gastrin had no significant effect at 24 or 48 h (figure 5b). However, treatment with 10 ng/ml and 100 ng/ml $[Leu^{15}]$-gastrin for 24 h (figure 5c) in the $OE33(E)_{GR}$ cell line, caused a small but significant decrease in the serum deprivation-induced G_0 phase fraction (by 7.5% and 7.8%, respectively) when compared with the untreated control samples as well as an increase in S phase cells by 10% ($p<0.05$, paired t-test). The restoration of the cell cycle, ie, more cells in S phase induced by 10 ng/ml but not 100 ng/ml $[Leu^{15}]$-gastrin was reversed using the CCK_2 receptor antagonist JB93182 (50 and 100 nmol/l) (figure 5c) (paired t-test). These gastrin-induced effects were confirmed using an alternate form of gastrin, by incubating the $OE33(E)_{GR}$ cell line with pentagastrin (0.1 to 100 ng/ml) for 24 h (figure 5d). Treatment with 10 and 100 ng/ml of pentagastrin for 24 and 48 h induced a significant decrease in the G0/G1 phase fraction caused through serum deprivation ($p<0.05$) and even

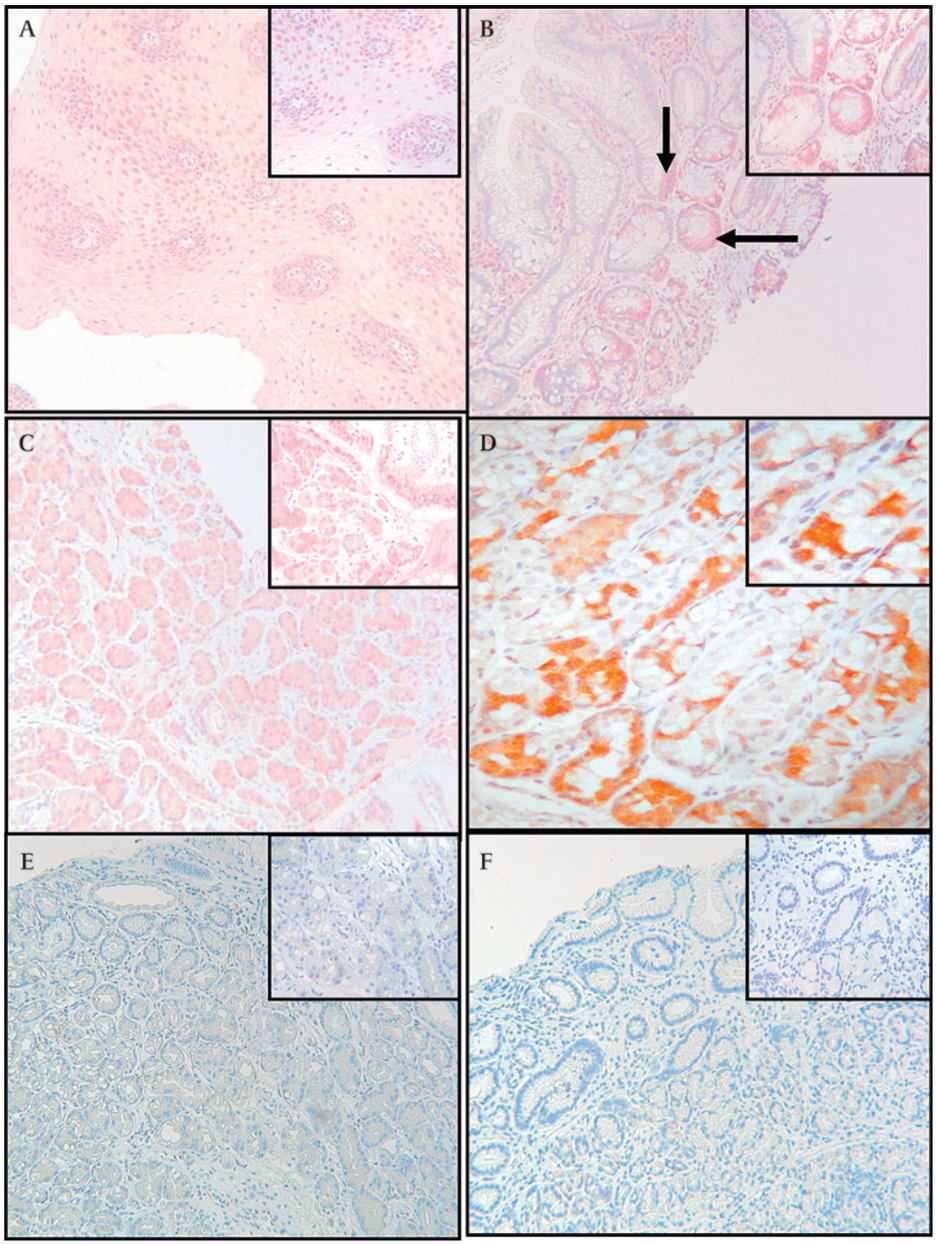

Figure 2 The in vivo tissue localisation of CCK_2 receptor as determined by immunocytochemistry, ×40. The relative protein levels and distribution of CCK_2 receptor in benign Barrett's, normal oesophagus and gastric epithelium was analysed using immunohistochemistry. The immunohistochemistry shown is representative of at least five matched biopsy specimens, and showed more intense staining in the stomach (D then C), followed by the Barrett's oesophagus (B) and then normal oesophagus (A). The fundus (D) was strongly positive, whereas the isotype matched and no antibody controls (E, F) were negative (F).

1 ng/ml caused an increase in the S phase cells at 24 and 48 h ($p<0.05$). Co-incubation with the CCK_2 receptor antagonist JB93182 partly reversed the hypergastrinaemia effect evoked through pentagastrin (figure 5d).

Gastrin-induces an increase in cellular migration in vitro

In order to assess the implications of gastrin stimulation on restitution in mucosal ulceration we conducted a series of in vitro experiments. First, a wound assay was performed whereby the migration of cells was measured after 24 h. There was no observed significant change in the motility of the squamous cell lines OE21(E) and $OE21(E)_{GR}$ (figure 6a) following exposure to [Leu^{15}]-gastrin (100 ng/ml). There was, however, a significant induction in cellular motility in the $OE33(E)_{GR}$ (from 16.5 A.U.±2 to 92±6, $p<0.005$, paired t-test) cell line compared with the parental OE33(E) (figure 6a). This migration induced by [Leu^{15}]-gastrin in $OE33(E)_{GR}$, was maximal at 100 ng/ml in 0.1% FCS and was higher than HGF (figure 6b). The gastrin inhibitor successfully abrogated the gastrin response (figure 6c). However, when the more sensitive filter assay was used, migration in $OE33(E)_{GR}$ was induced, from 10±1 to 58%±3, by 1 ng of gastrin 17 ($p<0.01$, paired t-test) (figure 7) or factor ($p<0.05$, paired t-test) (figure 6b) using the same assays.

In order to see if the migration was a direct consequence of increased proliferation we inhibited DNA synthesis by aphidicolin (1.5 μmol/l). The motogenic effects of [Leu^{15}]-gastrin on $OE33(E)_{GR}$ were independent of proliferation as aphidicolin-treated and untreated cells had similar wound healing (figure 6b). In addition we verified that $OE33(E)_{GR}$ cells had an absence of Ki67 staining in the aphidicolin-treated cells (data not shown). The [Leu^{15}]-gastrin-induced increase in migration was partially and completely reversed by pre-treatment with the CCK_2 receptor antagonist compound A 1 nmol/l and 10 nmol/l, respectively (figure 6c).

DISCUSSION

Accurate and detailed tissue analysis

This study is the largest and longest detailed morphological analysis of gastrin and CCK_2 expression and biology in benign

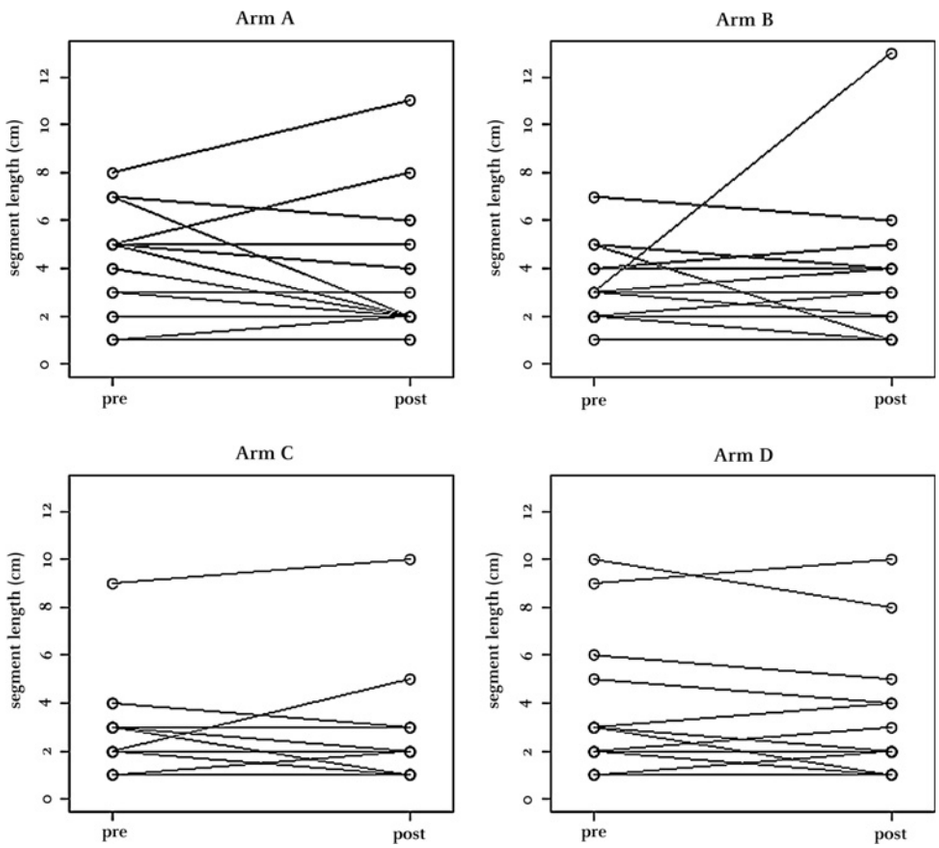

Figure 3 The change in Barrett's length in 70 patients in the AspECT trial. The change from the baseline measurements are shown for low-dose PPI (A: 20 mg esomeprazole) and for high dose PPI (B: 80 mg esomeprazole). There was no significant difference in the change in length between the low-dose and high-dose regimes over 2 years (t-test p=0.8).

Barrett's oesophagus. In addition, we have exploited the recently published updated histological and endoscopic classification of Barrett's oesophagus, and so previous reports may be inadequate, especially confusing hiatus hernias with Barrett's oesophagus.[24] Moreover, we have obviated the confounding issue of *H pylori* infection on gastrin levels. Furthermore, we have specifically concentrated on benign Barrett's oesophagus rather than cancer and used matched samples from both SqE and stomach as well as Barrett's oesophagus in all patient samples as it is here that hypergastrinaemia may have the commonest occurrence.[25 26]

Gastrin expression is increased by PPIs

There was 2-fold more gastrin protein in Barrett's oesophagus compared with SqE in protein expression by Elisa and immunocytochemistry. This is important as the concentration of gastrin in Barrett's oesophagus is at the lower end of a physiological effective dose ~0.1–1 ng/ml. Consistent with previous studies, we showed a 2- to 4-fold increase in plasma gastrin with short-term PPI treatment, and this leads to hypergastrinaemia (up to 400 pg/ml) over a prolonged treatment.[27 28] It has been previously reported that fasting serum gastrin can reach levels of 200 pg/ml[27] and in excess of 4 ng/ml in conditions such as

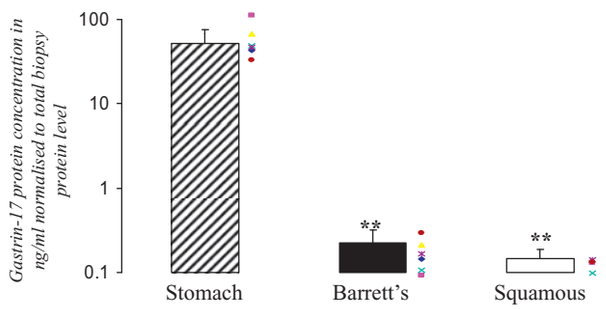

Figure 4 The relative ex vivo protein levels of gastrin-17 in benign Barrett's, normal oesophagus and gastric epithelium. The relative protein levels of gastrin-17 in benign Barrett's, normal oesophagus and gastric epithelium were analysed using an ELISA. The data are the means (with standard deviation) of three replicates taken on 10 matched biopsy tissues, with ** indicating significance ($p<0.05$) when comparing with stomach.

Table 2 A summary of the fasting serum gastrin-17 levels in a cohort of seven patients before and 2 months after commencement of proton pump inhibitor (PPI) therapy

Patient I.D.	Length of Barrett's oesophagus	Concentration (pg/ml) of gastrin-17 before PPI therapy	Concentration (pg/ml) of gastrin-17 after PPI therapy	Increase in gastrin (%)
1	8 cm	27	91	~240
2	4 cm	13	143	~1000
3	4 cm	12	47	~290
4	2 cm	11	32	~190
5	8 cm	23	69	~200
6	3 cm	6	41	~590
7	10 cm	165	299	~80
Mean value		36 (±57)	103 (±94)	

Table 3 A summary of the expression profile of gastrin and CCK2 receptor in vitro

		OE21 (E)	OE21 $(E)_{GR}$	OE21 $(E)_{EV}$	OE21 $(E)_{IV}$	OE33 (E)	OE33 $(E)_{GR}$	OE33 $(E)_{IV}$
mRNA	Gastrin	+++	+++	+++	+++	+	+	–
	CCK_2 receptor	+	+++	+	+	–	+++	–

– No expression (equivalent to negative control).
+ Weak expression (equivalent to positive oesophageal tissue control).
+++ Strong expression (equivalent to positive gastric tissue control).

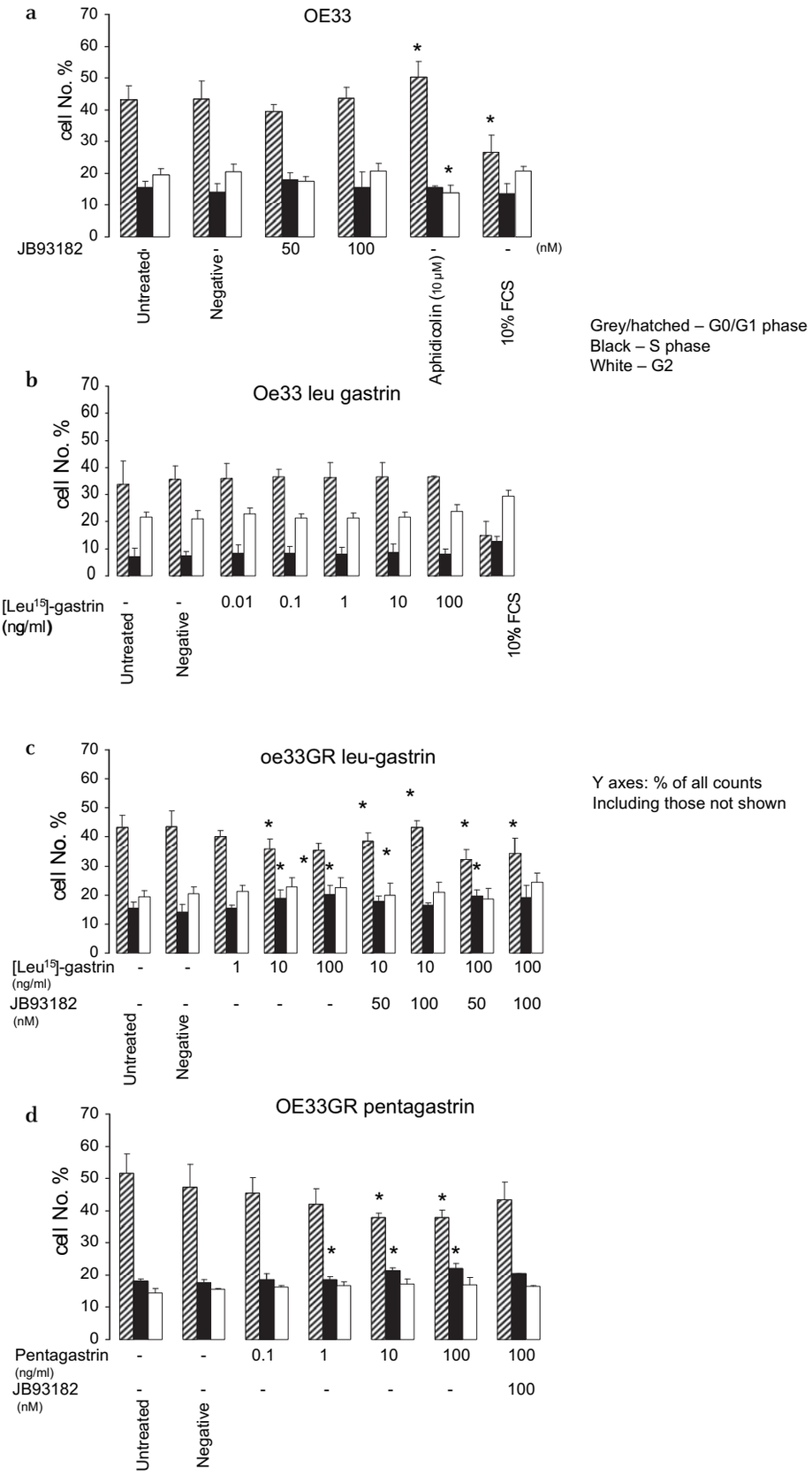

Figure 5 Influence of gastrin on cell proliferation in a panel of oesophageal cancer cell lines using flow cytometry. The OE33(E) cells transfected with the empty vector were not stimulated by gastrin (a). The CCKR inhibitor alone had no effect. Amphidocolin resulted in increased G0/G1 fraction and decreased G2/M. Ten per cent FCS resulted in decreased G0/G1 fraction (a). Cell proliferation was examined in OE33(E) (b), and $OE33(E)_{GR}$ (c,d) using flow cytometric propidium iodide staining. Both the full growth medium (RPMI supplemented with 10% FCS) and aphidicolin (10 μmol/l) treatment were used as additional positive and negative controls respectively. Cells were cultured in serum-free RPMI containing [Leu^{15}]-gastrin (0.01—100 ng/ml) in the presence and absence of the CCK_2 receptor antagonist JB93182 (50 and 100 nmol/l), for 24 h (a, c and d). The experiments have been repeated using pentagastrin stimulation (0.1—100 ng/ml) over 24 h (d). Coulter FACScan, with the percentage of cells in each phase of the cell cycle calculated using an XL System II software programme. The Y axes indicate the number of cells in each phase of the cell cycle. Data are the means (with standard deviations) of at least three different experiments with hatched, black and clear bars representing G_0/G_1, S and G2/M phase cells respectively. *Indicates significant difference ($p<0.05$) compared with the untreated control (media without gastrin) in the same group.

Zollinger—Ellison syndrome resulting in gastric hypertrophy.[29] Perhaps it has not been fully appreciated until now that the combined tissue and circulating gastrin concentration is now approaching the physiological range capable of inducing epithelial migration in concentrations over 1 ng/ml. It is, however, important to emphasise that even at these levels the biological

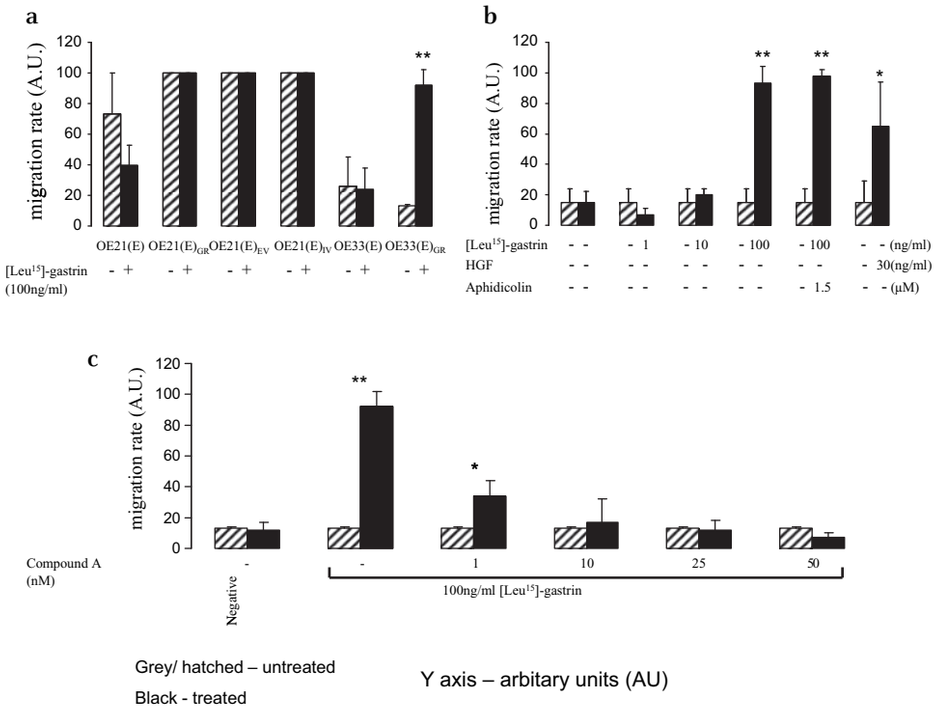

Figure 6 Influence of gastrin on the migration of a panel of oesophageal cancer cell lines. Cell migration was examined using an in vitro wound or 'scratch' assay, and was a measure of the cells' ability to move into the cleared cell-free area. The relative distances moved were measured in arbitrary units (AU) from the cell front of the 'wounded area' from baseline (hatched bars) over a period of 24 h [Leu^{15}]-gastrin exposure (black bars). (a) The effect of [Leu^{15}]-gastrin (100 ng/ml) on OE21(E), OE21(E)$_{GR}$, OE21(E)$_{EV}$, OE21(E)$_{IV}$, OE33(E), and OE33(E)$_{GR}$ cell lines; (b) the effect of [Leu^{15}]-gastrin (1—100 ng/ml) with or without aphidicolin treatment (1.5 μmol/l), or hepatocyte growth factor (30 ng/ml); (c) the effect of [Leu^{15}]-gastrin treatment (100 ng/ml) with or without the pre-treatment with the CCK2 receptor antagonist compound A (1—50 nmol/l) for 24 h. Data are the pooled results of at least five independent experiments, with * and ** indicating significant difference ($p<0.05$ and $p<0.01$ respectively) from the control (untreated) group as determined by the Mann—Whitney test of significance.

effects in vivo over 2 years had no statistically significant effect on migration, proliferation or apoptosis. Furthermore CCK2R levels are known to increase in acutely damaged tissues, but there was no change up or down from baseline in the 70 patients on long term PPIs implying that there was a differential effect of low- and high-dose PPIs on the chronic damage seen in the gastrointestinal tract.[17 30 31]

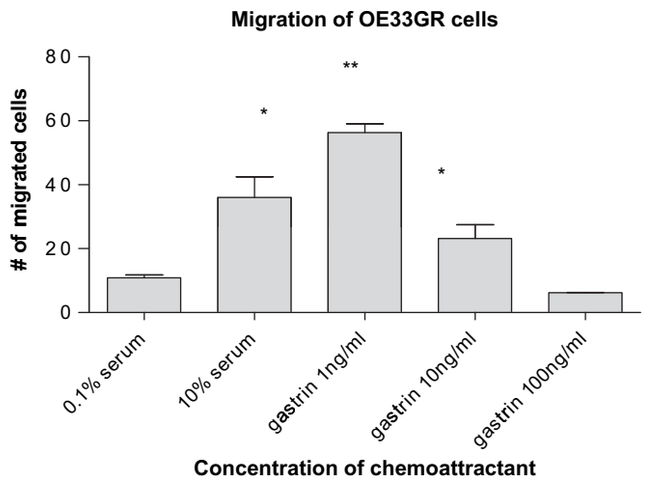

Figure 7 The effects of gastrin (1—100 ng/ml in 0.1% FCS) on the migration of OE 33GR oesophageal adenocarcinoma cell line following 22 h exposure using polycarbonate membrane filters with a 0.45 μm pore size. Ten per cent FCS and 0.1% FCS were used as positive and negative controls, respectively. A significantly ($p<0.05$) higher number of cells migrated in response to 1 ng/ml gastrin when compared to 0.1% FCS, 10 ng/ml gastrin and 100 ng/ml gastrin. Data are the result of at least three independent experiments, with * and ** indicating significant differences ($p<0.05$ and $p<0.01$ respectively) from the control (untreated) group as determined by the Mann—Whitney test of significance.

Gastrin-17 simulation is a potent motogen but a weak mitogen in vitro

In this study we have shown that gastrin signalling has some biologically significant sequelae, particularly cell migration, at physiological concentrations found in Barrett's oesophagus ~1 ng/ml. Migration of epithelial cells in tissue reflects an important property of the Barrett's oesophagus phenotype.[21] Importantly, our in vitro experiments showed that gastrin treatment induced a significant increase in cellular migration/motility in the OE33(E)$_{GR}$ cell line, but not in the OE21(E)$_{GR}$ or the parental control cell lines. This raises the question of whether squamous cells lack the functional parts of the signalling machinery, such as gastrin phosphorylation, to respond to gastrin stimulation even when CCK$_2$R is transfected.[31] The data here are similar to studies which showed a gastrin-induced increase in cellular migration using the AGS$_{GR}$ cells.

Using an in vitro cell model for Barrett's oesophagus, gastrin had no effect on anchorage-independent growth, or apoptosis (data not shown). However, it was clear from our flow cytometric data that gastrin also induced a modest increase in the S phase cells (10%) and a decrease in the G_0/G_1 cells but only in CCK$_2$R-positive cell lines. Interestingly, the concentration of gastrin required for proliferation was generally higher than that needed for migration ~100 times higher. Therefore, these data are consistent with a very modest gastrin-induced increase in proliferation in cells positive for CCK$_2$R, which is supported in the literature.[8 9] The concentration-dependent response on cell growth suggests that gastrin may only be physiologically, or even pathologically, trophic in the stomach where it is found at ~50—100 ng/ml.

In conclusion, the in vitro effects of gastrin lead to a significant biological effect, namely migration. Whether this translates into a clinical effect is as yet unclear. In patients on long-term PPI therapy there is no evidence that a concomitant raised gastrin level leads to any significant expansion of Barrett's oesophagus segments in vivo. There are many confounding

factors for which we have controlled, including *H pylori* infection, in increasing serum gastrin[32–34] and oesophagitis above the Barrett's oesophagus segments which could confound diagnosis.[35] Therefore while it has been shown in the short term that PPI therapy and/or hypergastrinaemia can increase proliferation and induce COX-2 expression in susceptible cells,[36] this study has also shown conclusively in a large randomised cohort that there is no evidence of longer-term harm with surrogate histological biomarkers of proliferation, apoptosis and surrogates of migration in vivo (data not shown). The power of this study was also sufficient to detect a change in the most relevant endoscopic biomarker—spread as a surrogate of migration (a 1 cm change in Barrett's oesophagus length in 10% of patients over 2 years). Since we showed the motogenic effects of gastrin were confined to Barrett's oesophagus cells and not squamous cells in vitro, this would be expected to allow (over 2 years) spread of Barrett's oesophagus at the expense of squamous mucosa into ulcerated areas in oesophagitis. It is therefore still theoretically possible that a very weak effect of PPI therapy may occur in a small subgroup of cases and this will need a trial of even greater power to detect changes of <0.5 cm in $\sim$5% of patients. In this regard we must therefore await the results of the 10-year 2513-patient randomised controlled clinical trial AspECT (Aspirin Esomeprazole Chemoprevention Trial),[37] due to report in 2014, before the long-term clinical effect of gastrin is fully elucidated.[38]

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