An Antiapoptotic Role for Gastrin and the Gastrin/CCK-2 Receptor in Barrett’s Esophagus

Joseph C. Harris,¹ Philip A. Clarke,¹ Altaf Awan,¹ Janusz Jankowski,² and Susan A. Watson¹

¹Academic Unit of Cancer Studies, University of Nottingham, Nottingham, and ²Department of Cancer Biomarkers, University of Leicester, Leicester, United Kingdom

Abstract
Mechanisms by which premalignant Barrett’s metaplasia (BM) progresses to esophageal adenocarcinoma are currently being sought. This study investigated the role played by the polypeptide hormone gastrin, specifically its antiapoptotic effects through activation of protein kinase B/Akt (PKB/Akt). In esophageal cell lines with low basal levels of activated PKB/Akt, phosphorylation could be induced by exogenous amidated gastrin. High basal levels of activated PKB/Akt were linked to endogenous gastrin expression and were reduced by treatment with a cholecystokinin-type 2 receptor (CCK-2R) antagonist. Expression of a constitutively active splice variant of the CCK-2R additionally increased basal activation of PKB/Akt. It is proposed that gastrin acting in an autocrine and endocrine manner via a CCK-2R isofrom may activate PKB/Akt and that with expression of gastrin and CCK-2-R isoforms increasing in BM samples, gastrin may aid progression of BM through amplification of antiapoptotic pathways. Evidence for this proposal was provided through the observed specific up-regulation of PKB/Akt in BM samples.

Introduction
Barrett’s esophagus (BE) is a premalignant condition of the lower esophagus caused by prolonged gastroesophageal reflux and characterized by a metaplastic change from normal squamous to columnar intestinal-type epithelium (1). The prevalence of Barrett’s metaplasia in the population is estimated to be between 5 and 9% and is associated with an increased risk of progression to esophageal adenocarcinoma (2). This study aimed to investigate a link between gastrin and antiapoptotic mechanisms in the metaplastic esophageal environment.

Gastrin has recently been shown to increase transcription of a number of target genes, such as ligands of the epidermal growth factor receptor (3) and cyclooxygenase 2 (4), as well as exhibiting angiogenic (5) and antiapoptotic properties (6). Up-regulated gastrin production through a number of scenarios, including use of proton pump inhibitors (7), may therefore aid the progression of BE to esophageal adenocarcinoma. Gastrin acts via the cholecystokinin type-2 receptor (CCK-2R), a member of the 7-transmembrane domain G-protein-coupled receptor superfamily. A novel splice variant named CCK-2Ri4sv has recently been described, which exhibits constitutive activation (8).

An important downstream effect of CCK-2R activation is the phosphorylation/activation of the potent antiapoptotic factor, protein kinase B (PKB/Akt) (6). Once phosphorylated PKB/Akt can itself inactivate a range of proapoptotic factors, including caspase-9, Bad, and the forkhead/winged-helix transcription factors important in the transcription of the cell death ligand Fas, as well as activating the antiapoptotic inhibitor αB kinase cascade (9). This study was aimed at determining the antiapoptotic potential of increased gastrin, CCK-2R, and CCK-2Ri4sv expression in human BE samples, using transfected human esophageal adenocarcinoma cell lines as in vitro models.

Materials and Methods

Human Tissue Sample Collection. Endoscopic biopsy samples were collected by both Professor Janusz Jankowski (Leicester Royal Infirmary, Leicester, United Kingdom) and Altaf Awan (Derby Royal Infirmary, Derby, United Kingdom). Samples were made anonymous according to Medical Research Council good clinical research practice guidelines and were collected after local National Health Service Ethical Committee guidelines in both centers. They were analyzed by a pathologist and verified as being normal or Barrett’s metaplasia.

Cell Culture. OE19 (esophageal adenocarcinoma pathological stage III), OE21 (esophageal squamous carcinoma pathological stage III), OE33 (Barrett’s metaplasia-derived esophageal adenocarcinoma pathological stage II), and AR42J (rat exocrine pancreatic) cells known to express the classical CCK-2R were cultured in RPMI 1640 (Sigma, Poole, United Kingdom) with the addition of 10% fetal bovine serum (Sigma). The cells were maintained in a humidified environment at 37°C and 5% CO₂.

Transfection with the CCK-2Ri4sv. OE33 cells were stably transfected with a pcDNA3.1 vector containing a CCK-2Ri4sv insert (obtained from Mark Hellmich; Department of Surgery, University of Texas, Galveston, TX) using the Promega Transfast Transfection Reagent (Promega, Madison, WI).

RNA Extraction and Reverse Transcription. Total RNA was extracted from human biopsy and in vitro cell samples using RNA-Beec (Biogenesis; Poole, Dorset, United Kingdom) with reverse transcription being carried out using Superscript II reverse transcriptase reagents (Invitrogen, United Kingdom) as described previously (10).

Real-Time PCR. mRNA expression in cell lines and tissue samples was determined via real-time PCR, using fluorescent SYBR green dye to allow semiquantitative analysis of gene expression levels. Optimized primers designed to bind the gastrin, CCK-2R, and CCK-2Ri4sv genes were used in conjunction with reagents from the qPCR Core kit for SYBR Green I (Eurogentec, Romsey, United Kingdom). PCR assays were carried out on a 5700 Sequence Detection System (PE Biosystems, Warrington, United Kingdom).

Western Blotting. The effect of 10⁻⁸ m exogenous gastrin stimulation on PKB/Akt phosphorylation was assessed via Western blotting using specific anti-PKB/Akt and anti-phospho-PKB/Akt antibodies (Cell Signaling Technologies, Beverly, MA). The manufacturer’s protocol was followed, with horseradish peroxidase labeled swine antirabbit secondary antibodies (Dako, Carpinteria, CA) and ECL reagents (Amersham, Buckinghamshire, United Kingdom). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control and semi-quantitatively assessed using the densitometry software of the computerised analysis system (NIH Image, Bethesda, Maryland, USA). The band intensities were normalized to GAPDH band intensities and their ratios were compared between groups.

Immunohistochemical Evaluation of Gastrin and CCK-2R in OE Cells. OE cells were cultured in 8-well SuperCell chamber slides (Menzel-Gläser, Braunschweig, Germany) for 24 h then fixed in 4% paraformaldehyde. Immunohistochemistry (IHC) was performed on paraffin sections using goat antirabbit primary antibody (Aption Corporation, Woodland, CA) raised against the first extracellular domain of the receptor (11). The CCK-2R antagonist YM022 used at 10⁻⁸ m was kindly donated by the James Black Foundation (Dulwich, London, United Kingdom).

Received 8/29/03; revised 1/21/04; accepted 1/23/04.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Susan A. Watson, Academic Unit of Cancer Studies, D Floor, West Block, Queen’s Medical Centre, University Hospital, Nottingham, NG7 2UH, United Kingdom. Phone: 44-0-115-9709248; Fax: 44-0-115-9709902; E-mail: sue.watson@nottingham.ac.uk.
Essary controls were also prepared. Slides were treated with Slow Fade Light Antifade Solution with 4',6-diamidino-2-phenylindole (Molecular Probes) before fluorescent microscopy analysis.

Immunohistochemical Evaluation of PKB/Akt and Phospho-PKB/Akt in Human Normal and Barrett's Samples. Immunohistochemical labeling using murine PKB/Akt and rabbit polyclonal phosphorylated PKB/Akt anti-

Fig. 1. Gene expression of three genes in human Barrett's versus normal biopsy samples via real-time PCR. A, gastrin. B, cholecystokinin-type 2 receptor (CCK-2R). C, CCK-2Ri4sv. Significant up-regulation of all three genes was observed in the premalignant Barrett's samples compared with their paired normals as shown in the figures (Wilcoxon signed rank test). Samples in which expression could not be detected are shown as having relative expression levels of 0.00001.

Fig. 2. Expression of cholecystokinin-type 2 receptor (CCK-2R) and gastrin protein in three esophageal cell lines. A, using immunohistochemistry, OE cell lines were stained with a monoclonal anti-CCK-2R antibody, representative staining of the OE19 cell line is shown. The CCK-2R is localized at the plasma membrane and clustered at intracellular locations. B, Western blotting using an anti-CCK-2R antibody suggests that it is the classical isoform of the receptor, which is being expressed by all three esophageal cell lines. C, immunohistochemical staining of OE19 cells using an anti-progastrin antibody. Gastrin is observed at high levels in an intracellular perinuclear granular pattern.

bodies (both Cell Signaling Technology) was carried out on formalin-fixed, paraffin-embedded esophageal biopsy tissue (n = 8 for each sample type) by indirect streptavidin biotin-labeling technique (StreptABC system; DakoCytomation, Ely, United Kingdom) according to manufacturers instructions. Visualization was via diaminobenzidine tetra hydrochloride chromogen (Liquid DAB system; DakoCytomation). In the case of the murine PKB/Akt antibody, nonspecific cross-reactivity was controlled for using an irrelevant IgG1 monoclonal antibody at matched concentration. Nonspecific cross-reactivity for the polyclonal phosphorylated PKB/Akt antibody was controlled for by preabsorption with the Ser473 blocking peptide as per manufacturer's instructions (Cell Signaling Technology). Staining intensity was assessed in 10 fields of view for each normal and Barrett's sample using the QWin Standard image analysis system (Leica Microsystems, Cambridge, United Kingdom).

Results

Tissue Sample Analysis. Paired human normal and Barrett’s metaplasia biopsy samples were analyzed via semiquantitative real-time PCR for relative expression levels of gastrin (n = 16), CCK-2R
mRNA levels for these key genes were expressed relative to levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Significant increases in gene expression (calculated via the Wilcoxon signed rank test) for all three genes were observed \[\text{gastrin, } P = 0.0076, 29.2\text{-fold increase (Fig. 1A)}; \text{CCK2R, } P = 0.0068, 7.8\text{-fold increase (Fig. 1B)} \] and there was de novo expression of CCK2Ri4sv; \[P = 0.0077 \text{ (Fig. 1C)} \] in the BE samples compared with their paired normals.

Evaluation of CCK-2R and Gastrin Expression in Esophageal Cells. CCK-2R protein expression was confirmed via immunohistochemical staining, with the presence of the receptor established in all three esophageal cell lines, predominantly at the plasma membrane. Fig. 2A shows a representative staining pattern with OE19 cells. Western blot analysis using a specific CCK-2R antibody confirmed that the classical 74-kDa isoform of the receptor was expressed (Fig. 2B). There was no evidence of the intron 4 splice variant, which the NH2-terminal CCK-2R antiserum would have detected at a molecular weight of 82–85kDa, which was confirmed by gene expression studies (results not shown).

Both immunohistochemical staining and real-time PCR analysis (results not shown) were used to confirm expression of gastrin in the esophageal cell lines. Fig. 2C shows OE19 cells fluorescently labeled with anti-progastrin antibodies, with gastrin localization showing a perinuclear granular clustering pattern. Relative gastrin gene expression values were observed to be 0.65 for OE19 compared with 0.23 for the other adenocarcinoma line, OE33.
Effect of Exogenous Gastrin on PKB/AKT Phosphorylation in Esophageal Cells. The effect of exogenous gastrin on PKB/Akt phosphorylation was examined after induction of apoptosis via 24-h serum withdrawal. Ten nM gastrin were added to the medium, and Western blot analysis was then performed using specific anti-PKB/Akt and anti-phospho-PKB/Akt antibodies. Serum-starved AR42J cells showed no PKB/Akt phosphorylation in the absence of gastrin but demonstrated an increase in phosphorylation after gastrin stimulation, an event that reached its maximal level at 30 min (Fig. 3A), as confirmed via densitometry. Similar results were obtained for the three esophageal lines. Maximal phosphorylation was observed at 30 min in the OE33 cells and 60 min in the OE21 cells and was observed to be at constitutively high levels in the OE19 cells (Fig. 3B).

Transfection of OE33 Cells with the CCK-2Ri4sv. Chosen for having the lowest basal PKB/Akt phosphorylation after serum withdrawal, OE33 cells were stably transfected with the constitutively active isoform of the gastrin receptor CCK-2Ri4sv. Western blot analysis of these cells showed an increase in basal phosphorylation status of the antiapoptotic protein compared with that observed in the vector control transfected cells. Phosphorylation increased additionally after gastrin stimulation for 60 min (Fig. 4A). Phosphorylation of PKB/Akt in the CCK-2Ri4sv-transfected lines could be reduced after YM022 treatment, indicating a role for the receptor in the process (Fig. 4B).

Effect of YM022 blockade of the CCK-2R on PKB/Akt Phosphorylation in OE19 Cells. OE19 cells shown to exhibit constitutive PKB/Akt phosphorylation were serum starved and then treated with the CCK-2R antagonist YM022 at 10 nM. This treatment resulted in a reduction in the levels of basal Akt phosphorylation observed in the cell line. Phosphorylation was almost completely inhibited after a 60-min incubation period (Fig. 3C) with no effect on cell viability.

The Effect of YM022 Blockade of the CCK-2R on PKB/Akt Phosphorylation in OE19 Cells. OE19 cells shown to exhibit constitutive PKB/Akt phosphorylation were serum starved and then treated with the CCK-2R antagonist YM022 at 10 nM. This treatment resulted in a reduction in the levels of basal Akt phosphorylation observed in the cell line. Phosphorylation was almost completely inhibited after a 60-min incubation period (Fig. 3C) with no effect on cell viability.
Evaluation of PKB/Akt Phosphorylation Status in Normal and Barrett’s Samples. Using immunohistochemical methods, paired normal and Barrett’s samples (n = 8) were assessed for levels of total and phosphorylated PKB/Akt using specific antibodies. Quantification of immunohistochemistry was carried out using image analysis software macro routines. A binary mask was used to highlight positive antibody staining on a digitized color picture of the tissue, the gray level intensity of each pixel measured, and the mean value found for 10 representative fields of view. Higher gray level intensities correspond to higher levels of antibody binding. These averages could hence be compared and the total mean for all samples calculated. Levels of total PKB/Akt staining were consistently intense in both normal and Barrett’s samples; however, a significant difference in phospho-PKB/Akt staining was observed (Paired Samples T Test, P < 0.008 for each pair). Negligible phospho-Akt levels (mean gray level intensity = 7.5) were present in all normal samples (Fig. 5A) with high levels (mean gray level intensity = 59.9) observed in all metaplastic Barrett’s samples (Fig. 5B). With regard to the latter, staining was focused in the metaplastic intestinal crypts.

Discussion

Amidated gastrin increases phosphorylation/activation of the anti-apoptotic factor PKB/Akt in a time-dependent fashion in three esophageal cell lines. Additionally, compared with the AR42J control cell line, higher basal phosphorylation levels were observed in the esophageal cell lines, a phenomenon attributed to their relatively higher level of endogenous gastrin expression, which could act in an autocrine/juxtacrine fashion on the classical CCK-2R. Evidence supporting this theory was provided by blocking the CCK-2R of the esophageal adenocarcinoma cell line OE19, which resulted in a decrease in PKB/Akt phosphorylation. No exogenous gastrin was present in the cell growth media, and hence, it is concluded the basal Akt phosphorylation may partially result from autocrine gastrin stimulation. The three esophageal lines were chosen for their exclusive expression of the classical CCK-2R isoform to gain a clearer understanding of the functionality of the CCK-2Ri4sv after transfection into the cells.

A similar high level of constitutive PKB/Akt phosphorylation was observed in OE33 cells after transfection with the splice variant receptor CCK-2Ri4sv. This increase is attributed to the apparent constitutive signaling capability of the CCK-2Ri4sv that results in Ca2+ oscillation even in the absence of ligand binding (8).

Previous research using human BE metaplastic samples has shown an increase in CCK-2R expression levels (12) compared with normal mucosa, with this current research additionally showing an increase in gastrin and CCK-2Ri4sv gene expression in the same sample type. These findings correlate with the observed significant increase in activated PKB/Akt seen in BE tissue compared with normal tissue. It is concluded that gastrin, in conjunction with CCK-2R isoforms, may reduce cell death in metaplastic BE through increased activation of PKB/Akt, thus aiding malignant progression.

References

An Antiapoptotic Role for Gastrin and the Gastrin/CCK-2 Receptor in Barrett's Esophagus

Joseph C. Harris, Philip A. Clarke, Altaf Awan, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/6/1915

Cited articles
This article cites 8 articles, 2 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/6/1915.full#ref-list-1

Citing articles
This article has been cited by 17 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/64/6/1915.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.