Glycine-extended gastrin inhibits apoptosis in Barrett’s oesophageal and oesophageal adenocarcinoma cells through JAK2/STAT3 activation

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Abstract

Barrett’s oesophagus (BO) and oesophageal adenocarcinoma (OAC) are regarded as complications of gastro-oesophageal reflux disease, although all the factors that contribute to the development of these lesions are unknown. Acid suppressive drugs are widely used for symptomatic therapy of reflux disease but may induce hypersecretion of gastrin peptides. Amidated gastrin (G-17) has been shown to be a growth factor for OAC cells. We have examined the effects of glycine-extended gastrin (G-Gly), an alternative product of progastrin processing on apoptosis in the QhERT Barrett’s oesophageal cell line and OE33 and BIC-1 OAC cells. G-Gly inhibited serum-starvation and camptothecin-induced apoptosis in all three cell lines, G-17 was only effective in OE33 cells. By contrast to the effects of G-17, the anti-apoptotic effect of G-Gly was independent of both the CCK2 receptor and cyclo-oxygenase-2 activity. G-Gly stimulated JAK2 phosphorylation and kinase activity and JAK2-dependent STAT3 phosphorylation and transcriptional activity. G-Gly also increased mRNA and protein levels of the anti-apoptotic proteins survivin and BCL2L1 but did not affect the levels of BAD, BAX or BCL-2. Novel small molecule inhibitors of JAK2 and STAT3 as well as STAT3 siRNA blocked the anti-apoptotic effects of G-Gly and inhibited the induction of survivin and BCL2L1 in OE33 cells. We conclude that G-Gly inhibits apoptosis in BO and OAC via mechanisms distinct from those activated by G-17 and involving JAK2 and STAT3 activation. Release of gastrin peptides in response to acid suppressive therapy may adversely influence the dynamics of the epithelium in BO.

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Introduction

Glycine-extended gastrin (G-Gly) is a product of processing of the preprogastrin peptide product of the gastrin gene. It is becoming apparent that the different peptide products of this processing have different profiles of biological activities and contributions to diseases states (Dockray et al. 2001). The role of amidated gastrins (gastrin-17 and gastrin-34) in regulating the secretion of gastric acid is well documented but increasingly roles for the different peptides in stimulating the growth of various normal and transformed cell types and promoting the development of cancers have been described (Dockray et al. 2001).

Carboxy-terminal amidated gastrin stimulates the growth of oesophageal, gastric, pancreatic and colon cancer cell lines in culture (Iwase et al. 1997, Stepan et al. 1999, Haigh et al. 2003), inhibits apoptosis in pancreatic cancer cells (Todisco et al. 2001), and transgenic over-expression of gastrin induces gastric atrophy and promotes the development of gastric cancer (Wang et al. 2000, Fox et al. 2003). The larger precursor 80 amino acid progastrin peptide also stimulates proliferation of colon cancer cell lines (Singh et al. 2007), inhibits pancreatic cancer apoptosis (Rengifo-Cam et al. 2007), and transgenic mice over-expressing progastrin exhibit colonic hyperproliferation, increased mitosis after irradiation and adenomata and carcinomas after exposure to the chemical carcinogen azoxymethane (Cobb et al. 2004, Ottewell et al. 2005).

G-Gly is an alternate processing product that also seems to have a role in promoting cancers of the GI tract. In cell culture systems, G-Gly stimulates the proliferation of gastric, pancreatic and colon cancer cells (Seva et al. 1994, Iwase et al. 1997, Ogunwobi & Beales 2006) and inhibits apoptosis in HT-29 colon cancer cells (Beales & Ogunwobi 2006). Transgenic over-expression of G-Gly has been reported to stimulate colonic epithelial proliferation (Koh et al. 1999, Ferrand et al. 2005) and also increases the incidence of cancers in the APC min (±) mouse model of colon cancer (Koh et al. 2000). G-Gly has also been shown to promote the growth of lung cancer (Koh et al. 2003). However, much less is known of the intracellular
mechanisms mediating the effects of G-Gly, probably related to lack of identification of a specific receptor. Proliferative and anti-apoptotic responses to amidated gastrin are mediated via the classical gastrin CCK$_2$ receptor (CCK$_2$R) and activation of various signalling pathways (including Ca$^{++}$, extra-cellular signal-related kinase and Akt) (Todisco et al. 1997, Stepan et al. 2004) and at least some of the responses to progastrin are mediated via binding to annexin II and subsequent activation of the canonical nuclear factor-$kappa$B (NF-$kappa$B) pathway (Rengifo-Cam et al. 2007). By contrast, much less is known about G-Gly induced signalling. The hyperproliferative colonic mucosa of G-Gly expressing transgenic mice showed increased activation of various pathways including janus kinase 2 (JAK2) and signal transducer and activator of transcription-3 (STAT3) (Ferrand et al. 2005) and in HT-29 cells, we have previously shown that G-Gly inhibited apoptosis that was blocked by the commonly used small molecule JAK inhibitor AG490 (Beales & Ogunwobi 2006).

Although these results were very suggestive of pro-carcinogenic G-Gly signalling via a JAK2/STAT3 pathway, these were indirect inferences and there are also no data showing what the possible downstream targets of this pathway are. Therefore in this study, we have examined the effect of G-Gly on non-transformed Barrett’s oesophageal and oesophageal adenocarcinoma (OAC) cells. While effects of G-Gly on several gastrointestinal tissues have been reported, the effect on oesophageal epithelial cells has not been examined: but we have hypothesised that metaplastic glandular (Barrett’s) oesophageal epithelium may be an important target for G-Gly. The incidence of OAC has increased markedly (by over 600%) in the developed world over the last 30 years (Buttar & Wang 2004). In nearly all cases, OAC develops from the precursor metaplastic lesion, Barrett’s oesophagus (BO) (Buttar & Wang 2004). The factors that drive the transformation of BO to cancer remain to be understood although hormones or growth factors which increase proliferation or inhibit apoptosis could be involved (Buttar & Wang 2004). Gastro-oesophageal reflux of gastric acid and bile is thought to be central to the pathogenesis of BO and drugs that inhibit gastric acid secretion are extensively used for symptomatic treatment of reflux disease. The most commonly used drugs are the proton-pump inhibitors (PPIs) which inhibit acid secretion by binding to the H$^+$/K$^+$/ATPase in the gastric parietal cell; in doing so a negative feedback mechanism is stimulated, leading to increased secretion of gastrin peptides from the gastric antrum in response to the increased gastric pH (Dockray et al. 1991, Nemeth et al. 1992). This hypergastrinaemia could adversely affect the metaplastic oesophageal mucosa.

The limited available data support this hypothesis: amidated gastrin stimulates proliferation and inhibits apoptosis in OAC cells via the CCK$_2$R (Haigh et al. 2003). As G-Gly is stored and secreted in common with amidated gastrin (Sugano et al. 1987), it is highly plausible that patients with BO taking PPI therapy are also exposed to elevated G-Gly levels. It has proved difficult to develop assays that reliably distinguish between the gastrins and as a result there are very few data specifically examining G-Gly levels related to human disease states, although G-Gly secretion and levels have been reported to approach those of amidated gastrin (Del Valle et al. 1987, 1989, Sugano et al. 1987). The majority of in vitro studies have suggested that biological effects of G-Gly can be seen at lower concentrations (sometimes greater than 10-fold) than seen with amidated gastrin (Stepan et al. 1999, Dockray et al. 2001, Beales & Ogunwobi 2006): meaning that relatively mild G-Gly hypersecretion may be biologically important.

Therefore, we have examined the effect of G-Gly on apoptosis in both non-neoplastic Barrett’s oesophageal cells as well as two different OAC cell lines, with specific attention to the JAK/STAT pathway, which has also been recently implicated in the progression of OAC (Dvorak et al. 2007). We have examined this utilising novel, recently described, specific small molecule JAK2 (tyrene CR4 (Grumberger et al. 2003), curcubitacin I (Blaskovich et al. 2003)) and STAT3 (stattic (Schust et al. 2006)) inhibitors as well as a cell-permeable STAT3 inhibitory phospho-peptide (Turkson et al. 2001) and STAT3 RNA interference. We have used protein phosphorylation, kinase assays and promoter reporter assays to confirm that G-Gly inhibits apoptosis via a JAK2/STAT3 pathway in BO and adenocarcinoma.

Materials and methods

The OE33 human OAC cell line was obtained from ECACC (Wiltshire, UK), the BIC-1 human oesophageal adenocarcinoma cell line was a gift from Dr D Beer (University of Michigan, Ann Arbor, MI, USA) and the QhERT non-neoplastic Barrett’s oesophageal cell line transformed with the human telomerase catalytic subunit was a gift from Dr P Rabinovitch (Fred Hutchinson Cancer Center, Seattle, WA, USA). The characteristics of this cell line have been described previously (Palanca-Wessels et al. 2003). G-Gly-17 was from NeoMPS (Strasbourg, France) and amidated gastrin-17 was from Bachem (St Helens, UK). Indomethacin and NS-398 were from Alexis Biochemicals (Nottingham, UK). SC 791, AG1478, camptothecin, curcubitacin I, proglumide, AG490, PP2 and the cell permeable STAT3 inhibitor peptide were from Merck. Stattic (6-nitrobenzo[b]thiophene 1,1-dioxide), a small molecule STAT3 inhibitor was from Tocris Bioscience (Bristol, UK). CR2945 and all other general reagents were from Tocris Bioscience (Bristol, UK).
were from Sigma. All other cell culture reagents were from Invitrogen. All inhibitors and antagonists were added 60 min before stimulation with peptides. Inhibitor concentrations were chosen using data from our own laboratory or other published sources as indicated (Turkson et al. 2001, Souza et al. 2002, Blaskovich et al. 2003, Grunberger et al. 2003, Ogunwobi et al. 2006, Schust et al. 2006, Langmesser et al. 2007).

Cell culture and assessment of apoptosis

OE33 and BIC-1 cells were cultured in DMEM containing 4500 mg/l glucose, 100 mg/l penicillin, 100 mg/l streptomycin and 2 mM L-glutamine and supplemented with 10% foetal bovine serum (FBS) as previously described (Ogunwobi et al. 2006). QhERT cells were maintained in MCDB 153 medium supplemented with 5% FBS, 20 ng/ml epidermal growth factor (Bachem), 140 µg/ml bovine pituitary extract, 5 µg/ml insulin, 5 µg/ml transferrin, 0.4 µg/ml hydrocortisone, 100 pM cholera toxin, 20 µg/ml adenine, 4 mM glutamine, 0.25 µg/ml amphotericin B, 100 µg/ml penicillin and 100 µg/ml streptomycin (Palanca-Wessels et al. 2003).

For apoptosis experiments, cell lines were cultured in complete media in 96-well plates for 48 h and then the medium was changed to serum free medium, supplemented as appropriate with test peptides. Inhibitors and receptor antagonists were added 60 min prior to the addition of peptides. When appropriate camptothecin (10 µM) was added to induce apoptosis after 42 h as previously described (Rengifo-Cam et al. 2007). After 48 h, apoptosis was quantified using the Biocolor APOPercentage assay according to the manufacturer’s instructions (Biocolor Ltd., Newtonabbey, UK). This utilises a colormetric reagent that shows uni-directional uptake into apoptotic committed cells consequent on the transfer of phosphatidylserine to the outer cell membrane leaflet (Johnson et al. 2003). The dye accumulates within cells and is released using the APOPercentage Dye release reagent. Apoptosis was quantified by reading the absorbance at 550 nM.

Confirmatory studies were also performed using quantification of intracellular nucleosomes as an alternative measurement of apoptosis: this was performed using a specific ELISA Cell Death detection kit (Roche) (Ogunwobi & Beales 2007a).

Immunoblotting

Sample preparation, western blotting and visualisation were performed as described previously (Ogunwobi et al. 2006). The primary antibodies used were goat polyclonal anti-CCK2R and rabbit polyclonal anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), used at 1:200 with detection using peroxidase conjugated secondary antibodies (1:2000, Santa Cruz).

JAK2 and STAT3 phosphorylation

OE33 cells were grown in 96-well plates and serum starved for 24 h. After stimulating with G-Gly (1 nM) cells were fixed with 4% formaldeyde. Subsequently, levels of total and phosphorylated (tyrosine-1007/tyrosine-1008) JAK2 were quantified using a cell-based ELISA as previously described (Beales & Ogunwobi 2006). Total and tyrosine-705 phosphorylated STAT3 were quantified by a commercially available cell-based ELISA (CASE assay, Tebu-Bio, Peterborough, UK).

JAK2 kinase assay

Immunoprecipitates and kinase assays were performed as described previously with minor modifications (Blaskovich et al. 2003, Ferrand et al. 2006). Serum-starved OE33 were stimulated with 1 nM G-Gly and after 5 min, cells were lysed with lysis buffer (HEPES 50 mM pH 7.5, NaCl 150 mM, EDTA 10 mM, Na3P2O7 10 mM, NaF 10 mM, Na3VO4 2 mM, NP 40 1%, PMSF 0.5 mM, 20 µM leupeptin, 10 µg/ml aprotinin). Equal amounts of protein (500 µg) were incubated with 1 µg anti-JAK2 antibody (Santa Cruz) for 2 h at 4°C. Protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad). Aliquots of protein A agarose (Santa Cruz) (20 µl) were then added and solutions mixed for 1 h at 4°C. Immunoprecipitates were collected by microfuging for 5 min at 4°C. After centrifugation, the pellets were washed with lysis buffer and twice with kinase buffer. Immunoprecipitated JAK2 was used to phosphorylate the biotinylated FLT3 substrate peptide (Cell Signalling Technology, Hitchin, UK). Kinase reactions were carried out in a final volume of 50 µl kinase buffer (60 mM HEPES pH 7.5, 5 mM MgCl2, 5 mM MnCl2, 3 mM Na3VO4, 1.25 mM DTT) containing 1-5 µM substrate peptide and 20 µM ATP. After incubating at room temperature for 30 min, the reaction was terminated by adding 50–50 mM EDTA, pH 8.0. Subsequently, biotinylated FLT3 was captured by transferring 25 µl of the reaction mixture and 75 µl H2O to the wells of a streptavidin-coated 96-well plate (Pierce, Northumberland, UK) and incubating for 60 min at room temperature. After washing three times with PBS/0.05% Tween (PBS/T), specific FLT3 phosphorylation was detected by incubating with a mouse monoclonal anti-phosphotyrosine antibody at 1:1000 (Cell Signalling Technology) at room temperature for 60 min. After three further washes with PBS/T, wells were incubated for 30 min at room temperature.
with a HRP-conjugated goat anti-mouse antibody (1:10000) (Santa Cruz). After five further PBS/T washes, colour development with Sigma Fast OPD substrate solution (Sigma) was quantified by reading the optical density at 450 nm with a reference OD at 600 nm. The background optical density was determined from control experiments using biotin (Sigma) instead of the biotinylated peptide.

G-Gly-induced STAT3 transcriptional activity
OE33 cells were seeded into 96-well plates and STAT3 transcriptional activity was measured using the Rapid Reporter pRR-High-STAT3 Gaussia luciferase reporter system (Active Motif, Rixensart, Belgium) according to the manufacturer’s instructions. Briefly, ~60% confluent OE33 cells grown in 10 cm dishes were transfected with the Rapid Reporter pRR-High-STAT3 plasmid using FuGENE 6 (Roche) as directed. This vector contains the STAT tandem response element upstream of the Gaussia luciferase gene. After 6 h, cells were trypsinised and replated at 50 000 cells per well into 96-well plates. After 24 h culture the cells were serum-starved for a further 24 h and then stimulated with G-Gly; where appropriate inhibitors were added 60 min prior to G-Gly. After 3 h, the media was removed and replaced with lysis buffer. Luminescence was measured after the addition of Gaussia assay buffer and substrate.

mRNA quantitation
Serum-starved OE33 cells were stimulated with 1 nM G-Gly and after 4 h cells were lysed and total mRNA extracted and cDNA synthesised as described previously (Ogunwobi et al. 2006). Quantitative real-time PCR (qRT-PCR) was performed using an Applied Biosystems ABI prism 7700 sequence-detection system (Applied Biosystems, Warrington, UK). Specific commercially available primer pairs for BAD, BAX, BCL-2, BCL2L1 and survivin with 18s rRNA used as the internal control were obtained from SuperArray Bioscience Corporation (Tebu-Bio). PCR reactions were performed in 96-well optical plates using 1 µl cDNA, 12.5 µl RT2 SYBR Green/ROX qPCR Master Mix (SuperArray), 10.5 µl H2O and 1.0 µl 10 µM primer pair stock. Amplification consisted of 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Quantitation of mRNA was performed by determining the cycle number at which amplification entered the exponential phase (termed the Ct or cycle threshold) and converting these to cDNA concentrations using standard curves and comparing with the corresponding 18s rRNA levels to give arbitrary relative units as described (Ogunwobi & Beales (2008a)).

Survivin and BCL2L1 protein quantitation
Serum-starved OE33 cells were treated for 24 h with 1 nM G-Gly and subsequently lysed as described previously (Ogunwobi et al. 2006). Survivin and BCL2L1 protein levels were quantified in equal amounts of cell lysates using specific ELISA kits (R and D Systems, Abingdon, UK).

STAT3 RNA interference
OE33 cells were transfected with 80 nM STAT3 siRNA sequence or scrambled siRNA sequence (both Santa Cruz) using Santa Cruz siRNA transfection reagent and transfection medium according to the manufacturer’s instructions (Ogunwobi & Beales (2008b)). After 72 h, STAT3 relative protein levels were compared using the STAT3 CASE cell-based ELISA. For apoptosis and apoptosis protein assays, siRNA transfected cells were serum starved after 48 h and experiments performed as described above. For STAT3 transcription assays, cells were additionally transfected with the STAT3 reporter plasmid after 24 h and then experiments performed as described.

 Statistical analysis
Apoptosis studies were done in triplicate wells. Protein and mRNA studies were performed in duplicates with each condition assayed in duplicate. Experiments for direct detection of other signalling intermediates were performed in triplicate wells. Each experiment was repeated 3–5 times. Results are expressed as mean ± s.e.m. One way ANOVA was used for dose–response curve and Mann–Whiney U-test was used to analyse the effect of inhibitors. A P value <0.05 was considered significant.

Results
Effect of G-Gly on apoptosis
G-Gly caused a dose-dependent reduction in serum-starvation induced apoptosis in the OE33, BIC-1 and QhERT cells. The dose–response and magnitude of effect was similar in all three cell lines. Serum-starvation induced apoptosis was higher in QhERT cells (10.7%) than either BIC-1 (7.4%) or OE33 (5.9%). Maximal effects of G-Gly were reductions of 33% (OE33), 37% (BIC-1) and 22% (QhERT) and were seen at concentrations of 1 nM and above (Fig. 1a). A similar pattern was seen when apoptosis was quantified with the ELISA of intracellular nucleosomes: G-Gly significantly reduced apoptosis by between 27 and 39%
G-Gly also significantly reduced camptothecin-induced apoptosis in all three cell lines; in this situation the reduction in apoptosis was 24% (BIC-1), 45% (QhERT) and 46% (OE33) (Fig. 1c).

Pre-treatment with either the non-selective CCK receptor antagonist proglumide or the CCK2R antagonist CR2945 (Langmesser et al. 2007) did not reduce the anti-apoptotic effects of G-Gly in any of the cell lines (Fig. 2b–e). OE33 but not BIC-1 or QhERT cells expressed detectable levels of CCK2R protein (Fig. 2a) and in keeping with this gastrin-17 (10 nM) significantly reduced serum-starvation induced apoptosis (by 18%) in OE33 cells (Fig. 2e) but had no effect in either the BIC-1 or QhERT cells (data not shown). Pre-treatment of OE33 cells with either proglumide (1 mM) or CR2945 (1 μM) blocked the anti-apoptotic effect of gastrin-17, confirming that this effect was mediated via the CCK2R (Fig. 2e). In OE33 cells, the combination of G-17 and G-Gly had a greater additive effect in reducing serum-starvation induced apoptosis (combined 56% reduction) (Fig. 2f).

Previous clinical and experimental studies have implicated cyclo-oxygenase-2 (COX-2) as a potentially important mediator of anti-apoptotic signals in BO and oesophageal cancer (Buttar et al. 2002a, b, Cheong et al. 2003, Buttar & Wang 2004, Harris et al. 2004). We investigated the involvement of COX-2 by pre-treating the cells with either the non-selective COX-2 inhibitor indomethacin (10 μM) or two different COX-2 selective inhibitors (NS-398 (5 μM) or SC 791 (400 nM) (Hardy et al. 2003)). All three agents induced a small but statistically insignificant increase in basal apoptosis in serum-starved cells (data not shown), but none of these COX inhibitors ameliorated the anti-apoptotic effects of G-Gly in OE33, BIC-1 or QhERT cells (Fig. 3a). However, all three inhibitors did abolish the anti-apoptotic effects of gastrin-17 in OE33 cells (Fig. 3b).

Pre-treatment of all three cell lines with either of the two different small molecule JAK2 pathway inhibitors (AG490 (25 μM) or curcubitacin I (10 μM)), a small molecule STAT3 inhibitor (stattic, 20 μM)) or a cell permeable STAT3 inhibitor peptide (1 mM) blocked the anti-apoptotic effects of G-Gly. By contrast, pre-treatment of the cells with either the SRC kinase inhibitor PP2 (10 μM) or the EGFR-kinase inhibitor AG1478 (250 nM) did not affect the anti-apoptotic effects of G-Gly (Fig. 4a and b). Stattic and the cell permeable STAT3 inhibitory peptide had small non-significant (10–15% increase) effects, AG1478 and PP2 had no effects and AG490 (+26%) and curcubitacin I (+24%) small but statistically significant effects on serum-free apoptosis (data not shown). In view of the similarity in responses between all cell lines, further detailed studies were continued in the OE33 cells.

Effect of G-Gly on JAK2 and STAT3 activation

G-Gly significantly increased JAK2 activation in OE33 cells as assessed by quantitation of JAK2 phosphorylation: G-Gly increased phosphorylation to 239% above basal. This was blocked by both AG490 and curcurbitacin I but unaffected by the two STAT3 inhibitors (Fig. 5a).

Similarly, G-Gly significantly increased tyrosine-705 STAT3 phosphorylation to 277% above basal. This increase

Figure 1: Effect of glycine-extended gastrin on Barrett’s esophageal and esophageal adenocarcinoma cell apoptosis. (a) OE33, BIC-1 and QhERT cells were treated for 48 h with increasing concentration of G-Gly. Apoptosis was assessed by the BioColor APOPercentage assay and compared with control (serum-free cells). (b) Cells were cultured for 48 h in control (serum-free medium) or supplemented with 1 nM G-Gly. Apoptosis was quantified using an ELISA for intracellular nucleosomes. (c) Cells were cultured for 42 h in serum-free medium or supplemented with 1 nM G-Gly. Camptothecin 10 μM was then added and apoptosis in a further 6 h quantified with the BioColor APOPercentage assay. Results expressed as mean ± S.E.M. N=3, *P<0.05 versus control, **P<0.05 versus G-17 treated.
was abolished by both AG490 and cucurbitacin I and significantly reduced by the cell permeable STAT3 inhibitory peptide and completely blocked by stattic, the small molecule STAT3 inhibitor (Fig. 5c). G-Gly induced STAT3 phosphorylation was unaffected by inhibition of SRC with PP2 or the EGFR with AG1478 (data not shown).

Similar results were seen with assays of JAK2 and STAT3 functional activities: G-Gly increased JAK2 kinase activity, measured in G-Gly stimulated-OE33 cell JAK2 immunoprecipitates, by 283% (Fig. 5b). This was abolished by AG490 and cucurbitacin I but was unaffected by G-Gly combined with 10 nM gastrin-17 (e) or 1 nM G-Gly combined with 10 nM gastrin-17 (f). Apoptosis was assessed by the Biocolor APOPercentage assay and compared with control (serum-free cells). Results expressed as mean ± S.E.M., N=3–5, * P<0.05 versus control, ** P<0.05 versus gastrin-17 treated, *** P<0.05 versus G-17 and G-Gly treated.

**Figure 2** Effect of CCK receptor antagonists on glycine-extended gastrin inhibited apoptosis. (a) CCK2-R expression in OE33, BIC-1 and QhERT cells was assessed using western blotting with a specific antibody. HT-29 cells transfected with wildtype CCK2-R cDNA in the pcDNA3.1+ expression plasmid (Missouri S &T cDNA resource center) were used as a positive control. OE33 (b, e, f), BIC-1 (c) and QhERT (d) cells were pre-treated with the CCK2 receptor antagonist CR2945 (1 μM) or the non-selective CCK receptor antagonist proglumide (1 mM) for 1 h before culturing for 48 h with 1 nM G-Gly (a,b,c) or 10 nM gastrin-17 (e) or 1 nM G-Gly combined with 10 nM gastrin-17 (f). Apoptosis was assessed by the Biocolor APOPercentage assay and compared with control (serum-free cells). Results expressed as mean ± S.E.M., N=3–5, * P<0.05 versus control, ** P<0.05 versus gastrin-17 treated, *** P<0.05 versus G-17 and G-Gly treated.

**Effect of G-Gly on mediators of apoptosis**

To examine the possible downstream mediators of the G-Gly mediated STAT3 anti-apoptotic effects, we performed quantitative real-time PCR analysis for apoptosis-related proteins on G-Gly stimulated OE33 cells. G-Gly significantly increased the mRNA levels of survivin by 219%, and BCL2L1 by 116% but did not significantly affect the mRNA levels of BAD, BAX, or BCL-2 (Fig. 6a). These increases in survivin and BCL2L1 were abolished by pre-treating the cells with 20 μM stattic, the small molecule STAT3 inhibitor (Fig. 6b).

Consistent with the mRNA data, G-Gly increased the protein levels of both survivin and BCL2L1 in OE33 cell lysates; again this effect was blocked by inhibition of STAT3 with the small molecule Stat3 inhibitor (Fig. 6c).
Effect of STAT3 RNA interference

To confirm the effects of the small molecule inhibitors, we used a siRNA STAT3 oligonucleotide in complementary experiments. The STAT3 siRNA sequence, but not a scrambled control RNA, reduced STAT3 protein levels by 88% (Fig. 7a) and inhibited both G-Gly induced STAT3 transcription (Fig. 7b) and the anti-apoptotic effect of G-Gly in serum-starved cells (Fig. 7c).

Similarly, the STAT3 siRNA sequence significantly inhibited the G-Gly stimulated increases in both BCL2L1 and survivin (Fig. 7d and e).

Discussion

In this study, we have shown that G-Gly inhibits apoptosis in Barrett’s oesophageal cells and OAC cells. This effect is mediated via activation of the JAK2/STAT3 pathway and is associated with upregulation of the anti-apoptotic proteins survivin and BCL2L1. We believe that these data suggest that progastrin-derived peptides from the gastric antrum may have a role in promoting the development of OAC. Factors that inhibit apoptosis in BO are likely to perpetuate genetic changes and promote the establishment of mutations that lead to the development and progression of OAC (Buttar & Wang 2004). We have recently shown that G-Gly can induce cell proliferation and DNA synthesis in the OE33 and OE19 oesophageal cell lines (Ogunwobi & Beales (2008)), so it appears
that G-Gly may act in the promotion of OAC both by inhibiting apoptosis and by stimulating proliferation. Similar effects have been documented for other putative growth factors in the BO-dysplasia-cancer sequence (Buttar & Wang 2004, Ogunwobi et al. 2006). This is the first study to show an anti-apoptotic effect of G-Gly in oesophageal cells but perhaps more importantly, this is the first study in any system to describe in detail the cell signalling pathway activated by G-Gly leading to anti-apoptotic effects. This further strengthens the implications that endocrine, paracrine or autocrine secretion of G-Gly is a growth factor or anti-apoptotic factor for a variety of gastrointestinal cancers. These data support the previous studies in colon cancer cells (Beales & Ogunwobi 2006) and transgenic mice (Ferrand et al. 2005) that inferred that the JAK/STAT pathway was activated by G-Gly but here we have shown direct stimulation of the pathway by G-Gly, that survivin and BCL2L1 are downstream effectors and that inhibition of the pathway abolishes the anti-apoptotic effect of G-Gly.

We used a selection of recently developed novel small molecule inhibitors and specific RNA interference directed against STAT3 to confirm the importance of the JAK2/STAT3 pathway. We used AG490, which is probably the most widely used JAK2 inhibitor, although it has also been reported to inhibit other JAKs and the EGFR (Blaskovich et al. 2003). Similar results were obtained with a further inhibitor, tyrene CR4, which is reported to be extremely specific for JAK2 (Grunberger et al. 2003). Cucurbitacin I, also provided similar inhibition of G-Gly induced signalling and effects. The mode of action of this agent is not fully defined; in intact cellular systems it inhibits JAK2 activation, but it does not inhibit JAK2 kinase activity in isolated enzyme preparations, suggesting that it may target inhibitory phosphatases or JAK2/STAT3 binding proteins (Blaskovich et al. 2003). No effect of AG1478, the EGFR inhibitor was seen and G-Gly activation of JAK2 was confirmed with both a kinase assay and quantitation of JAK2 tyrosine phosphorylation. Although the cell surface receptor(s) for G-Gly have
still to be identified, our data clearly show that JAK2 activation is an early event in G-Gly induced signalling. Recently, annexin II was reported to be a functional cell surface receptor for progastrin (Singh et al. 2007) and intracellular signalling coupled to NF-kB anti-apoptotic signalling in pancreatic AR42-J cell was demonstrated (Rengifo-Cam et al. 2007). Although G-Gly appears to be able to bind to annexin II and displace radiolabelled amidated gastrin, there are as yet no published data showing whether G-Gly functionally signals via annexin II. While the specific aims of this study were to examine the intracellular mechanisms mediating the anti-apoptotic effects of G-Gly in oesophageal cells, it will be imperative to determine in the future, whether annexin II is an upstream mediator of these systems.

Janus kinase-2 is the prototypical upstream activator of STAT3, although other tyrosine kinases particularly SRC have been implicated in the initial tyrosine-705 phosphorylation of STAT3 which leads to dimerisation and nuclear localisation (Bowman et al. 2000). Phosphorylation and transcriptional activation of STAT3 was abolished by all three JAK2 inhibitors but unaffected by PP2 the SRC inhibitor, suggesting that JAK2 is the important upstream STAT3 activator in this system. G-Gly has been shown to activate SRC in HC116 colon cancer cells and it is possible there are alternative signalling pathways downstream of the putative G-Gly receptor(s) (Ferrand et al. 2006).

Normally, following receptor activation and tyrosine phosphorylation STAT3 proteins dimerise and translocate to the nucleus where they act as transcription factors for various genes carrying the STAT3 motif (T TCT GGG AAT T) in their promoter regions (Bowman et al. 2000). Using three different approaches to STAT3 inhibition; siRNA, a cell permeable phosphopeptide that prevents STAT3 interacting with SH2 domains on both receptors and other STAT3 monomers (Turkson et al. 2001) and a novel small molecule inhibitor (Schust et al. 2006), we confirmed that STAT3 is essential to the anti-apoptotic effects of G-Gly. It is now accepted that STAT3 can act as an oncogene; increased expression of STAT3 has been reported in several cancers and it is believed that STAT3 can drive the transcription of various genes implicated in cancer (Bowman et al. 2000). Increased STAT3 activation does not usually stem from activating mutations, much more commonly STAT3 overactivity is driven by upstream stimulation (Bowman et al. 2000). Further studies are warranted to determine if G-Gly driven STAT3 activity contributes to the growth factor effects of G-Gly in other tissues. Our data show that the anti-apoptotic proteins survivin and BCL2L1 were upregulated by G-Gly via JAK2/STAT3 signalling. The survivin promoter is known to contain a STAT3 consensus sequence and BCL2L1 has also been reported to be regulated by STAT3 (Gritsko et al. 2006).

In this study, we have used a carefully chosen selection of small molecule inhibitors of the JAK2/STAT3 pathway to determine the significance of this pathway in the effects of G-Gly: the consistency of the results, using agents with completely different modes of action, attests to the importance of this pathway and minimises the likelihood of non-specific effects of the inhibitors being responsible. We also used RNA interference to confirm the importance of STAT3 in the effects. The siRNA sequence used effectively reduced STAT3 protein levels, while the control sequence was ineffective. While it is recognised that RNA duplexes may have non-specific off-target effects,
the overall consistency in our results suggests that
STAT3 is central to the anti-apoptotic effects of G-Gly in
OE33 cells. Similarly, we have used validated ELISA
methods to quantify protein phosphorylation and
supported this with kinase and transcriptional activity
assays: all with consistent results. Again suggesting
specificity of the methods utilised.

Recently, G-Gly has been shown to inhibit apoptosis
in gastric IMGE-5 cells via upregulation of BCL2L1
protein (survivin was not examined) via pathways
requiring the small GTPases Rho and cdc42 (He et al.
2008) and further studies will be required to determine
the inter-relationships between GTPase signalling and
STAT activation. Interestingly, this latter study also
confirmed that amidated gastrin and G-Gly inhibited
apoptosis via different and complementary intracellular
signalling pathways.

Recent studies using immunohistochemistry have
shown increased expression of activated phospho-
STAT3 and BCL2L1, and increased survivin expression
by both immunohistochemistry and RT-PCR in BO,
with increased expression along the dysplasia-carcinoma
sequence (van der Woude et al. 2002, Vallbohmer
Our data show that G-Gly may be one putative driver of
STAT3 expression and our data may also be pertinent
to explain the mode of action of other putative growth
factors of BO and OAC that activate JAK2/STAT3
signalling such as leptin, transient acid exposure or IL-6
(Ogunwobi et al. 2006, Beales & Ogunwobi 2007,
Ogunwobi & Beales 2008b). Cucurbitacin I is a natural
plant product derived from the Cucurbitaceae and
Cruciferae families and has been used in traditional
Indian and Chinese remedies for many years. Our data
confirming the role of activated JAK2/STAT3 signalling
in BO and OAC suggest that exploring the potential of
both natural and synthetic small molecule inhibitors
would be worthwhile.

In this study, we confirmed the previously reported
anti-apoptotic effects of gastrin-17 in OE33 cells;
however, G-17 failed to reduce apoptosis in either the
BIC-1 OAC line or the QhERT Barrett’s cell line. The
OE33 cells had detectable CCK2R protein expression
but the receptor was undetectable in the BIC-1 and
QhERT cells. Previous studies have reported conflicting
data concerning CCK2R protein expression by OE33 cells; while
Haigh et al. (2003) were only able to demonstrate effects
after transfecting the cells with expression plasmids
carrying the CCK2R, three other studies using RT-PCR,
immunocytochemistry, western blotting and functional

Figure 7 small interfering RNA directed against STAT3 reverses the anti-apoptotic effects of glycine-
extended gastrin. OE33 cells were transfected with a specific STAT3 siRNA sequence or a control
scrambled sequence. (a) After 72 h, STAT3 protein levels were quantified using a specific cell-based ELISA
(one experiment performed), OD 450/595 is proportional to total STAT3 abundance. (b) 24 h after
transfection, cells were additionally transfected with the RapidReporter pRR-High-STAT3 Gaussia
luciferase reporter plasmid and subsequently stimulated with 1 nM G-Gly. After 3 h stimulation luciferase
activity was quantified. (c) 48 h after siRNA transfection, OE33 cells were treated with control serum-free
medium or supplemented with 1 nM G-Gly for 48 h and apoptosis was quantified with the Biocolor
APOPercentage assay. (d and e) After 48 h siRNA transfected cells were serum-starved and then
stimulated with 1 nM G-Gly, after 24 h BCL2L1 (d) and survivin (e) protein levels in cell lysates were
quantified with specific ELISA assays. Results expressed as mean ± S.E.M., N = 3, *P < 0.05 versus control,
**P < 0.05 versus G-Gly treated, † P < 0.01 versus control.
studies have shown that OE33 cells express CCK_{2R} (Abdalla et al. 2004, Harris et al. 2004, Konturek et al. 2004). Presumably, this difference reflects subtle differences in the expression patterns of subclones of the original cells line. Our data with amidated gastrin are also in keeping with the majority of the experimental data showing an anti-apoptotic effect (reviewed by Grabowska & Watson (2007)); there are data showing that amidated gastrin may actually have a proapoptotic effect in colon cancer cells (Muerkoster et al. 2005). The reasons for this disparity are not fully explained but it has been proposed that the cellular signalling environment (particularly, the NF-κB activation state) may influence the outcomes of pro/anti-apoptotic signalling via the CCK_{2R} and that there may be cell-type dependent signalling. Against this it should be noted that, although less data are available, only anti-apoptotic actions have been reported with both G-Gly and progastrin.

The general patterns of responses to G-Gly, including the dependence on JAK2/STAT3 signalling were similar in the two OAC lines and the non-malignant Barrett’s line. For convenience and cost, we only further characterised the JAK2/STAT3 signalling complex in OE33 cells and it remains plausible that different downstream signalling is utilised in non-transformed Barrett’s cells, although the in vivo correlations suggest this is not the case.

It is worth noting both that the maximal anti-apoptotic effect of G-Gly (33%) was greater than that of G-17 (18%) and also that effects of G-Gly were seen at lower concentrations, as has been reported previously for the growth factor effects (Todisco et al. 1997, Ogunwobi & Beales 2006). The effect of G-Gly, unlike that of G-17 was not mediated via the CCK_{2R}. A further difference in the signalling induced by the two peptides was apparent in the involvement of the COX-2 system. We confirmed the COX-2 dependence of the anti-apoptotic effect of G-17 (Abdalla et al. 2004) but the there was no involvement of this pathway in the G-Gly effect. The anti-apoptotic effect of G-Gly was unaltered by pre-treatment with either the non-selective cyclooxygenase inhibitor indomethacin, the commonly used experimental COX-2 inhibitor NS-398 or the even more COX-2 selective agent SC 791 and G-Gly had a similar degree of effect in BIC-1 cells, which do not express COX-2 (Souza et al. 2000, Buttar et al. 2002a,b). The combination of G-Gly and gastrin-17 had a greater than additive anti-apoptotic effect. These data are all consistent with the two gastrin-derived peptides mediating their anti-apoptotic effects via separate pathways. As gastrin (CCK_{2}) receptors are not expressed in all OAC (expression in 0, 58 and 75% has been variously reported (Clerc et al. 1997, Haigh et al. 2003, Abdalla et al. 2004)), and CCK_{2R} expression was only found in 60% of samples from dysplastic BO using RT-PCR (Abdalla et al. 2004), G-Gly may still be an important anti-apoptotic factor in tissues that are not gastrin-17 responsive.

The major hypothesis underlying our study is that the use of potent acid-suppressive medications for gastroesophageal reflux disease leads to a degree of negative feedback hypersecretion of gastrin peptides from the gastric antrum. Several other studies have begun to explore this area, suggesting that the released amidated gastrin may promote the growth of OAC cells (Sanduleanu et al. 1999, Abdalla et al. 2004, Harris et al. 2004, Konturek et al. 2004). Our data extend these by showing that G-Gly is also an anti-apoptotic factor that may promote the progression of OAC but also that as G-Gly inhibits apoptosis in non-malignant Barrett’s oesophageal cells, it may promote the development of cancer from non-neoplastic epithelium. Local expression of gastrin has been documented in both BO and OAC; it remains unclear whether this represents a terminally differentiated endocrine cell population or ectopic production by tumour cells (Buchan et al. 1985, Abdalla et al. 2004, Konturek et al. 2004). It remains possible that local production of G-Gly and other gastrins are also paracrine and autocrine growth factors for BO. Further studies both in vivo and in vitro as well as animal models will be required to unravel the complex inter-relationship between acid-reflux, acid-suppression and secretion of gastrins in the pathogenesis of BO and OAC.

Considerable data have implicated COX-2 in BO and OAC: COX-2 expression increases in BO with the development of dysplasia and cancer (Cheong et al. 2003), COX-2 inhibitors reduce proliferation and induce apoptosis in OAC cell lines in culture (Buttar et al. 2002a) and COX-2 inhibitors reduce progression in animal models of BO (Buttar et al. 2002b). However, the expression of COX-2 and prevention of disease by COX-2 inhibitors are not universal and factors such as G-Gly driving the avoidance of apoptosis by alternative pathways could contribute to progression of cancer. Further exploration of the role of G-Gly and the possible therapeutic roles of small molecule JAK2/STAT3 inhibitors in BO and OAC deserve further study.

The general degree of suppression of apoptosis in both the serum-starvation and camptothecin-induced systems (22–46%) were of the same order of magnitude reported for the other peptide anti-apoptotic factors in a variety of systems. Amidated gastrin reduced serum-starvation induced apoptosis by 1/3 in pancreatic AR42J cells (Ramamoorthy et al. 2004), leptin reduced serum-starvation induced apoptosis by 20–40% and celecoxib-induced apoptosis by 48% in colon cancer cells (Rouet-Benzineb et al. 2004, Hoda et al. 2007, Ogunwobi & Beales 2007b) and by 39% in serum-starved oesophageal OE33 cells (Beales & Ogunwobi 2007). Gastrin-17 and G-Gly had similar effects (30–40%).
reduction in serum-free apoptosis in IMGE-5 cells (He et al. 2008) and G-Gly reduced serum-free apoptosis by 44% in HT-29 cells (Beales & Ogunwobi 2006). IL-6 reduced bile acid induced apoptosis in oesophageal SEG-1 cells by 40% (Dvorak et al. 2007), leptin reduced both serum-free (30%) and docetaxel-induced (39%) apoptosis in MCF-7 breast cancer cells (Jiang et al. 2008) and serum-free apoptosis by 25% in human SH-SV5Y neuroblastoma cells (Russo et al. 2004), while transient acid exposure reduced OE33 cell apoptosis by 22% (Beales & Ogunwobi 2007). The same general degree of inhibition of apoptosis, always significantly less than complete inhibition, has also been reported using anti-gastrin immunoneutralisation, receptor antagonists and RNA interference and anti-sense strategies (Grabowska et al. 2007), confirming that gastrin-peptides are but one of a complex array of growth factors and signalling pathways activating survival signals in cancer cells.

In this study, we have concentrated on the signalling pathways activated by G-Gly and it will be important to establish how these interact with other putative growth factors for BO and OAC such as transient acid exposure, bile acids, leptin and IL-6 as well as adiponectin, which appears to inhibit OAC proliferation. In conclusion, we have described the anti-apoptotic effect of G-Gly in both OAC cells and non-malignant Barrett’s cells. This effect is mediated via mechanisms independent of both the CCK2R and COX-2 but involves immediate activation of JAK2. Subsequent activation of STAT3 leads to increased mRNA and protein levels of the anti-apoptotic proteins survivin and BCL2L1.

Declaration of interests

Neither author has any conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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