Gastrin increases mcl-1 expression in type I gastric carcinoid tumors and a gastric epithelial cell line that expresses the CCK-2 receptor

D. M. Pritchard,1 D. Berry,1 S. M. C. Przemeck,1 F. Campbell,2 S. W. Edwards,3 and A. Varro4

1Division of Gastroenterology, School of Clinical Sciences, University of Liverpool; 2Department of Pathology, Royal Liverpool and Broadgreen University Hospitals National Health Service Trust, Liverpool; and 3School of Biological Sciences and 4Division of Physiology, School of Biomedical Sciences, University of Liverpool, Liverpool, United Kingdom

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Gastrin increases mcl-1 expression in type I gastric carcinoid tumors and a gastric epithelial cell line that expresses the CCK-2 receptor. Am J Physiol Gastrointest Liver Physiol 295: G798–G805, 2008. First published August 21, 2008; doi:10.1152/ajpgi.00015.2008.—Elevated serum concentrations of the hormone gastrin are associated with the development of gastric carcinoid tumors, but the mechanisms of tumor development are not fully understood. We hypothesized that the antipapoptotic effects of gastrin may be implicated and have previously investigated the role of antipapoptotic members of the bcl-2 family of proteins. AGS-G8 human gastric carcinoma cells stably transfected with the CCK-2 receptor were used to assess changes in the expression of bcl-2 family members following gastrin treatment and the function of mcl-1 during apoptosis was investigated by use of small-interfering RNA (siRNA). Treatment of AGS-G8 cells with 10 nM gastrin for 6 h caused maximally increased mcl-1 protein abundance. Gastrin-induced mcl-1 expression was inhibited by the transcription inhibitor actinomycin D and by the protein synthesis inhibitor cycloheximide. Downstream signaling of mcl-1 expression occurred via the CCK-2 receptor, protein kinase C, and MAP kinase pathways, but not via PI 3-kinase. Transfection with mcl-1 siRNA significantly suppressed mcl-1 protein expression and abolished the antipapoptotic effects of gastrin on serum starvation-induced apoptosis. Mcl-1 protein expression was also specifically increased in the type I enterochromaffin-like cell carcinoid tumors of 10 patients with autoimmune atrophic gastritis and hypergastrinemia. Gastrin therefore signals via the CCK-2 receptor, protein kinase C, and MAP kinase pathways, but not via PI 3-kinase. Transfection with mcl-1 siRNA significantly suppressed mcl-1 protein expression and abolished the antipapoptotic effects of gastrin on serum starvation-induced apoptosis.

Apoptosis or programmed cell death is a tightly regulated cellular process and several families of proteins are thought to be involved. Gastrin has previously been shown to exert antipapoptotic effects in a number of cell lines including cells of the pancreas (e.g., AR42J) and gastric (e.g., IMGE-5) origin, and gastrin-induced alterations in the expression of several members of the bcl-2 family of proteins have also previously been demonstrated (10, 11, 16, 31–33). Increased immunohistochemical expression of antipapoptotic bcl-2 has also been demonstrated in biopsies obtained from human subjects with atrophic gastritis (23) and type I gastric carcinoid tumors (1).

We have therefore hypothesized that gastrin may regulate apoptosis by specifically altering the expression of individual members of the bcl-2 family of proteins. Mcl-1 is one antipapoptotic member of this family of proteins and it is thought to inhibit apoptosis by inhibiting mitochondrial cytochrome c release (reviewed in Ref. 24). To assess whether gastrin can alter the expression of antipapoptotic members of the bcl-2 family of proteins we used a human gastric epithelial cell line that has been stably transfected with the CCK-2 receptor and in which gastrin has previously been shown to exert multiple effects (AGS-G8) (28, 29, 38). This cell line is derived from a gastric adenocarcinoma and is not of neuroendocrine origin. Significantly increased expression of antipapoptotic mcl-1, a protein not previously demonstrated to be gastrin regulated, was demonstrated and subsequent experiments were therefore
performed to investigate the signaling mechanisms involved and the functional consequences of this observation in the cell line. We subsequently investigated whether mcl-1 expression was also increased in gastric epithelial cells that express the CCK-2 receptor in vivo. To do this we used endoscopic biopsies obtained from human patients with hypergastrinemia-associated type I gastric ECL cell carcinoid tumors.

MATERIALS AND METHODS

Materials. Ro-32-0432, PD-98059, LY-294002, and wortmannin were all from Calbiochem (Nottingham, UK), YM022 was from Tocris Bioscience (Bristol, UK), and gastrin-17 was from Bachem (St. Helens, UK). All other routine chemicals were from Sigma (Poole, UK), unless stated.

Tissue culture. The AGS human gastric carcinoma cell line and a transfectant stably expressing the CCK-2 receptor (AGS-GR) were used as previously described (35, 38). Cells were cultured in Ham’s F12 medium supplemented with 10% fetal calf serum (GIBCO, Paisley, UK), 2 mM L-glutamine, and 1% penicillin-streptomycin at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂.

Western blotting. Protein extracts were prepared and electrophoresed on 10% SDS-polyacrylamide gels followed by transfer onto nitrocellulose membrane (Protran, Schleicher & Schuell). Nonspecific antibody binding was blocked by incubating the membrane in 1% nonfat milk in PBS-Tween-20. Membranes were incubated with the following primary antibodies: mouse monoclonal anti-mcl-1 antibody (Calbiochem) at a dilution of 1:200, mouse monoclonal anti-bcl-2 (DakoCytomation, Cambridge, UK) at a dilution of 1:100, mouse monoclonal anti-bcl-X₀ (Calbiochem) at a dilution of 1:25, or mouse monoclonal anti-actin antibody (Neomarkers, Freemont, CA) at a dilution of 1:1,000. The secondary antibody was horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins from DakoCytomation. Membranes were developed by using Supersignal (Pierce, Tattenhall, UK) and chemiluminescence was detected by use of a Fluor-S molecular imager (Bio-Rad, Hertfordshire, UK). Densitometry was performed using Quantity One software and results were normalized to the expression of actin.

Transfection. AGS-Gₖ cells were transfected with siGENOME SMARTpool reagent M-004501-04 against human mcl-1, or siCONTROL nontargeting small-interfering RNA (siRNA) pool D001206-13 (both from Dharmacon) for 24 h according to the manufacturer’s instructions and with the use of DharmaFECT 1 transfection reagent. Medium was then changed to serum-free medium and cells were treated with 10 nM gastrin-17 for 6 h prior to harvest and assessment of apoptosis.

Assessment of apoptosis. Apoptosis was assessed by counting the numbers of floating cells (>95% with apoptotic morphology follow- ing staining with Hoechst dye) and the number of adherent cells (>95% with normal morphology following staining with Hoechst dye) as previously described and validated for this cell line (37). Apoptosis was also independently assessed by the Caspase-Glo 3/7 assay (Promega) by following the manufacturer’s instructions and using 96-well plates seeded with 10,000 cells per well.

Patients. Pinch biopsies of gastric mucosa were obtained by a single endoscopist (D. M. Pritchard) from 10 adult patients attending for routine diagnostic endoscopy for the investigation of pernicious anemia or surveillance of gastric carcinoid tumors. Immediately prior to endoscopy, a fasting venous blood sample was obtained and serum was assayed for gastrin concentration. Serum was also assayed for the presence of anti-Helicobacter pylori IgG and antiinflammatory parietal cell antibodies at the microbiology and immunology laboratories of Royal Liverpool and Broadgreen University Hospitals National Health Service Trust as part of each patient’s routine diagnostic investigations. During endoscopy, two to four biopsies were obtained from each of antral and corpus mucosa and from any nodules; these were subse-
tidyinositol 3-kinase (PI 3-kinase) inhibitors LY-294002 and wortmannin caused no significant changes in gastrin-induced mcl-1 expression (Fig. 3, B and C). These studies suggest that following binding to the CCK-2 receptor, gastrin signals via protein kinase C and MAP kinase pathways, but not via the PI 3-kinase pathway to cause increased mcl-1 protein abundance.

Mcl-1 siRNA transfection of AGS-GR gastric epithelial cells abolishes the antiapoptotic effects of gastrin. Transfection of AGS-GR cells with siRNA targeted against human mcl-1 caused a significant reduction in basal mcl-1 protein expression, and no increase in mcl-1 expression was observed following treatment with 10 nM gastrin for 6 h (Fig. 4, A and B). By contrast, transfection of AGS-GR cells with control siRNA had no significant effect on basal or gastrin-induced mcl-1 protein abundance compared with untransfected AGS-GR cells (Fig. 4, A and B). Following transfection, cells were transferred into serum-free medium for 6 h to induce apoptosis. Treatment with 10 nM gastrin significantly inhibited serum starvation-induced apoptosis in control siRNA-transfected AGS-GR cells. However, 10 nM gastrin treatment of mcl-1 siRNA-transfected AGS-GR cells did not result in inhibition of serum starvation-induced apoptosis but actually caused a significant increase in the percentage of apoptotic cells (Fig. 4C). Apoptosis was also assessed by another independent technique, namely assessment of effector caspase activity using the Caspase-Glo 3/7 assay. Using this method, we demonstrated a significant 25% decrease in caspase 3/7 activity in control siRNA-transfected cells following 6 h treatment with 10 nM gastrin in the presence of serum-free medium (P < 0.01, n = 8), whereas mcl-1 siRNA-transfected cells conversely showed a significant 16% increase in caspase 3/7 activity following the same treatment (P < 0.05, n = 8).

Mcl-1 is expressed in type I ECL cell gastric carcinoid tumors. We hypothesized that the gastrin-induced increase in mcl-1 protein abundance observed in AGS-GR cells in vitro also occurred in vivo in the stomach of human patients with hypergastrinemic states. We obtained endoscopic biopsies from the gastric corpus and nodules of 10 patients (8 women, mean age 57.7 yr, range 43–80 yr) who all had atrophic gastritis, achlorhydria, marked hypergastrinemia, and evidence of type I gastric carcinoid tumor development (Table 1). In all cases (although not absolutely confirmed in case 10), the gastric atrophy was thought to have an autoimmune cause, because of the presence of positive antigastic parietal cell antibodies and/or vitamin B12 deficiency, with absence of H. pylori by histological analysis in all cases and in 8 of the 10 cases by serology. No patients were taking acid-suppressing medications. In addition, gastric corpus biopsies were also obtained from four control patients (2 women, mean age 44.8 yr, range 27–60 yr), who all had normal esophagogastroduodenoscopy, normal gastric histology, normogastrinemia (fasting serum gastrin <20 pM in each case), and absent H. pylori infection by histological and serological criteria and who were not taking acid-suppressing medications.

Tissue sections were stained with H and E and immunostained for mcl-1 and chromogranin A and were then assessed...
by an experienced specialist gastrointestinal pathologist (F. Campbell). No positive signal was detected in control slides processed without the addition of primary antibody. Weak diffuse cytoplasmic mcl-1 expression was observed in a few cells in the upper portion of the gastric corpus glands of the control normogastrinemic patients (see Fig. 5A with chromogranin A immunohistochemistry of a serial section shown in Fig. 5B) as described in previous reports (18). Six of the 10 hypergastrinemic patients had microscopically confirmed carcinoid tumor in the biopsy specimens obtained. In five of these six cases there was distinct cytoplasmic mcl-1 expression in the gastric carcinoid tumor cells (see Fig. 5C for representative mcl-1 staining, with chromogranin A immunohistochemistry of a serial section shown in Fig. 5D), and one case showed weak cytoplasmic mcl-1 expression in the carcinoid tumor. All 10 cases additionally showed the presence of ECL cell hyperplasia in both linear and nodular forms. Eight of the 10 patients showed distinct cytoplasmic mcl-1 expression in areas of nodular ECL cell hyperplasia (see Fig. 5, E and G, for representative mcl-1 staining and Fig. 5, F and H, for chromogranin A staining of serial histological sections). The remaining two patients had weak mcl-1 staining in areas of nodular ECL cell hyperplasia.

We also immunostained serial sections of each sample for cleaved caspase 3 as a marker of apoptosis. Although occasional cleaved caspase 3-positive cells were seen in the gastric mucosa (Fig. 6, B and F), virtually no cleaved caspase 3-positive cells were seen in areas of nodular ECL cell hyperplasia (Fig. 6E) or of gastric carcinoid tumor (<1 cleaved caspase 3-positive cell per 1,000 cells in all 10 patients).

Fig. 3. A and B: representative Western blots. C: densitometric analysis of individual Western blots from 3–6 separate experiments showing effects of treatment with the CCK-2 receptor antagonist YM022 (10 nM), the protein kinase C (PKC) inhibitor Ro-32-0432 (1 μM), the MAP kinase (MAPK) inhibitor PD-98059 (20 μM), and the PI 3-kinase (PI3K) inhibitors LY-294002 (20 μM) and wortmannin (500 nM) on mcl-1 expression in AGS-G_6 cells following treatment for 6 h with 10 nM gastrin-17. *P < 0.05, **P < 0.01 compared with gastrin treatment alone.

Fig. 4. Representative Western blot (A) and densitometric analysis (B) of individual Western blots from 3 separate experiments showing effects of 24 h transfection with mcl-1 or control small-interfering RNA (siRNA) on mcl-1 protein expression in AGS-G_6 cells following treatment for 6 h with 10 nM gastrin-17. *P < 0.05 compared with gastrin-treated control siRNA-transfected cells. C: percentage apoptosis of AGS-G_6 cells transected for 24 h with either mcl-1 or control siRNA, then treated for 6 h with serum-free medium in the absence or presence of 10 nM gastrin-17. *P < 0.05 between gastrin-treated and non-gastrin-treated cells following siRNA transfection.
Serial sections of each sample were also immunostained for bcl-2. There appeared to be more bcl-2-positive cells in all 10 hypergastrinemic patients samples compared with the four normogastrinemic control patients (data not shown). However, whereas mcl-1 expression appeared to occur predominantly in areas of ECL cell hyperplasia and of carcinoid tumor (Fig. 6D), bcl-2 expression in all 10 patients was predominantly seen in lymphoid and stromal cells rather than in epithelial cells (Fig. 6, C and G).

**DISCUSSION**

In summary, we have demonstrated that gastrin induces mcl-1 protein expression in AGS-GR cells in a time-, dose-, and concentration-dependent manner.

**Table 1. Results of mcl-1 immunohistochemistry in 10 patients with type I gastric carcinoid tumors secondary to atrophic gastritis and hypergastrinemia**

<table>
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<th>Patient Number</th>
<th>Sex</th>
<th>Age</th>
<th>Anti GPC antibody</th>
<th>Vitamin B12 Replacement</th>
<th>H. pylori Serology</th>
<th>H. pylori on Histology</th>
<th>Fasting Serum Gastrin, pM</th>
<th>Number of Nodules</th>
<th>Maximum Nodule Size, mm</th>
<th>Atrophic Gastritis</th>
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Mcl-1 immunohistochemistry was graded as ++ (strong), + (distinct), +/− (weak), or − (absent). GPC, gastric parietal cell; M, male; F, female; ND, blood test not done; N/A, not applicable. *Result of antiparietal cell antibody could not be reported because of the presence of positive antimitochondrial antibody.

Fig. 5. Immunohistochemical analysis of mcl-1 (A, C, E, G) and chromogranin A (B, D, F, H) expression in gastric biopsies from human patients with no pathology (A and B) or autoimmune atrophic gastritis, hypergastrinemia, and either nodular ECL cell hyperplasia (E, F, G, H) or frank gastric carcinoid tumors (C, D). Low-power (×10 objective) (A, B, E, F) and high-power (×40 objective) (C, D, G, H). Boxes shown in E and F are shown at higher magnification in G and H.
and CCK-2-dependent manner (Fig. 1). Signal transduction occurs via protein kinase C and MAP kinase pathways, but not via PI 3-kinase (Fig. 3). Gastrin-induced mcl-1 expression is functionally important, since transfection with mcl-1 siRNA abolished the antiapoptotic effects of gastrin in this cell line (Fig. 4). Finally, we have demonstrated specific mcl-1 expression in the ECL cell nodular hyperplasia and type I carcinoid tumors of human subjects with autoimmune atrophic gastritis, achlorhydria, and hypergastrinemia (Fig. 5, Table 1).

Gastrin has previously been shown to exert antiapoptotic effects in a number of cell lines, and the involvement of various downstream mediators has been demonstrated in these studies (10, 11, 16, 31–33). However, other studies, which initially appear to be contradictory, have demonstrated pro-apoptotic effects of gastrin. For example, increased gastric epithelial apoptosis has been reported in hypergastrinemic INS-GAS mice following both γ-radiation and Helicobacter infection (4, 30), in H2 antagonist-treated hypergastrinemic African rodents Mastomys (Praomys natalensis) (15) and in H. pylori-infected humans with moderate degrees of hypergastrinemia (30). However, most of these reports have studied normal untransformed gastric epithelial cells in vivo. We have previously therefore suggested that the effects of gastrin on apoptosis may be cell type specific and may actually differ between transformed and untransformed cell types (30). It is also possible that the effects of gastrin on apoptosis in the normal gastric epithelium in vivo may not be a direct result of hypergastrinemia but may be due to indirect effects resulting from gastrin-induced alterations in other proapoptotic growth factors. Further studies are required to clarify the precise mechanisms involved. In the present study we have specifically assessed the direct effects of gastrin on transformed CCK-2 receptor-bearing cells in vitro and in vivo.

Gastrin-induced changes in the expression of several members of the bcl-2 family of proteins have previously been reported. Gastrin-induced inhibition of apoptosis in AR42J pancreatic adenocarcinoma cells has previously been shown to be associated with the phosphorylation and subsequent inhibition of proapoptotic bad (31). A similar mechanism has also been demonstrated in IMGE-5 gastric epithelial cells as a result of gastrin activation of Rac/Cdc42/PAK (11). Gastrin has also been shown to inhibit proapoptotic bax expression and to induce antiapoptotic bcl-xL expression in IMGE-5 cells via a Rho/ROCK pathway (11). Gastrin has also been reported to increase the expression of bcl-2 and survivin in KATO III gastric adenocarcinoma cells (16). Increased immunohistochemical bcl-2 expression has been demonstrated in autoimmune as well as H. pylori-associated atrophic gastritis (23) and in hypergastrinemia-associated ECL cell gastric carcinoid tumors (1, 14). We also demonstrated increased bcl-2 expression in patients with atrophic gastritis and hypergastrinemia, but this bcl-2 expression appeared to occur predominantly in stromal and lymphoid cells rather than in ECL cells (Fig. 6). Increased gastric bcl-2 expression may therefore contribute toward the development of gastric carcinoid tumors, but in view of the lack of bcl-2 expression by ECL cells the effects may not be due to direct inhibition of apoptosis in this cell type. Bcl-2 is also unlikely to contribute toward the antiapoptotic effects of gastrin described in AGS-G8 cells in this paper, since we could detect no expression of bcl-2 protein in this cell type.
tein expression has also previously been detected in human gastric glands (18). Immunohistochemical mcl-1 protein expression observed at 6 h was no longer seen at 24 h (Fig. 1). Mcl-1 is weakly expressed in the pit region of normal human gastric glands (18). Immunohistochemical mcl-1 protein expression has also previously been detected in ~70% of patients with gastric adenocarcinoma and expression has been shown to be associated with a poorer prognosis (17, 20). CagA expressing strains of H. pylori have also been shown to increase mcl-1 expression in the gastric pits of infected Mongolian gerbils (25). Interleukin-6 has also been shown to induce mcl-1 expression in AGS gastric carcinoma cells and this was shown to protect cells against apoptosis induced by reactive oxygen species (19). Treatment of gastric carcinoma cell lines with antisense oligonucleotides directed toward mcl-1 has also been shown to significantly increase the apoptosis induced in these cell lines by chemotherapeutic drugs (39).

Cultured isolated rat ECL cells have been shown to undergo apoptosis following addition of proinflammatory cytokines such as TNF-α and interleukin-1β (13, 21, 22). This process was inhibited by several growth factors including TGF-α and basic FGF (22). However, in these studies, although gastrin also appeared to partially inhibit cytokine-induced apoptosis, the results did not reach statistical significance and only a low concentration of gastrin (10 pM) was assessed (22). It is, however, possible that although direct effects were not demonstrated in isolated rat ECL cells at a single low concentration of gastrin, significantly elevated serum concentrations of this hormone may regulate ECL cell apoptosis in vivo when other cell types are also present.

In conclusion, therefore, we have made the novel observation that gastrin increases the expression of antiapoptotic mcl-1 protein in a gastrin-responsive CCK-2 receptor expressing gastric epithelial cell line. In addition, increased mcl-1 expression has been demonstrated in ECL cell tumors from human hypergastrinemic patients. Gastrin-induced mcl-1 expression may therefore be one factor leading to inhibition of apoptosis in gastric ECL cells, thus promoting the development of gastric carcinoid tumors.

GRANTS

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