Gastrin-induced gastric adenocarcinoma growth is mediated through cyclin D1

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Gastrin-induced gastric adenocarcinoma growth is mediated through cyclin D1. Am J Physiol Gastrointest Liver Physiol 285: G217–G222, 2003. First published February 26, 2003; 10.1152/ajpgi.00516.2002.—Gastrin is a gastrointestinal (GI) peptide that possesses potent trophic effects on most of the normal and neoplastic mucosa of the GI tract. Despite abundant evidence for these properties, the mechanisms governing gastrin-induced proliferation are still largely unknown. To elucidate the mechanisms by which gastrin might influence mitogenesis in gastric adenocarcinoma, we analyzed its effects on the human cell line AGS-B. Amidated gastrin (G-17), one of the major circulating forms of gastrin, induced a concentration-dependent increase in [3H]thymidine incorporation of cells in culture, with the maximum effective concentration occurring with 20 nM G-17. This effect was significantly attenuated by the gastrin-specific receptor antagonist L-365260. In addition, we found that G-17 induced a significant increase in the levels of cyclin D1 transcripts, protein, and promoter activity. The results of these studies indicate that gastrin appears to exert its mitogenic effects on gastric adenocarcinoma, at least in part, through changes in cyclin D1 expression.

Gastrin-17; AGS-B cells; proliferation

Although its incidence continues to decline in Western nations, gastric adenocarcinoma remains the second most common lethal malignancy in the world (9). The development of gastric and other malignancies of the gastrointestinal (GI) tract requires a multistep process involving genetic mutations combined with environmental cofactors, whereby normal epithelial cells undergo metaplastic and dysplastic transformation, followed by proliferation and eventual histological progression to neoplasia (8, 21, 25). The discovery of the bacterium Helicobacter pylori and its crucial role in the development of GI disorders, including gastroduodenal ulcer and gastric adenocarcinoma (3, 32, 36), provided an important impetus for studies aimed at elucidating the pathogenesis of this common malignancy. Infection with H. pylori leads to numerous alterations in gastric physiology, including the development of hypergastrinemia.

The polypeptide hormone gastrin is still considered the most potent substance known to stimulate gastric acid secretion (41). However, another biological property attributed to gastrin is its trophic effect on GI mucosa (14, 30). Numerous studies have demonstrated that gastrin stimulates not only growth of normal GI epithelial cells but also malignant cell lines of colorectal, gastric, and pancreatic etiology (2, 7, 18, 19, 21, 31, 39). A recent large epidemiological study by Thorburn et al. (34) found that prolonged hypergastrinemia comprises a risk factor for the development of colorectal cancer. Moreover, studies by Wang et al. (38) using transgenic mice overexpressing amidated gastrin (G-17) demonstrated a synergistic effect between this important peptide and H. pylori infection in the progression of gastric adenocarcinoma. These studies all suggest a potential role for gastrin in the pathophysiology of these malignancies of the GI tract, whereby elevated levels of circulating gastrin could provide a stimulus for the growth of these tumors.

Despite abundant evidence that gastrin may play an integral role in promoting tumor growth in the stomach, as well as malignancies in the GI tract, the precise mechanisms by which gastrin mediates its trophic properties have not been elucidated. In addition, previous studies aimed at determining a potential role for gastrin in gastric carcinogenesis have yielded conflicting results. These studies involved either the measurement of serum gastrin concentrations in patients with gastric cancer or conversely a determination of the number of individuals with hypergastrinemia who have cancer. Unfortunately, these studies have ignored the fact that gastrin is not mutagenic but rather is mitogenic. This important hormone probably does not cause malignancies to arise, but it does stimulate the growth of preexisting gastric tumors. Size appears to comprise a factor in determining the biological behav-
ior of these tumors, and any factor that enhances their growth could thereby incite their malignant degeneration.

One potential mechanism for the development of gastric and other GI malignancies involves the multifunctional cytoplasmic protein β-catenin, which under normal physiological circumstances plays a major role in cell-cell adhesion (20). In addition to its role in cell-cell adhesion, β-catenin is also a pivotal component of the Wnt/Wingless (Wg) signaling pathway, which plays a key role in an array of developmental processes (6, 40). Postnatally, mutations of the adenomatous polyposis coli gene, which occur not only in approximately 80% of sporadic colorectal carcinomas but also in other GI malignancies, result in a truncated protein incapable of forming a complex with β-catenin. As a result, phosphorylation of β-catenin by the inhibitory complex does not occur, causing β-catenin to accumulate and the Wg pathway to be activated (20). In addition, the increased cytoplasmic β-catenin leads to its association with members of the T cell factor/lymphocyte enhancer binding factor (TCF/LEF) family of transcription factors (20). This β-catenin-TCF/LEF complex is translocated to the nucleus, where it stimulates the transcription of a variety of downstream target genes including c-myc and cyclin D1 (6, 11, 20, 29, 33). In particular, cyclin D1, an important cell cycle regulator, has been reported to be upregulated in human colorectal tumors with adenomatous polyposis coli (6, 11, 20, 29, 33). In particular, cyclin D1, an important cell cycle regulator, has been reported to be upregulated in human colorectal tumors with altered β-catenin expression (37). Abnormalities in cyclin D1 expression constitute one of many possible mechanisms of growth may include the dysregulation of cyclin D1 expression.
onto PVDF membranes and incubated with primary antibodies to cyclin D1 (Pharmingen, Chicago, IL) and β-catenin (Transduction Laboratories, Lexington, KY). After incubation with the primary antibodies, membranes were washed thoroughly in TBS-Tween buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20), and appropriate secondary antibodies conjugated with horseradish peroxidase were used to detect the primary antibodies. Immunoreactive bands were visualized by chemiluminescence in signaling solution (Pierce, Rockford, IL).

**Cyclin D1-luciferase assay.** For cyclin D1 promoter analysis, AGS-B cells were first transfected in the presence of Lipofectamine (GIBCO BRL) with the full-length cyclin D1 promoter-luciferase (29) and renilla-luciferase constructs for 5 h, followed by an overnight recovery of the cells in serum-containing medium. Cells were then split into six-well plates, serum starved, and treated in triplicate either with vehicle or with different concentrations of G-17 for 4 and 24 h before being harvested for luciferase assays. Equal volumes of lysate (10 μl) were used to assess cyclin D1-dependent luciferase activity by using the luminometer and were normalized to renilla activity. Respective substrates used in these studies were purchased from Promega (Madison, WI). Each transfection was repeated at least three times, and the samples were analyzed in duplicate.

**Statistical analysis.** Using SAS 8.0, we performed one-way ANOVA to compare various culture conditions, followed by Tukey’s procedure for paired comparisons. Statistical significance was assigned if \( P < 0.05 \).

**RESULTS**

**Changes in cell morphology in response to G-17 incubation of AGS-B cells.** To determine whether gastrin might affect cellular morphology and cell-cell adhesion, AGS-B cells were incubated in the presence of 10 nM G-17 for 4 h. After staining with rhodamine phalloidin, a fluorescent ligand that binds to actin filaments, alterations were noticed at 4 h of G-17 treatment. Cells assumed a spindle shape and appeared to display more intense staining of filamentous actin compared with cells before incubation (Fig. 1).

**G-17 increases proliferation in AGS-B cells.** To examine whether gastrin augments cell proliferation, AGS-B cells were grown in the presence of either vehicle alone, with 1 and 20 nM G-17, or with 20 nM G-17 and the gastrin-specific receptor antagonist L-365260. A fourfold increase in \([3H]\)thymidine uptake was evident after 2 days in cells treated with G-17, an effect that was attenuated significantly by incubation in the presence of L-365260 (Fig. 2).

**G-17 induces a concentration-dependent induction of cyclin D1 and β-catenin mRNA in AGS-B cells.** To determine the molecular mechanisms governing the trophic effects of gastrin in gastric adenocarcinoma, AGS-B cells were used to determine whether cyclin D1, a protein important for G1-S transition, was involved in the process. In addition, β-catenin, an important coactivator of Wnt signaling pathway (which has been implicated in increased transcription of cyclin D1), was also analyzed. Total RNA samples isolated from the AGS-B cells treated with increasing concentrations of G-17 (10, 20, and 100 nM) in the presence or absence of 1 μM L-365260 were analyzed by Northern blot analysis. In response to the incubation of AGS-B cells in the presence of G-17, both β-catenin and cyclin D1 transcripts were increased, effects that were inhibited by L-365260 (Fig. 3), suggesting that enhanced proliferation induced by gastrin may involve these two trophic factors.
G-17 causes time-dependent induction of cyclin D1 and β-catenin mRNA in AGS-B cells. AGS-B cells were incubated with or without 10 nM G-17, and total RNA was extracted at different time intervals (1, 4.5, and 24 h). Northern blot analyses were performed by using radiolabeled cyclin D1 and β-catenin cDNA probes; GAPDH was used as a loading control (Fig. 4A). When normalized to GAPDH (Fig. 4B), a significant increase in the levels of cyclin D1 and β-catenin mRNA was detected in RNA extracted from cells that were incubated in the presence of G-17 at the time points examined.

G-17-mediated induction of cyclin D1 protein levels in the gastric carcinoma cell line AGS-B. In addition to mRNA induction, to determine whether gastrin also augments protein levels of these components, Western blot analysis was performed on protein extracts isolated from AGS-B cells incubated for 4.5 h in the absence or presence of 10 nM G-17. G-17 induced a significant induction of cyclin D1, whereas it did not increase β-catenin protein levels at 4.5 h (Fig. 5).

G-17 significantly increases cyclin D1 promoter activity. To determine whether gastrin directly affects cyclin D1 transcription, cyclin D1 promoter activity was analyzed. AGS-B cells were transfected with full-length (−1745) cyclin D1 promoter-luciferase (−1745CD1Luc) and renilla-luciferase constructs and were analyzed for the effects of gastrin on promoter activity. Following normalization of cyclin D1-dependent firefly luciferase to renilla luciferase, a fourfold induction of promoter activity was observed in G-17-treated samples, an effect that was abolished with L-365260 (Fig. 6). In a separate study, we examined the effects of G-17 on LEF-1-dependent transcriptional activity in AGS-B cells. In contrast to its effects on cyclin D1 promoter activity, G-17 did not enhance LEF-1 activity (data not shown).

DISCUSSION

The development of malignancies of the GI tract results from a complicated process that includes several
eral genetic alterations. As mentioned above, one potential mechanism involves the multifunctional cytoplasmic protein β-catenin, which normally is intimately involved in the process of cell-cell adhesion (20) but has also recently been identified as a critical oncogene. The capacity of β-catenin to form a complex with members of the TCF/LEF family induces stimulation of the transcription of a variety of proliferation factors, including intermediary and functional targets such as c-jun and fra-1 and c-myc, cyclin D1, matrix metalloproteinase-7, and p53, respectively (6, 11, 20, 29, 33).

In the present study, the incubation of AGS-B cells in the presence of G-17 not only caused morphological changes but also altered the growth characteristics of the cells. Recent studies by Kirton et al. (17) have demonstrated that gastrin stimulates the movement of parietal cells along the gastric gland axis. Moreover, using AGS cells, Pagliocca et al. (23) reported that stimulation of the CCK-2 (gastrin) receptor promoted branching morphogenesis. These studies, as well as our observations, all suggest that β-catenin, a dual modulator of cell-cell adhesion and oncogenic events, or its downstream targets, may be involved in mediating these processes. Interestingly, in the present study, the upregulation of β-catenin transcripts by G-17 did not extend to the protein level, as measured by Western blot analysis. We also determined that G-17 did not enhance LEF-1-dependent transcriptional activity in these cells. The reasons for this divergence are not clear, but several possibilities exist. One possibility is that the processing of β-catenin mRNA to protein might be disrupted in this cell line. Another possible explanation is that the components associated with ubiquitin-related degradation mechanism, which has been reported to rapidly degrade β-catenin protein (1), or any inhibitor of β-catenin stability may be enhanced as a result of stable overexpression of the CCK-2 (gastrin) receptor. Under such conditions, β-catenin would have been susceptible to degradation at the time point examined in the present studies. Future studies in which the proteasome machinery is inhibited will be necessary to determine whether β-catenin is more susceptible to degradation in these cells. It is also possible that a barely discernible alteration in the total β-catenin protein pool resulted in undetectable differences due to saturated basal levels. Finally, the induction of cyclin D1, one of the target genes of β-catenin-dependent transcription, by gastrin might be mediated directly by Sp1 and/or cAMP-responsive element sites in the cyclin D1 promoter, independent of any effect on β-catenin expression. Hocker et al. (12) recently reported that in AGS-B cells gastrin-dependent transcriptional response of chromogranin A in enterochromaffin-like cells is mediated by Sp1 and cAMP-responsive element binding sites in its promoter.

Gastrin-induced upregulation of cyclin D1, an important component involved in G1-S transition, suggests that this peptide may enhance the proliferation of AGS-B cells through constant turnover of cell cycle machinery. Chen et al. (4) recently reported that the inhibition of cyclin D1 by L-365260 directly by Sp1 and/or cAMP-responsive element binding sites in its promoter.

In conclusion, the results of the present studies have demonstrated that gastrin stimulated the proliferation of gastric adenocarcinoma cells and incited a concomitant increase in β-catenin mRNA levels without any detected increase at the protein level. Western blot analysis of AGS-B cell protein extracts did demonstrate significant changes in cyclin D1 transcripts and protein levels in response to the incubation of AGS-B with G-17. These observations indicate that gastrin appears to exert its mitogenic effects on gastric adenocarcinoma, at least in part, via changes in cyclin D1 expression. Further studies will be required to elucidate the precise intracellular and molecular pathways that mediate the trophic properties of gastrin in gastric adenocarcinoma.
REFERENCES