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 promotor 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 attempted to determine 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-

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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 play a role in the normal progression of cells through G1-S transition (29). Abnormalities in cyclin D1 expression constitute one of many possible mechanisms of growth that could lead to the development of uncontrolled cell proliferation. It has been proposed that the regulation of cyclin D1 may be critical to the progression of cells through G1-S transition by involving cyclin D1-dependent regulatory proteins (27, 42). In addition, in a study utilizing a mouse model in which hyperproliferation/hyperplasia was induced, protein levels of both β-catenin and cyclin D1 measured in tissue extracts were significantly enhanced (26). Moreover, a clinical analysis demonstrated that nearly 50% of tissue samples examined from 70 colorectal cancer patients expressed increased levels of β-catenin and cyclin D1 (35). These studies suggest that one of the potential mechanisms of growth may include the dysregulation of these proteins.

In the present study, we explored the potential roles of β-catenin and cyclin D1 in mediating the trophic effects of gastrin in gastric adenocarcinoma. We observed that G-17 stimulated the proliferation of AGS-B cells and incited a concomitant increase in cyclin D1 and β-catenin mRNA levels. Western blot analysis demonstrated significant changes in cyclin D1, but not β-catenin, levels in response to the incubation of AGS-B cells with G-17. Furthermore, G-17 enhanced activity of the full-length cyclin D1 promoter. These observations indicate that gastrin appears to exert its mitogenic effects on gastric adenocarcinoma, at least in part, through changes in cyclin D1 expression.

### MATERIALS AND METHODS

#### Cell culture

AGS-B cells (13), an altered human gastric carcinoma cell line stably expressing the CCK-2 receptor, were used for the following studies. Cells were maintained in DMEM (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin. For the Northern and Western blot analyses, G-17 (Peninsula/Bachem, Belmont, CA) was added at the end of 24 h of serum starvation, and RNA or protein was extracted respectively at the indicated time points. Gastrin antagonist L-365260 (1 μM; kindly provided by Dr. L. Iverson, Oxford, UK) was used in conjunction with G-17 in the indicated experiments.

#### Immunocytochemistry

Equal amounts of cells were cultured overnight and were serum starved for 24 h, after which they were further incubated in the presence of 10 nM G-17 for 4 h. After a 4 h incubation, the cells were fixed in paraformaldehyde, washed, and stained for F-actin with rhodamine phalloidin (Molecular Probes, Eugene, OR) for 20 min at 37°C. After being thoroughly washed, the cells were mounted on the slide with Gelvatol and observed under a Nikon epifluorescence microscope.

#### Proliferation assay

Equal amounts of cells (1 × 10^6 cells/well) were plated before proliferation assay in a 12-well plate. Following a 24-h serum starvation, cells were pulsed with 1 μCi/well (final concentration) [3H]thymidine for 4 h in the absence or presence of G-17 (1 or 20 nM) and harvested. In addition, to examine specificity, 1 μM L-365260 was added to samples treated with 20 nM G-17. At the end of the incubation period, cells were trypsinized and equal amounts of cellular lysates were individually dispensed into scintillation vials. After the addition of scintillation fluid, synthesis of [3H]thymidine-incorporated DNA was measured in triplicate by using an automatic Beckman liquid scintillation counter and was normalized against the corresponding cell numbers.

#### Northern blot analysis

Cells were serum starved for 24 h and treated with increasing concentrations of G-17 (10–100 nM) in the absence or presence of 1 μM L-365260. After incubating them for different periods of time, RNA was extracted by using a modified method of Chomczynski and Sacchi (5, 10, 15, 16, 24). Briefly, cultured cells, cells were washed in 1% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin. For the Northern and Western blot analyses, G-17 (Peninsula/Bachem, Belmont, CA) was added at the end of 24 h of serum starvation, and RNA or protein was extracted respectively at the indicated time points. Gastrin antagonist L-365260 (1 μM; kindly provided by Dr. L. Iverson, Oxford, UK) was used in conjunction with G-17 in the indicated experiments.

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#### Western blot analysis

To extract cellular proteins from cultured cells, cells were washed in 1× PBS, directly lysed in the plate at 4°C, and recovered with a cell scraper. Following lysis in radioimmunoprecipitation assay protein extraction buffer (20 mM Tris, pH 7.5, 140 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100, and 10% glycerol) supplemented with protease inhibitors, cell debris was pelleted at 4°C and the supernatant was collected for protein quantification. Bicinchoninic acid protein assay was used to estimate protein concentration according to the manufacturer’s instructions. Equal amounts of protein were diluted with 5× sample loading buffer, boiled, and loaded onto polyacrylamide gels. Following electrophoresis, gels were transferred

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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 firefly 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.

Fig. 1. Amidated gastrin (G-17) induces morphological change as evidenced by actin staining. AGS-B cells were treated with 10 nM G-17 and harvested either at time 0 of G-17 addition (A) or after 4 h of incubation (B). Rhodamine phalloidin was used to stain filamentous actin, and alterations in the morphology were observed under a Nikon epifluorescence microscope.

![Fig. 1](image1.jpg)

![Fig. 2](image2.jpg)

Fig. 2. G-17 enhances uptake of [3H]thymidine in gastric carcinoma cell line AGS-B. 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. Amount of [3H]thymidine uptake was measured under each condition at 24 and 48 h. Results are expressed as counts per minute (cpm)/10^5 cells (means ± SE) and were measured in triplicate. * \( P < 0.01 \).
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 sev-
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 inhibitor 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 through stable overexpression of antisense RNA to cyclin D1 can reverse the transformed phenotype of human gastric cancer cells. In one particular study (22), the degree of overexpression of cyclin D1 mRNA correlated with invasive stages of gastric cancer. Furthermore, following H. pylori infection, one of the major risk factors for developing gastric cancer, the hyperproliferative response observed in the gastric mucosa was associated with increased expression of cyclin D1 (28). These studies all indicate that the upregulation of cyclin D1 appears to be associated with an increase in the malignant potential in gastric adenocarcinoma.

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


