The Effect of Gastrin on Growth of Human Stomach Cancer Cells

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Gastrin is known as a trophic factor for some stomach and colorectal cancer cells; however, the roles of gastrin receptors and the intracellular signal transduction pathways by which gastrin regulates cell growth are still unknown. The authors examined the effect of synthetic human gastrin-17 on growth of human stomach cancer cells (the parent line, AGS-P, and two different clones, AGS-10 and AGS-12), which were established (and have been maintained) in our laboratory. Gastrin stimulated growth of AGS-P and AGS-10 cells, which have gastrin receptors, in a dose-dependent fashion. A highly selective gastrin receptor antagonist, JMV 320, inhibited the growth-stimulatory effect of gastrin on AGS-P cells in a dose-dependent fashion. Concentrations of gastrin (10^{-8} to 10^{-6} M), which stimulated growth of AGS-P cells, did not affect either cyclic adenosine monophosphate production or phosphatidylinositol hydrolysis. Gastrin (10^{-11} to 10^{-5} M) mobilized calcium from the intracellular organelles to increase intracellular calcium level in AGS-P cells. The AGS-12 clone has no gastrin receptors, and gastrin did not affect growth or mobilization of intracellular calcium in these cells. Our findings indicate that gastrin stimulates growth of AGS cells through a mechanism that involves binding to specific gastrin receptors that are linked to the system for mobilization of intracellular calcium.

GASTRIN IS SYNTHESIZED and secreted from G cells in the antrum of the stomach and stimulates gastric acid secretion. There is no question that gastrin is also a trophic hormone. Johnson[1] clearly demonstrated the trophic effects of gastrin on mucosa of the rat gastrointestinal tract. Patients with the Zollinger-Ellison syndrome, which is characterized by hypergastrinemia, are known to show massive fundic mucosal hyperplasia.[2] The trophic effect of gastrin on a number of established stomach cancer cells has been well documented *in vivo* and *in vitro*.[3-6] The intracellular mechanisms of signal transduction by which gastrin affects growth of cancer cells are, however, still unclear. To determine the precise mechanism by which gastrin affects the growth of stomach cancer cells will undoubtedly be valuable for developing a new therapeutic strategy for patients with stomach cancer. Therefore, the purpose of the current study was to examine in-depth the effect of gastrin on the growth of human stomach cancer cells in terms of receptor–ligand interaction and postreceptor signal-transduction pathways.

A human stomach cancer cell line (termed AGS), which was established and has been maintained in our laboratory,[6] was employed as a target cell to examine the effect of gastrin in the current study. We have maintained two different cloned cell lines (AGS-10 and AGS-12) in addition to the polyclonal AGS parent (AGS-P) cell line. We have reported previously that both AGS-P and AGS-10 cells have gastrin receptors (AGS-P: 25.1 ± 5.7 fmol/100 μg DNA and AGS-10: 28.0 ± 0.7 fmol/100 μg DNA), and AGS-12 cells have none.[7]

Materials and Methods

AGS-P, AGS-10, and AGS-12 cells have been maintained in culture medium (F-10), containing fetal calf serum (FCS) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Stock cultures have media changed every 3 days (the doubling times are approximately 20 hours for AGS-P and AGS-10 and 35 hours for AGS-12). Human gastrin-17 was purchased from Bachem, Torrance, California. Gastrin receptor antagonists were synthesized as previously reported[8-11]; JMV 56 is a phenethyl ester

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derivative analog of the C-terminal tetrapeptide of gastrin.[8] JMV 76 is a pseudo-peptide analog of tetragastrin.[9] JMV 97 is a partially modified retro-inverso pseudopeptide derivative of tetragastrin.[10] JMV 320 is a cyclic cholecystokinin analog that is highly selective for a central cholecystokinin receptor or a gastrin receptor.[11] The amino acid structures of all gastrin receptor antagonists employed in the current study are shown in Table 1. All experiments were performed using six dishes or wells (one cover slip for the calcium study) in each experiment, and each study was repeated on at least three separate occasions. Representative data from each experiment are shown in this paper.

Effect of Human Gastrin-17 and Gastrin Receptor Antagonists on Growth of AGS-P, AGS-10, and AGS-12 Cells

AGS-P, AGS-10, and AGS-12 cells (2×10^4) were plated in F-10 containing 5% FCS. After 48 hours, cells had medium replaced by F-10 containing 1% FCS. After 24 hours, gastrin-17 (10^{-8} to 10^{-6} M) and a gastrin receptor antagonist, either JMV 56 (10^{-5} M), JMV 76 (10^{-5} M), JMV 97 (10^{-5} M) or JMV 320 (10^{-7} to 10^{-5} M), were added alone or in combination. Each peptide or vehicle (0.03 N NH_4OH for gastrin and distilled water for gastrin receptor antagonists) was added only once at the first day of the experiment or added every other day. Every other day, cells were detached from culture dishes with trypsin (1:250, Gibco) and diluted 10-fold with Isoton (Curtin Matheson Scientific, Houston, Texas). The cell number was counted by a Coulter counter, which was electronically set to count cells under 100 μm in size. The number of cells determined by the Coulter counter was similar (less than 5% difference) to the number of viable cells determined by a dye (trypan blue)-exclusion method using a hemocytometer. Also, the coefficient variances of low and high counts determined by this electric counter were less than 2%.

Effect of Human Gastrin-17 and JMV 320 on Phosphatidylinositol Hydrolysis in AGS-P Cells

Phosphatidylinositol (PI) hydrolysis was measured following a protocol adapted from Hawkins and colleagues.[12] In brief, AGS-P cells (3×10^5), which were plated and cultured in 24-well tissue culture plates for 2 days, were incubated with F-10 containing 5% FCS supplemented with myo-3H-inositol (10 μCi/mL) for 16 hours. Cells were then incubated for 10 minutes with oxygenated Krebs-Ringer bicarbonate buffer (pH 7.4) containing HEPES (10 mM), bovine serum albumin (0.1%), glucose (2.5 mM) and LiCl (10 mM). After another 1-minute incubation in the presence of human gastrin-17 (10^{-7} to 10^{-5} M), cells were extracted with 10% perchloric acid and immediately neutralized with 6 N KOH. Fractions of inositol monophosphate (IP_1), inositol bisphosphate (IP_2), inositol trisphosphate (IP_3), and inositol tetrakisphosphate (IP_4) were extracted by anion exchange chromatography (Dowex AG-1 $\times$ 8; formate form; 200 to 400 mesh) using a mixture of formic acid and ammonium formate as an eluent. After a fraction of inositol was eluted by an excess of water, IP_1, IP_2, IP_3, and IP_4 were eluted by 0.1 M formic acid ± 0.2 M ammonium formate, 0.1 M formic acid ± 0.4 M ammonium formate, 0.1 M formic acid ± 1.0 M ammonium formate, and 0.1 M formic acid ± 1.6 M ammonium formate, respectively, in a stepwise fashion. Radioactivity of each fraction was measured by a liquid scintillation counter.

TABLE 1. *Amino Acid Structures of Gastrin-Receptor Antagonists*

Antagonist	Structure
JMV 56	Boc-Trp-Leu-Asp-2-phenethyl ester
JMV 76	Boc-Trp-LeuΨ(CH_2NH)-Asp-Phe-NH_2
JMV 97	Boc-Trp-Leu-gAsp-CO-$CH_2CH_2C_6H_5$
JMV 320	Acetyl-Tyr-Lys-Gly-Trp-Lys-Asp-Phe-NH_2

Effect of Human Gastrin-17 and JMV 320 on the Production of Cyclic Adenosine Monophosphate in AGS-P Cells

AGS-P cells (5×10^5) were plated and cultured in 35- $\times$ 10-mm tissue culture dishes for 2 days, incubated in oxygenated Krebs-Ringer bicarbonate buffer (pH 7.4) containing HEPES (10 mM), bovine serum albumin (0.1%), glucose (2.5 mM), and IBMX (0.1 mM). After 30-minute incubation in the presence of human gastrin-17 (10^{-8} to 10^{-5} M) alone or with JMV 320 (10^{-5} M), cells were extracted with 5% trichloroacetic acid and neutralized with an excess of $CaCo_3$ just before radioimmunoassay.[13] After acetylation of samples with acetic anhydride and triethylamine, intracellular cyclic adenosine monophosphate (cAMP) levels were measured by cAMP radioimmunoassay kit (Amersham Corporation, Arlington Heights, IL).

Effect of Human Gastrin-17 and JMV 320 on the Mobilization of Intracellular Calcium in AGS-P and AGS-12 Cells

AGS cells were grown on 25-mm diameter glass coverslips at 37 C in F-10 medium supplemented with 5% FCS. The coverslips were coated with 25% Matrigel (Collaborative Research Inc., Bedford, MA) solution to enhance attachment of cells. The cells were incubated for 60 minutes at 25 C with 2 mL Krebs Ringer Henseleit (KRH) buffer containing 10 μM of fura-2/acetoxylmethyl ester. Krebs Ringer Henseleit contains, in millimoles per liter: NaCl, 125; KCl, 5; $MgSO_4$ and KH_2PO_4, 1.2; $CaCl_2$, 2; glucose, 6; Hepes 25, pH 7.4. Loaded cells were washed

twice with fresh KRH and reincubated in KRH with 0.1% bovine serum albumin at 5 C for 30 minutes before fluorescence microscopy. The cells were placed in an open perfusion microincubator (PDMI-2) (Medical System Corp., Greenvale, NY) and stimulated by human gastrin-17 (10^{-12} to 10^{-5} M) diluted in either 0.5% FCS or normal saline at 37°C. Furthermore, to examine the effect of human gastrin-17 on mobilization of intracellular calcium, a calcium-free KRH solution with ethylene glycol tetra-acetic acid (5 mM) was employed as an incubation buffer instead of KRH. Fura-2 fluorescence intensity was measured using a dual-wavelength excitation spectrofluorometer (Spex Industries, Inc., Edison, NJ). The excitation light was obtained from a xenon high-pressure lamp at wavelengths of 340 nm and 380 nm (band width: 10 nm). Intracellular calcium was calibrated by lysing cells with 0.03% Triton X-100 and by using 5 nM ethylene glycol tetra-acetic acid. Concentration of intracellular calcium was calculated by the method of Grynkiewicz et al.,[14] using a Kd for calcium as 224 nM.

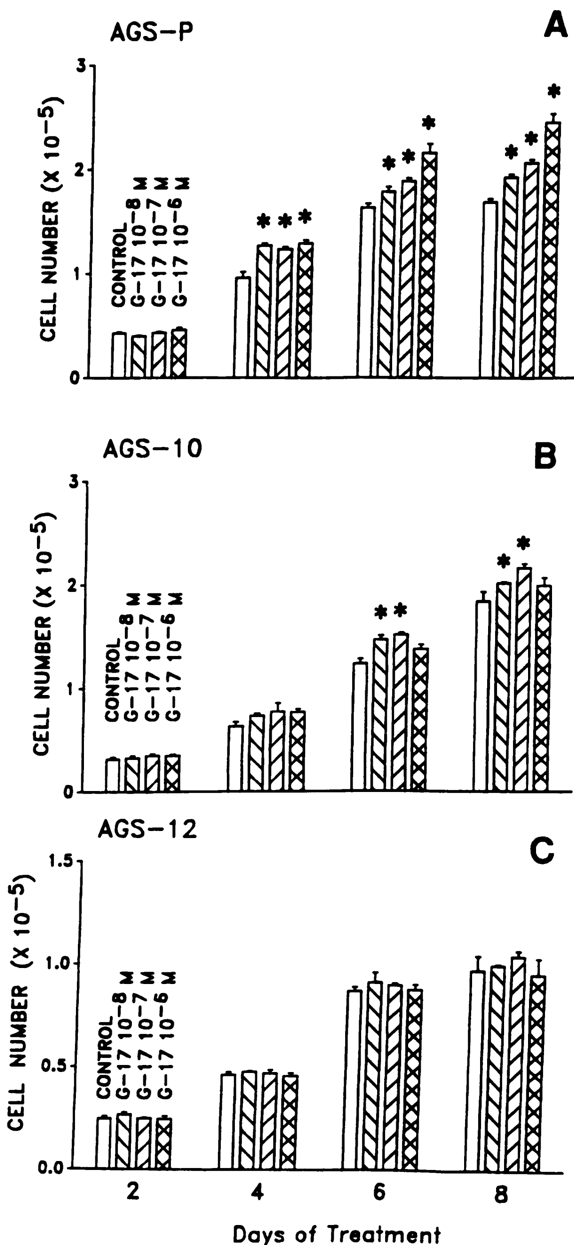

FIG. 1. Effect of human gastrin-17 on growth of (A) AGS-P, (B) AGS-10, and (C) AGS-12. In this figure and the following figures, error bars indicate standard error of the mean. *$p < 0.05$ *vs.* control.

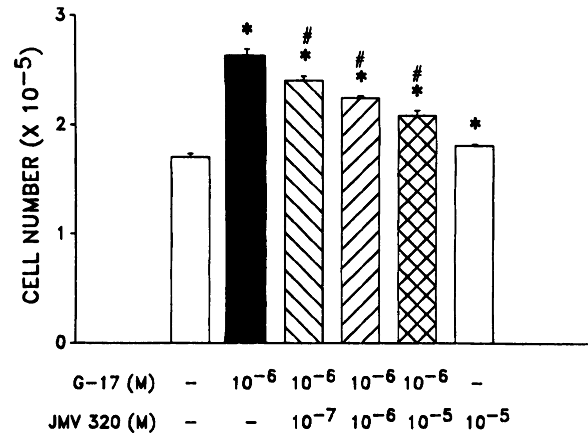

FIG. 2. Effect of JMV 320 on growth-stimulatory effect of gastrin on AGS-P cells. *$p < 0.05$ *vs.* control; #$p < 0.05$ *vs.* gastrin alone.

Statistical Analysis

Data from each study were analyzed by unpaired t test, and significance was assumed for p value less than 0.05.

Results

Effect of Human Gastrin-17 and Gastrin Receptor Antagonists on Growth of AGS-P, AGS-10, and AGS-12 Cells

Human gastrin-17 stimulated growth of AGS-P cells, which have gastrin receptors, in a dose-dependent fashion. Stimulatory effects became apparent at the fourth day after addition of gastrin and continued to the eighth day, when growth of cells without gastrin became confluent (Fig. 1A). Addition of gastrin every other day showed results similar to those of single addition of gastrin (data not shown). Growth of AGS-10 cells, which also have

TABLE 2. *Effect of Gastrin on Phosphatidylinositol (PI) Hydrolysis in AGS-P Cells*

	IP_1	IP_2	IP_3	IP_4
Control	1595 ± 37	657 ± 127	589 ± 58	350 ± 64 dpm
Gastrin 10^{-7} M	1450 ± 23	586 ± 100	564 ± 18	295 ± 29
Gastrin 10^{-6} M	1427 ± 70	599 ± 81	533 ± 28	278 ± 18
Gastrin 10^{-5} M	1507 ± 269	737 ± 35	598 ± 130	255 ± 45

IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; IP_4, inositol tetrakisphosphate.

TABLE 3. *Effect of Gastrin on Cyclic AMP Production in AGS-P Cells*

	Cyclic AMP Level
Control	42.4 ± 1.2 pmole/10^6 cells
Gastrin 10^{-8} M	44.8 ± 3.4
Gastrin 10^{-7} M	44.3 ± 0.8
Gastrin 10^{-6} M	45.4 ± 2.8
Gastrin 10^{-5} M	43.2 ± 2.8

gastrin receptors, was similarly stimulated by gastrin, although a high concentration (10^{-6} M) of gastrin did not stimulate growth of AGS-10 cells (Fig. 1B). Conversely, growth of AGS-12 cells, which have no gastrin receptor, was not affected by gastrin at any doses tested (Fig. 1C).

The stimulatory effects of gastrin on AGS-P cells were inhibited by JMV 320 in a dose-dependent fashion (Fig. 2), but other gastrin-receptor antagonists (JMV 56, JMV 76, and JMV 97), did not affect the stimulatory effect of gastrin (data not shown). A high concentration (10^{-5} M) of JMV 320 itself also stimulated growth of AGS-P cells (Fig. 2), whereas other gastrin receptor antagonists did not affect growth of AGS-P cells (data not shown).

Effect of Human Gastrin-17 and JMV 320 on Phosphatidylinositol Hydrolysis in AGS-P Cells

With a short (1 minute) stimulation by human gastrin-17, PI hydrolysis in AGS-P cells did not occur; that is, the radioactive fractions of IP_1, IP_2, IP_3, and IP_4 in AGS-P cells stimulated by gastrin were similar to those in AGS-P cells without gastrin (Table 2). JMV 320 did not affect PI hydrolysis in AGS-P cells, either by itself or by gastrin (data not shown).

Effect of Human Gastrin-17 and JMV 320 on the Production of Cyclic Adenosine Monophosphate in AGS-P Cells

Gastrin (at all concentrations tested) failed to affect the production of intracellular cAMP (Table 3). Similarly, JMV 320 did not affect the production of cAMP (data not shown).

Effect of Human Gastrin-17 and JMV 320 on the Mobilization of Intracellular Calcium in AGS-P and AGS-12 Cells

Intracellular calcium level in a basal condition was 135 ± 22 nM in AGS-P cells, and the level in AGS-12 cells was similar (129 ± 18 nM). In the absence of 0.5% FCS, any concentration of gastrin did not affect intracellular calcium level in AGS-P cells at all; however, in the presence of 0.5% FCS, which by itself did not affect intracellular calcium level, gastrin stimulated the mobilization of intracellular calcium. Intracellular calcium level was increased by a range between 10^{-11} and 10^{-5} M of gastrin. Response to gastrin, however, was dependent on the concentration of gastrin. Both 10^{-10} M and 10^{-7} M of gastrin quickly increased intracellular calcium level, whereas 10^{-11}, 10^{-9}, 10^{-8}, 10^{-6}, and 10^{-5} M gastrin increased intracellular calcium level to a lesser degree (Fig. 3). Furthermore, the intracellular calcium levels increased with a time lag of response to these concentrations of gastrin, as shown in Figure 3. That is, intracellular calcium level of AGS-P cells responded to gastrin in a biphasic dose-dependent fashion (Fig. 4).

To further clarify whether the effect of gastrin is dependent on extracellular calcium, response to gastrin was

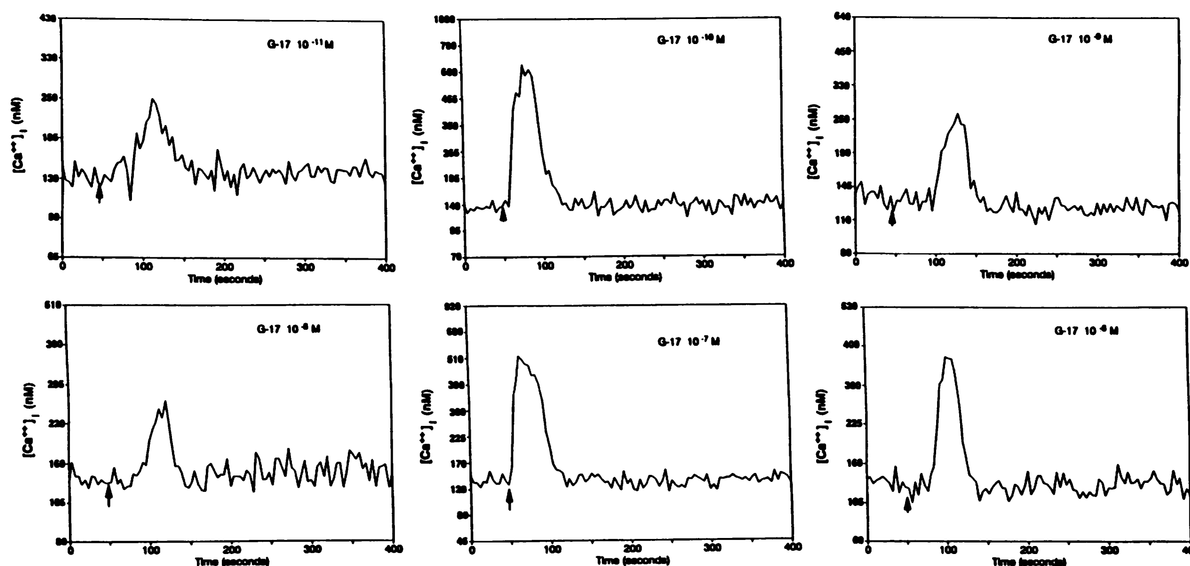

FIG. 3. Mobilization of intracellular calcium ($[Ca^{++}]_i$) in AGS-P cells in response to gastrin. Arrows indicate the time point when gastrin was added.

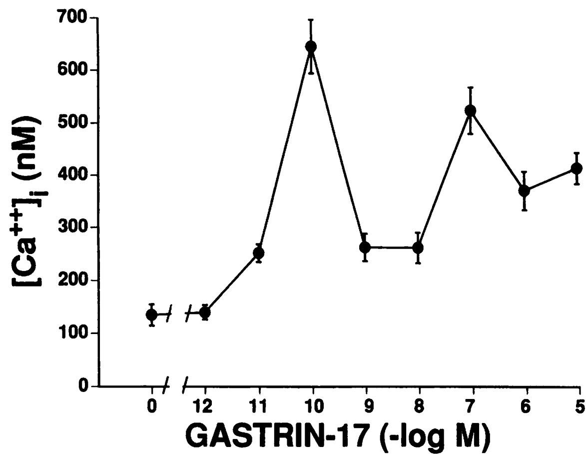

FIG. 4. Correlation between intracellular calcium level ($[Ca^{++}]_i$) and doses of gastrin in AGS-P cells.

examined in a calcium-free condition. As a result, even in a calcium-free condition, gastrin increased intracellular calcium level to a similar level to that in the presence of calcium in the extracellular fluid (Fig. 5).

Conversely, gastrin failed to increase the intracellular calcium level in AGS-12 cells that do not possess gastrin receptors (Fig. 6).

Discussion

To express their effects on cells, peptides must bind to specific cell membrane receptors that are linked to various intracellular signal transduction pathways, such as the cAMP pathway, the tyrosine kinase pathway, PI hydrolysis, or mobilization of intracellular calcium. The present findings indicate that growth-stimulatory effect of gastrin on human stomach cancer cells, AGS, are mediated through specific gastrin receptors that appear to be linked to the signal transduction pathway that involves mobilization of intracellular calcium.

In the current study, four different types of gastrin receptor antagonists were employed. Among those, only JMV 320 inhibited the growth-stimulatory effect of gastrin. JMV 320 is a cholecystokinin analog that is a highly selective antagonist for brain type of cholecystokinin receptor (Type II). Our findings, however, suggest that AGS-P cells have a third class of cholecystokinin receptor, which is found on parietal cells, gastric glands, and gastrointestinal smooth muscle cells.[15-21] This receptor has been reported to have approximately equal affinity for cholecystokinin and gastrin and is referred to as a gastrin receptor.[15-21] Thus, we have demonstrated that AGS-P cells have specific, functional gastrin receptors.

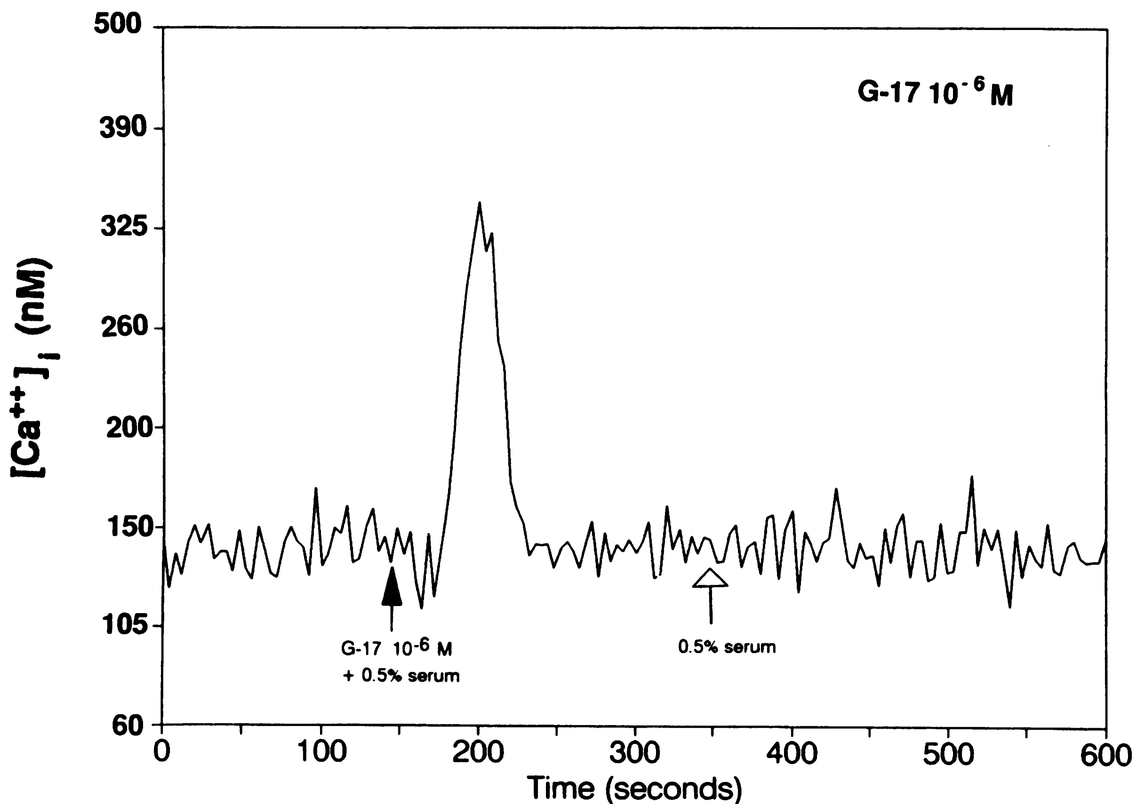

FIG. 5. Mobilization of intracellular calcium ($[Ca^{++}]_i$) in AGS-P cells in response to gastrin in calcium-free medium containing 5 mM ethylene glycol tetra-acetic acid.

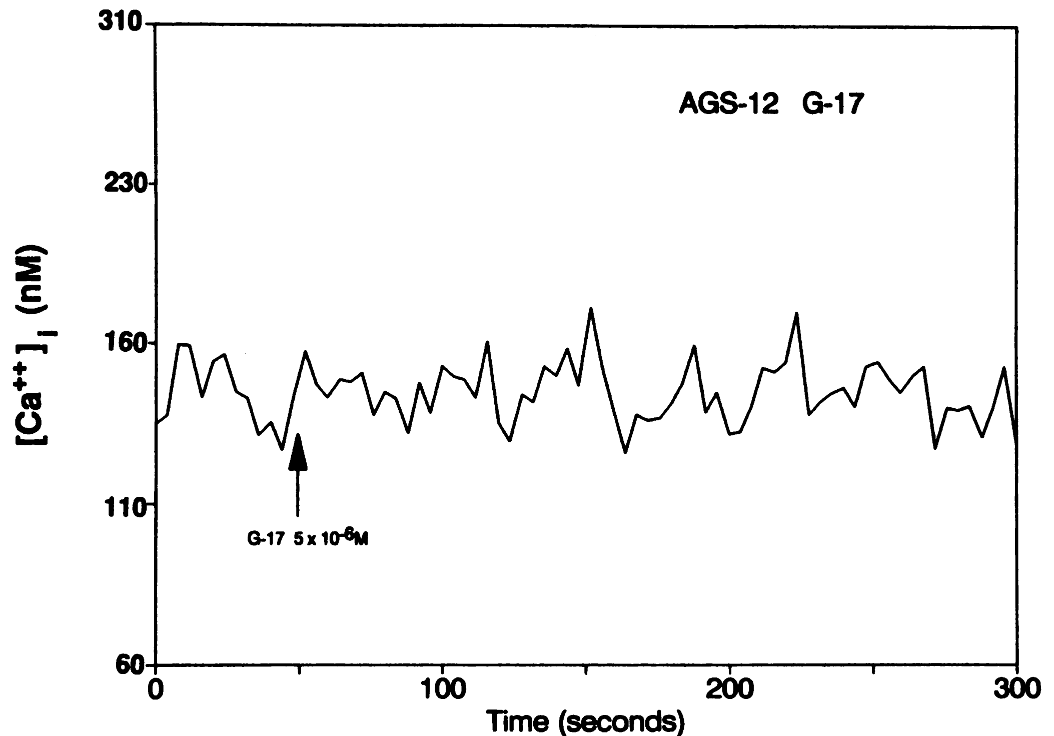

FIG. 6. Response of intracellular calcium ($[Ca^{++}]_i$) in AGS-12 cells to gastrin.

Kim and colleagues[22] have reported that vasoactive intestinal polypeptide inhibited growth of AGS-P cells through the intracellular second messenger, cAMP. Thus, when we found the growth-stimulatory effect of gastrin on AGS-P cells, we speculated that gastrin might inhibit the production of cAMP in AGS-P cells to express its growth-stimulatory effect. However, gastrin failed to affect the production of cAMP.

Phosphatidylinositol hydrolysis has been reported to play an important role in growth of certain cells.[23-25] In the current study, however, we found that gastrin did not affect PI hydrolysis in AGS-P cells.

Intracellular calcium is known to play an important role in growth of certain cells.[26] Gastrin clearly increased intracellular calcium levels in AGS-P cells in an unique, biphasic fashion. The reason for this unique response to gastrin is still unclear, but one explanation may be that AGS-P cells consist of at least two different populations of cells that have gastrin receptors with different sensitivities to gastrin. Although preliminary, we have found that the growth-stimulatory effect of gastrin is parallel to the mobilization of intracellular calcium (unpublished data). The product of PI hydrolysis, IP_3, is known to stimulate mobilization of intracellular calcium,[23] but our findings suggest that gastrin receptors on AGS-P cells are directly linked to the mobilizing system of intracellular calcium and are independent of PI hydrolysis. Furthermore, results obtained from the experiments using calcium-free medium indicate that gastrin elevated levels of intracellular calcium by mobilizing calcium mainly from the intracellular organelles, but not from the extracellular fluid through calcium influx.

There is no question that growth of normal and cancer cells are regulated by various factors through various intracellular signal transduction pathways. In the current study, we showed that growth of human stomach cancer cells was stimulated by human gastrin-17 through a specific gastrin receptor, and that the stimulatory effect of gastrin was mediated, at least in part, through mobilization of intracellular calcium from the intracellular organelles. Although the complete growth-stimulatory mechanism of gastrin remains to be clarified, this is the first report that gastrin stimulates growth of human stomach cancer cells through specific gastrin receptors that are linked to the system for mobilization of intracellular calcium.

Acknowledgments

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Discussion

Dr. Kirby I. Bland (Gainesville, Florida): President Ochsner, Dr. Jones, Fellows and Guests, I wish to begin the discussion of this important paper by thanking Dr. Townsend and his co-authors for allowing me to review this manuscript in advance. Their laboratory in Galveston has been responsible over a number of years for elucidating the stimulatory and inhibitor roles of a variety of polypeptides for several gastrointestinal cell lines. To my knowledge, however, this is the first report to document that gastrin stimulates the growth of human gastric cancer cells through specific gastrin receptors. We have seen in the presentation by Dr. Townsend that specific gastrin receptors are linked to the system that initiates mobilization of intercellular calcium. This paper, as have others from their laboratory, documents that growth of normal and neoplastic cells are regulated by various factors. These include interleukins and cytokines, and these pathways may augment or suppress signal transduction. I encourage the membership to review this manuscript because, again, a lot of data are included in that manuscript that Courtney did not have time to present.

Dr. Townsend also has emphasized that synthetic human gastrin 17 stimulates growth of the parent cell line, the AGSP, as well as the two clones, the variants 10 and 12, to varying degrees. Further, the highly selective receptor antagonists, JMV 320, statistically inhibit growth stimulatory effects on gastrin of this parenteral cell line. This effect is sustained in a dose-dependent fashion. Your data have convinced me, and they document that hormonal effects of gastrin by the PI hydrolysis as well as production of intracellular cyclic adenosine monophosphate are not responsible for the trophic or the stimulatory effects of growth of gastrin on human gastric cell lines. Also, the antagonist failed to effect PI hydrolysis and had no effect on cyclic adenosine monophosphate production. It is interesting, however, that of the four different types of gastrin receptor antagonists used, you found that only the 320 variant inhibited this growth stimulatory effect on gastrin 17. It is appropriate to note — and Courtney brought this out very clearly — that this receptor has equal affinity for gastrin as well as the cholecystokinin receptor, but we happen to refer to it in this model as the gastrin receptor. This laboratory has, therefore, documented that at a molecular level the parent neoplastic cell line has specific sites that function on gastrin receptors.

Courtney, I have a number of questions from these important observations you have made. Because intracellular calcium plays a prominent role linked to signal transduction pathways, this observation may account for the stimulatory role of gastric neoplastic cells in this model as well as, perhaps, other cell lines. Could you provide us with more data that explain the unique biphasic responses of gastrin to increased intracellular calcium? And linked to that question, secondly, is this biphasic response because of the heterogenous population of cell lines and their variation and binding sensitivity to gastrin?

And the next question, because we are interested, obviously, in transferring this technology to the bedside — and this is an *in vitro* model — have you completed any studies, at least any models generated thus far, as an *in vivo* preparation? And, finally, it would be interesting to know, would you anticipate other agents such as Losec (omeprasol, Merck, Sharp, and Dohme) or H_2 blockers to have a similar inhibitory role to block the trophic effects of gastrin *in vivo*? I enjoyed this paper very much, and I thank the Association for the privilege of the floor.

Dr. Josef Fischer (Cincinnati, Ohio): President Ochsner, Secretary Jones, Every medical student, even the ones that we currently educate — and that is saying quite a bit — knows that achlorhydria is associated with an increased incidence of carcinoma of the stomach. And I think this paper today probably goes a long way into elucidating that relation-