Antiproliferative Effects of Gastrin Receptor Antagonists and Antibodies to Gastrin on Human Colon Carcinoma Cell Lines

Naseema M. Hoosein, Peter A. Kiener, Robert C. Curry, Lucio C. Revati, Donnie K. McGilbra, and Michael G. Brattain

Bristol-Baylor Laboratory, Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030 [N. M. H., D. K. M., M. G. B.]; Pharmaceutical Research and Development Division, Bristol-Myers Company, Wallingford, Connecticut 06492 [P. A. K., R. C. C., M. G. B.]; and Rotta Research Laboratory, 20050 S. Frattuoso Monza, Milano, Italy [L. C. R.]

ABSTRACT

The gastrointestinal hormone gastrin has been shown to stimulate the growth of normal colonic mucosa. To examine for a possible role of gastrin in the proliferation of cultured colon tumor cells, we have studied the effects of two gastrin receptor antagonists, proglumide and benzotript, and of antibodies to gastrin. We find that proglumide (50% effective concentration, 2 to 5 mM) and benzotript (50% effective concentration, 0.4 to 0.8 mM) inhibit the monolayer growth of six human colon cancer cell lines. Addition of exogenous gastrin abrogated the growth-inhibitory effect of proglumide. The anchorage-independent growth of colon carcinoma cells was also inhibited by the two gastrin antagonists. Also, a dose-dependent increase in carcinoembryonic antigen secretion was observed upon treatment with proglumide and benzotript in three cell lines examined. Half-maximal inhibition of labeled gastrin binding was observed at concentrations of 0.4 mM benzotript and 8.6 mM proglumide. In addition, antigastrin antiserum added to HCT 116 cells adapted to growth in serum-free medium resulted in a concentration-dependent inhibition of cellular proliferation. These data suggest that gastrin may function as an autocrine growth factor in colon carcinoma.

INTRODUCTION

Gastrin is a gastrointestinal peptide (1, 2) that has been reported to have trophic effects on the mucosa of the digestive tract (3, 4). A pleiotropic response including stimulation of RNA, protein, and DNA synthesis has been shown to occur in response to gastrin in mammalian gastric, duodenal, as well as colonic mucosa (3–5). In addition, gastrin promotes growth of the exocrine pancreas (6). Proglumide, a derivative of glutamic acid (7, 8), inhibits gastrin-stimulated acid secretion (7) and the trophic effect of gastrin on normal gut mucosa (9). Benzotript, a tryptophan derivative, has been shown to have actions similar to those of proglumide (10, 11). Gastrin, CCK, and the amphibian skin peptide caerulein are members of a family of peptides that share a common COOH-terminal pentapeptide amide (1) and exert their effects on a particular target tissue by interacting with the same class of receptors (10). The antagonistic effects of proglumide and benzotript are thought to be due to their ability to inhibit binding of these peptides to their cell surface receptors (9, 10, 12).

In addition to its actions on normal gut tissue, gastrin has been reported to promote the growth of colonic tumors in vivo (13, 14) and of some colon tumor cell lines (15, 16). The growth of a transplantable mouse colon cancer (MC-26), which has gastrin receptors (17), is stimulated by exogenous pentagastrin (13). Proglumide was shown to inhibit the growth of MC-26 cells in vivo and thereby prolong survival of the tumor-bearing mice (18). The inhibitory effect of proglumide on the growth in vivo of MC-26 mouse colon cancer could be due to a direct effect on the tumor cells or due to an indirect effect via the tumor host. In order to ascertain this, it is necessary to examine the effect of proglumide on colon carcinoma cells in culture. Also, to assess the potential use of gastrin antagonists in colon cancer treatment, their effects on more colon tumor cells, preferably of human origin, need to be examined.

We have previously established a large bank of human colon carcinoma cell lines which reflects the high degree of heterogeneity observed in patients with this disease, thereby providing a good model system for the evaluation of therapeutic agents (19, 20). The colon carcinoma cell lines in this bank have been divided into three groups based on characterizations such as tumorigenicity in athymic mice, ability to grow with anchorage independence, growth rate in monolayer culture, formation of transport domes, and secretion of carcinoembryonic antigen (19–21). In this study we have used several colon cancer cell lines to examine effects of the gastrin antagonists, proglumide and benzotript, on the growth of colon tumor cells. We have also tested the effect of antigastrin antiserum on cellular proliferation. Our results described below show that the two gastrin antagonists as well as antigastrin antiserum have strong antiproliferative effects, suggesting an autocrine growth-stimulatory role for gastrin in these cells.

MATERIALS AND METHODS

Proglumide and benzotript were from Rotta Research Laboratory, Monza (Milano), Italy. Pentagastrin and nonsulfated CCK-8 were purchased from Peninsula Laboratories (Belmont, CA). Cell Culture. The HCT 116, RKO, MO5ER, JVC, FET, and CBS colon cancer cell lines were established in vitro from separate human tumor specimens as previously described (19). Working cultures of the lines were maintained at 37 °C in a humidified atmosphere of 5% CO2 in McCoy’s Medium 5A (Flow Laboratories) supplemented with 10% FBS and antibiotics (streptomycin-penicillin). Cells grown in the absence of serum were cultured in McCoy’s Medium 5A containing twice the normal concentrations of sodium pyruvate, vitamins, and amino acids. Also included in the serum-free medium were transferrin (4 ßg/ml), insulin (20 /in/ml), epidermal growth factor (10 ng/ml), sodium selenite (1 x 10~8M), hydrocortisone (2 í<g/ml), and triiodothyronine (4 x 10~10 M). Cells adapted to growth in serum-free medium were continuously maintained in the serum-free medium and are designated “serum free” to distinguish them from those grown in serum-supplemented medium which are designated “10% FBS.” Growth Inhibition Assays. For examination of dose response, 10,000 cells were plated in 35-mm wells in 2 ml of growth medium. After 2 days, cells were treated with varying concentrations of proglumide and benzotript as indicated. Cells grown in serum-supplemented medium were counted after 6 days of treatment, and serum-free cells were counted after 5 days. Conditioned media from serum-free cell lines were retained for CEA assays.

For growth curves, 10,000 HCT 116-10% FBS cells were inoculated into triplicate 35-mm dishes (Corning) in 2 ml of serum-free growth medium with or without 5 mM proglumide. Starting 2 days after plating, cells were counted using a hemocytometer at 24-h intervals for the next 5 days.
Soft agarose assays were performed as described previously (22). HCT 116-10% FBS cells were plated at 3500 cells/well with proglumide and benzotript at the indicated concentrations. Cells were stained with a vital dye after 9 days, and colonies containing greater than 20 to 25 cells were scored with the aid of an inverted microscope.

CEA Assay. A solid-phase direct binding radioimmunoassay was used to determine relative CEA content in serum-free conditioned medium. The procedure used was essentially similar to one described previously for fibronectin radioimmunoassay (22) with the following adaptations: 50 µl of conditioned medium were assayed; and the primary antiserum was antihuman CEA antisera (Dako Corporation, Santa Barbara, CA) at a 1:1000 dilution. Relative CEA increases observed using the above radioimmunoassay procedure were similar to those obtained using a monoclonal enzyme-linked immunosorbent assay for CEA (Abbott Laboratories, N. Chicago, IL).

Gastrin Labeling. [Leu'

3]gastrin,17 (Bachem) was labeled using Enzymobeads (Bio-Rad) according to the manufacturer's instructions. Labelled peptide was purified by gel filtration on a Sephades G-10 column followed by adsorption to a C-18 Sep-Pak (Waters). The bound peptide was eluted with water:acetonitrile (33:67) and evaporated to dryness on a speed vac (Savant). The labeled gastrin was dissolved in the sterile binding buffer, divided into aliquots, and stored frozen until used. The specific activity of the peptide prepared this way was 3.8 x 10⁵ to 1.1 x 10⁶ cpm/µg.

Binding Assay. Cells were trypsinized, plated out at 4 x 10⁵ cells per 175-cm flask, 24 h prior to initiation of the assay. One h before the assay the cells were harvested by scraping, dispersed by passage through a pipet, and then assessed for viability (greater than 85% by trypan blue exclusion). The cells were sedimented, 200 x g for 10 min, and then resuspended in sterile binding buffer (RPMI containing 5 mg/ml of bovine serum albumin and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) at about 5 x 10⁶ cells/ml. For the competition binding assay tubes were set up in triplicate, containing 0.1 µg of [125I]-gastrin, 5 x 10⁶ cells, and various concentrations of ligands, at a final volume of 220 µl. The samples were incubated for 1 h at 25°C, 200 µl were then removed and layered over 750 µl of phthalates (dibutyl:diethyl, 1:1) in 1.5-ml Eppendorf tubes at 4°C, and then samples were centrifuged for 1 min at 12,000 x g. Following this the tubes were immediately frozen in dry ice and then transferred to liquid nitrogen. The cell pellets were obtained by cutting of tips off the tubes, still frozen, and then counted in an LKB gamma counter. Nonspecific binding determined in the presence of 225 µM [Leu'

8]gastrin,17 was about 20% of total binding.

In preliminary experiments, gastrin binding affinity obtained with monolayers of HCT 116 cells attached to the substratum was of the same order as that obtained with the method using cells in suspension described above.

Antigasstrin Antiserum Preparation. Antibody to gastrin,17 was raised in rabbits by immunization with a gastrin-rabbit albumin conjugate. The polyclonal serum was purified by chromatography on a Protein A-Sepharose column, dialyzed against phosphate-buffered saline, and filter sterilized prior to use. The protein concentration of the affinity-purified antibodies was determined by spectrophotometry at 280 nm. Antibody to gastrin,17 was further enriched by adsorption to a C-18 Sep-Pak. The bound antibody was eluted with water-acetonitrile (33:67) and evaporated to dryness. The labeled gastrin was dissolved in the sterile binding buffer containing 5 µg/ml of bovine serum albumin and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4. The labeled gastrin was then resuspended in sterile binding buffer, divided into aliquots, and stored frozen until used. The specific activity of the peptide prepared this way was 3.8 x 10⁵ to 1.1 x 10⁶ cpm/µg.

RESULTS

Inhibition of Proliferation. The effects of proglumide and benzotript on the growth of six human colon carcinoma cell lines in monolayer culture are shown in Fig. 1. Both compounds inhibited growth in a dose-dependent manner. Half-maximal inhibition of growth was observed between 2 and 5 mM proglumide and between 0.4 and 0.8 mM benzotript. Thus, benzotript was the more potent growth-inhibitory agent.

Similar results (Fig. 2) were obtained when colon carcinoma cells adapted to growth in serum-free, defined medium were treated with proglumide and benzotript. Thus, the presence of serum in the growth medium did not affect the response observed. Growth curves obtained with the poorly differentiated Group I cell line HCT 116 in the presence and absence of 5 mM proglumide indicated that the doubling time of untreated cells was 22.6 h, and this was increased to 33.6 h in cells treated with 5 mM proglumide. Removal of cells from proglumide-containing growth medium after 4 and 5 days of treatment resulted in acceleration of the rate of cellular proliferation to control untreated levels (data not shown), indicating a reversal of the antiproliferative effect.

Growth in soft agarose of HCT 116 cells was also inhibited in a dose-dependent manner by both proglumide and benzotript (Fig. 3). There were 81%, 50%, and 33% reductions in colony formation at 10 mM, 5 mM, and 2.5 mM proglumide, respectively, compared to untreated HCT 116 cells. With benzotript (Fig. 3, bottom row) the reductions in number of colonies were 92.2%, 50%, and 18% at concentrations of 1 mM, 0.5 mM, and 0.25 mM, respectively, compared to untreated cells. Thus, near-maximal inhibition in anchorage-independent growth is observed at a concentration of 10 mM for proglumide and at a 10-fold lower concentration of 1 mM benzotript.

The antiproliferative effects of proglumide on HCT 116 cells (serum free) growing in monolayer cultures were blocked by exogenously added gastrin,17 (Fig. 4). In the presence of 50 µM exogenous gastrin, no effect of 2 mM proglumide could be observed.

A polyclonal antigastrin antibody also inhibited the growth of the poorly differentiated HCT 116 cells (serum free) in a dose-dependent fashion (Fig. 5), whereas nonimmune rabbit...
Fig. 3. Anchorage-independent growth of HCT 116 cells in the presence of proglumide (top) and benzotript (bottom). Top, control cells (a) and cells treated with 2.5 mM (b), 5 mM (c), and 10 mM (d) proglumide. Bottom, control cells (a) and cells treated with 0.5 mM (b), 1 mM (c), and 2 mM (d) benzotript. A repeat assay gave identical results.

Fig. 4. Abrogation of the antiproliferative effect of proglumide by exogenous gastrin. HCT 116 cells were plated at 10,000 cells/well in triplicate wells (24-well plates). After 24 h cells were treated as shown. Five days later cells were counted using a hemocytometer. Columns, mean; bars, SD (n = 4). Similar results were obtained with two additional experiments.

IgG had no significant effect on proliferation. Preincubation of the antibody with gastrin abolished its inhibitory activity. Dose-dependent inhibition by antigastrin antibodies was also observed with the well-differentiated CBS (serum-free) cells (data not shown).

Elevation of Carcinoembryonic Antigen Secretion. Higher expression of the glycoprotein CEA has been shown to correlate with better differentiated colorectal tumor cells (20, 23–26). We therefore determined CEA levels in the conditioned medium of a representative cell line from each of the 3 groups of colon carcinoma cells. Results are shown in Fig. 6. In all three cell lines examined a concentration-dependent elevation in CEA secretion was observed. Basal levels of CEA secretion were the highest in the well-differentiated CBS cells (Fig. 6) which displayed a 52-fold and 110-fold increase after exposure to 10 mM proglumide and 1.6 mM benzotript, respectively. In the poorly differentiated HCT 116 cells, CEA secretion was enhanced 27-fold and 48-fold by 10 mM proglumide and 1.6 mM benzotript, respectively. Somewhat lower increases of 5-fold (10 mM proglumide) and 43-fold (1.6 mM benzotript) were observed with the moderately well-differentiated MOSER cell line.

Inhibition of Radiolabeled Gastrin Binding. In competition studies proglumide and benzotript inhibited the binding of 125I-[Leu15]gastrin to HCT 116 colon carcinoma cells in a dose-dependent manner (Fig. 7). The half-maximal concentration of proglumide (IC50 = 8.6 mM) required for inhibition of gastrin binding to HCT 116 cells was higher than that of benzotript (IC50 = 0.4 mM). For both antagonists the half-maximal concentrations required for inhibiting growth (proglumide, EC50 = 2 to 5 mM; benzotript, EC50 = 0.4 to 0.8 mM; Fig. 1) are close to those required for competing with radiolabeled gastrin binding. Benzotript and proglumide also inhibited gastrin binding to MOSER cells with IC50s of 0.4 mM and 5.9 mM, respectively (not shown). Half-maximal inhibition of 125I-labeled [Leu15]-
PROGLUMIDE AND BENZOTRIPT EFFECTS ON COLON CARCINOMA

Fig. 6. Increase in conditioned medium CEA levels upon treatment with proglumide and benzotript. HCT 116 cells (1 x 10⁴ cells/well), MOSER (1 x 10⁴ cells/well), and CBS (2 x 10⁴ cells/well) that had been adapted to growth and continuously maintained in serum-free medium were plated in triplicate 35-mm wells. Two days later cells were treated with proglumide and benzotript at the indicated concentrations. Five days later conditioned medium was collected, and cells were counted by a hemocytometer. Relative CEA levels in the conditioned medium were determined by a solid-phase radioimmunoassay. Points, mean; bars, SD (n = 3). An additional assay gave similar fold increases in CEA levels in the proglumide- and benzotript-treated cells.

gastrin₁₇ binding to HCT 116 cells was observed at 1.6 μM for [Leu₁⁵]gastrin₁₇, at 13.2 μM for nonsulfated cholecystokinin₈, and 100 μM for pentagastrin (Fig. 7). The antiproliferative effects of 2 mM proglumide were blocked by gastrin₁₇ partially at 0.5 μM and 5 μM and completely at a concentration of 50 μM (Fig. 4). This correlates well with micromolar [Leu₁⁵]gastrin₁₇ binding affinity.

DISCUSSION

Antiproliferative effects of gastrin antagonists on colon carcinoma cells growing in defined, serum-free growth medium (Fig. 2) suggest that gastrin or a gastrin-like peptide which may be synthesized and secreted by these cells could function as an autocrine growth factor. The role of gastrin as an autocrine growth factor in these cells is also suggested by the results that antibodies to gastrin can inhibit cell proliferation (Fig. 4). Malignant transformation of cells has been linked to aberrant production and/or response to growth-modulating polypeptides (27–29). Immunohistochemical studies have demonstrated the presence of gastrin in human antral mucosa (30). Gastrin-like immunoreactivity is also found in the proximal small intestine, but not in the oxyntic mucosa, ileum, or colon (31). Thus, it is possible that ectopic production of gastrin in the colon as described for human chorionic gonadotropin (32) is involved in the pathogenesis of colon cancer. Also, hypergastrinemia in rats, induced by antral exclusion or by administration of pentagastrin, is known to promote chemically induced colon cancer.

(14) Abnormal expression and/or response to gastrin by colon carcinoma therefore needs further examination.

In receptor studies with radiolabeled gastrin, benzotript displayed an approximately 10-fold higher binding affinity to the gastrin binding site compared to proglumide (Fig. 7). This correlates well with the greater potency of benzotript in inhibiting growth of HCT 116 cells (Figs. 1 to 3) as well as the other cell lines examined (Figs. 1 and 2). The order of potency obtained with the gastrin agonists [Leu₁⁵]gastrin₁₇ > cholecystokinin₈ > pentagastrin (Fig. 7) concurs with that described for normal rat gastric mucosal membranes (33). However, in contrast to micromolar binding affinity (IC₅₀ = 1.6 μM) of gastrin₁₇ to colon tumor cells, gastrin receptors in normal gastrointestinal tissue have high nanomolar affinity for binding gastrin (33–35). In this regard, there is some evidence that the gastrin receptor may exist in two conformational states with different binding affinities (36, 37). In agreement with our data Weinstock and Baldwin (36) have recently reported the presence of low affinity (Kₐ 0.2 to 3.1 μM) gastrin receptors on gastric and colonic carcinoma cell lines.

As it is true for solid tumors, a high degree of heterogeneity is observed in colon tumors (20, 38, 39). Subpopulations of many tumor cells (38) including those from colon carcinoma (39) differ in their susceptibilities to various antineoplastic agents, providing an obstacle to effective therapy. However, we find that colon carcinoma cell lines of heterogeneous phenotypes examined in this study displayed nearly equal sensitivities to the growth-inhibitory effects of the two gastrin antagonists (Figs. 1 and 2). Also, CEA levels, often used as a marker for induction of differentiation in cultured colorectal tumor cells (23–25), were increased in the poorly, moderately, and well-differentiated colon carcinoma cells examined.
REFERENCES


Antiproliferative Effects of Gastrin Receptor Antagonists and Antibodies to Gastrin on Human Colon Carcinoma Cell Lines

Naseema M. Hoosein, Peter A. Kiener, Robert C. Curry, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/24_Part_1/7179

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.