Inhibition of Pentagastrin-stimulated Up-Regulation of Gastrin Receptors and Growth of Mouse Colon Tumor in Vivo by Proglumide, a Gastrin Receptor Antagonist

Pomila Singh, Sy Le, R. Daniel Beauchamp, Courtney M. Townsend, Jr., and James C. Thompson
Department of Surgery, The University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT

We have recently demonstrated that gastrin stimulates growth of mouse colon cancer (MC-26) in vivo by regulation of gastrin receptors (GR). In the present study, we have tested the effect of proglumide (PGL), a GR antagonist, on the trophic and GR-regulatory effects of gastrin on MC-26 tumors. Four groups of 12 mice each were inoculated with $5 \times 10^6$ MC-26 cells and given injections of either normal saline (control), pentagastrin (PG), PGL, or both PG + PGL for 21 days. At the end of the treatment period, body, tumor, fundic, and colon weights were recorded and GR measured. Two types of specific gastrin-binding sites were found on tumor cell membranes of control mice, one with high binding affinity ($K_d = 0.1 \text{ nm})$ and low capacity (GR), and the other with a very high capacity and a low affinity ($K_d > 0.1 \mu M$) (type 2 gastrin-binding sites). Only the type 1 GR were observed on the fundic mucosal and colon membranes. PG treatment resulted in a significant weight increase of the tumors with an up-regulation of only type 1 GR. On the other hand, PG had no significant effect on fundic mucosal and colonic GR levels, but caused a significant increase in fundic mucosal weights. PGL completely inhibited both the trophic and GR up-regulatory effects of PG on tumors, but incompletely reduced the PG-stimulated fundic mucosal weight gain, indicating differential sensitivity of tumor and normal tissues to PGL. PGL, in the absence of PG, was slightly trophic for normal fundic mucosa, but had no effect on MC-26 tumors and normal colon. The one striking effect of PGL, in the presence of PG, was the significant lowering of the binding affinity of type 1 GR for gastrin on both the tumor and normal gastrointestinal tissues. This effect may be another mechanism by which PGL interferes with the actions of PG on MC-26 tumors and fundic mucosa of mice.

INTRODUCTION

Proglumide, a synthetic derivative of glutaramic acid [DL-4-benzamido-N,N-dipropyl-glutaramic acid (1)], antagonizes the effects of gastrin on acid secretion (2-4), mildly inhibits gastric secretion in patients with the ZE syndrome (5), and inhibits the trophic effects of PG, on both normal GÌ mucosa in rats (6) and mouse colon cancers (MC-26) (7). Gastrin and cholecystokinin are structurally similar at the C-terminal end; for this reason, PGL has also been found to antagonize actions of CCK on exocrine pancreatic secretion in rats (8), dogs (9, 10), and guinea pigs (11) and to inhibit CCK-stimulated growth of rat pancreas (12). PGL inhibits the specific binding of gastrin to GR on fundic mucosal membranes from rats (13), rabbits (14), and dogs (15). PGL also inhibits binding of CCK to CCK receptors on pancreatic acini (11-16). These studies support the contention that PGL inhibits the actions of gastrin and CCK at the receptor level (11, 13-16). Yamaguchi and coworkers have postulated that PGL at very high doses may have nonspecific effects of its own (12).

We have established a mouse colon cancer cell line (MC-26) which is stimulated to grow by administration of gastrin (17) and which possesses specific GR (18, 19). We have reported that the trophic response of MC-26 tumors to gastrin, in vivo, is closely related to gastrin-mediated up-regulation of GR levels on MC-26 tumors and also appears to be dependent on gastrin regulation of GR binding affinity for gastrin (20). Furthermore the growth of MC-26 cancers was inhibited by PGL in vivo, and survival of PGL-treated mice with MC-26 tumors was prolonged (7). The purpose of the present study was to examine the mechanism of PGL antagonism of the effects of PG at the receptor level on MC-26 tumors. In addition, the inhibitory effects of PGL on PG-stimulated growth of MC-26 tumors were compared to those on normal GI tissues to determine whether tissue-specific or cancer-specific effects of PGL can be delineated. Since there are no specific methods, other than surgical excision, to treat colon cancer, an understanding of the mechanism of PGL inhibition of the trophic effects of gastrin on MC-26 tumors may ultimately provide new therapeutic strategies in the treatment of colon cancer.

MATERIALS AND METHODS

Transplantation of Mouse Colon Cancer Cells in Mice. A transplantable mouse colon cancer cell line, CT-26, was obtained from Mason Research Laboratory (Worcester, MA); from it, a tissue culture cell line, MC-26, was established in our laboratory (17). Single-cell suspensions of MC-26 cells ($5 \times 10^6$) were inoculated s.c. into the flank of male BALB/c mice (20 to 25 g). This cell number produces cancers in all mice by 21 days, and 100% of the inoculated mice survive for 25 days.

Treatment of Animals. After inoculation of the mice with MC-26 cells, mice were given injections i.p. of either PG (250 µg/kg body) in the presence or absence of PGL (250 mg/kg body) (treated group) or of saline (control group). Injections were given 3 times a day for 21 days. The dose of PG used in these studies was previously found to be optimal for stimulation of tumor growth (17), and the dose of PGL used has been found to effectively inhibit the growth of MC-26 tumors (7).

Collection of Tissues. At the end of 21 days of treatment, small (-1 cm diameter) well-defined tumors were palpable in all mice. On Day 21, the mice were sacrificed, and the fundus, colorectum, and s.c. tumors were quickly removed, placed in ice-cold 0.9% NaCl solution, and washed extensively to remove all contaminating material. The tumors had well-defined margins and were dissected free without difficulty. All excised tissues were removed by individuals who were unaware of which treatments each mouse had received.

Further steps were carried out at 4°C unless otherwise stated. Tissues were then washed in Buffer A [Tris (10 mM), NaCl (137 mM), KCl (5 mM), CaCl$_2$ (2 mM), MgCl$_2$ (2.5 mM), and sucrose (0.25 mM), pH 7.4], which contained 0.1% bovine serum albumin (Fraction V; Sigma), 0.1% soybean trypsin inhibitor (Worthington), and 0.1% bacitracin (Sigma) (BSB). Each tissue sample was blotted with Whatman No. 41 filter paper to remove excess buffer and was weighed before and after mucosal scrapings. Mucosa from the normal fundic and colorectal portion of the alimentary tract was scraped on ice with an ice-cold glass slide, and
the scrapings were collected in storage vials kept on dry ice. Nonmucosal tissue (fundic muscle) was not scraped, and mucosal tissue was quantitatively recovered by this method. Tumor from 2 to 4 animals, colon mucosa from 4 animals, and fundic mucosa from 4 to 6 animals were pooled and processed independently as separate samples. Each sample was given a code number and processed in a double blind manner. Tissue samples thus collected were stored at -70°C in an ultradry freeze (Revco).

Preparation of Membranes. Frozen tissues were weighed and pulverized with a Thermovac autopulverizer cooled in liquid nitrogen. The resulting powder was homogenized in 5 volumes of Buffer A + BSB, using a precooled Polytron homogenizer (PG-10ST; Brinkman Industries, Westbury, NY). The homogenate was filtered through a double layer of cheese cloth presoaked in ice-cold Buffer A and was centrifuged at 200 × g for 10 min to remove any cell debris. For the preparation of total crude membrane fractions, the supernatant (200 × g for 10 min) was subjected to centrifugation (30,000 × g for 45 min) in a fixed-angle SM-24 rotor. Pellets (30,000 × g for 45 min) were washed once again with Buffer A + BSB and repelleted at 30,000 × g for 45 min. The pellet obtained was resuspended in Buffer B (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (25 mM), MgCl₂ (2.5 mM), KCl (5 mM), NaCl (137 mM), NaHPO₄ (0.7 mM), glucose (10 mM), pH 7.4) + BSB, was homogenized manually with a glass Teflon homogenizer, and processed for measurement of protein and GRs. In order to determine whether comparable amounts of membranes were being used as substrate for the binding studies, the levels of both the total and ouabain-sensitive Na⁺-K⁺-ATPase were measured in the laboratory of Dr. E. Labelle, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX, as described previously (19). No significant difference was observed in ouabain-sensitive ATPase levels in the tumor and normal tissues from different groups receiving different treatments; this indicates that the receptors were measured on essentially comparable substrates, and that the different treatments had no measurable nonspecific effects on the cell membranes.

Gastrin-binding Assay. Synthetic human gastrin-17-1 (BACHEM, Torrance, CA) was iodinated with Iodo-Gen (Pierce Laboratory, IL) as described previously (19). Briefly, Iodo-Gen (5.8 nmol) in 10 μl of CH₂Cl₂ was dried under nitrogen in a reaction tube. Potassium phosphate buffer (50 μl of 0.5 M, pH 7.4), 5 μl of 0.1 N HCl, and 1 nmol of gastrin were added. The reaction tube was tightly capped for 6 min on ice, and the reaction was stopped by the addition of 100 μl of 0.05 M NH₄HCO₃. Monoiodinated gastrin was next separated on an anion-exchange column, and the specific activity was calculated to be approximately 1500 dpm/fmol of gastrin. The biological activity of iodinated gastrin molecules was determined as described previously (19) and was found to be similar to that of an equimolar concentration of noniodinated gastrin.

Binding to Membranes. Aliquots of membrane suspension in Buffer B + BSB, containing approximately 200 μg of protein, were used as the substrate in each tube. A multipoint (7 to 10 point) saturation analysis was carried out, using increasing concentrations of radiolabeled gastrin (with a high specific activity = 1 to 2 × 10⁶ dpm/fmol) (0.01 nM to 0.5 nM), either in the presence (for nonspecific binding) or in the absence (for total binding) of a 1000-fold excess of unlabeled gastrin, to measure high affinity binding sites. In a separate set of studies, increasing concentrations of 125I-gastrin (with a low specific activity = 1 × 10⁴ dpm/pmol) (0.05 μM to 50 μM) ± 1000-fold excess unlabeled gastrin were also used to measure low affinity binding sites. We have previously described this technique for measuring low affinity estradiol binding sites in rat pancreas (21). In each case, binding was analyzed by a Scatchard plot of the specific binding data (22). The binding assay was performed at 30°C in a water bath for 30 min. At the end of the incubation, the binding assay tubes were chilled on ice, and 100 μl of ice-cold Buffer B + BSB were added. The peptide hormone bound to the substrate was separated from the excess, unbound hormone by filtration over cellulose acetate filters (Celotape; Millipore) using multfiltration units (Model 1225 sampling manifold; Millipore). The membrane filters containing the substrates were placed in a glass tube and counted in a gamma counter (Beckman Model 5550, with 78% efficiency for 125I counting).

RESULTS

Weights of Fundus, Colon, and MC-26 Tumors in Control and Treated Mice. The body weights of treated and control groups of mice were not significantly different. The effects of PG and PGL treatment on tumor, fundus, and colon weights are shown in Table 1 and Fig. 1. PG treatment resulted in a significant increase in the weights of the tumor (approximately 1.5 times) and fundic mucosa (approximately 1.7 times), but no significant effect was observed on colon and fundic muscle weights. PGL, in the presence of PG, significantly reduced the MC-26 tumor weights, both in comparison to the saline control group (approximately 0.6 times) and the PG-treated groups (approximately 0.4 times). These findings indicate that PGL not only effectively reduces PG-stimulated growth, but further reduces the growth of tumors to less than control values in the PG + PGL treated group, possibly by an independent mechanism. On the other hand, PGL was less effective in suppressing the trophic effects of gastrin on normal GI mucosal cells in PG-treated mice. PGL reduced the trophic effects of PG on fundic mucosa only slightly (approximately 0.8 times), but the effect
was not statistically significant. PGL, by itself, had no significant effect on either the MC-26 tumors or the normal GI tissues. PGL appeared to have a slight, but not significant, trophic effect on fundic mucosa (approximately 1.3 times).

Binding Affinity of Gastrin and GR Level in Control and Treated Mice. In MC-26 tumors from control (saline injected) mice, two types of specific gastrin-binding sites were detected (Fig. 2), which we have arbitrarily termed type 1 and type 2 gastrin-binding sites. The two types of binding sites appeared to be independent entities with no apparent cooperative effects between the two sites. The type 1 binding sites were saturable at doses of <0.05 nM gastrin and demonstrated a high binding affinity for gastrin ($K_d = <0.5 \text{ nM}$); we have termed these sites GRs. The type 2 sites were nonsaturable at physiological concentrations of gastrin (Fig. 2), but were saturated at pharmacological concentrations of 10 to 50 $\mu$M. A modified binding assay (described in “Materials and Methods”) was used to measure the binding affinity of type 2 sites. The binding affinity of type 2 sites was found to be very low ($K_d = 0.2$ to 0.5 $\mu$M), and at saturation, a total number of 2 to 10 pmol of specific gastrin binding sites/mg of protein could be measured. A representative Scatchard plot, depicting type 2 sites (termed arbitrarily as gastrin binding sites to differentiate them from GR) on control tumor membranes, is shown in Fig. 3.

The effect of PG and PGL on the GR levels and on the binding affinity of GR for gastrin on the MC-26 tumor membranes is summarized in Table 2 and graphically presented as the percentage of change in Fig. 1. PG treatment of mice resulted in a significant increase (approximately 1.99 times) in the level of type 1 GR on MC-26 tumors compared to the levels of type 1 GR on tumors from controls receiving saline injections. There was no significant effect on the binding affinity of type 1 GR. PGL prevented the up-regulatory effect of PG on type 1 GR. This effect of PGL may be related to a significant decrease in the binding affinity of GR for gastrin (approximately 1.64 times) in the same group (PG + PGL injected) of mice. PGL given alone had no significant effect on either the type 1 GR levels or the binding affinity of GR for gastrin. Levels of type 2 gastrin binding sites on MC-26 tumors did not change significantly in any of the treated groups, indicating the nonspecific presence of type 2 sites on the tumor membranes (data not shown). We have similarly observed type 2 specific estradiol binding sites in normal rat pancreas, with a very large capacity (21); the physiological significance of these sites is not known.

In normal GI tissues, unlike MC-26 tumors, we found only the high-affinity type 1 binding sites (GR) in all groups of control and treated mice. The effect of PG and PGL on GR levels and binding affinity of GR for gastrin on cell membranes from normal GI tissue is summarized in Table 3 and is graphically presented in Fig. 1. Treatment with either PG or PGL, alone, resulted in approximately 1.3 to 1.5 times increase in GR levels in fundic mucosa, but because of the variability of results, the effect was not statistically significant. PG treatment...
binding affinity of GR. These combined actions apparently of both the binding of gastrin to GR and reduction of the actions of gastrin on MC-26 tumors may be due to inhibition compared to 100% of gastrin and 0.3% of PG. These findings GR on MC-26 cells with a relative binding affinity of 0.0003%

The in vivo growth of MC-26 cells presently, however, had no significant effect on the binding affinity of GR for fundic mucosal membranes, but it had no effect on the binding affinity of GR on colonic membranes.

**DISCUSSION**

PGL is an antagonist of the acid secretory and trophic effects of gastrin on normal gastrointestinal cells (1–6). We have previously reported that PGL inhibits the growth of mouse colon tumors (MC-26) in vivo and prolongs survival of tumor-bearing mice (7). The inhibitory effects of long-term treatment with PGL in vivo are thought to be mediated by inhibition of binding of endogenous gastrin to GR. PGL completely inhibits the binding of gastrin to GR on fundic mucosal cells in vitro(13-15) at concentrations higher than 10^-5 M. In the present study, we did not observe an inhibition of tumor growth in the presence of PGL alone. Our previous (7) and present studies were done more than 2 yr apart, and it is possible that the in vivo growth of MC-26 cells in previous studies was affected significantly by endogenous gastrin itself, which was inhibited by PGL. The in vivo growth of MC-26 cells presently, however, is significantly up-regulated only in the presence of exogenous gastrin, which may explain our present observation of an absence of inhibitory effects by PGL alone. In the present study, we observed that PGL completely inhibited the gastrin-mediated growth of the MC-26 tumors, reduced the gastrin-stimulated GR levels to control values, and in the presence of PG, significantly reduced the binding affinity of GR for gastrin on the tumor membranes. In preliminary studies (data not shown) we have observed that PGL inhibits the binding of gastrin to GR on MC-26 cells with a relative binding affinity of 0.0003% compared to 100% of gastrin and 0.3% of PG. These findings provide evidence that the antagonism produced by PGL for the actions of gastrin on MC-26 tumors may be due to inhibition of both the binding of gastrin to GR and reduction of the binding affinity of GR. These combined actions apparently resulted in the loss of the ability of gastrin to regulate GR.

Mechanisms controlling the binding affinity of membrane receptors are not fully understood. Pertussis toxin has been found to reduce the binding affinity of somatostatin (SRIF) receptors for SRIF analogues on a mouse pituitary tumor cell line (24). On the other hand, a gut hormone, gastric inhibitory polypeptide, was recently found to increase the receptor affinity for insulin and the cellular sensitivity to insulin in adipocytes (25). These findings and the findings of our present studies provide further evidence that the antagonistic and agonistic actions of hormones and hormone-receptor antagonists may be a consequence of changes in receptor affinity by mechanisms which are not yet clear. The mechanism of action of PGL may be directly mediated by inhibition of gastrin binding to GR. An independent, intracellular postreceptor mechanism, however, cannot be excluded at this point. We have also observed a similar reduction in the binding affinity of GR on MC-26 tumor membranes by the long-acting somatostatin analogue (SRIF-201-995; Sandoz Co., East Hanover, NJ) (26). SRIF, unlike PGL, however, does not inhibit gastrin binding to GR on MC-26 tumors, which supports the possibility that other (indirect or direct) mechanisms, perhaps at the intracellular level, may be operative in reducing the binding affinity of GR on tumor membranes.

The present study provides evidence that PGL-mediated attenuation of gastrin effects is tissue specific. Both the trophic and GR-regulatory effects of gastrin on MC-26 tumors were significantly reduced (to control or less than control levels) by PGL treatment. In contrast, the trophic effects of gastrin on fundic mucosal cells were only partially or insignificantly reduced by PGL. A possible explanation of this effect may be that endogenous gastrin competes successfully with the antagonistic actions of exogenous PGL on normal fundic mucosal cells, but may be less effective as a competitor in the tumor cells.

Gastrin receptor levels on fundic mucosal cells were not significantly up-regulated (increased) by gastrin. This could explain failure to detect a significant effect of PGL treatment on GR-regulatory actions of gastrin on fundic mucosa. Furthermore, no significant trophic or GR-regulatory effects of gastrin were observed on the colonic cells; this was unchanged in the presence of PGL. On the other hand, PGL combined with PG significantly reduced the binding affinity of GR for gastrin on all three tissue membranes examined. This reduction indicates that the differential antagonism afforded by PGL for PG actions on the tumor and normal cells may not be related to the common effect on the binding affinity. Alternatively, the loss of GR binding affinity induced by PGL may be more readily expressed in tumor cells and reflects on the growth and GR stimulatory actions of gastrin in tumor cells that are rapidly dividing and not on normal cells that divide less rapidly. Tumor cells are more sensitive to the actions of PG and require PG for maintaining the high affinity type 1 GR, which are otherwise lost during progressive tumor growth (20). In the same manner, the MC-26 tumors appear to be more sensitive to the antagonistic actions of PGL, and the effects of PGL are magnified on the tumor cells, in comparison to that on normal cells.

In the absence of exogenous PG, PGL did not significantly affect the weight, the GR levels, or the binding affinity of GR on MC-26 tumors and normal colon. In the fundus, however, PGL significantly reduced binding affinity of GR, but did not cause significant up-regulation of GR or growth of fundic mucosa. The significant loss in binding affinity of GR on the fundic mucosa produced by PGL further supports our view that the mechanism of receptor affinity changes may be independent and separate and not mediated via binding to GR. Since PGL can interact with GR, it may act as a weak agonist at the high concentrations we have used and cause a slight up-regulation of GR which would lead to a slight trophic effect on the fundic

| ***Table 3: Effect of PG and PGL on the GR levels and binding affinity of GR for gastrin on the fundic and colonic membranes from mice*** |
|-------------------------------------------------|----------|----------|----------|----------|
| **Treatment** | **GR (fmol/mg protein)** | **K<sub>d</sub> (nM)** | **GR (fmol/mg protein)** | **K<sub>d</sub> (nM)** |
| Saline   | 45.6 ± 4.2<sup>a</sup> | 0.48 ± 0.07 | 52.7 ± 4.4 | 0.25 ± 0.05 |
| PG      | 60.0 ± 11.9 | 0.58 ± 0.06 | 62.3 ± 5.4 | 0.38 ± 0.09 |
| PG + PGL| 39.2 ± 2.9  | 0.74 ± 0.04<sup>a</sup> | 64.9 ± 9.1 | 0.78 ± 0.04<sup>a</sup> |
| PGL     | 69.0 ± 10.5 | 1.8 ± 0.38<sup>a</sup> | 48.7 ± 6.9 | 0.3 ± 0.11 |

<sup>a</sup> Mean ± SE of data from 3 to 6 observations from one experiment.  
<sup>b</sup> P < 0.05 compared with saline-treated group.  
<sup>c</sup> P < 0.05 compared with PC-treated group.
mucosa. PGL alone has been reported to be slightly trophic for rat pancreas (12, 27). The effect of proglumide on pancreas may be produced by the interaction of PGL with CCK receptors, which in turn may cause the weak agonistic effects.

We do not yet understand the disparity between the significant negative effect of PGL alone on binding affinity of GR on fundic mucosa compared to no effect on MC-26 tumors or normal colon. We speculate that the lack of PGL effects on these two tissues may be due to several reasons. Since PGL is at best only a weak agonist, its trophic and GR regulatory effects on tumor cells may have been camouflaged by the rapid growth of tumor cells. The normal mouse colon appears to be less sensitive to regulatory effects of PG and may therefore be less sensitive to PGL as well. In order to observe a change in the expression of binding affinity of GR by PGL, an interaction between PGL and some PG-mediated intracellular event may be required. In normal fundic mucosal cells, endogenous gastrin itself may be sufficient for PGL action on binding affinity of GR, while in the case of MC-26 tumors, additional priming with exogenous PG may be required before the tumor becomes functional by itself, and may require priming by gastrin before it becomes capable of antagonizing the growth of tumor cells. The normal mouse colon appears to be less sensitive to PGL as well. In order to observe a change in the expression of binding affinity of GR by PGL, an interaction between PGL and some PG-mediated intracellular event may be required. In normal fundic mucosal cells, endogenous gastrin itself may be sufficient for PGL action on binding affinity of GR, while in the case of MC-26 tumors, additional priming with exogenous PG may be required before the tumor becomes susceptible to the inhibitory effects of PGL on binding affinity of GR.

From our present studies it appears that, in the presence of gastrin, PGL is a strong antagonist of tumor growth, but is not functional by itself, and may require priming by gastrin before achieving the ability to express antagonistic effects. In some uterine cancers, prostogesterone is used as a therapeutic agent and sometimes requires priming with estradiol to initiate important intracellular events (including synthesis of progesterone receptor), before it becomes capable of antagonizing the growth of uterine cancer (28). PGL may similarly require intracellular priming by gastrin, including GR regulation, before tumor growth can be antagonized.

ACKNOWLEDGMENTS

We wish to thank Professor Luigi Rovati of Rotta Laboratories, Monza, Italy, and Joseph Barrows of Barrows Research Group, New York, NY, for their generous gift of proglumide. The authors gratefully acknowledge the technical assistance of A. Laridjani and the excellent secretarial help of Kay Smith.

REFERENCES


Inhibition of Pentagastrin-stimulated Up-Regulation of Gastrin Receptors and Growth of Mouse Colon Tumor in Vivo by Proglumide, a Gastrin Receptor Antagonist

Pomila Singh, Sy Le, R. Daniel Beauchamp, et al.

*Cancer Res* 1987;47:5000-5004.

Updated version: Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/47/19/5000

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.