Role of Gastrin and Gastrin Receptors on the Growth of a Transplantable Mouse Colon Carcinoma (MC-26) in BALB/c Mice

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ABSTRACT

We recently reported trophic response of transplantable mouse colon cancer cells (MC-26) to pentagastrin, in vivo, and demonstrated gastrin receptors on MC-26 cells, in vitro. In the present study, growth of MC-26 cells in mice, in response to pentagastrin, was studied in relation to binding kinetics and capacity of gastrin receptor. Gastrin receptor levels on mouse fundic and colonic membranes and on MC-26 cellular membranes were determined before MC-26 cell inoculation and designated as Day 0 levels. Four groups of mice were next inoculated with MC-26 cells and given injections of either pentagastrin (treated) or normal saline (control) for 10 or 15 days. At the end of the treatment periods, body, tumor, fundic, and colon weights were noted and gastrin receptor measured. Tumor and fundic weights increased significantly within 15 days of pentagastrin treatment, compared to control values. In control (non-pentagastrin treated) mice, the binding affinity of gastrin receptor on tumor membranes was significantly decreased and associated with the complete loss of high-affinity gastrin receptor \( K_d = <0.5 \text{ nM} \) by Day 15 of tumor growth. On the other hand, both the binding affinity and gastrin receptor levels of tumor membranes were maintained at Day 0 values by pentagastrin treatment. Endogenous gastrin was therefore ineffective in maintaining high-affinity gastrin receptor on control tumors. A significant number of low-affinity gastrin-binding sites \( K_d = >2 \text{ nM} \) appeared in control tumors by Day 15, which could reflect rapid differentiation or conformational changes of gastrin receptor in the absence of high levels of normal regulatory hormones. These studies demonstrate that the trophic effects of gastrin on MC-26 cells are probably mediated by its regulation and maintenance of the binding affinity and capacity of gastrin receptor on the cancer cells, in vivo.

INTRODUCTION

Carcinoma of the large bowel accounts for 14.7% of all cancers and is one of the most common causes of death in both men and women (1). One of the hormones that affects growth of the mucosal cells lining the GI tract is gastrin (2). A biologically active analogue of gastrin, PG, has been shown to stimulate DNA and protein synthesis in duodenal and gastric mucosa of rats (3). On the other hand, antrectomy, which removes the major source of endogenous gastrin, results in atrophy of the mucosa of the gastric fundus that can be reversed by exogenous gastrin (4).

An increased incidence of cancers of the small and large bowel, in response to dimethylhydrazine or azoxymethane, has been shown in the rat, after small bowel resection, which is known to result in elevated gastrin levels (5–7). In addition, we have reported a significant increase in tumor weight and tumor DNA content of a transplantable mouse colon cancer (MC-26), mediated by pentagastrin (9). These observations suggest that proliferation of normal colorectal cells is a gastrin-dependent process, and that growth of some colorectal and stomach cancer may also be influenced by gastrin. Other tumors arising from hormone-responsive tissues, notably breast cancer, have been treated successfully by endocrine-related procedures, when the tumor has been shown to have specific receptors for the hormone(s) that stimulates growth of the tissue (10, 11). It may not be beyond the realm of possibility to consider, in the future, similar maneuvers to modify the growth of colon cancer.

Gastrin is believed to act by way of specific membrane receptors in a manner similar to other peptide hormones. GR are known to be present on the fundic mucosa of rats, dogs, and rabbits (12–15). We have recently reported the presence of GR on MC-26 cells in vitro (16, 17). In a preliminary communication, we have reported the presence of GR in a significant percentage of human cancers arising from the large bowel and stomach (18). These findings suggest that the observed trophic effect of gastrin may be mediated by its interaction with the gastrin receptor on GI cancer cells. In order to test this possibility, we have further examined the growth of MC-26 cells, in vivo, in response to pentagastrin, in relation to levels of GR.

MATERIALS AND METHODS

Transplantation of Mouse Colon Cancer Cells in Mice. A transplantable mouse colon cancer cell line, CT26, was obtained from Mason Research Laboratory (Worcester, MA); from it, a tissue culture cell line, MC-26, was established in our laboratory. A large number of these cells were frozen in 10% dimethyl sulfoxide and were later thawed and grown in tissue culture as needed. Single-cell suspensions were inoculated into male BALB/c mice (20–25 g). Cancer cells (1 x 10⁶) were injected s.c. in the flank, as this number was found to be sufficient to produce cancer in all the animals tested so far; 90% of inoculated mice survived for 21 days (data not presented).

Treatment of Animals. After the inoculation of the mice with MC-26 cells, mice were given injections i.p. of pentagastrin (250 μg/kg body weight) (treated group) or of saline (control group) 3 times a day. This dose of PG was previously found to be optimal for stimulation of tumor growth (9).

Collection of Tissue. At the end of the treatment period, which ranged from 7–15 days postinoculation of MC-26 cells, the mice were decapitated. Gastric fundus, colorectum, and s.c. tumors were quickly removed from mice; placed in ice-cold 0.9% NaCl solution; and washed extensively to remove all extraneous contamination. The tumors had well-defined margins and dissected from surrounding normal tissues without difficulty. All tissues were removed by one individual who did not know what variant had been given. All other steps were carried out at 4°C unless otherwise stated. Tissues were then washed in Buffer A (10 mM Tris, 137 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2.5 mM MgCl₂, 0.25 mM sucrose, pH 7.4), containing 0.1% bovine serum albumin (Fraction V; Sigma), 0.1% soybean trypsin inhibitor ( Worthington), and 0.1% bacitracin (Sigma) (BBS). Each tissue sample was blotted with Whatman No. 1 paper to remove excess buffer and 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.

Tumor and colon mucosa samples from 2–4 animals were pooled as one sample, while fundic mucosa from 4–6 animals was pooled as one sample.
sample and processed independently. Each sample was given a code number and processed in a double blind manner, so that the results could be evaluated objectively. Tissue samples thus collected were stored at —70°C in an ultradry freeze (Revo).

Preparation of Membranes. Frozen tissues were weighed and pulverized with a Thermovac autopulverizer cooled in liquid nitrogen. The resulting powder was homogenized in five volumes of Buffer A plus BSB, using a precooled polytron homogenizer (PG-10ST; Brinkman Industries, Westbury). The homogenate was filtered through a double layer of cheese cloth, presoaked in ice-cold Buffer A, and subjected to 200 × g 10-min centrifugation to remove any cell debris. For the preparation of total crude membrane fractions, the 200 × g 10-min supernatant was subjected to 30,000 × g 45-min centrifugation in a fixed-angle SM-24 rotor. Pellets (30,000 × g, 45 min) were washed once again with Buffer A plus BSB and repelleted at 30,000 × g for 45 min. The pellet thus obtained was resuspended in Buffer B [25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 5 mM KCl, 137 mM NaCl, 0.7 mM NaHPO4, 10 mM glucose, pH 7.4] plus BSB, homogenized manually with a glass Teflon homogenizer, and processed for measurement of protein and gastrin receptors.

Gastrin-binding Assay. Synthetic human Gastrin-17-1 (Bachem, Torrance, CA) was iodinated with Iodo-Gen (Pierce Laboratory, IL) as we have previously described (17). Briefly Iodo-Gen (5.8 nmol) in 10 µl of CH3Cl2 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 NHCI, and 1 nmol of gastrin were next added. The reaction tube was lightly tapped for 6 min on ice, and the reaction was stopped with 100 µl of 0.05 M NH4HCO3. Moniodinated 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 published (17) and found to be similar to that of an equimolar concentration of noniodinated gastrin.

Binding to Membranes. Aliquots of membrane suspension in Buffer B plus BSB, containing approximately 200 µg of protein, were used as substrate in each tube. A multipoint saturation analysis (7-10 point assay), using increasing concentrations of radiolabeled gastrin (0.01-150 fmol/mg protein) and processed for measurement of protein and gastrin receptors.

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Binding of Gastrin to Membranes. Aliquots of membrane suspension in Buffer B plus BSB, containing approximately 200 µg of protein, were used as substrate in each tube. A multipoint saturation analysis (7-10 point assay), using increasing concentrations of radiolabeled gastrin (0.01-0.5 nmol), with (nonspecific binding) or without (total binding) 1000-fold excess of cold gastrin, was carried out, and the binding was analyzed by a Scatchard plot of the specific binding data (19). The binding assay was performed at 30°C in a water bath for 30 min, which was found optimal for measuring the maximum number of binding sites (data not presented). At the end of the incubation, the binding assay tubes were chilled on ice, and 100 µl of ice-cold Buffer B plus BSB were added. The peptide hormone bound to the substrate was separated from the excess, unbound hormone by filtration over cellulose acetate filters (Celotate; Millipore) using multifiltration units (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 131I counting).

Table 1  Effect of pentagastrin on GR levels and on binding affinity of gastrin for GR on fundic, colorectal, and MC-26 tumor membranes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Day</th>
<th>GR levels (fmol/mg protein)</th>
<th>Kd (nm)</th>
<th>GR levels (fmol/mg protein)</th>
<th>Kd (nm)</th>
</tr>
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<tbody>
<tr>
<td>MC-26</td>
<td>0</td>
<td>38.2 ± 2.6</td>
<td>0.46 ± 0.03</td>
<td>49.0 ± 2.9</td>
<td>0.39 ± 0.03</td>
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<td></td>
<td>10</td>
<td>7.5 ± 0.2</td>
<td>0.35 ± 0.02</td>
<td>48.7 ± 3.7</td>
<td>0.45 ± 0.04</td>
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<tr>
<td></td>
<td>15</td>
<td>44.8 ± 3.5</td>
<td>2.73 ± 0.48</td>
<td>28.9 ± 1.9</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>Fundus</td>
<td>0</td>
<td>33.3 ± 1.9</td>
<td>0.46 ± 0.01</td>
<td>39.7 ± 3.8</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>37.4 ± 3.3</td>
<td>0.45 ± 0.01</td>
<td>28.9 ± 1.9</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>34.6 ± 3.1</td>
<td>0.39 ± 0.02</td>
<td>30.0 ± 2.4</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>Colon</td>
<td>0</td>
<td>43.1 ± 1.2</td>
<td>0.53 ± 0.03</td>
<td>44.4 ± 6.7</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>38.7 ± 1.4</td>
<td>0.42 ± 0.03</td>
<td>30.0 ± 2.4</td>
<td>0.47 ± 0.02</td>
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*Control mice were given injections of saline, while treated mice received PG (250 µg/kg of body weight, 3 times daily), as given in "Materials and Methods."

*GR and Kd values were determined on MC-26 cellular membranes before inoculation and on normal fundic and colorectal membranes from control mice before inoculation. The values thus obtained are depicted as Day 0 values.

*Mean ± SE of 4 or more independent observations from pooled tissue samples, taken from 2–6 animals/experiment. Twelve mice/group were studied in 2 separate experiments.

*P < 0.05 compared to either Day 0, 10, or 15 control groups or all the control and treated groups as shown.
GR on the MC-26 cells was similar to that on the normal colonic membranes (Fig. 1).

Effect of PG Treatment on Body and Tumor Weights and GR Levels. After inoculation of MC-26 cells, a viable tumor was evident in 90% of the mice 10 days postinoculation. At this point, a second group of control (not treated with PG) and a group of treated (PG-treated) mice were decapitated. The body weights of both the control and treated groups of mice, at Day 10, were not significantly different from each other or from that of mice on Day 0 of cancer cell inoculation, (Table 2). Tumor weights of treated mice, at Day 10, although slightly decreased (approximately 1.3 times) compared to those from control mice, were not statistically significant.

The number of GR in both the treated and control groups of animals is shown in Table 1. The binding affinity and number of GR on normal fundic and colonic membranes from the treated and control groups of mice at Day 10 were similar to that observed at Day 0, indicating that high circulating levels of PG were without significant effect on normal cell GR levels. On Day 10, GR levels on MC-26 tumor cells, on the other hand, were significantly reduced (approximately 80%) in the control (non-PG-treated) mice and insignificantly increased in the treated group, compared to Day 0. The binding affinity of gastrin for GR remained unchanged in the control and treated tumor cells on Day 10, compared to that on Day 0. A representative Scatchard plot of the specific gastrin-binding data from control and treated mice on Day 10 is shown in Fig. 2. This indicates that the number of GR on tumor cells, in vivo, is acutely under gastrin control and requires the presence of high levels of gastrin to maintain the full complement of GR. The endogenously produced gastrin is apparently insufficient to maintain GR levels on the tumor cells in non-PG-treated mice.

A third group of control and treated mice were sacrificed on Day 15 and were analyzed as described above for Day 10 mice. The body weights, once again, did not change significantly in either the control or treated groups of mice on Day 15 compared to those of either Day 0 or Day 10 mice (Table 2). The tumor weights, on the other hand, demonstrated a significant increase in the control and treated mice on Day 15, compared to those in the corresponding Day 10 mice. The tumor weights of Day 15-treated mice were, in addition, significantly higher compared to those of Day 15-control mice (Table 2), indicating a positive trophic effect of PG, at the dose given, within 15 days of treatment.

By Day 15, dramatic changes in the number of GR and the binding affinity were apparent in the tumors of nontreated mice (Table 1; Fig. 3). The binding affinity of gastrin for GR was significantly decreased (approximately one-tenth) in the control mice, at Day 15, compared to that in all other groups. A significantly high number of these low-affinity sites were, however, measurable in the tumors of control mice at Day 15. This could indicate a possible degradation or conformational change of the type of gastrin-binding sites being coded in the tumor cells in the absence of PG. In the normal cells in rats, on the other hand, a change in the binding affinity, on removal of endogenous gastrin, has not been observed, although the number of binding sites was reduced significantly (22).

In the PG-treated mice in the present study, the number and affinity of GR were maintained in the tumor cells on Day 15 at levels similar to those observed at Day 10 and Day 0, indicating that the rapid growth of the tumor did not alter the GR response of the tumor cells to PG. The number of GR on the normal fundic and colonic membranes was slightly increased at Day 15 in the treated animals, compared to that at Day 10 and Day 0, but the difference was not statistically significant. An absence of significant effect of high levels of PG for 15 days on the number of GR on normal cells indicates that either the exogenous PG did not reach the GI tract in appreciable amounts, or the GR response of the normal cells cannot be increased beyond the physiological level per cell but is decreased in the absence of gastrin (22). The significant increase in the growth response of normal tissues at Day 15 in the presence of PG (Table 2) indicates that exogenous PG effectively reaches the GI tract but results only in weight increases and not in GR levels per unit of protein or unit of tissue weight.

DISCUSSION

We have previously described the presence of gastrin receptors on gastric and colonic cancer cell lines (8, 17). One of

![Fig. 1. Scatchard plot of specifically bound $^{125}$I-SHG-17 to crude membranes prepared from MC-26 cells, fundus, and colon on Day 0, before inoculation into BALB/c mice. Details are given in "Materials and Methods." Amounts of membrane protein used per assay point were 78 µg for the MC-26 tumor cells, 150 µg for the fundus, and 245 µg for colon samples. Gastrin receptor levels calculated from the Scatchard plots were 32.6, 34.3, and 41.4 fmol/mg of protein for tumor, fundus, and colon, respectively. Each point is the mean of duplicate observations from a representative experiment.](image-url)
**GASTRIN AND GASTRIN RECEPTORS IN MOUSE COLON CARCINOMA**

Fig. 2. Scatchard plot of specifically bound $^{125}$I-SHG-17 to crude membranes prepared from MC-26 cells, fundus, and colon on Day 10 after cancer cell inoculation into control (non-PG treated) BALB/c mice. Amounts of membrane protein used per assay point were 170 $\mu$g for the MC-26 tumor cells and 300 $\mu$g for the fundus and colon samples. Gastrin receptor levels calculated from the Scatchard plots were 5.9, 43.3, and 56.7 fmol/mg of protein for tumor, fundus, and colon, respectively. Each point is the mean of duplicate observations from a representative experiment.

Fig. 3. Scatchard plot of specifically bound $^{125}$I-SHG-17 to crude membranes prepared from MC-26 cells taken from control and PG-treated mice at Days 10 and 15 after cancer cell inoculation into mice. The Scatchard plot of the specific gastrin binding data to tumor membranes from PG-treated mice at Day 10 is not shown and was found to be similar to that shown for the Day 15 PG-treated group. The amounts of membrane protein used per assay point were 170 $\mu$g for Day 10 control tumors, 195 $\mu$g of protein for Day 15 PG-treated tumors, and 270 $\mu$g of protein for Day 15 control tumors. Gastrin receptor levels calculated from the Scatchard plots were 5.9, 46.1, and 51.8 fmol/mg of protein for Day 10 control tumors, Day 15 pentagastrin-treated tumors, and Day 15 control tumors, respectively. Each point is the mean of duplicate observations from a representative experiment.

These, the MC-26 cells, was found to be highly positive for the presence of specific gastrin binding sites, with a high binding affinity for gastrin [$K_d = 0.5$ nM (16)]. In the present study, we have further demonstrated that the specific gastrin-binding capacity of the MC-26 cancer cells is maintained 10 days postinoculation into mice, although at significantly reduced levels. The gastrin-binding system of the cancer cells appears to undergo a significant change, in vivo, and requires the presence of high circulating levels of PG in order to maintain the full complement of binding sites. The reason for this new acquisition of dependency on the trophic hormone, in vivo, is not understood, since we have shown the maintenance of specific gastrin-binding activity by the MC-26 cells in vitro, even in the absence of any hormone in the culture medium (16). Similarly, the presence of receptors for GI hormones, such as vasoactive intestinal peptide (23–25), secretin, and caerulein (26) on pancreatic, fundic, and colonic cancer cell lines in vitro has been repeatedly shown in the absence of peptide hormones. The tumor cells may rapidly dedifferentiate, in vivo, in the absence of the sustaining hormones, which could explain the present findings of a loss of binding affinity and concomitant significant loss in the levels of high-affinity gastrin binding sites within 15 days of growth in vivo.

In the presence of PG, however, the binding affinity and high-affinity gastrin-binding sites were maintained by the tumor cells in vivo. This would explain the observed trophic response of the tumors to PG in vivo. Administration of PG to mice, 15 days after cancer cell inoculation, was ineffective in initiating trophic response, further corroborating the possibility that loss of binding affinity and high-affinity sites, within 15 days, in vivo, makes the tumor independent of hormone action and nonresponsive to PG. These studies may thus have future clinical significance in the light of our knowledge in breast cancer. Breast cancers are known to be either hormone dependent or hormone independent, and only the former tumors respond to endocrine manipulation by regression (27, 28). Recently, we have, in fact, observed a significant decrease in the growth rate of MC-26 tumors in mice in the presence of proglumide (an antigastrin drug) (29). In addition we have observed a significant inhibition by proglumide of pentagastrin effects on both the growth and gastrin receptor up-regulation of MC-26 cells in vivo. We thus speculate that, in the future, it may be possible to identify hormone-dependent GI cancers and, possibly, to treat them by a suitable endocrine regimen. A word of caution, however, needs to be applied at this point. These results, so far, are limited to our observations on only one tumor cell line and may not apply to other GI cancer cell lines from other animal models or human sources. Studies therefore need to be conducted on other GI cancer cell lines, in a similar manner, before the precise role of gastrin in growth control of GI cancers can be established.

**REFERENCES**


P. Singh, J. P. Walker, C. M. Townsend, Jr., and J. C. Thompson, unpublished data.
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