Aberrant expression of gastrin-releasing peptide and its receptor by well-differentiated colon cancers in humans

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Carroll, Robert E., Kristina A. Matkowskyj, Subrata Chakrabarti, Thomas J. McDonald, and Richard V. Benya. Aberrant expression of gastrin-releasing peptide and its receptor by well-differentiated colon cancers in humans. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G655–G665, 1999.—Epithelial cells lining the adult human colon do not normally express gastrin-releasing peptide (GRP) or its receptor (GRPR). In contrast, approximately one-third of human colon cancers and cancer cell lines have been shown to express GRP-binding sites. Because GRP coexpression causes the proliferation of many cancer cell lines, GRP has been presumed to act as a clinically significant growth factor. Yet GRP has not been shown to be expressed by colon cancers in humans nor has the effect of GRP and/or GRPR coexpression on tumor behavior been investigated. We therefore determined GRP and GRPR expression by immunohistochemistry in 50 randomly selected colon cancers resected between 1980 and 1997, all 37 associated lymph node and liver metastases, and 20 polyps. Tumor sections studied were those that contained the margin and adjacent nonmalignant epithelium. Overall, 84% of cancers aberrantly expressed GRP or GRPR, with 62% expressing both ligand and receptor, whereas expression was not observed in adjacent normal epithelium. Consistent with the previously established mitogenic capabilities of GRP, tissues coexpressing GRP and GRPR were more likely to express proliferating cell nuclear antigen than tissues not expressing both ligand and receptor. Yet GRP/GRPR coexpression was seen with equal frequency in stage A as in stage D cancers and was only detected in 1 in 37 metastases. Furthermore, Kaplan-Meier analysis did not reveal any difference in patient survival between those whose tumors did or did not express GRP/GRPR. In contrast, GRP/GRPR coexpression was found in all well-differentiated tumor regions, whereas poorly differentiated tissues never coexpressed GRP/GRPR. Overall, these data indicate that, although GRP is a mitogen, it is not a clinically significant growth factor in human colon cancers. Rather, the strong association of GRP/GRPR coexpression with tumor differentiation raises the possibility that these proteins primarily act in vivo as morphogens.

adenocarcinoma; bombesin; mitogen; morphogen

GASTRIN-RELEASING PEPTIDE (GRP) is the mammalian homologue of bombesin, a tetradecapeptide originally isolated from the skin of the frog Bombina bombina (8). GRP and/or bombesin is important in regulating a number of normal physiological processes within the gastrointestinal (GI) system, including modulating secretion of the exocrine pancreas and other GI peptide hormones as well as altering smooth muscle contractility and intestinal transit (18). GRP mediates its effects in humans by binding to a specific seven transmembrane-spanning G protein-coupled receptor that has been cloned and sequenced (6).

Although GRP receptors (GRPR) are found on intestinal smooth muscle cells (46), they are not normally expressed by epithelial cells lining the human colon (9). In contrast, two studies each with relatively few patients showed that 24% (32) to 40% (36) of surgically resected colon cancers aberrantly expressed GRP binding sites. Because GRP expression has been associated with the proliferation of all human cancer cell lines in which it is expressed, including those derived from the lung (7, 24–26), breast (15), prostate (30, 38), and colon (12, 13, 35, 37), GRP has been proposed to act as an autocrine growth factor. However, except for small-cell lung cancer cell lines (7), GRP has yet to be shown to be present in any human cancer or cancer cell line. Furthermore, the clinical contribution of GRP and/or GRPR expression by any human cancer has not been elucidated.

To specifically investigate the extent and significance of GRP/GRPR expression in adenocarcinomas of the human colon, we evaluated 50 randomly selected cancers, along with adjacent normal tissue, all 37 associated metastases, as well as 20 polyps. We herein demonstrate that aberrant expression of GRP and GRPR is common but that our evidence does not support this peptide hormone acting as a clinically significant growth factor. Rather, because GRP/GRPR coexpression is found only in the most well-differentiated tumor regions, irrespective of cancer stage, we propose the novel hypothesis that GRP may act in an autocrine fashion as a morphogen in human colon cancer.

METHODS

Materials. Ammonium hydroxide, Harris' modified hematoxylin with acetic acid, hydrochloric acid, 10% formalin (wt/vol), Permount, and xylene (histology grade) were purchased from Fisher Scientific (Pittsburgh, PA). Absolute and 95% anhydrous ethanol were purchased from Pharmco Products (Brookfield, CT). PBS was purchased from Gibco BRL (Grand Island, NY). Unless otherwise indicated, all immunohistochemical reagents including large volume DAKO LSAB(R)2 kit and DAKO liquid DAB substrate-chromogen system were from DAKO (Carpenteria, CA), and all other reagents were obtained from Sigma (St. Louis MO). All reagents and solvents were used at reagent-grade purity.

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GRP expression was determined using a polyclonal rabbit anti-porcine whole peptide antibody (11), whereas GRPR expression was evaluated using a polyclonal rabbit anti-peptide antibody (20). The latter antibody (generously provided by Dr. J. Battey, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) was designed using a sequence corresponding to the distal third intracellular loop of the mouse and human GRPR (CVEGNIHKQIESRKR). In all instances, antibodies were used at a 1:250 dilution, predetermined as optimal by dilution titration using human small cell lung carcinoma tissue as a positive control. Proliferating cell nuclear antigen (PCNA) was from Boehringer Mannheim (Indianapolis, IN) and used at 1:200 as directed by the manufacturer.

Patient and tumor block selection. The Chicago Veterans Affairs Medical Center (CVAMC) (West Side Branch) Gastrointestinal Tissue Bank contains surgical pathology specimens from all patients undergoing surgical resection between 1980 and 1997. From this data base, we used a random number generator to select hospital identification numbers to identify 50 patients undergoing colectomy between 1990 and 1997 (10 Dukes stage A, 10 stage B1, 10 stage B2, 10 stage C, and 10 stage D, along with all associated cancer-containing lymph nodes (n = 30) and liver or peritoneal metastases (n = 7)). An additional 20 randomly selected polyps resected either endoscopically (n = 18) or surgically (n = 2) were included in the study sample (5 hyperplastic, 5 tubular, 5 villous, 5 villous with high-grade dysplasia). Pertinent clinical information was obtained from the Veterans Information System and Technical Architecture computer system. The University of Illinois-CVAMC combined Institutional Review Board approved this study.

Tissue preparation and classification. Specimens previously fixed in paraffin-embedded blocks were freshly sectioned at a thickness of 5 µm and mounted on poly-L-lysine-coated slides. Slides were heat fixed at 75°C for 30 min to promote adherence and stained with hematoxylin and eosin using standard techniques (2). Blocks including the tumor margin and containing both cancer and adjacent normal mucosa were identified and used for immunohistochemical analyses. In this fashion, all slides contained regions of nonmalignant epithelium and thus possessed an internal negative control.

Immunohistochemistry. For GRP and GRPR detection, a standard three-stage indirect immunoperoxidase technique was used (17). Briefly, fixed tissue sections were rehydrated in graded alcohols and then rinsed in a running water bath. To quench endogenous peroxidase activity, slides were preincubated in 3% hydrogen peroxide in a light impermeable chamber. After they were washed in PBS, slides were incubated in blocking solution [5% skim milk (vol/vol) and 0.15% H2O2 (vol/vol) in deionized water]. After slides were washed again in PBS, primary antibody was applied, and the tissue was incubated for 1 h in a humidity chamber (control tissues were processed similarly except that primary antibody was not applied). To evaluate for PCNA positivity, we treated slides similarly except that primary antibody was incubated overnight at 4°C. After being washed again in PBS, the tissues were incubated with biotinylated anti-rabbit IgG (DAKO) for 15 min. After they were washed in PBS, the slides were incubated with streptavidin conjugated to horseradish peroxidase (DAKO) for 15 min and washed again in PBS buffer. Incubating slides with the liquid DAB substrate-chromogen system (DAKO) for 5 min identified bound antibody. After a final wash in PBS and distilled water, the slides were counterstained with either Gill’s or Harris’ modified hematoxylin for 4 min, dehydrated in graded alcohols, and mounted with a coverslip using Permount.

Microscopic analysis. All specimens were evaluated using a Nikon E600 microscope with Axioplan objectives connected to a Microluma ultraresolution scanning digital camera (3.380 × 2,700 pixels (Leaf Systems, Fort Washington, PA)).

Assessment of tumor differentiation was performed using a three-grade classification system as previously described (21). Well-differentiated tumors were defined by the presence of well-formed glands containing malignant columnar cells displaying small regular nuclei. The complete absence of gland formation, or the presence of bizarrely shaped glands, identified poorly differentiated tumors. Moderately differentiated tumors possessed well-formed glands, but the cells were less columnar or frankly cuboidal, with reduced cell polarity and more dysplastic nuclei than those observed in well-differentiated tumors.

The geographic extent of staining in each section was determined and scored independently by three investigators (Benya, Carroll, and Matkowsky). Each observer evaluated 10 or more (10 100 high-power fields (hpf)) containing tumor as well as an equal number of normal mucosal fields at 400 magnification. Fields were scored as 1 = <25%, 2 = 25–50%, 3 = 50–75%, and 4 = >75% cells/hpf positive for chromogen.

Statistical analysis. Statistical evaluations were performed using StatView (Abacus Concepts, Berkeley, CA). Survival distribution was estimated by Kaplan-Meier analysis; the data were stratified by tumor stage, with censored and uncensored observations segregated in calculating the hazard function. Comparisons between groups were otherwise performed by ANOVA or $\chi^2$ analysis. All data in this paper are reported as means ± SE.

RESULTS

Clinical characteristics of the patient population. Fifty patients with adenocarcinoma of the colon were randomly selected by stage from the CVAMC Gastrointestinal Tissue Bank (Table 1). Consistent with a CVAMC population, all patients were male and of relatively advanced age (mean = 68.9 ± 3.4 yr; range 36–87 yr). Post Surgery, only five patients were lost to follow-up (at 1, 1.5, 2, 12, and 30 mo post surgery).

Overall, 17 patients died during follow-up (2 stage A, 3 stage B1, 1 stage B2, 2 stage C, and 9 stage D). In 6 of

Table 1. Clinical characteristics of 50 patients with adenocarcinoma of the colon evaluated by immunohistochemistry

<table>
<thead>
<tr>
<th>Tumor Stage</th>
<th>Age (Range), yr</th>
<th>Duration (Range) of Follow-up, mo</th>
<th>Number Lost to Follow-up</th>
<th>Number of Colon Cancer Deaths</th>
<th>Number of Other Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>71.1 ± 1.6</td>
<td>37.2 ± 6.5 (12–71)</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>B1</td>
<td>68.1 ± 3.2</td>
<td>33.5 ± 5.6 (11–63)</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>B2</td>
<td>70.8 ± 3.1</td>
<td>38.5 ± 7.5 (16–73)</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>70.2 ± 4.5</td>
<td>31.0 ± 10.9 (2–71)</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>64.6 ± 2.6</td>
<td>12.3 ± 5.3 (1–58)</td>
<td>0</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>68.9 ± 3.4</td>
<td>30.7 ± 6.5 (1–73)</td>
<td>5</td>
<td>11</td>
<td>6</td>
</tr>
</tbody>
</table>

Age and duration values are means ± SE. Patients were randomly selected from those seen between 1980 and 1997.
17 patients, death was unrelated to their underlying malignancy. One patient each died from respiratory failure associated with pneumonia (stage A), advanced chronic obstructive pulmonary disease (stage A), congestive heart failure associated with coronary artery disease (stage B1), and acute myocardial infarction (stage B1). In an additional two instances, deaths were due to the development of new lung cancers unrelated to their colon primaries [1 small cell lung cancer (stage B1), 1 non-small cell lung cancer (stage D)]. No patient showed evidence of colon cancer recurrence or progression.

In contrast, 11 of 17 deaths were directly attributable to colon cancer recurrence or progression. Eight of these deaths were in patients that had metastatic disease at the time of their initial presentation (i.e., stage D). In the three other patients, death was directly due to tumor recurrence, including one each from malignant bowel obstruction (stage B2), brain metastasis (stage C), and tumor cachexia associated with peritoneal carcinomatosis (stage C).

Antibody sensitivity and specificity. Because small cell lung cancer cells have previously been shown to express GRP and GRPR, we used paraffin blocks containing this tumor to establish the optimal dilutions for immunohistochemistry. Optimal antibody dilution was determined to be 1:250 by dilution titration to stain tumor tissue but not adjacent noncancerous tissue (Fig. 1, A–C). The specificities of the antibodies for GRP (11) and GRPR (20) have been previously shown. We further evaluated antibody specificity by evaluating a number of negative control tissues not suspected to express GRP or GRPR. Because we have previously shown that epithelial cells lining the colon do not normally express

![Fig. 1. Determination of gastrin-releasing peptide (GRP) and its receptor (GRPR) antibody specificity in resected human tissues. Optimal dilution of GRP and GRPR antibody for use in this study was established by using small cell lung cancer (SCCL) tissue as a positive control. Positive staining is seen as the brown color indicated by arrowheads. A: GRP antibody applied to a SCCL tumor at 1:250 dilution; strong staining of the tumor cells (arrowhead) is shown. B: SCCL tissue treated similarly except for the absence of primary antibody as a negative control. C: GRPR antibody applied to a SCCL tumor at 1:250 dilution. Similar to A, tumor cells stain positively (arrowhead). Treatments of normal human colon epithelium (D) and colon removed for ischemic colitis (E) or diverticulitis (F) with GRP antibody at the same dilution are also shown. Similar lack of positivity for these colonic tissues was also observed when treated with GRPR antibody. Tissues were processed as described in METHODS. Magnification = ×1,000 (A–C) and ×400 (D–F).]
GRPR mRNA (9), we tested our two antibodies on normal colon tissue (Fig. 1D) and on colons resected for diverticulitis (Fig. 1F) and ischemic colitis (Fig. 1E). Normal and diseased colons devoid of underlying malignancy did not show any evidence of immunostaining for either GRP or GRPR (Fig. 1, D–F). Thus GRP/GRPR expression does not occur in nonmalignant disorders nor does inflammation per se allow for nonspecific binding of the primary antibodies.

Aberrant GRP/GRPR expression in cancer. In contrast to normal colonic epithelium, markedly increased ligand and receptor immunostaining was observed in the majority of the adenocarcinomas we evaluated. Overall, 84% of tumors expressed either GRP or GRPR, with 35 of 50 (70%) cancers immunopositive for GRP and 38 of 50 (76%) for GRPR (Table 2). This staining was predominantly cytoplasmic for both (Fig. 2, and 3; 38 of 50 (76%) for GRPR (Table 2). This staining was markedly increased contrast to normal colonic epithelium, markedly increased binding of the primary antibodies.

Nor does inflammation per se allow for nonspecific expression does not occur in nonmalignant disorders approximated equal numbers of stage A tumors and stage D tumors expressed GRP and/or GRPR (50–90% vs. 60–70%) (Table 2). Because we studied consecutive histological sections for both ligand and receptor, we could assess whether the same tumor regions expressed both proteins. Overall, 31 of 50 (62%) tumors expressed both GRP and GRPR, with both proteins always coexpressed in the same histological area. In contrast, 4 of 50 (8%) tumors expressed only GRP and 7 of 50 (14%) expressed only GRPR. In only 8 of 50 (16%) tumors was ligand or receptor not detected at all (Table 2). Thus aberrant GRP/GRPR expression is common in adenocarcinomas of the colon but show no evidence of increasing expression as a function of stage, as might be expected if expression provided tumors with a growth advantage.

Because of the near-equal rates of expression of GRP/GRPR in tumors irrespective of stage (Table 2), we looked for evidence of receptor or ligand expression in premalignant lesions. A total of 20 polyps were examined (5 hyperplastic, 5 tubular adenomas, 5 villous adenomas, and 5 villous adenomas with dysplasia). GRPR immunostaining could not be identified in any polyp, whereas GRP was detected in two of five villous adenomas containing regions of high-grade dysplasia. Significantly, GRP expression was only detected in severely dysplastic cells (Fig. 3A).

We likewise evaluated all metastatic lesions from patients with stage C and D tumors. This included all lymph nodes (n = 30), liver biopsies (n = 5), and serosal implants (n = 2) containing tumors. One lymph node (7%) was strongly positive for both GRP and GRPR (Fig. 3B), whereas one other node showed evidence of only GRPR expression (Table 2). These two lymph nodes originated from primary tumors that were strongly positive for both ligand and receptor. Of the seven non-lymph node metastases, one to the serosa expressed GRPR and not GRP. Similar to the positive lymph nodes, this positively staining metastasis arose from a primary tumor that also stained strongly for both GRP and GRPR. However, 5 of 15 primary tumors stained for GRP/GRPR with similar intensity but gave rise to metastases that showed no evidence of expressing either protein. Thus ligand and receptor expression is uncommon in metastatic disease and does not necessarily correlate with the degree of immunostaining detected in the primary tumor.

GRP acts as a mitogen. Because GRP has been proposed to act as an autocrine growth factor in cancer (7, 24–26), including those originating in the colon (13, 27, 32, 36, 37), we were interested to see if tumor regions coexpressing GRP and GRPR were associated with increased amounts of cell proliferation. To do this, we selected five separate histologically distinct regions positive for both GRP and GRPR, either GRP or GRPR, and neither GRP nor GRPR. We then counted the PCNA-positive nuclei in three different high-powered

Table 2. GRP or GRPR expression in premalignant and malignant tissues by immunohistochemistry

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sample Size</th>
<th>Number Positive</th>
<th>Either GRP or GRPR</th>
<th>Both GRP and GRPR</th>
<th>Neither GRP nor GRPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyp type</td>
<td></td>
<td></td>
<td>Percent Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplastic</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tubular</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Villous</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>High-grade dysplasia</td>
<td>5</td>
<td>2/5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cancer stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>0</td>
<td>4/10</td>
<td>5/10</td>
<td>0%</td>
</tr>
<tr>
<td>B1</td>
<td>10</td>
<td>1/10</td>
<td>2/10</td>
<td>5/10</td>
<td>90%*</td>
</tr>
<tr>
<td>B2</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10/10</td>
<td>50%†</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>2/10</td>
<td>1/10</td>
<td>5/10</td>
<td>80%*</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>1/10</td>
<td>0</td>
<td>6/10</td>
<td>100%*</td>
</tr>
<tr>
<td>Total cancer</td>
<td>50</td>
<td>4/50</td>
<td>7/50</td>
<td>31/50</td>
<td>70%*</td>
</tr>
<tr>
<td>Metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>30</td>
<td>0</td>
<td>1/30</td>
<td>1/30</td>
<td>3%</td>
</tr>
<tr>
<td>Liver/peritoneal</td>
<td>7</td>
<td>0</td>
<td>1/7</td>
<td>0</td>
<td>14%</td>
</tr>
</tbody>
</table>

Polyps and cancers are from individual patients, whereas lymph nodes and metastases represent the total number of resected lesions containing malignant cells from the patients with stage C and D adenocarcinomas of the colon. GRP, gastrin-releasing peptide; GRPR, GRP receptor. For the “both GRP and GRPR” group, in all cases, the same histological regions of the tumor were found to coexpress both GRP and GRPR.* Statistically significant difference compared with tumors not expressing either GRP or GRPR, P < 0.05, Friedman test. †Statistically significant difference compared with tumors not expressing either GRP or GRPR, P < 0.05, Friedman test.
fields in each of these areas (a total of 1,545 cells were counted) (Table 3). Whereas 37% of nuclei were PCNA positive in regions expressing both GRP and GRPR, <15% of nuclei were positive in regions not expressing both ligand and receptor (Table 3, Fig. 4). Thus these data support a role for GRP as a mitogen acting in an autocrine manner in human colon cancer.

Survival data. Because we had failed to find evidence of increasing rates of GRP/GRPR expression with more advanced and metastatic tumors, we were particularly interested to determine if this mitogenic peptide hormone and its receptor had any impact on patient survival. We determined if GRP/GRPR coexpression influenced patient outcome by performing Kaplan-Meier analysis on the survival data (Fig. 5). Complete information was available for 45 of 50 patients whose tumors were evaluated, since 5 were lost to follow-up after surgery. We compared survival of patients whose tumors expressed both ligand and receptor (n = 29) compared with those whose tumors did not coexpress both proteins (n = 16). We grouped patients whose tumors expressed only GRP or GRPR with those whose tumors expressed neither protein, since we postulated that a difference in survival, if present, should only be seen if tumors coexpressed both ligand and receptor. Censored data were primarily used, since only 16 deaths occurred in the statistical sample. Overall, no significant difference in survival could be detected between either group by log rank (Mantel-Cox) analysis (P = 0.81) (Fig. 5). Thus patient survival is not altered when tumors coexpress GRP and GRPR and where an autocrine growth loop could conceivably be present. In combination with our observation that there is no increase in GRP/GRPR expression as a function of tumor stage, these data suggest that this peptide may not be acting as a clinically important growth factor.
Receptor/ligand expression and tumor differentiation. We observed that GRP and GRPR immunostaining was always focal in nature and was never diffusely observed throughout a tumor. The 50 tumor sections that we evaluated contained a total of 158 separate and distinct histological regions comprised of well-differentiated, moderately differentiated, or poorly differentiated cells. Because stage A and B1 tumors tended to contain only a single histological region, this means that there were between 1 and 4.6 separate regions present within any given section. When these 158 sections were evaluated independently, we found that the extent of both GRP and GRPR immunostaining was positively associated with the degree of tumor region differentiation (Fig. 6). The greatest extent of immunostaining was observed in well-differentiated tumor regions (Figs. 6 and 7) irrespective of tumor stage (Fig. 6). To determine if the converse applied, we then evaluated regional histology as a function of the immunopositivity status (Table 4). Tumor regions expressing

![Fig. 3. GRP and/or GRPR expression in polyps and lymph nodes. A: tubulovillous polyp containing high-grade dysplasia treated with GRP antibody. Immunopositivity is present only in the most severely dysplastic cells. Inset: compare immunopositive dysplastic cells on left vs. the nonstaining and nondysplastic cells on right of the crypt. Similar results were not seen when treated with GRPR antibody. Magnification = ×100 and ×400 (inset). B: metastatic tumor in a lymph node from a patient with Dukes stage C cancer treated with GRPR antibody. Similar result was obtained when treated with antibody for GRP. Magnification = ×100.]

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total Nuclei Counted</th>
<th>PCNA Negative</th>
<th>PCNA Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. GRP/GRPR negative</td>
<td>415</td>
<td>382 91%</td>
<td>33 9%</td>
</tr>
<tr>
<td>II. Either GRP or GRPR</td>
<td>447</td>
<td>383 86%</td>
<td>64 14%</td>
</tr>
<tr>
<td>III. GRP/GRPR positive</td>
<td>683</td>
<td>431 63%</td>
<td>252 37%</td>
</tr>
</tbody>
</table>

As described in RESULTS, all nuclei were scored for proliferating cell nuclear antigen (PCNA) immunopositivity in 3 separate crypts from 5 different tumors in areas that were positive only for GRP or GRPR, both GRP and GRPR, and areas not expressing either GRP or GRPR. Data were evaluated by ANOVA using StatView (Berkeley, CA). P values are shown as a matrix, condition I vs. II or III, condition I vs. III. ND, not determinable; NS, not significant.
either ligand or receptor alone tended to be moderately or poorly differentiated, although some were well differentiated (Table 4). In contrast, no region was found to be well differentiated that expressed neither protein and no region expressing both proteins was poorly differentiated (Table 3). When moderately differentiated tumors are excluded from analysis, all tumor regions expressing both GRP and GRPR were well differentiated and none were poorly differentiated, whereas all regions expressing neither protein were poorly differentiated.

**DISCUSSION**

Epithelial cells lining the human GI tract outside of the gastric antrum do not normally express GRPR (9). In contrast, previous studies have shown that GRP binding sites are present in 24–40% of resected colon cancers (32, 36), whereas approximately one-third of human colon cancer cell lines expresses functional GRPR (13). Because it was first reported that GRP causes the growth of most human small-cell lung cancer cell lines by an autocrine mechanism (7), it has been assumed that this ligand acts as an autocrine growth factor in all tumors where its cognate receptor is aberrantly expressed. However, aside from lung cancer cell lines (7, 24–26), studies investigating GRPR expression by various tumors have not documented the presence of ligand. Furthermore, because GRP acts as a mitogen in all cancer cell lines in which GRPR are expressed, including those derived from GI tumors (13, 27, 32, 36), it has been assumed but never proven that these proteins are clinically important for tumor growth and progression. Indeed, we have previously shown that introduction of the GRPR alone into a nonmalignant human colon cell line resulted in receptor constitutive activation and ligand-independent cell proliferation (10). These findings clearly indicate that GRP/GRPR can act as mitogens; we therefore set out in this study to quantify the extent and significance of GRP and GRPR expression by adenocarcinomas of the colon.

Our results show that, whereas normal and nonmalignant colonic epithelia do not express GRP or GRPR, 84% of colon cancers aberrantly express either one of these proteins. Because 62% of colon cancers studied contain regions coexpressing both ligand and receptor and regions coexpressing these proteins contained greater numbers of PCNA-positive cells, it might appear that GRP acts as an autocrine growth factor, as has been previously postulated (13, 27, 32, 36, 37). Yet, surprisingly, our observations do not support the hypothesis that GRP/GRPR acts as a clinically significant growth factor in colon cancer. First, no increase in GRP/GRPR expression as a function of tumor stage could be detected (Table 2). Second, only 2 of 30 lymph nodes containing tumor and 1 of 7 liver and peritoneal metastases expressed either protein (Table 2). If GRP...
acts as a clinically significant growth factor, the presence of ligand and its receptor should provide cancers with a growth advantage such that increased frequency of expression would be observed with more advanced stage tumors and in metastases. However, similar levels of GRP/GRPR were detected in stage A as in stage D cancers, whereas 34 of 37 (92%) metastases did not express either protein (Table 2). Finally, there was no difference in survival between patients whose tumors expressed both GRP and GRPR and those whose...
Table 4. Differentiation as a function of GRP/GRPR immunopositivity

<table>
<thead>
<tr>
<th>Immuno-positivity Status</th>
<th>Separate Areas Evaluated</th>
<th>Well Differentiated</th>
<th>Moderately Differentiated</th>
<th>Poorly Differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP only</td>
<td>18</td>
<td>5%</td>
<td>62%</td>
<td>33%</td>
</tr>
<tr>
<td>GRPR only</td>
<td>27</td>
<td>4%</td>
<td>68%</td>
<td>28%</td>
</tr>
<tr>
<td>Neither</td>
<td>50</td>
<td>0%</td>
<td>42%</td>
<td>58%</td>
</tr>
<tr>
<td>Both</td>
<td>63</td>
<td>94%</td>
<td>6%</td>
<td>0%</td>
</tr>
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Total numbers of separate areas immunopositive for GRP only, GRPR only, neither protein, and both proteins in all 50 tumor samples evaluated were assessed for the degree of histological differentiation as defined in METHODS.

Tumors did not express both proteins (Fig. 5). In aggregate, therefore, these data indicate that, despite the ability of GRP to cause cell proliferation in vitro, this peptide hormone does not act as a clinically significant oncogenic growth factor in vivo.

Instead, we make two observations in this study regarding aberrant GRP/GRPR expression that suggest a novel function for these proteins in colon cancer. First, our data show that GRP/GRPR expression is common to all colon cancers regardless of stage and occurs early in malignant transformation. As such, the differentiation associated with tumors assuming a more primitive intestinal phenotype appears to involve expression of GRP and GRPR. Evidence for this being the case is found in fetal rats, the only species so studied, in which epithelial cells lining the GI tract transiently express GRPR from embryonic days 13–16 until birth (3, 44). Although the role of GRP/GRPR in the development of the GI tract has yet to be determined, the transient nature of this expression suggests a possible role for these proteins in gut differentiation and/or maturation. Thus expression of GRP/GRPR may well reflect tumor assumption of a more primitive phenotype, as occurs during malignant transformation.

Second, and directly related to our first observation, GRP/GRPR expression was only detected in well-differentiated tumor areas (Figs. 6 and 7, Table 4). Expression of GRP or GRPR alone was as likely to be expressed by poorly differentiated as by well-differentiated tissues (Table 4). In contrast, all well-differentiated tumor regions coexpressed GRP and GRPR, whereas no poorly differentiated tissue coexpressed both proteins. The association of tissue differentiation and GRP/GRPR coexpression was also independent of tumor stage (Fig. 6). Because the association with differentiation was only observed when both ligand and receptor were coexpressed, these findings suggest the possibility that these proteins act in an autocrine fashion regulating cellular differentiation.

Differentiation factors are more commonly known as morphogens and were first described in the regulation of normal embryological development (reviewed in Ref. 16). More recently, morphogens have been shown to be important in cancer. In the GI tract, perhaps the best-described morphogen is hepatocyte growth factor (HGF), important in altering the behavior of gastric adenocarcinomas (reviewed in Ref. 42). HGF is a weak mitogen synthesized by stromal tissues that binds to the tyrosine kinase receptor c-met expressed by gastric cancer cells (5, 31). When gastric cancers concomitantly express high levels of E-cadherin, important in regulating cell-to-cell attachment, HGF causes these cells to adopt a more differentiated phenotype (23). However, when E-cadherin levels are low, HGF instead acts as “scatter factor” and causes cancer cell migration (4, 42). Thus HGF in gastric cancer can act as a mitogen, motogen, or morphogen, depending on the cellular situation. Similar to HGF, GRP is a mitogen. Furthermore, GRP is known to activate multiple different intracellular signaling pathways, including those that modulate cell-to-cell attachment. Depending on the cell type, the GRPR couples to multiple different G proteins, including members of the p21 ras superfamily (33). GRP activation of these G proteins, including p21 ras, alters p125 fak phosphorylation and influences the integrity of focal adhesions (22). Thus GRP is similar to HGF insofar as a theoretical mechanism exists for it being able to alter cell-to-cell attachment and thereby act as a morphogen in cancer.

The case for GRP/GRPR acting as a morphogen in colon cancer is strengthened by recent reports indicating that these proteins are important in normal fetal organogenesis. In mice, mRNA for GRPR is observed in lung buds starting at embryonic day 12 (19). Branching of explanted buds, a marker of increasing lung differentiation, was significantly increased in the presence of bombesin, a pharmacological homologue of GRP (19). Likewise, in rabbits, GRP is synthesized by pulmonary neuroendocrine cells and acts on GRPR expressed by distal airway epithelial tubes only at the time of peak airway growth and differentiation (45). Interestingly, in both cases, administration of GRP/bombesin also increased airway cell proliferation (19, 45). Thus, during at least normal lung development, GRP acts as both a mitogen and a morphogen, suggesting that these two properties are linked.

Because in normal development many morphogens act via heptaspanning receptors (16), it is not surprising that some have now been shown to perform this role in cancer. Of the heptaspanning receptors associated with differentiation in cancer, the best described is vasoactive intestinal peptide (VIP). Similar to GRP, VIP has been shown to act as an autocrine growth factor in various cancer cell lines, including neural crest tumors such as neuroblastomas (28). Yet VIP also induces neuroblastoma cell line differentiation in vitro (29), whereas expression has been shown to correlate with the presence of more differentiated neuroblastomas (34) and other neural tumors (1) in vivo. In contrast to our data, however, VIP expression by these neural tumors is associated with improved patient survival.

Unlike other GI tumors, the prognosis for patients with colon cancer does not correlate with the tumor differentiation status (40, 41). This is probably due to the fact that, unlike other GI tumors, colon cancers contain multiple, different histological regions. In this study, larger tumors contained on average 4.6 histologi-
Gastricly distinct regions, irrespective of tumor stage. When the pathologist describes a colon cancer's stage of differentiation, they are providing an overview of the predominant tumor histology and are not stating that such differentiation is exclusively present. Thus a "well-differentiated" tumor may also contain regions of moderately and/or poorly differentiated cells (an example of this is shown in Fig. 7). Because there is no way to know with certainty which cells in a primary tumor give rise to the metastatic lesion, it is not surprising that the predominating histology does not convincingly correlate with patient outcome in colon cancer (40, 41).

The association of GRP/GRPR expression with tumor differentiation does not, of course, prove that differentiation is due to the aberrant expression of these proteins. At this point, our observations serve only to 1) question whether GRP acts as a clinically significant growth factor in colon cancer and 2) suggest the possibility that this peptide hormone acts in a completely novel fashion as a morphogen. The association of GRP/GRPR coexpression with tumor differentiation is novel and serves to underscore the need for additional studies into the normal and abnormal roles of these proteins in the GI tract.

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