Expression of the Gastrin Gene in the Normal Human Colon and Colorectal Adenocarcinoma

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ABSTRACT

Gastrin, produced in the G-cells of the gastric antrum and regulating acid secretion in the stomach, also acts as a trophic factor in the gastrointestinal tract. Because of its possible role in colon adenocarcinoma and differentiation, evidence for its presence in normal colorectal mucosa and adenocarcinoma was sought. Utilizing tumors and matched normal mucosa from 26 patients, mature gastrin and progastrin were studied by immunohistochemistry. In normal colon mucosal crypts, occasional cells stained concordantly for gastrin, progastrin, and chromogranin A, suggesting that they are of neuroendocrine origin. Adenomatous polyps stained neither for gastrin nor chromogranin A. In 22 of 23 adenocarcinomas, more than 50% of tumor cells stained for gastrin and progastrin. The expected gastrin transcript was demonstrable by polymerase chain reaction and RNase protection in tumors and by polymerase chain reaction in normal mucosa. Its identity was confirmed by sequencing the polymerase chain reaction product. A larger transcript containing Intron II was present in both cancers and normal mucosa but was barely discernible in the gastric antrum. Aberrant expression of gastrin may contribute to deregulated proliferation of many colorectal carcinomas.

INTRODUCTION

The treatment of disseminated colorectal cancer is unsatisfactory and patients with this disease almost invariably succumb. Over the years a wide variety of antineoplastic chemotherapeutic agents, surgical procedures, and radiation therapy has been utilized therapeutically with only modest success in stabilizing the disease, without clear evidence that life has been consistently prolonged. These modalities have been reviewed recently (1). While investigative work in these areas continues, the advances in cell and molecular biology present opportunities for developing alternative therapies. An array of proliferation-promoting peptides appears to play a crucial role in the growth and differentiation of the normal colon. Selective inhibition of one or more of these may provide a novel and effective mode of treatment.

Growth factors mediating cell proliferation within particular organs may be identified in tumors removed from these organs. Tumors obtained at surgery are readily available, often in relatively large amounts for direct study or for establishing cell lines which can facilitate identification of growth factors. Thus, from colorectal cancers and cell lines derived from them, mRNAs encoding a variety of growth promoting peptides have been identified, including TGF-α3 and -β, EGF, gastrin, insulin-like growth factors I and II, PDGF, and fibroblast growth factor (2-4). Moreover, receptors such as c-erbB-1, c-erbB-2, the PDGF receptor, and the insulin-like growth factor I receptor have been shown to be present, suggesting the existence of autocrine loops (5-8). Paracrine stimulation may also promote growth in colorectal cancers and normal mucosa. For example, proliferation factors may be produced by the sparse but ubiquitous neuroendocrine cells dispersed throughout the gastrointestinal tract (9-11). Pericycral fibroblasts located in the lamina propria adjacent to the crypt (12) or even lymphoid cells diffusely present throughout the intestine (13) may be sources of growth and differentiation factors.

Gastrin, a peptide hormone produced by the G-cells of the gastric antrum, was initially thought to control only acid secretion by the stomach. The structure of the peptide and its mRNA and genomic sequence have been established (14, 15). In recent years it has been suggested that gastrin might also function as a trophic factor for the entire gastrointestinal mucosa. This role is clearly separable from its effect on acid secretion and is summarized in recent reviews (3, 16, 17).

Gastrin expression has been detected in colon cancer cell lines (18, 19). Most of the gastrin studies on colon carcinoma cell lines tested the effects of adding gastrin to medium and assessing cell proliferation (18, 20-23). The results have varied. In a recent study, four different human colon cancer cell lines were tested for growth stimulation by added pentagastrin and only one showed growth stimulation (24). Such experiments are difficult to interpret. Since gastrin or an analogue is usually added to the medium, lack of a proliferative response may indicate that the peptide does not act as an effector for proliferation, that the receptor for gastrin is absent, or that the cells are already producing gastrin. Quantitative PCR measurements detected gastrin mRNA in several colon cancer cell lines (25).

Whether gastrin is involved in the pathogenesis and progression of colon cancer remains unclear. Watson et al. (26), using flow cytometric detection with an anti-gastrin 17 antibody, demonstrated the presence of intracellular gastrin in 6 of 28 human colorectal tumors. In these tumors, more than 20% of the cells stained positively. The gastrin peptide and gastrin-like intermediates have also been found in neural tumors (27, 28), pituitary tumors (29), gastrinomas (30), lung tumors (31), and certain endocrine tumors (32, 33). Conflicting data have been published as to whether blood levels of gastrin are elevated in colorectal cancer (34, 35).

Limited data are available to support a role for gastrin as a positive growth stimulus for colorectal carcinoma in vivo and these have been reviewed (17). In this report, we provide additional evidence that gastrin may be an effector for proliferation, and perhaps differentiation, in both normal colon and colorectal tumors. We demonstrate that progastrin and gastrin are present in neuroendocrine cells of normal colorectal mucosa and that colorectal tumors comprise cells that commonly express gastrin.

MATERIALS AND METHODS

Immunohistochemistry. Human colorectal surgical specimens were fixed in formalin, embedded in paraffin, and cut into 4-μm sections. Representative samples of normal mucosa and tumors were evaluated by routine pathological studies and these samples were also stained with an indirect immunoperoxidase method (36) using Vectastain Elite ABC Kits (Vector Laboratories, Burlingame, CA) with 3,3'-diaminobenzidine substrate and Mayer's hematoxylin counterstain. Polyclonal rabbit anti-human progastrin antibody (Cambridge Research Biochemicals, Wilmington, DE) directed against the peptide Ser-Ala-

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1 Supported in part by a Merit Review Grant from the Veterans Administration and by Amgen, Inc.
2 To whom requests for reprints should be addressed at VA Medical Center, University Drive C, Pittsburgh, PA 15240.
3 The abbreviations used are: TGF, transforming growth factor; cDNA, complementary DNA; PCR, polymerase chain reaction; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

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Glu-Asp-Glu-Asn (amino acids 96–101 of preprogastrin) was used at a 1:2000 dilution. This peptide is cleaved off progastrin during normal processing and is not present in biologically active G-34 and G-17 gastrins. Polyclonal rabbit anti-human gastrin-17 antibody and polyclonal normal rabbit IgG were obtained from DAKO (Carpenteria, CA) and monoclonal antibody against chromogranin A was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). These were used as recommended by the manufacturers. Sections of paraffin-embedded gastric antrum were obtained from DAKO and used as positive controls.

**RESULTS**

Immunohistochemical Demonstration of Gastrin and Progastrin in Colorectal Adenocarcinoma and Normal Mucosa. The human preprogastrin peptide comprises 101 amino acids including: a 19 amino acid NH₂-terminal signal peptide sequence; a 37 amino acid spacer region; a sequence containing the major bioactive forms of gastrin (gastrin 34 and gastrin 17); and a COOH-terminal flanking peptide (Fig. 1). The molecule undergoes extensive posttranslational processing (42) to yield: (a) progastrins extended beyond glycine at COOH-terminus; (b) glycine-extended progastrins, the immediate precursors of mature gastrins; and (c) amidated, biologically active gastrins in which the COOH-terminal phenylalanine residue is amidated using glycine as the amide donor. In gastrins and progastrins, tyrosine 87 has variable degrees of sulfation (43). In the gastric antrum and duodenum about 50% of the gastrin molecules are sulfated. The antibodies used in this study include rabbit anti-human gastrin-17 and anti-progastrin antibodies directed against different regions of the gastrin primary translation product (Fig. 1).

Previous gastrointestinal immunohistochemical studies localized gastrin and progastrin to gastric antrum G-cells and duodenal neuroendocrine cells (44, 45). Consistent with these reports, we find strong immunohistochemical staining of gastric antrum G-cells with both anti-gastrin and anti-progastrin antibodies (Fig. 2). This staining is absent when a negative control rabbit IgG antibody is used (data not shown).

In normal colonic mucosa, serial 4-μm sections demonstrate that occasional crypt cells stain concordantly for gastrin (Fig. 3a) and progastrin (data not shown). Staining is mostly cytoplasmic and was absent when a negative control rabbit IgG antibody was used. In Fig. 3, b and c, successive sections were stained for gastrin and human chromogranin A, a neuroendocrine marker, suggesting that the cells containing gastrin are of neuroendocrine origin. All cells which stained for gastrin also stained for chromogranin A, but the converse is not true. All normal mucosa studied were from patients who had a neoplasm somewhere in the colon or rectum. The specimens were at least 5 cm from the border of the tumor. We have not yet had an opportunity to study normal colon from a normal patient.

In contrast to the infrequent staining of normal colorectal mucosal cells, we found that in 22 of 23 colorectal adenocarcinomas, more than 80% of the tumor cells stained for gastrin (Table 1). Of these 23 tumors, 20 were also stained for progastrin and all of these were positive. As in the case of normal mucosa, there was concordant cellular staining for gastrin and progastrin in the same cells. Fig. 4 shows a typical example of a colonic tumor stained for gastrin and progastrin. Staining is predominantly cytoplasmic. Virtually every tumor cell stains, but the intensity of staining may vary between tumors and within a given tumor. In Fig. 4a, the staining is diffuse and uniform throughout as is true for the progastrin staining shown in Fig. 4b. This was not always the case, and in some tumors the pattern of staining was more heterogeneous. In these tumors some cells did not stain at all and variation in staining intensity was seen from one sector of the tumor to another (data not shown). Nevertheless, in no case were less than 50% of the cells stained. A negative control utilizing rabbit IgG showed no staining (Fig. 4c). Interestingly, although not always the case, the majority of these tumors which stained positively for gastrin showed no staining for chromogranin A, including the matched tumor of the chromogranin A positive normal mucosa shown in Fig. 3b.
Because of a lack of chromogranin A staining in tumors, we examined staining for chromogranin A and gastrin in six adenomatous polyps. These polyps varied in size from less than 1 cm to more than 2 cm. No evidence of positive staining was detected (data not shown). Normal mucosa stains for chromogranin A and gastrin, polyps stain for neither, and the tumors we examined stained positively for gastrin and negative for chromogranin A. As is evident from Table 1, gastrin staining correlated neither with the degree of tumor differentiation nor Dukes stage. Normal mucosa directly adjacent to tumors showed no evidence of increased staining (data not shown).

Polymerase Chain Reaction of Gastrin mRNA Transcripts in Adenocarcinoma and Normal Mucosa. Ten tumors and their matched normal mucosa were assayed for gastrin mRNA by PCR. The predicted gastrin cDNA PCR product based on the primers used was 299 base pairs (Fig. 1). In fact, PCR amplified a 299-base pair product, as well as a larger 428-base pair product from all 20 colorectal specimens (Fig. 5). The larger product could barely be detected in matched normal mucosa (Figs. 5 and 6). The yield of the 299-base pair PCR product is consistently higher from tumor tissue than from matched normal mucosa (Figs. 5 and 6). To confirm that the PCR reaction products from the colon were indeed gastrin related, they were subjected to Southern blot analysis shown in Fig. 6. These results indicate that gastrin-related mRNAs are present in both normal and neoplastic colonic tissue.

To further characterize the colonic gastrin PCR products, the 299- and 428-base pair DNAs from colonic mucosa and from a colon adenocarcinoma were gel purified, cloned into M13 mp 8 and 9, and sequenced. This revealed the 299-base pair fragment to be identical in sequence to the published gastrin cDNA sequence (14). The longer cDNA was also encoded by the human gastrin gene but contained the 129-base pair Intron II sequence indicated in Fig. 1. This larger product apparently is amplified from unspliced gastrin mRNA present in colonic mucosa and tumors but largely absent from gastric antrum. It is unlikely to be due to genomic DNA contamination of the colonic RNA used to prepare cDNA for PCR since the RNA used as a target in PCR failed to produce any visible product without reverse transcription.

Direct Detection of Gastrin mRNA in Colon Tumors by Ribonuclease Protection. Despite numerous attempts, Northern blot analysis of colorectal carcinomas and normal mucosal tissue utilizing up to 20 µg of whole cellular RNA failed to reveal the presence of gastrin mRNA although the transcript was easily demonstrable in RNA prepared from the gastric antrum (Fig. 7). Ribonuclease protection assays provide considerably more sensitivity in detecting mRNA than Northern hybridization analysis (41) and are more quantitative than PCR amplification. Four colorectal tumors were assayed for the presence of gastrin mRNA by this method. Ten µg of polyadenylated mRNA were used from each specimen. For two of these tumors, sufficient corresponding normal mucosa was available to provide RNA for the assay. Neither of the normal mucosal

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**Fig. 1.** Human gastrin cDNA sequence. Oligonucleotide primers are shown with brackets indicating restriction enzyme recognition sites added to facilitate cloning. EcoRI-[GAATTTC], BamHI-[GGATCC]. HindIII restriction sites in the native sequence are indicated. Boxes, dibasic processing sites for trypsin-like proteases which cleave the preprogastrin. The circled amino acids in the gastrin-17 sequence are homologous to the region surrounding tyrosine 315 in polyoma virus middle T-antigen. The tyrosine residue in gastrin-17 (double circle) is the site of sulfation and in vitro phosphorylation. The circled amino acids in the gastrin-17 sequence are homologous to the region surrounding tyrosine 315 in polyoma virus middle T-antigen. The tyrosine residue in gastrin-17 (double circle) is the site of sulfation and in vitro phosphorylation.
samples showed a protected band, but two of the tumors contained detectable message (Fig. 8).

DISCUSSION

Our results support the conclusion that gastrin is present in normal colonic mucosa and in colorectal tumors. The concordant staining for gastrin and progastrin in the same cells allows us to infer gastrin synthesis in situ. Moreover, the presence of the progastrin hexapeptide SAEDEN (amino acids 96–101) serves to distinguish gastrin from the physiologically active cholecystokinin octapeptide, also produced in the gastrointestinal tract. This is of concern since the gastrin antibody we have utilized cross reacts with cholecystokinin (46).

The coincident staining for gastrin and chromogranin A in normal colonic mucosa strongly suggests that gastrin expression in colonic crypts is restricted to neuroendocrine cells (28). In the common colonic neoplasms, adenomatous polyps and adenocarcinomas, the former stain neither for gastrin nor chromogranin A while the latter stain for gastrin but frequently not for chromogranin A. This suggests that increased gastrin expression occurs at a late stage in malignant

Fig. 2. Immunohistochemical staining of the normal gastric antral mucosa (×400). a, staining with anti-gastrin antibody. b, same tissue stained with anti-progastrin antibody showing identical staining pattern to a.

Fig. 3. Immunohistochemical staining of normal colonic mucosa (×400). a, colonic mucosa stained with anti-gastrin antibody showing occasional cell staining positively. b and c, consecutive sections stained with anti-gastrin and anti-chromogranin A antibodies, respectively. The coincident staining pattern suggests that the gastrin-containing cells are of neuroendocrine origin (see text).

A ••• .......
B

A ••• •••
B

A ••• ••••
B
GASTRIN IN HUMAN COLORECTAL TUMORS

Table 1  Expression of gastrin and progastrin in colorectal cancers

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dukes stage</th>
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<th>Gastrin#</th>
<th>Progastrin#</th>
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<td>MWDA</td>
<td>Tr - 3+</td>
<td>3+</td>
<td>D:49M</td>
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* WDA, well differentiated adenocarcinoma; MWDA, moderately well differentiated adenocarcinoma; PDA, poorly differentiated adenocarcinoma.

# Intensity of stained cells, in different areas of the tumor section on a scale of 0 (negative) to 4+. ND, not done.

† D, dead; A&W, alive and well; R, relapse; P, perioperative death within 6 weeks of surgery; M, months.

Transformation of colonic epithelial cells. This observation further suggests that the origin of the adenomatous polypl is not from neuroendocrine cells. Finally, PCR-detection of gastrin mRNA in normal mucosa and colorectal adenocarcinomas, much more abundant in the latter than in the former, and sequencing of the PCR products provides additional evidence for gastrin expression in these tissues. The ribonuclease protection assay confirms this conclusion.

Gastrin gene expression is regulated by multiple mechanisms. The gastrin promoter contains negative and positive elements to which trans-acting protein factors bind (47, 48). TGF-α, EGF (9) and gastrointestinal peptides such as somatostatin (49, 50) and bombesin/gastrin releasing peptide (51) appear to be mediators of gastrin transcription and release in the gastric antrum. TGF-α, which is expressed in most colorectal carcinomas (52) may promote gastrin gene expression in these tumor cells. It is also clear that gastrin mRNA processing is altered in the colon and colorectal tumors since the large PCR product from unspliced gastrin mRNA precursors is barely detectable in the antrum. Colorectal tumor cells, although the majority contain gastrin, have low levels of gastrin mRNA. In contrast, the gastric antrum, while comprising about 30% G-cells (53), produces enough gastrin mRNA to be seen on Northern analysis. The accumulation of gastrin in tumor cells suggests more efficient gastrin mRNA translation and/or less efficient secretion of gastrin than in G-cells.

The precise role of gastrin in colorectal cancer remains unclear. Table 1 shows that most adenocarcinomas contain gastrin immunoreactive protein irrespective of Dukes stage, morphology, or clinical outcome. Further, gastrin staining does not correlate with patient age, tumor size, or location. It is unlikely that an intestinal hormone as physiologically active as gastrin occurs in 22 of 23 colorectal tumors by chance alone and exerts no effect on tumor cell growth. Gastrin may act in a permissive capacity, necessary but insufficient for tumor development and progression. In the normal colon it seems likely that the neuroendocrine cells, acting in a paracrine manner, release gastrin and other peptides that play a role in the proliferation and differentiation of adjacent crypt cells. Studies using exogenously administered gastrin strongly suggest a role for gastrin in the growth of normal colonic mucosa (16, 17) which can be abrogated using a gastrin antagonist, proglumide (54, 55).

Colon tumor cell studies support an autocrine growth mediating role for gastrin. Hoosein, et al. (18) showed that the HCT116 colon cancer cell line contained mRNA that hybridized to a gastrin-specific probe, although the transcripts were much longer than those anticipated for gastrin mRNA. In another study, Baldwin, et al. (19) convincingly demonstrated gastrin mRNA in the human Okajima and HCT116 cell lines. Recently, they have demonstrated gastrin mRNA in seven additional cell lines (25). Moreover, both proglumide and gastrin strongly suggest a role for gastrin in the growth of normal colonic mucosa (16, 17) which can be abrogated using a gastrin antagonist, proglumide (54, 55).

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**Fig. 5.** Gastrin PCR products from colorectal tissues. *a.* Lane 1, positive control; PCR product from the linearized plasmid pHG529 which contains the full length human gastrin cDNA. This lane demonstrates the predicted 299-base pair band (middle arrow). Lanes 2 and 3, duplicate lanes demonstrating the amplification products from a human colon carcinoma cDNA. There is a prominent band at 299 base pairs (middle arrow) and a weaker band about 428 base pairs in length (top arrow). The larger product was not seen in the positive control. *Lane 4,* negative control; no added template DNA. *b.* PCR products from normal colonic mucosa. *Lane 1,* positive control; PCR product from the linearized plasmid pHG529 which contains the full length human gastrin cDNA. This lane demonstrates the predicted 299-base pair band. *Lane 2,* negative control; no added template DNA. *Lanes 3 and 4,* duplicate lanes demonstrating the amplification products from normal colonic mucosal cDNA. There are products of 299 and about 428 base pairs in length. The larger product was not seen in the positive control (*Lane 1*). In both *a* and *b* a primer dimer band is visible at the bottom of the gel (bottom arrow).

**Fig. 6.** Southern blot analysis of gastrin PCR products from three adenocarcinomas and matched normal mucosa. *Lane 1,* gastric mucosal cDNA, approximately 1 ng utilized as a target in the PCR reaction. Note predominance signal at 299 base pairs (bottom arrow) and faint signal at 428 base pairs (top arrow). *Lane 2,* positive PCR control utilizing of 20 pg of linearized gastrin cDNA plasmid (pHG529), demonstrating the single 299-base pair product. *Lane 3,* normal mucosa showing predominately the 428-base pair product. The 299-base pair fragment is poorly seen; *Lane 4,* adenocarcinoma from patient whose normal mucosa is shown in *Lane 3.* The signals from both the 299- and 428-base pair fragment are easily seen. *Lane 5 and 6,* normal mucosa and tumor, respectively, from a patient. Signals from both products are easily seen in both tissues. *Lane 7 and 8,* normal mucosa and tumor, respectively from a patient. The 299-base pair product is poorly seen in the normal mucosa and the 428-base pair product is poorly seen in the tumor. *Lanes 3–8* utilized approximately 5 ng of cDNA for each amplification reaction. Ten % of the amplified product was run on the gel in each instance (*Lanes 1–8*).
anti-gastrin antibodies inhibit the growth of the HCT116 colon tumor cell line in a concentration-dependent manner (18, 56).

It seems likely that gastrin in the stomach, and possibly in the colon, acts as an autocrine or paracrine peptide growth factor. The canine parietal cell gastrin receptor has recently been cloned and characterized (57). It appears to be a seven-transmembrane-segment receptor with significant homology to members of the \( \beta \)-adrenergic family of G-protein-coupled receptors. Interaction of this gastrin receptor transfected into COS-7 cells with gastrin results in phosphatidylinositol hydrolysis and calcium mobilization suggesting a role for the receptor in signal transduction (57). The recently cloned human CCK\(_B\) receptor, which appears to be identical or highly homologous to the antral gastrin receptor, also contains seven regions of hydrophobic residues corresponding to transmembrane domains of G-coupled receptors (58). Homology of human CCK\(_A\) and the canine gastrin receptor is greater than 90% although notable differences in function can be detected by radioligand binding studies with antagonists and agonists. The availability of antibodies to the receptor protein and cDNA probes to detect receptor message will allow clarification of the mode of action of gastrin in colonic mucosa and tumors.

The accumulation of gastrin within colorectal tumor cells suggests the possibility that the peptide may mediate proliferation without actually being secreted. The existence of internal autocrine loops, in which the effector molecule associates with its receptor within the secretory compartment of the cell, are by now well established. A notable example of this is in the action of PDGF and its oncogene counterpart, v-sis (59). Alternatively, gastrin may associate intracellularly with molecules known to be involved in the signal transduction process. Baldwin (60) made the intriguing observation that the amino acid sequence of human gastrin has homology to a carboxy-terminal region of the transforming middle T-antigen of polyoma virus that contributes to cell transformation. The homologous middle T region contains the sequence EEEEYMPM, while the corresponding region in gastrin contains the sequence EEEAAYWM. These regions of polyoma middle T protein and gastrin (Fig. 1) are also homologous to the consensus phosphatidylinositol 3-kinase binding domain of receptors tyrosine kinases (61). When the tyrosine in this polyoma middle T sequence is phosphorylated in vivo, the protein associates with and activates pp60\(^{src}\) as well as associating with phosphatidylinositol 3-kinase (61). We have clearly shown that most, if not all, colorectal tumors contain increased levels of src activity (62). Moreover, the corresponding gastrin tyrosine can be phosphorylated by the activated EGF receptor in vitro (63). We suggest that known signal transduction pathways culminating in cell division may be activated by intracellular gastrin in colorectal tumors. Although speculative, it is not inconceivable that the mode of action of gastrin in the stomach and normal colonic mucosa is through the more conventional cell surface receptor pathway, while tumor cells are stimulated by gastrin through tumor-specific intracellular mechanisms. These mechanisms may be amenable to therapeutic intervention. For example, somatostatin, or analogues of somatostatin, inhibit growth of human colon cancer cell lines (64). suppress gastrin gene expression (50), and induce tyrosine dephosphorylation of proteins phosphorylated in response to EGF (65). We are currently assessing the role of somatostatin analogues in suppressing gastrin-mediated growth of colon cancer cell lines.

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