The Binding of Bombesin and Somatostatin and Their Analogs to Human Colon Cancers

(43447)

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Abstract. **Specific receptors for bombesin/gastrin-releasing peptide, somatostatin, and EGF were investigated in 15 human colon cancer specimens. Eight of 15 clinical specimens (15%) of colon cancer showed the presence of somatostatin receptors. Octapeptide somatostatin analogs, RC-160 and RC-121, showed 10 times higher binding affinity for somatostatin receptors on colon cancer membranes than somatostatin. Analysis of ^{125}I-Tyr^4-bombesin binding data revealed the presence of specific binding sites in six (40%) specimens of human colon cancer. Scatchard analysis of ^{125}I-labeled bombesin indicated a single class of receptors in three specimens with an apparent K_d value of 2.5 n*M* and two classes of receptors with high (K_d = 0.4 ± 0.2 n*M*) and low affinity (K_d = 1.6 ± 0.4 μ*M*) in three other specimens. The ^{125}I-Tyr^4-bombesin binding capacities in the colon cancers for high affinity binding sites were from 6 to 228 fmol/mg protein and for low affinity binding sites 76 ± 15 pmol/mg protein. None of the membrane preparations made from normal colonic mucosa specimens showed specific binding for ^{125}I-Tyr^4-bombesin. Five pseudononapeptide (ψ^{13-14}) bombesin (6–14) antagonists, with different modifications at Positions 6 and 14, synthesized in our laboratory, inhibited the binding of ^{125}I-Tyr^4-bombesin in nanomolar concentrations. No correlation was found between the degree of differentiation and the presence of binding sites for somatostatin or bombesin. Specific binding of EGF was detected in 80% of colon cancer specimens. EGF binding capacity in colon cancer membranes was on average twice as high as in normal colon mucosa (50 ± 21 vs 28 ± 14 fmol/mg protein, respectively). Specific binding sites for somatostatin and EGF, but not bombesin, were also demonstrated in human colon cancer cell line HT-29. In HCT-116 colon cancer line only EGF receptors were found. These receptor findings and our *in vivo* studies on inhibition of colon cancer growth support the merit of continued evaluation of somatostatin analogs and bombesin/gastrin-releasing peptide antagonists in the management of colonic carcinoma.** [P.S.E.B.M. 1992, Vol 200]

Colorectal cancer is one of the most common malignant tumors in the United States, with a death rate second only to that of lung cancer. Surgical excision can be an effective treatment in early diagnosed patients, but the prognosis of patients with advanced colorectal cancer remains poor and new treatment modalities are needed. Recent evidence suggests that colon cancer may be, at least in part, hormone dependent. The incidence of colon cancer is increased in acromegalics, suggesting that excessive secretion of growth hormone or insulin-like growth factor-I may be a factor (1). Sex steroids, gastrointestinal hormones, and growth factors such as epidermal growth factor (EGF), insulin-like growth factor-I, and transforming growth factor-α may be involved in tumorigenesis of the colon (2, 3), but hormonal influence in colon cancer is, in part, documented only in the case of gastrin and insulin.

Somatostatin is a regulatory peptide with many biological actions and appears to be an endogenous antiproliferative agent (1, 4, 5). Somatostatin inhibits the release of growth hormone as well as that of gastrin, secretin, cholecystokinin, insulin, glucagon, and bom-

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Received October 8, 1991. [P.S.E.B.M. 1992, Vol 200]
Accepted January 29, 1992.

0037-9727/92/2003-0394$3.00/0

besin and suppresses gastrin-stimulated gastric, intestinal, and pancreatic DNA synthesis (6, 7). In addition, somatostatin may inhibit the action or secretion of growth factors, especially EGF, transforming growth factor-α, and insulin-like growth factor (1). Direct antiproliferative actions of somatostatin are mediated through specific somatostatin receptors (1). Recently, we have shown that somatostatin analog RC-160 inhibited the growth of transplanted colon cancers in rats (8) and nude mice (9, 10).

Bombesin (BN) and its mammalian counterpart, gastrin-releasing peptide (GRP), appear to be autocrine growth factors for small cell lung carcinoma and receptors for bombesin and GRP are found in many tissues. Among the effects of GRP on the gastrointestinal tract are the regulation of gut motility, modulation of gastrointestinal enteropancreatic hormone release, and stimulation of intestinal and pancreatic epithelial growth *in vivo* (11). GRP is known to release various gastrointestinal peptides, such as gastrin, cholecystokinin, insulin, and glucagon, but not secretin.

The expression of EGF receptor has been observed in cells of various normal and cancerous tissues, including gastrointestinal carcinomas (12–16). Western blotting using monoclonal anti-EGF receptor antibody showed various levels of EGF receptor expression in seven of the eight (87.5%) colonic carcinomas (14). In another study, EGF receptors were found in nine of the 18 (50%) colorectal tumor specimens (17). Interestingly, EGF mRNA was detected only in 28% of the colorectal tumors, but not in normal mucosa (17). Our recent findings indicate that the inhibition of growth of experimental pancreatic (18) and colon cancers (9, 10) by bombesin antagonists is associated with a major down-regulation of EGF receptors.

Altogether, these observations strongly suggest the need for continued evaluation of somatostatin analogs, with selective and prolonged activities, as well as new specific bombesin antagonists, as possible agents for the treatment of colon cancers. In this study, we report new findings on the presence of binding sites for somatostatin and bombesin in human colon cancer specimens as well as the determination of the affinity of octapeptide somatostatin analogs and nonapeptide bombesin antagonists to these receptors. Our results extend and complement the information on receptor for growth factors in colonic carcinomas (14, 15, 17).

Materials and Methods

Somatostatin (SS)-14, Tyr^{11}-SS-14, and D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2 (RC-160) were supplied by Debiopharm S. A. (Lausanne, Switzerland). SMS-201–995 (D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-OL) were kindly provided by Sandoz Ltd. (Basel, Switzerland). Octapeptide analogs of SS-14, as well as bombesin antagonists with reduced (ψ^{13-14}) peptide bond, were synthesized using standard solid phase procedures and purified by high-performance liquid chromatography (19, 20). Introduction of reduced peptide bond was carried out by utilizing the reductive alkylation reaction described by Sasaki and Coy (21). Cleavage and deprotection of the resin-bound peptide was achieved by treatment with liquid HF containing anisole as a scavenger. The structures of somatostatin analogs and bombesin antagonists tested in receptor binding assays on human colon cancers are presented in Table I.

Radioiodination of Tyr^{11}-SS-14, preparation of membranes, and receptor assays for SS-14 and EGF were conducted as described previously (22, 23). [^{125}I] EGF was purchased from Amersham, Arlington Heights, IL (1270 Ci/mmol). Tyr^4-bombesin, synthesized in our laboratory, was iodinated by the chloramine T method and purified on high-performance liquid chromatography as reported previously (20, 24). In some experiments, [^{125}I]-Tyr^4-BN (New England Nuclear, Boston, MA) was used, and it showed the same binding characteristics as our radioligand.

The binding assay of [^{125}I]-Tyr^4-BN in membrane preparations from colon cancer specimens from patients was carried out in a total volume of 150 μl of binding medium containing 25 m*M* HEPES, 5 m*M* $MgCl_2$, 1 μg/ml of bacitracin, and 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (pH 7.4). Membranes (50 μg) were incubated with ^{125}I-Tyr^4-BN (0.05 n*M*) in the presence of increasing amounts of unlabeled Tyr^4-BN (4×10^{-11}-4×10^{-6} *M*) for 60 min at 21°C. After rapid filtration on GF/B glass filters (1.0-μm pore size), the filters were washed with ice-cold phosphate-buffered saline containing 0.1% BSA (10-sec washing time). The specificity of Tyr-BN binding was tested by addition, in excess concentrations, of SS-14, insulin, or EGF into incubation medium.

Tissue samples were obtained from 15 patients with colonic adenocarcinomas who underwent segmental resection of the colon at various institutions in the United States located outside New Orleans (Mount Sinai Medical Center, New York City, and James Graham Brown Cancer Center, Louisville, KY). All samples were shipped to New Orleans on dry ice and stored at −70°C. All patients were diagnosed as having Dukes classification Stage C colon cancer. Portions of viable cancer were excised and normal mucosa was obtained from the specimen as far as possible from the tumor. The clinical features of 15 colon carcinomas are presented on Table II.

The preparation of membranes was carried out as described previously for various tumor and normal tissues (22, 23). Briefly, the samples were cleaned of fat and connective tissue, cut into small slices, and homogenized in 5 vol of sucrose buffer (0.3 *M* sucrose, 25 m*M* Tris base, 0.25 m*M* phenylmethylsulfonyl fluoride,

Table I. Structures of Somatostatin and Bombesin/GRP Analogs[a]

Code number	Structure
RC-121	D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2
RC-160	D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2
RC-95-I	D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Trp-NH_2
RC-98-I	D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2
Bombesin	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 1 2 3 4 5 6 7 8 9 10 11 12 13 14
RC-3125	D-Trp^6,$Leu^{13}\psi[CH_2NH]Leu^{14}$-BN(6-14)
RC-3095	D-Tpi^6,$Leu^{13}\psi[CH_2NH]Leu^{14}$-BN(6-14)
RC-3420	D-Trp^6,$Leu^{13}\psi[CH_2NH]Phe^{14}$-BN(6-14)
RC-3110[b]	D-Phe^6,$Leu^{13}\psi[CH_2NH]Phe^{14}$-BN(6-14)

[a] The analogs were synthesized in our laboratory and tested in receptor binding assays on human colon cancer.
[b] The analog with this structure was synthesized previously and reported by Coy *et al.* (13).

Table II. Clinical Features of 15 Colon Carcinomas

Patient	Age	Sex	Histology[a]	Stage[b]	Position[c]	Department[d]	Lymph node[e]	Somatostatin[f]	Bombesin[f]
1	65	M	Mod	A	A	pm	−	+	−
2	71	M	Poor	B	R_s	ps	+	+	+
3	72	F	Mod	C	R_s	S	−	−	−
4	39	M	Well	B	D	pm	−	−	−
5	63	F	Mod	C	S	s	+	−	−
6	90	M	Mod	C	A	s	+	+	+
7	62	F	Mod	C	D	pm	+	−	−
8	69	M	Mod	C	R_s	s	+	+	−
9	70	M	Poor	B	R_s	pm	−	−	−
10	74	M	Mod	D	S	s	+	+	−
11	64	M	Well	B	D	ss	+	−	+
12	65	F	Mod	C	D	s	+	+	+
13	63	F	Mod	C	S	s	+	−	+
14	71	M	Well	C	T	s	+	+	−
15	60	M	Mod	C	R_s	ss	+	+	+

[a] Well, well differentiated; mod, moderately differentiated; poor, poorly differentiated.
[b] Dukes' classification.
[c] C, cecum; A, ascending colon; T, transversing colon; D, descending colon; S, sigmoid colon; R_s, rectosigmoid colon.
[d] Tumor invasion: pm, tunica muscularis propria; ss, subserosa; s, tunica serosa.
[e] Positive (+) or negative (−) metastasis to regional lymph nodes.
[f] Presence (+) or absence (−) of somatostatin or bombesin binding sites.

1 m*M* EGTA, 10 m*M* monothioglycerol, and Trasylol at 10,000 kallikrein inactivator units/liter, pH 7.5) using an Ultra-Turrax homogenizer (Tissumizer; Tekmar, Cincinnati, OH) at maximal speed 5 × 5-sec strokes at 0°C. The homogenate was centrifuged at 500*g* for 10 min at 4°C. The supernatant containing the crude membrane fractions was then ultracentrifuged at 70,000*g* for 45 min at 4°C (Beckman Preparative Ultracentrifuge; Beckman Instruments, Inc., Palo Alto, CA). The resulting pellet was resuspended in binding medium without BSA.

The binding of Tyr^4-BN to human colon cancer cell lines HT-29 and HCT-116 was tried on confluent cells grown in RPMI 1640 medium supplemented by 10% colostrum-free bovine serum in 24-well tissue culture plates (Primaria, Falcon; Becton Dickinson, Lincoln Park, NJ). The binding buffer used was RPMI 1640 medium with 0.1% BSA and 100 μg/ml of bacitracin. Cells were incubated with labeled and unlabeled peptide in various experimental conditions for different incubation times at 4, 21, and 37°C. The specificity of Tyr^4-BN binding was investigated in a 10-point assay using competitive inhibition studies with unlabeled Tyr^4-BN SS-14 (from 6×11^{-11} to 6×11^{-6} *M*) in triplicate wells.

For the binding of ^{125}I-Tyr^4-BN to crude membranes from HT-29 and HCT-116 cells, the cells were subcultured in 1850-cm^2 Falcon roller bottles, grown to confluence, and harvested at 4°C by scraping. The cells were homogenized using the same procedure and buffers described by Sinnett-Smith *et al* (25) for membranes from Swiss 3T3 cells.

The Ligand-PC computerized curve-fitting program of Munson and Rodbard (26) was used to determine the types of receptor binding, dissociation constant (K_d), and the maximal capacity of receptors

(B_{max}). The data obtained were evaluated by Student's t test.

Results

SS-14 receptors were found in eight of 15 colon cancer specimens. The analyses of displacement curves of [^{125}I-Tyr^{11}]-SS-14 by unlabeled SS-14 in membranes from human colon cancer and colonic mucosa specimens indicated that labeled SS-14 was bound to one class of noncooperative binding sites. Binding of [^{125}I-Tyr^{11}]-SS-14 was specific, since unlabeled D-Trp^6-luteinizing hormone-releasing hormone (1.6 μmol) and EGF (100 ng) were unable to displace labeled SS-14 from its binding sites. Membrane preparations from three specimens of colon cancer were found to have receptors for SS-14 with similar affinity and binding capacity. Homologous displacement assay of [^{125}I-Tyr^{11}]-SS-14 by unlabeled SS-14 in membranes from normal colon and colon cancer specimens from these three patients revealed the presence of high affinity ($K_d = 0.3 \times 10^{-9}$ M for normal colon and $K_d = 0.7 \times 10^{-9}$ M for colon cancer) and low capacity binding sites (B_{max} = 147 ± 72 fmol/mg protein for normal colon and B_{max} = 186 ± 63 fmol/mg protein for colon cancer) (Table III). The fourth and fifth colon cancer specimens showed fewer SS-14 binding sites (B_{max} = 28 and 40 fmol/mg protein). The membranes from the first three patients were pooled and used for the testing of different somatostatin octapeptide analogs in a heterologous displacement assay. Analogs RC-160 and RC-121 showed the highest affinity to receptors for SS-14 in both normal colon and colon cancer membranes (Table IV). They were 10 times more potent than SS-14 in displacing [^{125}I]Tyr^4-SS-14 from somatostatin binding sites in colon cancer membranes (Table IV).

In six of 15 colon cancer specimens, we found specific binding of ^{125}I-Tyr^4-BN. The time course study of BN binding at 20°C showed that equilibrium of binding was reached after 45 min and remained constant for 60 min. The analysis of [^{125}I]-Tyr^4-BN binding was performed at 20°C for 60 min. SS-14, insulin, and EGF did not affect BN binding to membranes from colon cancers. The displacement of ^{125}I-Tyr^4-BN from binding sites in membranes of colon cancer specimens by increasing concentrations of unlabeled Tyr^4-BN and Scatchard analyses of these data suggested that the labeled peptide was bound to two classes of binding sites in three specimens and a single class of binding sites in three other specimens (Fig. 1). In the first three colon cancer membranes, the biexponential model gave a significantly better fit than monoexponential (F ratio test, $P < 0.05$). One class of binding sites had high affinity (K_d = 0.42 ± 0.2 × 10^{-9} M), low maximum binding capacity (B_{max} = 5.4 ± 1.6 fmol/mg protein)

Table III. Binding Capacity of ^{125}I-Tyr^{11}-SS-14, ^{125}I-Tyr^4-BN, and ^{125}I-EGF to Membranes of Normal Human Colonic Mucosa and Colon Cancer Specimens[a]

Radioligand	Normal colon (fmol/mg protein)	Colon cancer (fmol/mg protein)
^{125}I-Tyr^{11}-SS	147 ± 72	186 ± 63
^{125}I-Tyr^4-BN	NB	
Single class		6–228
Low affinity		76,000 ± 15,000
^{125}I-EGF	28 ± 14	50 ± 21

[a] Data are expressed as mean ± SE. NB, no specific binding.

Table IV. Binding Potency of Somatostatin Analogs and Bombesin Antagonists to Normal Colonic Mucosa and Colon Cancer

Peptide	Normal colonic mucosa[a] (IC_{50}, nM)	Colon cancer[a] (IC_{50}, nM)
SS-14	0.4	0.9
SMS-201-995	—	0.7
RC-160	0.07	0.08
RC-121	0.09	0.1
RC-95-I	—	5.3
RC-98-I	—	25.3
Tyr^4-bombesin	NB	0.47
RC-3125	NB	3.4
RC-3095	NB	15.7
RC-3420	NB	0.34
RC-3110	NB	3.4

[a] The ability of various peptides to inhibit half of the specific [^{125}I]Tyr^{11}-SS-14 or [^{125}I]Tyr^4-bombesin binding to normal colon and colon cancer membranes. SS analogs were tested in three pooled specimens with high affinity binding and bombesin antagonists in six pooled specimens. The mean value of three determinations is indicated. NB, no binding.

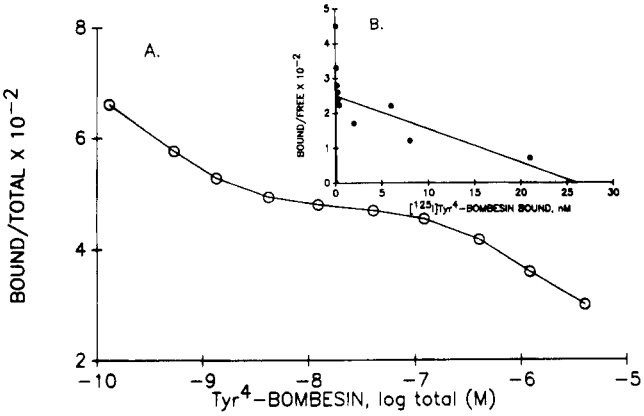

Figure 1. (A) Log-dose inhibition of specific binding of ^{125}I-Tyr^4-BN by increasing concentrations of bombesin to human colon cancer membranes in which a two-site model gave a significantly better fit. Aliquots of tumor membranes (50 μg) were incubated with 0.05 nM ^{125}I-Tyr^4-BN in the absence or presence of increasing amounts of Tyr^4-BN (4×10^{-11}–4×10^{-6} M) for 60 min at 21°C. (B) Scatchard plot of specific ^{125}I-Tyr^4-BN binding data from Figure 1A. The individual data points are means of triplicate determinations.

and the second class showed low affinity (K_d = 1.6 ± 0.4 × 10^{-6} M) and high capacity binding (B_{max} = 76 ± 15 pmol/mg protein). In three other specimens with single binding sites, apparent K_d was found to be approximately 2.5 nM and maximal [^{125}I]Tyr^4-BN binding capacity varied from 6 to 228 fmol/mg protein (Table IV). Bombesin/GRP antagonists, chosen from the list of analogs that showed the highest binding affinity constants in Swiss 3T3 cells, were tested for their ability to inhibit the specific radiolabeled Tyr^4-BN binding. Membranes from colon cancer specimens with positive specific binding for iodinated Tyr^4-bombesin were pooled and used for the testing of different pseudononapeptide bombesin antagonists. All these antagonists showed strong binding potency and exhibited IC_{50} in nanomolar concentrations (Table IV). Analog D-Trp^6,Leu$^{13}\psi$[CH$_2$NH]Phe^{14}-BN(6-14) (RC-3420) showed the highest binding affinity for bombesin/GRP binding sites, with IC_{50} less than 1 nM. None of the membrane preparations made from normal colon mucosa specimens showed specific binding for ^{125}I-Tyr^4-BN.

Specific ^{125}I-EGF binding was found in 12 of 15 (80%) investigated specimens of colon cancer. Analysis of the displacement curve of iodinated EGF by unlabeled EGF suggests that in normal colon and in colon cancer membranes, EGF binds with high affinity to a single class of noncooperative binding sites. The maximal binding capacity of EGF receptors in colon cancer specimens was twice as high as that found in normal colonic mucosa, but the difference was not statistically significant (Table III).

Specific SS-14 binding of low affinity was already described in colon cancer cell line HT-29 (10) and in the present study, no SS-14 binding was found in HCT-116 cells. Using cultured, intact colon cancer cell lines HT-29 and HCT-116 and membrane preparations from these cells, no specific binding for iodinated Tyr^4-BN was found. The analyses of displacement curves of ^{125}I-EGF by unlabeled EGF in HT-29, as well as in HCT-116 cells, suggested that the labeled EGF was bound to one class of noncooperative high affinity, low capacity binding sites. Binding of ^{125}I-EGF was specific, since unlabeled SS-14, insulin, GRP(14-27), and D-Trp^6-luteinizing hormone-releasing hormone, in concentrations of 10^{-7} M, were unable to displace labeled EGF from its binding sites. Affinity constants of EGF binding to HT-29 and HCT-116 (0.02 and 0.06 nM) were approximately 10 times higher than in human colon cancer membranes and EGF binding capacity in these cells was 15 and 20 fmol/mg protein, respectively.

Discussion

There are compelling reasons for investigating peptide and growth factor receptors in different tumors. Information on presence, characteristics, and content of receptors could be important for the therapy of certain tumors and might be also of significant prognostic value (1, 27). Findings about peptide receptor levels may make feasible the estimation of any possible direct effects of analogs on certain tumors.

Specific somatostatin receptors have been detected in many normal and malignant tissues (1, 23, 27, 28). High affinity somatostatin receptors have been recognized in brain and pituitary tumors, small cell lung carcinoma, and gastrinomas, as well as in pancreatic, prostate, and breast cancers (23, 27, 28). Binding of somatostatin to the membrane receptor in the MIA PaCa-2 cell line activates dephosphorylation of EGF receptor phosphotyrosyl membrane protein, the phosphorylation of which is promoted by EGF (1, 29). Superactive analogs RC-160 and RC-121 exhibit even higher activity than somatostatin on dephosphorylation of the EGF receptor. These observations indicate that somatostatin analogs can act as growth inhibitors in cancer cells through the activation of tyrosine phosphatase (29). The action of somatostatin analogs might involve the inhibition of not only endocrine, but also paracrine- and autocrine-mediated effects of growth factors (1, 29).

Our results indicate the presence of high affinity binding sites for SS-14 in 53% of the human colon cancer specimens. The membranes from normal human colon also showed the presence of SS-14 receptors. No correlation between the degree of differentiation and the presence of SS-14 binding sites on tumors was found (Table II). The maximal binding capacity (B_{max}) of SS-14 receptors in colon cancer seemed to be higher than in normal colon, but the difference was not statistically significant (Table III). However, the number of tumor specimens examined is too low to allow a firm conclusion. In contrast to human colon cancer membranes, SS-14 binds with low affinity to HT-29 cells (IC_{50} = 238 nM) (10) and no specific SS-14 binding was found on HCT-116 colon cancer cell line. The absence of somatostatin receptors in colon cancer cell lines could explain the lack of effects of somatostatin analog RC-160 on tumor growth *in vitro* of DHD/K12 rat colon adenocarcinoma observed by Qin *et al.* (8). In that study, RC-160 significantly inhibited the growth of DHD/K12 colon adenocarcinoma *in vivo* (8). Similarly, we did not find specific binding sites for bombesin/GRP in HT-29 and HC-116 cells.

Two of our long-acting octapeptide superactive analogs of somatostatin, RC-160 and RC-121, designed especially for antitumor activity (1, 19, 23), showed 10 times higher binding affinity for somatostatin receptors on colon cancer membranes than SS-14. These analogs also showed high affinity for somatostatin receptors on pancreatic and breast cancer (23). Analog SMS-201-995 had a binding affinity similar to SS-14 in colon cancer specimens. SMS-201-995 binds well to some

normal tissue where it is a good anti-secretory agent, but poorly to most tumors (23). Our observations suggest that RC-121 or RC-160, in addition to blocking the secretion or action of endocrine, autocrine, or paracrine growth factors, might inhibit colon cancer growth by direct action on somatostatin receptors. This would be in agreement with findings in pancreatic cancers (1, 23, 29).

EGF receptors were present in both HT-29 and HCT-116 cell lines, as well as in most of the colon cancer specimens. EGF binding capacities on colon cancer specimens in this study were similar to those recently described for gastric (30) and colonic carcinomas (14, 17). Our results, as well as the results of Yasui *et al.* (14), indicate that EGF receptors might be overexpressed in carcinomatous tissue compared with normal colonic mucosa.

Bombesin/GRP-like peptides are found in many different tumors and their role as autocrine growth factors in small cell lung carcinoma is well established (31, 32). Bombesin/GRP immunoreactive sites are abundant in the myenteric plexus and submucosal layer of the human colon (33). GRP is widely distributed in intrinsic neurons throughout the mammalian gastrointestinal tract. Price *et al.* (33) reported high levels of GRP immunoreactivity in human fundus, antrum, pylorus, and pancreas, with lower levels in the duodenum, jejunum, and colon. The highest levels of GRP immunoreactivity were reported in the stomach, but the greatest concentration of GRP mRNA was found in the colon. By autoradiography, GRP receptors are abundant in the circular muscle and myenteric plexus of the antrum, the longitudinal muscle of ileum and colon, and myenteric plexus in both the small and large bowel (33). In the present study, we found that some human colon cancers can have bombesin/GRP receptors. Our results also indicate that two types of bombesin/GRP receptors might exist in some human colon cancer. In an analogy to findings with somatostatin receptors, no correlation could be found between the degree of differentiation and the presence of binding sites for bombesin/GRP on colon cancer specimens. Our work demonstrates the heterogeneity of receptor content among individual cancers and shows that each colon cancer may express various combinations of receptors for bombesin and/or SS-14. This suggests the need to evaluate each tumor individually. It is important to emphasize that specific binding sites for bombesin were not found in normal colonic mucosa. It is intriguing why the finding of the receptors for bombesin seems to be linked with the presence of a malignancy. Further biochemical studies are needed to confirm these findings. In a very recent study, it was reported that two specific and probably functional binding sites for GRP were present in mouse colon cancer cells (34). It has been shown that bombesin can stimulate the growth of different rat colon adenocarcinoma cell lines (35). By using various classes of receptor antagonists, von Schrenck and co-workers (36) proved that different subtypes of bombesin receptors exist in esophageal muscle and pancreatic tissue. However, it is not known if bombesin/GRP receptors play a role in the growth of human colon cancer.

GRP was named in recognition of its promoting effect on gastrin release *in vivo*. In addition, intravenous administration of GRP stimulates the secretion of insulin, glucagon, pancreatic polypeptide, somatostatin, and gastric inhibitory peptide in dogs and humans. GRP stimulates gastrin and insulin from isolated gastrin cells, and insulin release from pancreatic islet cell lines, respectively (37). The stimulating effects of gastrin and insulin in the growth of colon cancer are well established *in vitro*, as well as *in vivo* (38–41). The possible stimulatory role of bombesin/GRP on growth of gastrointestinal tumors can be suspected, but remains to be firmly determined. As part of such studies, potent bombesin/GRP antagonists are being investigated *in vivo* for their possible effects on inhibition of colon cancer growth (9, 10). The synthesis of short-chain bombesin (6–14) nonapeptide analogs with a reduced peptide bond and higher potency was first reported in 1989 (42). We synthesized more than 40 pseudopeptide [ψ^{13-14}] bombesin (6–14) analogs with different modifications at positions 6, 7, and 14 (20, 43). Introduction of D-forms of 2,3,4,9,-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid (Tpi) and tryptophan (Trp) at Position 6 in [Leu$^{13}\psi$(CH_2NH)Leu^{14} or Phe^{14}]bombesin (6–14) produced analogs, which are active in the nanomolar range (20). We have demonstrated that at least two of these bombesin antagonists, RC-3095 and RC-3440, inhibited the growth of HT-29 human colon cancer lines xenografted in nude mice (9, 10). The present study indicates that our modern bombesin/GRP antagonists show high binding affinity to some human colon cancer specimens. Additional studies with bombesin/GRP antagonists and somatostatin analogs are needed to confirm their inhibitory effects on tumor growth in models of colon cancer. Subsequently, clinical trials could determine whether such analogs could possibly benefit patients with colon cancer.

This work was supported by the National Institutes of Health Grant CA 40077 and by the Medical Research Service of the Veterans Affairs (to A.V.S.). The contents of the manuscript are solely the responsibility of the authors and do not necessarily present the official views of the National Cancer Institute.

We are grateful to Elvira Monje and Robert O'Brien for experimental assistance. We thank Dr. Kate Groot for the iodination and for high-performance liquid chromatography separation of iodinated peptides and Dr. S. Brower and Dr. V. Hollander (Mount Sinai Medical Center, New York), and Dr. J. Wittliff (James Graham Brown Medical center, Louisville, KY), and Dr. Jane Gertler (East Jefferson Hospital, Metairie, LA) for colon cancer samples.

1. Schally AV. Oncological application of somatostatin analogues. Cancer Res **48**:6977–6985, 1988.
2. Townsend CM, Singh P, Thompson JC. Gastrointestinal hormone and gastronintestinal and pancreatic carcinomas. Gastroenterology **91**:1002–1006, 1986.
3. Lamers CBHW, Jansen JBMJ. Role of gastrin and cholecystokinin in tumors of the gastrointestinal tract. Eur J Cancer Clin Oncol **4**:267–273, 1988.
4. Schally AV, Coy DH, Meyers CA. Hypothalamic regulatory hormones. Annu Rev Biochem **47**:89–128, 1978.
5. Reichlin D. Somatostatin. N Engl J Med **309**:1495–1501, 1983.
6. Lehy T, Dubrasquet M, Bonfils S. Effect of somatostatin on normal and gastric-stimulated cell proliferation in the gastric and intestinal mucosae of the rat. Digestion **19**:99–109, 1979.
7. Morisset J, Genki P, Lord A, Solomon TE. Effects of chronic administration of somatostatin on rat exocrine pancreas. Regul Pept **4**:49–58, 1982.
8. Qin Y, Schally AV, Willems G. Somatostatin analogue RC-160 inhibits the growth of implanted colon cancer in rats. Int J Cancer **47**:765–770, 1991.
9. Radulovic S, Miller G, Schally AV. Inhibition of growth of HT-29 human colon cancer xenografts in nude mice by treatment with bombesin/GRP antagonist (RC-3095). Cancer Res **51**:6006–6009, 1991.
10. Radulovic S, Schally AV, Milovanovic S, Szepeshazi K, Groot K, Miller G, Yano T. Inhibitory effect of bombesin/GRP antagonists (RC-3095 and RC-3440) and somatostatin analog (RC-160) on growth of HT-29 human colon cancer xenografts in nude mice. Cancer (in press).
11. Tache Y, Melchiorri P, Negri L (Eds). Bombesin-like Peptides in Health and Disease. New York: New York Academy of Sciences, 1988.
12. Fitzpatrick SL, LaChance MP, Schultz GS. Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. Cancer Res **44**:3442–3447, 1984.
13. Merlino GT, Xu YH, Ishii S, Clark AJL, Semba K, Toyoshima K, Yamamoto T, Pastan I. Amplification and enhanced expression of the epidermal growth factor gene in A431 human carcinoma cells. Science **224**:417–419, 1984.
14. Yasui W, Sumiyoshi H, Hata J, Kameda T, Ochiai A, Ito H, Tahara E. Expression of epidermal growth factor receptor in human gastric and colonic carcinomas. Cancer Res **48**:137–141, 1988.
15. Ozawa S, Ueda M, Ando N, Abe O, Shimizu N. Epidermal growth factor receptors in cancer tissues of esophagus, lung, pancreas, colorectum, breast and stomach. Jpn J Cancer Res **79**:1201–1207, 1988.
16. Bennett C, Paterson IM, Corbishley CM, Luqmani YA. Expression of growth factor and epidermal growth factor receptor encoded transcripts in human gastric tissues. Cancer Res **49**:2104–2111, 1989.
17. Ito M, Yoshida K, Kyo E, Ayhan A, Nakayama H, Yasui W, Ito H, Tahara E. Expression of several growth factors and their receptor genes in human colon carcinoma. Virchows Arch [B] **59**:173–178, 1990.
18. Szepeshazi K, Schally AV, Cai R-Z, Radulovic S, Milovanovic S, Szoke B. Inhibitory effect of bombesin/GRP antagonist RC-3095 and high dose of somatostatin analog RC-160 on nitrosamine-induced pancreatic cancers in hamsters. Cancer Res **51**:5980–5986, 1991.
19. Cai RZ, Karashima T, Guoth J, Szoke B, Olsen D, Schally AV. Superactive octapeptide somatostatin analogs containing tryptophan residue in position 1. Proc Natl Acad Sci USA **84**:2502–2506, 1987.
20. Radulovic S, Cai R-Z, Serfozo P, Groot K, Redding TW, Pinski J, Schally AV. Biological effects and receptor binding affinities of new pseudononapeptide bombesin/GRP receptor antagonists with N-terminal D-Trp or D-Tpi. Int J Pept Protein Res **38**:593–600, 1991.
21. Sasaki Y, Coy DH. Solid phase synthesis of peptides containing the CH_2NH peptide bond isostere. Peptides **8**:119–121, 1987.
22. Fekete M, Redding TW, Comaru-Schally AM, Pontes JE, Connelly RW, Srkalovic G, Schally AV. Receptors for luteinizing hormone releasing hormone, somatostatin, prolactin and epidermal growth factor in rat and human prostate cancers and in benign prostate hyperplasia. Prostate **14**:191–208, 1989.
23. Srkalovic G, Cai R-Z, Schally AV. Evaluation of receptors for somatostatin in various tumors using different analogs. J Clin Endocr Metab **70**:661–669, 1990.
24. Moody TW, Kris RM, Fiskum G, Linden CD, Berg M, Schlessinger J. Characterization of receptors for bombesin/gastrin-releasing peptide in human and murine cells. Methods Enzymol **168**:481–493, 1979.
25. Sinnett-Smith J, Lehmann W, Rozengurt E. Bombesin receptor in membranes from Swiss 3T3 cells. Biochem J **265**:485–493, 1990.
26. Munson PJ, Rodbard D. Ligand: A versatile computerized approach for characterization of ligand-binding systems. Anal Biochem **107**:220–239, 1980.
27. Foekens JA, Portengen H, van Putten WLJ, Trapman AMAC, Reubi JC, Alexieva-Figusch J, Klijn JGM. Prognostic value of receptors for insuline-like growth factor 1, somatostatin, and epidermal growth factor in human breast cancer. Cancer Res **49**:7002–7009, 1989.
28. Reubi JC, Lamberts SWJ, Maurer J. Somatostatin receptors in normal and tumoral tissue. Horm Res **26**:65–69, 1988.
29. Liebow C, Reilly C, Serrano M, Schally AV. Somatostatin analogues inhibit growth of pancreatic cancer by stimulating tyrosine phosphatase. Proc Natl Acad Sci USA **86**:2003–2007, 1989.
30. Pfeiffer A, Rothbauer E, Wiebecke B, Pratschke E, Krämling H-J, Mann K. Increased epidermal growth factor receptors in gastric carcinomas. Gastroenterology **98**:961–967, 1990.
31. Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A, Minna JD. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer cells. Nature **316**:823–826, 1985.
32. Carney DN, Moody T, Cuttitta F. Bombesin: A potent mitogen for small cell lung cancer. Ann NY Acad Sci **547**:303–309, 1988.
33. Price J, Penman E, Wass JAH, Rees LH. Bombesin-like immunoreactivity in human gastrointestinal tract. Regul Pept **9**:1–6, 1984.
34. Narayan S, Guo J-S, Townsend CM, Singh P. Specific binding and growth effects of bombesin-related peptides on mouse colon cancer cells in vitro. Cancer Res **50**:6772–6778, 1990.
35. Maley MA, House AK, Jenkyn DJ, Agrez MV. Three new rat colon tumour cell lines from 1,2-dimethylhydrazine induced adenocarcinomas: Morphology, karyotype and clonogenicity. Br J Exp Pathol **69**:719–726, 1987.
36. Von Schrenck T, Wang L-H, Coy D-H, Villaneuva M-L, Mantey S, Jensen R-T. Potent bombesin receptor antagonists distinguish receptor subtypes. Am J Physiol **259**:G468–G473, 1990.
37. Sunday ME, Kaplan LM, Motoyama E, Chin WW, Spindel ER. Biology of disease, gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. Lab Invest **59**:5–24, 1988.
38. Takahashi T, Yamaguchi T, Bando K, Tanaka J, Ogata BN, Kohno K. Effects of gastrointestinal hormones on protein synthesis and growth of gastrointestinal cancers (Abstract). International Cancer Congress, 97, 1982.
39. Sirince KR, Levine BA, Moyer MP. Pentagastrin stimulates in vitro growth of normal and malignant human colon epithelial cells. Am J Surg **149**:35–39, 1985.
40. Singh P, Walker JP, Townsend CM Jr, Thompson TC. Role of

gastrin and gastrin receptors on the growth of a transplantable mouse colon carcinoma (MC-26) in BALB/C mice. Cancer Res **46**:1612–1616, 1986.
41. Lamote J, Willems G. Stimulating effect of pentagastrin on cancer cell proliferation kinetics in chemistry induced colon cancer in rats. Regul Pept **20**:1–9, 1988.
42. Coy DH, Taylor JE, Jiang N-Y, Kim SH, Wang L-H, Huang S-C, Moreau J-P, Gardner JD, Jensen RT. Short-chain pseudopeptide bombesin receptor antagonists with enhanced binding affinities for pancreatic acinar and Swiss cells display strong antimitotic activity. J Biol Chem **264**:14691–14697, 1889.
43. Schally AV, Srkalovic G, Szende B, Redding TW, Janaky T, Juhasz T, Korkut E, Cai RZ, Szepeshazi K, Radulovic S, Bokser L, Groot K, Serfozo P, Comaru-Schally AM. Antitumor effect of analogs of LH-RH and somatostatin: Experimental and clinical studies. J Steroid Biochem Mol Biol **37**:1061–1067, 1990.