ABSTRACT

In this study, we investigated the effect of bombesin/GRP antagonist RC-3095 on the growth of CFPAC-1 human pancreatic cancer cells transplanted to nude mice or cultured in vitro. Nude mice bearing xenografts of the CFPAC-1 cell line received s.c. injections of RC-3095 (10 μg twice a day) or the vehicle (control) for 25 days. Chronic administration of RC-3095 inhibited the growth of CFPAC-1 tumors in nude mice as shown by a significant decrease in tumor volume throughout the period of treatment. Tumor volume doubling time was prolonged by RC-3095 treatment from 7.2 days to 10 days, and the tumor growth rate was decreased by 49%. In mice treated with RC-3095, the tumor growth delay time was 5.8 days. Treatment with RC-3095 decreased the final tumor weight by 37% and reduced DNA and protein contents in tumor tissues by 44% and 39.9%, respectively, compared to the controls. In cultures of the CFPAC-1 cell line, the addition of bombesin(1-14) (1 pm-6.1 μM) to the medium induced a dose-dependent increase in cell number. RC-3095 at 1 nM concentration effectively inhibited the bombesin-stimulated growth of CFPAC-1 cells in culture. At 1 μM, RC-3095 in the absence of bombesin was completely ineffective. RC-3095 (1 μM) and bombesin (1-100 nM) inhibited DNA synthesis in the presence of bombesin(1-14) (1 μM). RC-3095 at 1 μM concentration completely inhibited the bombesin-stimulated incorporation of [3H]thymidine into CFPAC-1 cells. The addition of bombesin(1-14) to the cells in the presence of RC-3095 caused a 39-40% decrease in the [3H]thymidine incorporation by the cells compared to the values obtained with bombesin alone. Receptor binding assays showed the presence of two classes of specific binding sites on bombesin for CFPAC-1 cells, one with high affinity (K_d = 4.25 ± 0.77 nM) and low affinity (K_d = 321.70 ± 68.46 nM) and another with low affinity (K_d = 0.268 ± 0.052 pmol/10^6 cells) and high affinity (K_d = 3.991 ± 0.374 pmol/10^6 cells). Antagonist RC-3095 inhibited the binding of 125I-Tyr 4-bombesin to CFPAC-1 cell membranes in a dose-dependent manner. These observations suggest that bombesin acts as a growth factor and stimulates the proliferation of CFPAC-1 human pancreatic cancer cells through specific receptors for bombesin on the cell membrane. RC-3095 appears to inhibit the growth of CFPAC-1 cells by blocking the interaction of bombesin with its receptors. Bombesin/GRP receptor antagonist RC-3095 could be considered for the development of new approaches for treatment of human pancreatic cancers.

INTRODUCTION

Pancreatic cancer is one of the greatest challenges for oncologists (1-5). Carcinoma of the exocrine pancreas is the fifth leading cause of death from cancer in the United States (1). Most of pancreatic cancers are histologically ductal cell carcinomas, which constitute about 80-90% of the cases (2). In the past two decades, great efforts have been made to improve the therapies for pancreatic cancers. However, the overall prognosis of patients with pancreatic cancer is still very poor, and the 5-year survival rate is only 2-5% (2-4). Less than 15-20% of pancreatic tumors are resectable, mostly due to the difficulties in early diagnosis and the frequent occurrence of local or distal metastases, and fewer than 5% of the patients can survive for over 5 years postoperatively (3-5). Radiotherapy and chemotherapy are usually ineffective (2-5). Therefore, an urgent need exists to develop a new and effective therapy for treatment of patients with pancreatic cancers.

Recently, various investigations have demonstrated that gastrointestinal hormones and growth factors may play important roles in the regulation of growth of normal and malignant exocrine pancreas (5-10). In vitro studies have shown that gastrin, CCK,3 and secretin stimulate the proliferation of pancreatic adenocarcinomas in tissue cultures (6, 7). Carcereulin combined with secretin promotes the in vivo growth of H-2T pancreatic ductal adenocarcinoma cells in golden hamsters (8). Gastrointestinal hormones may also influence the phenotypic transformation of pancreatic cells (6, 8, 9). These findings suggest that the growth of pancreatic cancers might be possibly controlled by hormonal manipulations (4-10), and various peptidic analogues have been suggested for treatment (5-10).

Bombesin and its mammalian counterpart, GRP, are hormonal peptides which can exert diverse physiological or pharmacological actions in various systems (5, 11, 12). In the gastrointestinal tract, bombesin and GRP stimulate gastric and pancreatic secretions, enhance the release of several gastrointestinal hormones, and promote the growth of exocrine pancreas (5, 11, 12). Administration of bombesin to rats produces hyperplasia and hypertrophy of pancreatic acinar cells (13). Bombesin and GRP appear to function as autocrine or paracrine growth factors and stimulate the growth of some normal or malignant cells including Swiss 3T3 fibroblast cells (14), human small cell lung cancer cells (15), human mammary and gastric cancer cells (16), mouse and human colon cancer cells (17, 18), and CAPAN human pancreatic tumor cells (19). The trophic effect of bombesin/GRP has also been demonstrated on the growth of azaserine-induced pancreatic acinar-cell adenocarcinomas in rats (20, 21) and on the cell proliferation of ascites tumor in primary cultures (22). The action of bombesin/GRP is thought to be mediated through its specific receptors present on the target cells (19, 22). The discovery that bombesin appears to act as an autocrine growth factor in human small cell lung carcinoma (15, 23) and may also be involved in other cancers (16-22) has aroused major interest in the development of competitive bombesin/GRP receptor antagonists.

During the past few years, various bombesin/GRP antagonists including RC-3095 have been synthesized in our laboratory and evaluated for antitumor activity (24, 25). Receptor studies showed that these synthetic peptide analogues inhibit the binding of bombesin/GRP to specific receptors on the membranes of Swiss 3T3 cells, SCLC cells, and human gastric cancer cells (24, 25).3 Bombesin

The abbreviations used are: CCK, cholecystokinin; GRP, gastrin-releasing peptide; FCS, fetal calf serum; IMDM, isoosmotic modified Dulbecco’s medium; PIBS, phosphate-buffered saline; GHRH, growth hormone-releasing hormone; Tpi, 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid; EGF, epidermal growth factor; [α-251I]LHRH, α-tropophan-6-luteinizing hormone-releasing hormone.

3 The abbreviations used are: CCK, cholecystokinin; GRP, gastrin-releasing peptide; FCS, fetal calf serum; IMDM, isoosmotic modified Dulbecco’s medium; PIBS, phosphate-buffered saline; GHRH, growth hormone-releasing hormone; Tpi, 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid; EGF, epidermal growth factor; [α-251I]LHRH, α-tropophan-6-luteinizing hormone-releasing hormone.
antagonist RC-3095 has been shown to effectively suppress the growth of HT 29 human colon cancers (26), PC-32 human prostate cancers (27), MKN45 human gastric cancers xenografted in nude mice (28), MXT breast cancers in mice (29), and nitrosamine-induced pancreatic cancers in hamsters (30). The inhibitory effect of antagonist RC-3095 on the cell proliferation in vitro has also been demonstrated in the cultures of some of these cancer cell lines (16, 25, 28). 4 RC-3095 and other novel bombesin antagonists could be considered for the development of hormonal therapy for pancreatic cancers. However, the presence of specific receptors for bombesin/GRP has been reported so far only in CAPAN human pancreatic cancer cells (19), and the effects of bombesin/GRP and their antagonists on the growth of human pancreatic ductal cancer cells must be evaluated more extensively.

In the present study, we investigated the effect of bombesin antagonist RC-3095 on the growth of CFPAC-1 human ductal pancreatic cancer cells implanted in nude mice. Direct effects of bombesin and RC-3095 on the proliferation of CFPAC-1 cells were evaluated in cell cultures. Specific receptors for bombesin/GRP on CFPAC-1 cells were characterized, and the ability of RC-3095 to inhibit the binding of bombesin to the receptors was also studied.

**MATERIALS AND METHODS**

**Peptides and Chemicals.** Bombesin antagonist Tyr-Tpi6-Leu13-NMTI[CH2NH]-Leu14-bombesin (6–14) (RC-3095), originally synthesized by a solid phase method and characterized in this laboratory (24, 25), was provided by Asta Pharm Co. (Frankfurt/M, Germany) for this study. Tyr3-bombesin and somatostatin-14 were obtained from Bachem (Torrance, CA) and Wyeth-Ayerst (Philadelphia, PA), respectively. Bombesin (1–14), GRH-RH (1–29), cholecystokinin-8, and Tyr-Tpi6-LHRH were all synthesized by a solid phase method in this laboratory (25, 31). 125I-Na and [methyl-3H]thymidine were purchased from Amersham (Arlington Heights, IL). Enzymobead iodination reagent was obtained from Bio-Rad (South Richmond, CA). FCS, IMDM, PBS, and Hanks’ balanced salt solution were purchased from Gibco (Grand Island, NY). Universal scintillation cocktail was obtained from ICN (Costa Mesa, CA). Other chemicals were purchased from Sigma (St. Louis, MO).

**Cancer Cell Line.** CFPAC-1 human pancreatic cancer cell line, originally established from a well-differentiated ductal pancreatic adenocarcinoma of a 26-year-old white male with cystic fibrosis (32), was purchased from the American Type Culture Collection (Rockville, MD). The cancer cells were routinely maintained as a monolayer culture in Costar T75 culture flasks with IMDM medium containing 10% FCS, 0.5 g/liter l-glutamine, 25 mM HEPES, 3.7 g/liter NaHCO3, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin-B under humidified 5% CO2 at 37°C. The cells growing exponentially were harvested by an incubation with 0.25% Trypsin-EDTA in calcium- and magnesium-free Hanks’ balanced salt solution for 5 min at 37°C. For tumor implantation, a single cell suspension was prepared in serum-free IMDM by repeatedly passing the cells through a G-22 needle; then the cells were diluted to a concentration of 5 × 106 cells/ml.

**Implantation of Tumors in Nude Mice.** Male athymic BALB/c (nu/nu) 6-week-old mice were obtained from the National Cancer Institute (Bethesda, MD) and housed in a laminar airflow cabinet under pathogen-free conditions throughout the experiments.Implantation of CFPAC-1 tumors was performed according to a method described previously (33). Three nude mice received s.c. injections in the flanks with 0.2 ml of cell suspension (1 × 106 cells) and served as tumor donors. After 4 weeks, the implanted tumors grew to a size of about 5 mm in diameter and were removed from the mice. Tumor samples were dissected free of necrotic tissue and blood vessels and were cut into small fragments of about 8 mm3. Under ether anesthesia, two pieces of tumor fragments were implanted s.c. by trocars on both sides of the flanks for each mouse. The mice bearing the implanted tumors were randomly divided into two groups with 10 mice in each group.

**Treatment with Bombesin Antagonist RC-3095.** The nude mice with implanted tumors started to receive injections of bombesin antagonist RC-3095 7 days after the tumor cell injection. RC-3095 solution was freshly prepared by dissolving the peptide in 200 μl of 0.01 M acetic acid and diluted with 0.9% NaCl containing 0.2% bovine serum albumin to a concentration of 125 μg/ml. Each mouse in the treatment group was given s.c. injections of RC-3095 at a dose of 10 μg twice daily for 25 days. This dose of RC-3095 was chosen on the basis of a previous report of the inhibitory effect of RC-3095 on the growth of HT29 human colon cancers in nude mice (26). The animals in the control group were injected with the same volume of vehicle but without RC-3095 or bombesin.

**Evaluation of Tumor Growth.** During the treatment with RC-3095, the size of the implanted tumors was measured by calipers in each mouse at 3–4 day intervals for 25 days to construct the tumor growth curves in vivo. Tumor volume was calculated by the formula: Tumor volume = Length × width2 × 0.5. Tumor growth parameters, i.e., tumor volume doubling time, tumor growth delay time, and tumor growth rate, were calculated from the tumor growth curves as described previously (34). In this study, the tumor volume doubling time was defined as the time required for the tumors to grow from 50 mm3 to 100 mm3 for the control group and from 35 mm3 to 70 mm3 for the treatment group, respectively. The tumor growth delay time was estimated as the time difference for the treated tumors and the controls to reach a volume of 70 mm3. At the end of the treatment, the animals were sacrificed with an overdose of ether. The tumors were removed from the animals, weighed, and immediately frozen in liquid nitrogen for measurements of DNA and protein contents in tumor tissues.

**Determination of DNA and Protein in Tumor Tissues.** DNA in tumor tissues was determined by a method of Labara and Paigen (35, 36), which is based on the enhancement of fluorescence reaction upon binding bisbenzimidazole Hoechst 33258 (H33258) to DNA in cell nuclei in a high ionic strength solution. Tumors collected in each group were pooled and homogenized in 10 times their volumes of a buffer consisting of 0.05 M Na2PO4, 2.0 M NaCl, and 2 μg EDTA (pH 7.4). H33258 was dissolved in the same buffer at a concentration of 1 μg/ml and filtered before use. An aliquot of tumor homogenate (0.4 ml) was suspended in 4 ml of H33258 solution, followed by an incubation in a dark room for 30 min. The reaction was measured by a fluorescence spectrophotometer at excitation and emission wavelengths of 356 and 492 nm, respectively. Calf thymus DNA type I was used as a standard reference.

**Protein in Tumor Tissues.** Protein in tumor tissues was measured by a modified method of Bradford (37) using bovine serum albumin fraction V as a standard.

**Measurement of Cell Growth in Vitro.** The effects of bombesin and bombesin antagonist RC-3095 on the growth of CFPAC-1 human pancreatic cancer cells in vitro was evaluated by direct cell counting and [3H]thymidine incorporation assay.

**Direct Cell Counting.** CFPAC-1 cells collected from 60–70% confluent cultures were used for this study and seeded to 24-well culture plates (1 × 104 cells/well). After the cells were cultured in IMDM containing 10% FCS for 48 h, the medium was replaced by IMDM supplemented with 2.5% FCS and various combinations of bombesin (1–14), bombesin antagonist RC-3095, or a combination of both. The same volume of medium but without peptides was added to the control wells. Following another 24 h of incubation, the culture was terminated by aspiration of the medium from the wells and washing with PBS (0.5 ml/well). The cells were trypsinized by a 10-min incubation with (0.5 ml/well) 0.25% Trypsin-EDTA. The detached cells were dispersed by repeated pipetting using a G-22 needle and syringe. The number of cells collected from each well was counted by an automated electronic cell counter ( Coulter Counter Model-ZF; Coulter Electronics, Inc., Hialeah, FL).

**[3H]Thymidine Incorporation Assay.** DNA synthesis in tumor cells was measured by [3H]thymidine incorporation assay as reported previously (38). Single cell suspension was prepared in IMDM with 10% FCS and was seeded to 24-well culture plates (1 × 104 cells/well). After 48 h of culture, the medium was changed to IMDM (0.5 ml/well) containing 2.5% FCS and various concentrations of bombesin (1–14), bombesin antagonist RC-3095, or a combination of both. The control wells received the same medium without bombesin or RC-3095. After 24 h of culture, [methyl-3H]thymidine (radioactivity 25 Ci/ml) was added to each well (1 μCi/well) to pulse the cells. After a 4-h incubation, the medium was removed from the wells, and the cells were fixed by Camoy’s solution (1 ml/well; methanol/glacial acetic acid, 3:1 v/v) for 20 min. After washing three times with PBS, the cells in each well were dissolved with 0.5 ml of 0.3 M NaOH for 15 min at room temperature. The cell lysate was collected from each well and mixed with 3 ml of Universal scintillation cocktail. The radioactivity was measured for 1 min by a liquid scintillation beta counter (Mark III; Searle Analytic, Inc., Des Plaines, IL).
Receptor Binding Assay. Receptor binding assay was performed using intact CFPAC-1 cells in monolayer cultures. Tyr⁴-bombesin was radiolabeled with ¹²⁵I-Na using a Bio-Rad enzymobead iodination kit. Mono-¹²⁵I-Tyr⁴-bombesin was purified by high performance liquid chromatography as described (39). The specific activity of ¹²⁵I-Tyr⁴-bombesin was about 2,000 Ci/mmol. CFPAC-1 cells were seeded to 24-well culture plates (1 × 10⁴ cells/well) and cultured with IMDM containing 10% FCS for 48 h. The cells in subconfluent culture were washed once with serum-free IMDM supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM MgCl₂, 1 mM EGTA, 10 mM monothioglycerol, 0.25 mM phenylmethylsulfonyl fluoride, aprotonin 10,000 kallikrein inactivator units/liter, and 0.1% bovine serum albumin (pH 7.5), followed by an incubation for 2 h at 22°C with the same medium containing (0.5 nm) ¹²⁵I-Tyr⁴-bombesin in the presence or absence of various concentrations of unlabeled bombesin(1-14), bombesin antagonist RC-3095, or structurally unrelated peptides somatostatin-14, cholcecytokinin-8, GHRH(1-29), and 3-Trp³-LHRH. The binding reaction was terminated by adding 0.5 ml of ice-cold medium to each well. After washing four times with ice-cold PBS (pH 7.4), the cells in each well were dissolved with 0.5 ml of 0.3 N NaOH. The resultant cellular lysate was collected from each well for measurement of radioactivity by a gamma counter (APEX; Microdemic System, Inc., Huntsville, AL).

Statistical Analysis. All data are expressed as the mean ± SEM of duplicate or triplicate observations from at least 2–3 repeated experiments. Mean values between the RC-3095 treatment group and the control group were analyzed by the Student t test, Mann-Whitney U test, or one-way analysis of variance. The difference was statistically considered to be significant when a two-tailed value of P was less than 0.05.

RESULTS

Effect of RC-3095 on Growth of CFPAC-1 Tumors in Nude Mice. Nude mice with implanted CFPAC-1 human pancreatic cancers started to receive injections of bombesin antagonist RC-3095 7 days after the tumor inoculation when all animals had palpable tumors in the flanks. Injections of RC-3095 at a dose of 10 μg twice a day for 25 days did not show any obvious side effects on the growth of the treated animals. There was no significant difference in body weights between the RC-3095-treated and the control groups (P > 0.05).

Chronic administration of RC-3095 inhibited the growth of implanted CFPAC-1 tumors in nude mice. Fig. 1 shows the change in tumor volumes in the RC-3095-treated mice and the controls as measured at intervals of every 3–4 days during the treatment. A significant decrease in tumor volumes was observed in the RC-3095-treated mice throughout the treatment. At the end of the treatment, the final tumor volume in the RC-3095 treated mice was decreased by 38% compared to the controls (P < 0.01). Administration of RC-3095 resulted in a 49.3% reduction of the growth rate of CFPAC-1 tumors in the treated mice (Table 1). Tumor volume doubling time was prolonged by RC-3095 treatment from 7.2 days in the control group to 10 days for the treated group. The tumor growth delay time in the RC-3095-treated mice was estimated to be 5.8 days. The final tumor weight in the RC-3095-treated mice was decreased by 37% compared to the controls. (P < 0.01; Table 1).

Effect of RC-3095 on DNA and Protein Contents in CFPAC-1 Tumors. The inhibitory effect of bombesin antagonist RC-3095 on the growth of CFPAC-1 tumors was further examined by the determinations of DNA and protein contents in tumor tissues. As shown in Fig. 2, administration of RC-3095 induced a significant decrease in the amount of both DNA and protein in the RC-3095-treated tumors. After 25 days of RC-3095 treatment, DNA and protein contents of tumor tissues were decreased by 44.0 and 39.9%, respectively, in the treated mice compared to the controls (P < 0.01; Fig. 2).

Effects of Bombesin and RC-3095 on the Growth of CFPAC-1 Cells in Vitro. Bombesin stimulated the growth of CFPAC-1 human pancreatic cancer cells in vitro. Fig. 3 shows the cell number after CFPAC-1 cells were cultured in the presence or absence of bombesin(1-14) for 24 h. Addition of bombesin(1-14) at the doses of 1 pm-0.1 μM to the culture induced a dose-dependent increase in the number of cultured cells. Maximal stimulation of the cell growth by bombesin was observed at a dose of 0.1 μM, where the number of cells in the culture with bombesin was 53% higher than that of the control (P < 0.01).

Bombesin antagonist RC-3095 alone did not show any significant effect on the growth of CFPAC-1 cells in the culture (Fig. 3). Addition of RC-3095 alone at the doses of 1 pm-0.1 μM to the medium only induced a slight (6.1–11.8%) but not significant decrease in the number of cells in the cultures (P > 0.05). However, RC-3095 powerfully inhibited the bombesin-stimulated growth of CFPAC-1 cells in vitro (Fig. 4). Addition of RC-3095 at a dose of 1 nm or 1 μM to the cultures reduced or nullified the stimulatory effect of bombesin(1-14) on cell growth. In the cultures with both (1 nm) RC-3095 and (1 pm-1 μM) bombesin(1-14), the number of cells was significantly lower than that with bombesin(1-14) alone (P < 0.01). This inhibitory effect of RC-3095 on the bombesin-stimulated growth of CFPAC-1 cells appeared to be dependent on the doses of the antagonist added to the cultures. When a higher dose (1 μM) of RC-3095 was added to the culture, the cell growth was totally suppressed even though these cells were cultured simultaneously with effective doses (1 pm-1 μM) of bombesin(1-14). The number of cells in the cultures in which both (1 μM) RC-3095 and (1 pm-1 μM) bombesin(1-14) were present was much lower than that with bombesin alone or that with a combination of a lower dose (1 nm) of RC-3095 with bombesin (P < 0.01).

Effects of Bombesin and RC-3095 on DNA Synthesis of CFPAC-1 Cells in Cultures. Using the [³H]thymidine incorporation assay, bombesin was shown to have mitogenic effects on the proliferation of CFPAC-1 human pancreatic cancer cells in vitro (Fig. 5). Addition of bombesin (1-100 nm) to the cultures induced a 89–131% increase in the uptake of [³H]thymidine by the cells as compared to the controls (P < 0.01). In contrast, bombesin antagonist RC-3095 inhibited both the basal and bombesin-stimulated proliferation of CFPAC-1 cells in cultures (Fig. 5). Addition of RC-3095 alone to the cultures at doses of 10–100 nm decreased the [³H]thymidine uptake by 39–40% as compared to the controls (P < 0.05). When the cells were incubated with a combination of (1 μM) RC-3095 and (1-100 nm)
which causes 50% inhibition of I-Tyr-bombesin binding to the receptors on CFPAC-1 cells. When the cells were incubated with 0.5 nM I-Tyr-bombesin in the presence of increasing doses of unlabeled bombesin(1-14), the amount of [3H]thymidine incorporated by the membranes of CFPAC-1 cells was significantly lower than that with bombesin alone (Table 2).

Table 2. Effect of bombesin antagonist RC-3095 on the growth parameters of CFPAC-1 human pancreatic cancers in nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body weight (g)</th>
<th>Final tumor weight (g)</th>
<th>Tumor growth rate (mm^2/day)</th>
<th>Tumor volume doubling time (days)</th>
<th>Tumor growth delay time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.2 ± 0.78</td>
<td>0.133 ± 0.012</td>
<td>6.9</td>
<td>7.2</td>
<td>5.8</td>
</tr>
<tr>
<td>RC-3095</td>
<td>28.35 ± 0.31</td>
<td>0.086 ± 0.009</td>
<td>3.5</td>
<td>10.0</td>
<td>5.8</td>
</tr>
</tbody>
</table>

* a Nude mice with implanted tumors (10 in each group) received injections of RC-3095 (10 μg twice a day) or vehicle (control) for 25 days.

** Versus control, Student's t test, P < 0.01.

...the tumor volume throughout the treatment. At the end of the treat-

**DISCUSSION**

The present study demonstrates that pseudonanopeptide bombesin/GRP antagonist RC-3095 can effectively inhibit the growth of CFPAC-1 human pancreatic ductal cancers xenografted to nude mice. Chronic administration of RC-3095 induced a significant decrease in the tumor volume throughout the treatment. At the end of the treat-

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Fig. 2. DNA (A) and protein (B) contents in implanted tumors of CFPAC-1 human pancreatic cancer cells in nude mice after 25 days of treatment with bombesin antagonist RC-3095.

Fig. 3. Effects of bombesin and antagonist RC-3095 on the growth of CFPAC-1 human pancreatic cancer cells in vitro. CFPAC-1 cells (1 × 10^4 cells/well) were cultured in 24-well plates in the presence or absence of (1 pM-0.1 μM) bombesin(1-14) or RC-3095. After 24 h, the cells in each well were directly counted. The cell growth is expressed as the percentage of the cell number in the cultures in the absence of bombesin or RC-3095 (control). Points, the mean ± SEM of triplicate observations from 2-3 separate experiments.

Fig. 4. Inhibitory effect of bombesin antagonist RC-3095 on bombesin-stimulated growth of CFPAC-1 human pancreatic cancer cells in vitro. CFPAC-1 cells (1 × 10^4 cells/well) were cultured in 24-well plates with a combination of (1 pM-0.1 μM) bombesin(1-14) and (1 nM or 1 μM) RC-3095. After 24 h, the cells in each well were directly counted. The cell growth is expressed as the percentage of the cell number in the cultures in the absence of bombesin or RC-3095 (control). Points, the mean ± SEM of triplicate observations from 2-3 separate experiments.
Bombesin(1-14) and RC-3095 or a combination of both. After 24 h, the cells were pulsed with bombesin (control).

In this study, the lowered tumor growth rate, and the decrease in the growth parameters, including the prolongation in tumor volume doubling time, the lowered tumor growth rate, and the decrease in the final tumor weight. Chemical analyses further revealed that treatment with RC-3095 resulted in an apparent reduction in DNA and protein contents in the CFPAC-1 tumor tissues. Our results are in agreement with the previous observations from this laboratory on the inhibitory effect of RC-3095 on tumor growth in several experimental models including nitrosamine-induced pancreatic cancers in Syrian golden hamsters (30), MXT breast cancers in mice (29), and xenografts of PC-82 human prostate cancer (27), HT-29 colon cancer (26), and MKN-45 gastric cancer in nude mice (28). These observations suggest that bombesin/GRP antagonist RC-3095 is a potent inhibitor of the growth of pancreatic and other cancers in vivo.

Although trophic effect of bombesin/GRP has been demonstrated in various normal and malignant cells, the role of this class of peptides in the regulation of the proliferation of pancreatic cancer cells has not been fully established. In vivo studies have shown that bombesin/GRP enhanced the tumor growth of azaserine-induced pancreatic acinar cells in rats (20–22) but inhibited the development of nitrosamine-induced pancreatic ductal cell type lesions in hamsters (41) and growth of SK1 human pancreatic cancers in nude mice (42). These contradictory observations were suggested to be related to the origin of tumors, i.e., acinar versus ductal cell types (22). Hajri et al. (22) reported that GRP increased the [3H]thymidine incorporation in azaserine-induced rat pancreatic acinar carcinoma cells in primary cultures, indicating a stimulatory effect of bombesin/GRP on the proliferation of pancreatic acinar cancer cells in vitro. However, bombesin was found to stimulate the mitogenesis of both acinar and ductal cells in rat pancreas (43) and growth of CAPAN human ductal pancreatic cancer in vivo (19). A possible down-regulation of EGF receptors after chronic administration of bombesin may have been also involved in these phenomena (42, 44; also see below), and it is difficult to draw unambiguous conclusions from in vivo investigations since endogenous release of various hormones and growth factors may affect tumor growth in animals. In the present study, we observed that bombesin stimulated the growth of CFPAC-1 human ductal pancreatic cancer cells in culture. Addition of bombesin(1-14) to cell cultures induced a dose-dependent increase in the number of cells. The mitogenic effect of bombesin on the proliferation of CFPAC-1 cells was also confirmed by an enhanced DNA synthesis in bombesin-treated cells. Bombesin was observed to significantly increase the uptake of [3H]thymidine into DNA by the tumor cells in the culture. These results provide strong evidence that bombesin is a mitogen for the growth of human ductal pancreatic cancer cells in vitro.

The action of bombesin/GRP to stimulate cell growth is thought to be mediated through specific membrane receptors on the target cells (17, 18, 22–25). Our recent study (4) has demonstrated that high affinity receptors for bombesin/GRP are present on the membranes of HS746T human gastric cancer cells, which are coupled with G-protein in the signal transduction pathway. Although several previous studies have

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Table 2. Displacement of [125I]-Tyro bombesin binding to CFPAC-1 human pancreatic cancer cells by bombesin, antagonist RC-3095, and unrelated peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>IC50 (nM)</th>
</tr>
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<tbody>
<tr>
<td>Bombesin(1-14)</td>
<td>9.5 x 10^{-10}</td>
</tr>
<tr>
<td>RC-3095</td>
<td>1.8 x 10^{-9}</td>
</tr>
<tr>
<td>GRHR(1-29)</td>
<td>NB</td>
</tr>
<tr>
<td>Cholecystokinin-8</td>
<td>NB</td>
</tr>
<tr>
<td>Somatostatin-14</td>
<td>NB</td>
</tr>
<tr>
<td>D-Trp6-LHRH</td>
<td>NB</td>
</tr>
</tbody>
</table>

Note: IC50 is the dose causing 50% inhibition of specific binding of [125I]-Tyro bombesin to CFPAC-1. Structurally unrelated peptides GRHR(1-29), CCK-8, SS-14 and D-Trp6-LHRH could not displace the binding of [125I]-Tyro bombesin to CFPAC-1 cells, NB, no binding.
shown that bombesin/GRP receptors are widely distributed on the membranes of many benign and malignant tumor cells including rat pancreatic acinar cancer cells (17, 18, 22-26), the presence of bombesin/GRP receptors in human pancreatic cancer cells has been reported so far only by Avis et al. (19). In our previous study, antagonist RC-3095 did not inhibit the growth of MIA PaCa-2 human pancreatic cancer xenograft in nude mice (45). It is possible that our MIA PaCa-2 line has undergone some changes in morphology and receptor content. The absence of receptors may have been a contributing factor as specific binding sites for bombesin were not found in our cultures of MIA PaCa-2 cells and neither bombesin nor bombesin antagonist RC-3095 influenced cell growth in vitro (45). In the present study, we demonstrated that specific receptors for bombesin/GRP are present on the membranes of CFPAC-1 human ductal pancreatic cancer cells. In competitive receptor binding studies, we observed that bombesin (1-14) was able to effectively displace 125I-Tyr\(^2\)-bombesin from the binding sites on CFPAC-1 cells. In contrast, structurally unrelated peptides such as somatostatin-14, cholecystokinin-8, d-Trp\(^6\)-LHRH, and GHRH(1-29) did not affect the binding of 125I-Tyr\(^2\)-bombesin to the cell membranes. Scatchard analysis indicated that two types of bombesin/GRP receptors are present on the membranes of this cancer cell line, one class having a high affinity and low capacity and the other class having a low affinity and high capacity. Characteristics of bombesin receptors on CFPAC-1 cells are similar to those on MC-26 mouse colon cancer cells, which also show two classes of binding sites for bombesin/GRP (17). These receptors are apparently different from those on Swiss 3T3 cells (24) and many other tumor cell lines (18, 22, 23, 25) in which only one class of binding sites is found. Because bombesin was observed to have direct stimulatory effects on the proliferation of CFPAC-1 cells in cultures, our receptor studies suggest that the specific binding sites for bombesin/GRP on CFPAC-1 cells may represent functional receptors which mediate the action of bombesin on the growth of these tumor cells.

RC-3095 [d-Tpi\(^6\),Leu\(^1\),Phe\(^2\)-His\(^2\)-Leu\(^3\)-bombesin(6-14)] is a potent bombesin antagonist, which was synthesized in our laboratory specially for antitumor therapy. Previous studies have shown that this synthetic peptide inhibits the binding of 125I-Tyr\(^2\)-bombesin to the membranes of Swiss 3T3 cells (24) and H-345 human small cell lung cancer cells (25) and suppresses the basal and GRP-induced amylase release from rat pancreatic acini in a superfusion assay (24). RC-3095 was proven to be a potent tumor growth inhibitor in experimental models of nitrosamine-induced pancreatic cancers in hamsters (30), MXT breast cancers in mice (29), PC-82 human prostate cancers (27), MKN 45 gastric cancers (28), and HT-29 colon cancers in nude mice (26). The direct inhibitory effect of RC-3095 was observed on the cell proliferation of mammary (16) and gastric cancer cell lines (28). The results of the present study further demonstrate that the addition of bombesin antagonist RC-3095 alone to the medium produced only a slight but not significant decrease in the number of cells in the culture. This suggests that RC-3095 itself does not affect the growth of CFPAC-1 cells in vitro, but it can effectively inhibit the bombesin-stimulated growth of CFPAC-1 cells in cultures. The presence of RC-3095 in culture medium reduced or nullified the stimulatory action of bombesin on the proliferation of CFPAC-1 cells as evidenced by a lower cell count in cultures treated with both RC-3095 and bombesin as compared to that with bombesin alone. Furthermore, RC-3095 inhibited DNA synthesis in CFPAC-1 cells during the cell proliferation. Addition of RC-3095 to the cultures reduced the bombesin-enhanced DNA synthesis in the CFPAC-1 cells as measured by \(^{3}H\)thymidine incorporation assay. The inhibitory effect of RC-3095 on bombesin-stimulated proliferation of CFPAC-1 cells was apparently dependent on the doses of RC-3095 added to the cultures. On the basis of both cell number and \(^{3}H\)thymidine incorporation assay, addition of RC-3095 at a higher dose of 1 \(\mu\)M completely suppressed the bombesin-induced growth of CFPAC-1 cells in cultures, suggesting that the action of this antagonist is competitive. This view is supported by the observations of the inhibitory effect of RC-3095 on the binding of bombesin to its receptors on CFPAC-1 cells. In displacement binding assays, we noticed that bombesin antagonist RC-3095 blocked, in a dose-dependent manner, the binding of 125I-Tyr\(^2\)-bombesin to CFPAC-1 cells. Our findings indicate that the inhibitory effect of RC-3095 on bombesin-induced growth of CFPAC-1 cells is probably due to competitive occupation of the binding sites for bombesin/GRP on the cells.

However, the suppressive effect of RC-3095 on the growth of CFPAC-1 tumors in nude mice cannot be solely explained by the competitive action of this antagonist to displace the binding of bombesin/GRP to the receptors on the tumor cells. Several other regulatory mechanisms, such as a major down-regulation of EGF receptors, may also mediate the action of RC-3095 (26, 30, 44). It has been found that in vitro treatment with RC-3095 induced a down-regulation of receptors for both bombesin/GRP and EGF on the membranes of MKN45 gastric cancers in nude mice (28) and greatly reduced concentration of EGF receptors in pancreatic, mammary, colorectal, and prostatic cancers (27, 29, 30, 44). RC-3095 may also affect EGF binding through an action on the protein kinase C system (46) and inhibition of up-regulation of EGF receptors produced by bombesin/GRP (47). Whether the effect of RC-3095 on the growth of CFPAC-1 tumors is related to the down-regulation of bombesin/GRP and EGF receptors on the membranes of CFPAC-1 cancer cell line or the interference with the function of EGF receptors (46, 47) needs to be elucidated. In addition, since bombesin/GRP can powerfully stimulate the release of gastrointestinal hormones that may have trophic effects on the growth of pancreas and pancreatic cancers (5-12), the possible involvement of RC-3095 not only in the inhibition of action of bombesin/GRP but in the suppression of the release of these trophic hormones should be also considered. It is possible that the inhibitory action of RC-3095 on tumor growth of CFPAC-1 cells in vivo is mediated through several mechanisms.

In summary, our study shows that bombesin/GRP antagonist RC-3095 can powerfully suppress the growth of CFPAC-1 human ductal pancreatic cancer cells xenografted into nude mice as well as cultured in vitro. The effect of RC-3095 appears to be mediated by the interference with the binding of bombesin to its receptors on this cancer cell line. These findings suggest the merit of continued evaluation of bombesin/GRP antagonist RC-3095 for the possible development of new approaches for the treatment of pancreatic cancers.

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REFERENCES


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Yunfeng Qin, Tibor Ertl, Ren-Zhi Cai, et al.


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