Inhibitory Effect of Bombesin/Gastrin-releasing Peptide Antagonist RC-3095 and High Dose of Somatostatin Analogue RC-160 on Nitrosamine-induced Pancreatic Cancers in Hamsters

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ABSTRACT

Female Syrian golden hamsters with N-nitrosobis(2-oxopropyl)amine-induced pancreatic cancers were treated for 2 months with new pseudonanpeptide bombesin receptor antagonist [d-Tp6,Leu13(CH2NH)-Leu14]bombesin(6-14)(RC-3095), administered s.c. with implanted osmotic minipumps releasing 20 µg/day of the analogue. The results were compared to those obtained by treatment with somatostatin analogue RC-160 (35 µg/day and 150 µg/day) or [d-Tp6]luteinizing hormone-releasing hormone (25 µg/day), which inhibited the growth of pancreatic cancers in our previous studies. A new acetylated somatostatin analogue Ac-d-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH2 (RC-160-II) (30 µg/day) also was used for comparison of therapeutic response. All peptide analogues induced tumor inhibition by at least one of the measured parameters. Bombesin antagonist RC-3095 and high dose of RC-160 (150 µg/day) had the greatest inhibitory effect on pancreatic cancers: A significant decrease in the number of animals with tumors, reduced pancreatic weight, 87-89% inhibition of tumors pancreas weight, and a significant diminution in the number of tumor nodules and argyrophilic nucleolar organizer region count in tumor cell nuclei were observed in the groups treated with these regimens. We were able to detect receptors for bombesin in membranes of N-nitrosobis(2-oxopropyl)amine-induced pancreatic tumors and these receptors were not down-regulated after treatment with the bombesin antagonist. In hamsters treated with bombesin antagonists, tumor inhibition might be explained by a significant decrease in the binding capacity of epidermal growth factor receptors in pancreatic cancers. The acetylated somatostatin analogue RC-160-II had a similar inhibitory effect on the tumors as the original analogue RC-160. Our results suggest that the increase in the dose of RC-160 improves the therapeutic response, and this finding should be taken into account in clinical use of this somatostatin analogue. In view of its strong inhibitory effect on experimental pancreatic tumors, the bombesin antagonist RC-3095 might be considered as a possible new agent for the therapy of human exocrine pancreatic cancer.

INTRODUCTION

Bombesin and its mammalian counterpart gastrin releasing peptide, exert stimulatory effects on several mammalian tissues (1). Bombesin, GRP, and related peptides can influence the release of gastrointestinal hormones, stimulate gastric and pancreatic secretions, and gastrointestinal motility (1-3). Bombesin-like immunoactivity has been demonstrated in the brain, gastrointestinal tract, lungs, and mammary glands of various animals (1-3). Bombesin-binding sites have been found on the endocrine cells of antral mucosa, gastric fundus, the myenteric plexus, and pancreatic acinar cells (3, 4). Human small cell lung cancer cells possess significant endocrine paracrine activity and among the hormones produced, bombesin/GRP can act as autocrine growth factors in these tumors (5). A possible stimulatory role of bombesin/GRP on exocrine pancreatic cancer cells (6) also has been demonstrated.

As radiation and chemotherapy are of limited effectiveness in the treatment of human pancreatic cancer, other therapeutic approaches have to be explored. Various studies indicate that exocrine pancreatic cancer may be sensitive to sex steroids, gastrointestinal hormones, and growth factors (7-10). Experimental (11-15) and some clinical (16, 17) investigations have shown the beneficial effect of treatment with LH-RH and somatostatin analogues on pancreatic tumors. Gastrointestinal hormones such as gastrin, cholecystokinin, and secretin are involved not only in the regulation of pancreatic functions, but also can enhance the initiation and progression of pancreatic tumors in experimental animals (6, 18-20). The inhibitory effect of somatostatin analogues on pancreatic cancer may be mediated, at least partly, by suppression of gastrointestinal hormones (7, 10).

In view of a possible involvement of bombesin/GRP in pancreatic cancer (6, 9, 10) we decided to study the effect of a pseudonanpeptide bombesin/GRP receptor antagonist [d-Tp6,Leu13(CH2NH)-Leu14]bombesin(6-14)(RC-3095) on BOP-induced pancreatic cancers in hamsters. We have previously shown that this analogue in nanomolar concentrations is able to inhibit GRP-stimulated amylase release from dispersed rat pancreatic acini superfused in vitro (21, 22). RC-3095 also suppressed gastrin secretion in vivo and showed specific binding to human colon cancer cells (23). The effects of treatment with bombesin analogue were compared to those of [d-Tp6]LH-RH and somatostatin analogue RC-160. Both of these analogues inhibited the growth of pancreatic tumors in our previous studies. As raising the dose of RC-160 resulted in an improvement in the responses in our recent work (13), we increased the dose of RC-160 further to 150 µg/day in one animal group. Another group of animals was treated with a new acetylated somatostatin analogue, Ac-d-Phe1-RC-160 (RC-160-II), using a dose similar to that of the original RC-160 analogue, and the results were compared.

MATERIALS AND METHODS

Animals

Ninety female Syrian golden hamsters (CH:RGH) weighing 97.7 ± 5.3 g were obtained from the National Cancer Institute Frederick Cancer Research Facility (Frederick, MD). They were housed 2/cage at the Animal Research Facility of our institute in an air-conditioned room at 22 ± 1°C and 55 ± 5% humidity. The animals were kept under an automatic 12-h light, 12-h darkness schedule and were given Rodent Laboratory Chow 50001 (Purina Mills, Inc., St. Louis, MO) and tap water ad libitum.

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2 To whom requests for reprints should be addressed, at Veterans Affairs Medical Center, 1601 Perdido Street, New Orleans, LA 70146.

The abbreviations used are: GRP, gastrin-releasing peptide; BN, bombesin; LH-RH, luteinizing hormone-releasing hormone; BOP, N-nitrosobis(2-oxopropyl)amine; Tpi, 2,3,4,9-tetrahydro-1H-pyrrole[3,4-d]indol-3-carboxylic acid; HPLC, high-performance liquid chromatography; EGF, epidermal growth factor; AgNOR, argyrophilic nucleolar organizer region; [d-Tp6]LH-RH, d-tryptophan-6-luteinizing hormone-releasing hormone; CCK, cholecystokinin.

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The method of Pour et al. (24) was used with some modification (12). BOP was purchased from American Tokyo Kasei Inc. (Portland, OR), and Lot CRR890016-2 was used and stored at 4°C. The required amount of BOP was freshly dissolved in 0.9% NaCl solution and given s.c. at a dose of 10 mg/kg of body weight once weekly for 6 weeks. Eighteen weeks later (24 weeks from the start of the experiment), the surviving 70 hamsters were randomly divided into 6 groups.

Pepidies and Sustained Delivery Systems

Bombesin receptor antagonist RC-3095 [p-Tpi-Gln-Trp-Ala-Val-Gly-His-Leu(μ(CH2)NH)-Leu-NH2] was synthesized in our laboratory by solid-phase methods as described previously (22). The crude preparation was purified by HPLC. Batch BSS 1-89 was used for the treatment of animals. Aliquots of this RC-3095 solution in 50% propylene glycol were stored for 1 month at —70°C, at room temperature, and at 37°C. Samples of this solution were stored frozen in aliquots at —70°C. The LH-RH agonist [d-Trp3]LH-RH (pyro-Glu-His-Trp-Ser-Tyr-d-Trp-Leu-Arg-Pro-Gly-NH2) was synthesized by classical synthesis and was supplied by Debiopharm S.A. (Lausanne, Switzerland). Microcapsule formulation (8) of this peptide in biodegradable poly(DL-lactide-co-glycolide) was prepared by Dr. P. Orsolini at Cytotech S.A. (Martigny, Switzerland), using a phase-separation process. This sustained release formulation of (Lot G 476) an aliquot of 25 mg maintained a continuous liberation of 25 µg/day of the analog for 30 days.

Somatostatin analogue RC-160 (p-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH2) originally synthesized by solid-phase methods and evaluated in our laboratory, was made by classical synthesis by Novabiochem, Laufelfingen, Switzerland). Lot A 0569 was used for treatment with the higher dose of RC-160. Microgranules of RC-160 pamatoe in poly(DL-lactide-co-glycolide) were prepared by Cytotech S.A. Batch RGR 75-90-01 was designed to release about 25 µg/day of RC-160 for 2 weeks from an aliquot of 12 mg microgranules. RC-160-II (Ac-d-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH2), originally synthesized in our laboratory, was made by Novabiochem (Batch A06900) and was used in our experiments.

Pepidies without sustained delivery systems were administered by osmotic pumps (Alza Corp., Palo Alto, CA). Model 2ML4, releasing 2.5 µl/h for 4 weeks was implanted s.c. The filling of the pumps and the implantation were carried out according to manufacturer's recommendations. The peptides were dissolved in 50% propylene glycol in water. The hamsters were anesthetized with Metofane (Pitman-Moore, Washington Crossing, NJ) and the pumps were implanted s.c. in the dorsal area, using aseptic techniques. Control hamsters were implanted in our laboratory, was made by Novabiochem (Batch A06900) and was used in our experiments.

For treatment of groups 1, 2, 3, and 6, osmotic pumps were used that were implanted on day 1 and changed on day 29. The experiment was terminated 8 weeks after the first treatment with peptides, i.e., on day 57.

Autopsy and Histological Procedures

During the 8-week treatment period, pancreata and livers of animals that died were processed for histology. At the end of the eighth week, the hamsters were exsanguinated under Metofane anesthesia. Body weights were measured and any ascites was removed and measured. The average weight of all pancrea as well as the average weight of the tumorous pancrea (based on histology) in each group was recorded. Pancreas, liver, spleen, kidneys, uterus, and ovaries were removed, cleaned, and weighed. Pancreatic and liver tissue were fixed in buffered neutral formalin, specimens were embedded in Paraplast (Oxford Labware, St. Louis, MO), and step sections 6-µm thick were cut. Sections were stained with hematoxylin-eosin and Van Gieson's stains. For measurement of the apoptotic index, i.e., the percentage of tumorous glandular structures containing apoptotic cells, 200 glands were considered in each tumor. Similarly, the frequency of mitoses was characterized by the percentage of tumorous glandular structures containing mitotic cells. For the determination of connective tissue content of tumors, the point-counting method based on Chalkley's principles was used (25), and the crossing points of an ocular net that hid or coincided with connective tissue in sections were counted. The ratio of these points to the number of all points represented the ratio of the volume of connective tissue to the volume of the tumor tissue. For demonstration of the necrotic signal organizer region in tumor cell nuclei, the AgNOR number per cell was calculated. All counts were carried out without knowledge of treatment.

Receptor Assays

Radioiodination of Peptides. [Tyr4]Bombesin, synthesized in our laboratory, was iodinated by the standard chloramine-T method (28): 2 µg [Tyr4]BN in 40 µl 0.5 M phosphate buffer was mixed with 1 mCi Na125I (Amersham, Arlington, IL) and 1 µl chloramine-T. After a 60-s incubation at room temperature, the reaction was stopped with 2.1 µg sodium metabisulfite and diluted twice with 500 µl 0.05 M phosphate buffer containing 10% acetonitrile. The labeled hormone was purified by HPLC. A mixture of 0.1% trifluoroacetic acid in water (solvent A), and 0.1% trifluoroacetic acid in 70% aqueous acetonitrile (solvent B) was used at a flow rate of 1.2 ml/min in a linear mode (20-100% solvent B). One-half-min fractions (0.6 ml) were collected. The radioactivity in the effluent was monitored by a radiation detector (OCDM CDV-700) and plotted with a two-channel plotter (LKB). The method was able to separate reliably the mono- and diiodinated peptides. For the receptor assay, the monoiodinated compound was used which eluted at 64% of solvent B. After evaporating the acetonitrile, dithiothreitol was added and the sample was neutralized with 0.5 M phosphate buffer. The specific activity of the labeled [Tyr4]BN was about 1600 Ci/mmol as calculated by self-displacement method. The labeled compound was stored frozen in aliquots at —70°C. Iodinated EGF was purchased from Amersham (750—900 Ci/mmol).

Preparation of Membranes and Receptor Binding of Peptides. Specimens of BOP-induced pancreatic cancer from animals in control group and in the group treated with RC-3095 were used for receptor-binding experiments. Tumors from each group were pooled, cleaned of fat and connective tissues, weighed, cut into small slices, and homogenized in 0.1 M sodium metabisulfite and diluted twice with 500 µl 0.05 M phosphate buffer containing 10% acetonitrile. The homogenate was centrifuged at 500 x g for 10 min at 4°C. The supernatant containing the crude membrane fractions was then centrifuged at 64% of solvent B. After evaporating the acetonitrile, dithiothreitol was added and the sample was neutralized with 0.5 M phosphate buffer. The specific activity of the labeled [Tyr4]BN was about 1600 Ci/mmol as calculated by self-displacement method. The labeled compound was stored frozen in aliquots at —70°C. Iodinated EGF was purchased from Amersham (750—900 Ci/mmol).

Preparation of Membranes and Receptor Binding of Peptides. Specimens of BOP-induced pancreatic cancer from animals in control group and in the group treated with RC-3095 were used for receptor-binding experiments. Tumors from each group were pooled, cleaned of fat and connective tissues, weighed, cut into small slices, and homogenized in 5 volumes of buffer by using an Ultra-Turrax homogenizer (Tissumizer, Tekmar, Cincinnati, OH) at a maximal speed (five 5-s strokes) on ice. Homogenization buffer was made up of 0.3 M sucrose, 25 mM Tris base, 5 mM MgCl2, 0.25 mM phenylmethysulfonyl fluoride, 1 mM ethyleneglycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 10 mM monoothioglycerol, and Trasylol (aprotinin) at 10,000 kallikrein inactivator units/liter (pH 7.5). The homogenate was centrifuged at 500 x g for 10 min at 4°C. The supernatant containing the crude membrane fractions was then centrifuged at 70,000 x g for 45 min at 4°C by using a Beckman Preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The resulting pellet was washed twice by resuspending in wash buffer and spinning. Protein concentration was determined by the Bio-Rad protein assay kit according to the method of Bradford (29).

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The binding assay of [Tyr⁴]BN was performed in 12 x 75-mm polypropylene conical culture tubes, in a total volume of 150 μl. The incubation buffer consisted of phosphate-buffered saline (mm concentrations: NaCl, 138; KCl, 12.8; Na₂HPO₄, 8; KH₂PO₄, 1.45; CaCl₂, 0.91; MgCl₂, 0.49) containing 0.1% bovine serum albumin, 5 mm MgCl₂, 20 mm 4-(2-hydroxyethyl)-piperazinethanesulfonic acid, and 100 μg/ml bacitracin (pH 7.4). Labeled [Tyr⁴]BN (0.05 nm) was incubated with 25 μg/tube of the membrane protein in the presence or absence of different concentrations of competitor, unlabeled [Tyr⁴]BN or RC-3095 (4 x 10⁻⁹–4 x 10⁻⁶ M). Triplicate tubes were incubated for 30 min at 25°C, as this was found to give optimal binding. The reaction was terminated by rapid filtration through glass fiber filters (1.0-μm pore size) prewashed in assay buffer and presoaked for at least 3 h in 0.5% ice-cold polyethylenamine solution to minimize filter absorption. A semiautomatic cell-harvesting system (Cambridge Technology, Inc., Watertown, MA) with 10-s washing time was used. The washing buffer consisted of ice-cold phosphate-buffered saline containing 0.1% of bovine serum albumin. Radioactivity of the filters was counted.

The Ligand-PC computerized curve fitting program of Munson and Rodbard was used to determine the types of receptor binding, dissociation constant (Kᵦ), and the maximal binding capacity of receptors (Bₘₐₓ) (30).

Membrane preparation and receptor binding studies for EGF were performed as previously described (31).

Serum Gastrin Levels. Serum gastrin levels were determined by specific radioimmunoassay by using Gastrin¹²⁵I RIA kit from Becton Dickinson (Orangeburg, NY).

Statistical Analyses. Statistical evaluation of data was performed by Duncan’s multiple range test and Student’s t test by using a computer program.

RESULTS

Body, Liver, Kidney, Ovarian, and Uterine Weights. The average body weight of the hamsters was 97.7 g at the beginning of the BOP administration and 153.5 g at the initiation of treatments. No significant differences were found in body weights among groups at the end of the experiment. Ovarian and uterine weights were reduced significantly in the group treated with [D-Trp⁶]LH-RH. The liver and kidney weights were not changed by the various treatments. The weights are shown in Table 1. Fibrosis, proliferation, and sometimes cystic dilatation of bile ducts were found histologically in the liver of all animals due to BOP treatment.

Tumor Pathology. The number of animals that died during the treatment period was low even in the control group. All of the control hamsters had histologically verified pancreatic cancers. The number of animals with histological carcinoma was decreased in all treated groups. This reduction was statistically significant (P < 0.05) in the groups treated with bombesin analogue or the high dose, 150 μg/day, of RC-160. Two hamsters in the control groups and one in each treated group had invasive cancer; that is, the tumor infiltrated adjacent organs or tissues (stomach, liver, intestines, retroperitoneum). The average weight of the pancreata was decreased in all treated groups (Table 2). The greatest reduction (88–90%) was observed in the groups treated with bombesin analogue or with 150 μg/day of RC-160 (P < 0.05).

The diameters of the individual tumor nodules in pancreata varied from 2 to 30 mm. Because of the multinodularity and the confluence of nodules, the weight of the tumorous pancreas was recorded, on the basis of histological examination, when pancreatic cancers were detected histologically. The average weights of the tumorous pancrea are shown in Table 2. The average weight of the tumorous pancreata (including both tumor weight and the weight of the rest of the pancreas) was 4.32 g in the control group. Treatment with bombesin analogue resulted in an 87% inhibition of tumorous pancreas weight, while RC-160 in a dose of 150 μg/day caused about 89% inhibition, and treatment with [D-Trp⁶]LH-RH caused about 77% regression. RC-160 and RC-160-II, administered at an equivalent dose, caused a similar reduction, about 72–73%, of the tumorous pancreas weight.

Histologically, the pancreatic tumors were adenocarcinomas (Fig. 1) with infiltrative small or larger ductules, solid cell nests, or with cystic and papillary structures in some areas, as described previously (12). Preblastomatous lesions, similar to those described earlier (32) were found in all pancreata. For the

Table 1 Effect of treatment with bombesin/GRF antagonist RC-3095, somatostatin analogue RC-160, or [D-Trp⁶]LH-RH on body and organ weights and serum gastrin levels of hamsters with BOP-induced pancreatic cancer

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
<th>Kidney wt (g)</th>
<th>Ovarian wt (mg)</th>
<th>Uterine wt (mg)</th>
<th>Serum gastrin (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>162.6 ± 8.8*</td>
<td>7.98 ± 0.6</td>
<td>1.37 ± 0.1</td>
<td>39.64 ± 4.4</td>
<td>43.74 ± 5.8</td>
<td>254.6 ± 39.0</td>
</tr>
<tr>
<td>RC-3095</td>
<td>152.3 ± 11.7</td>
<td>7.01 ± 0.4</td>
<td>1.49 ± 0.2</td>
<td>44.00 ± 4.3</td>
<td>43.60 ± 53.9</td>
<td>254.6 ± 78.1</td>
</tr>
<tr>
<td>RC-160, 150 μg/day</td>
<td>139.8 ± 7.3</td>
<td>8.06 ± 0.7</td>
<td>1.59 ± 0.2</td>
<td>44.26 ± 4.5</td>
<td>321.55 ± 49.4</td>
<td>240.8 ± 29.2</td>
</tr>
<tr>
<td>RC-160, 30 μg/day</td>
<td>137.3 ± 6.5</td>
<td>7.38 ± 0.4</td>
<td>1.37 ± 0.1</td>
<td>39.25 ± 4.0</td>
<td>358.75 ± 30.5</td>
<td>225.0 ± 26.8</td>
</tr>
<tr>
<td>[D-Trp⁶]LH-RH</td>
<td>146.4 ± 7.5</td>
<td>7.02 ± 0.4</td>
<td>1.31 ± 0.0</td>
<td>26.08 ± 1.8</td>
<td>235.17 ± 19.5</td>
<td>189.4 ± 26.1</td>
</tr>
<tr>
<td>RC-160-II, 30 μg/day</td>
<td>137.0 ± 7.7</td>
<td>7.76 ± 0.7</td>
<td>1.44 ± 0.0</td>
<td>46.09 ± 2.9</td>
<td>428.18 ± 56.6</td>
<td>333.2 ± 36.7</td>
</tr>
</tbody>
</table>

* Mean ± SE.  
* P < 0.01.  
* P < 0.05.

Table 2 Effect of treatment with bombesin antagonist RC-3095, somatostatin analogue RC-160, or [D-Trp⁶]LH-RH on survival of animals, the frequency of pancreatic cancers, pancreatic weight, and tumorous pancreatic weight of hamsters with BOP-induced pancreatic cancers

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals which died during treatment/total no. of animals</th>
<th>No. of animals with histological carcinoma/total no. of animals</th>
<th>Pancreas wt (g)</th>
<th>Tumorous pancreas wt (g)</th>
<th>% of inhibition of tumorous pancreas wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2/12</td>
<td>12/12</td>
<td>4.32 ± 2.75*</td>
<td>4.32 ± 2.75</td>
<td>0</td>
</tr>
<tr>
<td>RC-3095</td>
<td>0/10</td>
<td>6/10*</td>
<td>0.52 ± 0.08*</td>
<td>0.56 ± 0.12</td>
<td>86.9</td>
</tr>
<tr>
<td>RC-160, 150 μg/day</td>
<td>1/12</td>
<td>7/12</td>
<td>0.46 ± 0.04*</td>
<td>0.49 ± 0.05</td>
<td>88.6</td>
</tr>
<tr>
<td>RC-160, 30 μg/day</td>
<td>1/12</td>
<td>10/12</td>
<td>1.05 ± 0.30</td>
<td>1.18 ± 0.35</td>
<td>72.7</td>
</tr>
<tr>
<td>[D-Trp⁶]LH-RH</td>
<td>1/12</td>
<td>9/12</td>
<td>0.87 ± 0.12</td>
<td>1.01 ± 0.12</td>
<td>76.6</td>
</tr>
<tr>
<td>RC-160-II, 30 μg/day</td>
<td>3/12</td>
<td>9/12</td>
<td>1.03 ± 0.23</td>
<td>1.20 ± 0.28</td>
<td>72.1</td>
</tr>
</tbody>
</table>

* Mean ± SE.  
* P < 0.05.
determination of the number of tumor nodules, the slides containing the largest tumorous field from step sections were used. In these slides the tumor nodules were counted. The number of tumor nodules per hamster, when all animals in a group are considered, except for those with huge infiltrative tumors where tumor nodules could not be counted, are shown in Table 3. The total number of tumor nodules was 24 in the control group, 7 in the group treated with bombesin analogue, 17 in the group treated with [D-Trp6]LH-RH, 19 in the group treated with a low dose of RC-160, 25 in hamsters receiving [D-Trp6]LH-RH, 17 in the group treated with a low dose of RC-160, and 15 after treatment with RC-160-II.

The amount of stroma, the percentage of tumorous glandular structures containing mitotic cell or apoptotic alterations (apoptotic index) and the AgNOR count in tumor cell nuclei were examined histologically in the tumors, and data are shown in Table 3. Some increase in the amount of stroma was observed in all treated tumors; this increase was statistically significant only after treatment with [D-Trp6]LH-RH. Figs. 2 and 3 show atrophic changes in tumorous glands and marked fibrosis in a pancreatic tumor after treatment with 150 μg/day of RC-160 or 20 μg/day of bombesin/GRP antagonist RC-3095. The percentage of tumorous glands containing mitotic cells was 12.8 in the control group. A slight nonsignificant decrease in mitoses was observed in all untreated tumors. The smallest decrease occurred in the group that received [D-Trp6]LH-RH. Apoptotic alterations including the shrinkage of isolated tumor cells, karyopyknosis, karyorrhexis, desquamated cell fragments in glandular lumina, or phagocytosed apoptotic bodies were more frequently found in treated tumors (Fig. 4). The apoptotic index was 18 in the control group and a significant increase was observed after treatment with [D-Trp6]LH-RH or a low dose of RC-160 (Table 3). The average number of AgNOR granules in tumor cell nuclei was 10.2 in the control group. This number was reduced in all treated groups, the greatest decrease occurring after treatment with the bombesin antagonist or high dose of RC-160.

Serum Gastrin Levels. Serum gastrin levels were not changed in groups given the bombesin antagonist, high and low dose of RC-160, [D-Trp6]LH-RH, RC-160-II as compared to control (Table 1).

Receptor Assays. The results of receptor assays are shown in Table 4. Specific binding sites for 125I-[Tyr4]bombesin were demonstrated in membranes of BOP-induced pancreatic cancer. Bombesin binding sites on tumor plasma membranes from animals treated with bombesin antagonist RC-3095 were present in the same numbers but had slightly lower binding affinity. RC-3095 was able to displace one-half of the specific 125I-[Tyr4]bombesin binding to membranes of BOP-induced pancreatic cancer with a 50% inhibitory concentration of 0.7 nm. EGF-binding sites were also present in the membranes of BOP-induced pancreatic tumors. A highly significant reduction in EGF-binding capacity and increase in affinity after treatment with bombesin antagonist RC-3095 could be observed. A decrease in EGF-binding capacity also occurred after treatment with high dose of RC-160, but it was smaller than that induced by RC-3095.

DISCUSSION

Bombesin and related peptides were found to stimulate pancreatic enzyme secretion by releasing cholecystokinin (33) or by direct excitation of acinar cells (34). Bombesin can stimulate insulin secretion of hamster pancreatic islet cells by binding to cell surface receptors, followed by internalization (35). Jensen et al. (4) studied the interaction of bombesin with specific receptors in guinea pig pancreatic acinar cells. Gastrointestinal hormones such as gastrin, CCK, secretin, neurotensin, bombesin/GRP can promote not only pancreatic functions, but also pancreatic growth (7, 36–38). Several groups investigated the effect of these peptides on pancreatic carcinogenesis, but some of the results are contradictory (18–20, 39, 40). Caerulein, CCK, and secretin were reported to enhance the carcinogenic effect of nitrosamines on pancreas (18, 19), but the inhibition of BOP cancers by exogenous CCK administration was found by others (39). Bombesin promoted azaserine-induced pancreatic cancer in rats (20) but was stated to inhibit the growth of human pancreatic cancer xenografts in nude mice (40). A growth stimulating effect of GRP and specific receptors for GRP were demonstrated in CAPAN human pancreatic cancer cell line (6). Bombesin receptors were also demonstrated in rat pancreatic acinar cancer cells AR 42J (41).

Table 3 Effect of treatment with bombesin/GRP antagonist RC-3095, somatostatin analogue RC-160, or [D-Trp6]LH-RH on histological parameters of BOP-induced pancreatic cancers in hamsters

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of tumor nodules in slides/animal</th>
<th>Stroma area (%)</th>
<th>No. of mitoses/100 glands</th>
<th>Apoptotic index</th>
<th>No. of AgNORs/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.40 ± 0.7(^*)</td>
<td>20.20 ± 2.8</td>
<td>12.8 ± 2.1</td>
<td>18.0 ± 2.4</td>
<td>10.23 ± 0.5</td>
</tr>
<tr>
<td>RC-3095</td>
<td>0.78 ± 0.3(^*)</td>
<td>30.17 ± 5.4</td>
<td>7.2 ± 3.2</td>
<td>28.0 ± 9.9</td>
<td>7.54 ± 0.4(^*)</td>
</tr>
<tr>
<td>RC-160, 150 μg/day</td>
<td>0.67 ± 0.19</td>
<td>32.14 ± 4.5</td>
<td>7.7 ± 2.5</td>
<td>22.7 ± 2.3</td>
<td>7.97 ± 0.2(^*)</td>
</tr>
<tr>
<td>RC-160, 30 μg/day</td>
<td>1.37 ± 0.31</td>
<td>28.90 ± 3.4</td>
<td>8.2 ± 2.2</td>
<td>33.6 ± 4.6(^*)</td>
<td>8.35 ± 0.2(^*)</td>
</tr>
<tr>
<td>[D-Trp6]LH-RH</td>
<td>2.09 ± 0.47</td>
<td>36.33 ± 4.1(^*)</td>
<td>11.6 ± 3.6</td>
<td>32.8 ± 3.7(^*)</td>
<td>8.03 ± 0.2(^*)</td>
</tr>
<tr>
<td>RC-160-II, 30 μg/day</td>
<td>1.37 ± 0.34</td>
<td>31.88 ± 3.4</td>
<td>7.6 ± 2.3</td>
<td>25.9 ± 2.9</td>
<td>8.29 ± 0.4(^*)</td>
</tr>
</tbody>
</table>

\(^*\) Mean ± SE.

\(^*\) \(P < 0.05\).

\(^*\) \(P < 0.01\).
RC-3095 AND RC-160 EFFECTS ON HAMSTER PANCREATIC CARCINOMAS

Fig. 2. Atrophic changes in tumorous glands and marked fibrosis in a pancreatic tumor after treatment with 150 ng/day of RC-160. H & E, × 100.

Fig. 3. Atrophic-fibrotic area in a pancreatic cancer of a hamster treated with 20 μg/day of bombesin/GRP antagonist RC-3095. H & E, × 100.

Because of the wide spectrum of pathophysiological activities of bombesin related peptides, several series of analogues have been developed. Lately, the synthesis of short chain bombesin antagonists with a reduced peptide bond between positions 13 and 14 was reported (42) and some of them were 100 times more potent than the previous antagonists. The new antagonists showed high binding affinities for pancreatic acinar cells and displayed strong antimitotic activity (42).

Recently, several pseudononapeptide (ψ13-14) bombesin(6-14) antagonists with different modifications at positions 6, 7, 8, and 14 have been synthesized in our laboratory (21–23). The substitutions such as the replacement of the D-Phe at position 6 by D-Trp analogue D-Tpi (2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid) resulted in very potent antagonists, including D-Tpi6,Leu13,ψCH2NHLeu14-bombesin(6-14)(RC-3095). These antagonists inhibited the binding of iodinated Tyr6-bombesin to Swiss 3T3 and SCLC H-345 cells and suppressed the response to GRP(14–27) as based on incorporation of [3H]thymidine into DNA of these cells. Nanomolar concentrations of antagonist RC-3095 inhibited GRP(14-27)-stimulated amylase release from rat pancreatic acini in a superfusion assay. Antagonist RC-3095 is active in vivo in rats, dogs, and cats in abolishing gastric and pancreatic responses (21). Our recent results demonstrated high activity of antagonist RC-3095, administered by different in vivo routes, in suppressing serum gastrin levels in rats. The effects of this bombesin/GRP antagonist on BOP-induced pancreatic cancers in hamsters were evaluated in our present study. This tumor model shows remarkable similarities to human pancreatic cancer in morphological, biological characteristics (43), in the receptors for hormones and growth factors (14, 31), and in blood group- or tumor-associated antigens (44, 45) expressed by the tumor cells. BOP-induced pancreatic carcinoma is of ductal origin, as is the majority of human pancreatic cancers (43). Gastrointestinal hormones have a physiological secretagogue effect on pancreatic acinar cells (22, 34, 36–38), but also can influence the growth of normal neoplastic cells of both acinar (20, 41) or ductal origin (6, 18, 19, 40).

Treatment with LH-RH and somatostatin analogs resulted in significant tumor regression in our previous experiments on BOP-induced pancreatic cancers in hamsters (11–15). Our present study confirms these results. A somewhat lower number of tumorous animals, fewer tumor nodules as seen histologically, a greatly reduced pancreatic and tumorous pancreas weight, increased amount of stroma, enhanced apoptosis, and decreased AgNOR count in tumors were observed after treatment with [D-Trp6]LH-RH or 35 μg/day of somatostatin analogue RC-160, as compared to controls. These results were contrasted with those produced by the treatment with our new compounds, bombesin antagonist RC-3095, acetylated analogue RC-160-II, as well as with a higher dose of RC-160.

The treatment was well tolerated by the animals in all groups. No changes were observed in body, liver, or kidney weights of hamsters among groups, not even in those receiving bombesin analogue or a high dose of RC-160. The reduced ovarian and uterine weights in groups treated with [D-Trp6]LH-RH indicate the inhibition of the pituitary-gonadal axis in these animals.

We have studied several parameters of the pancreatic tumors to determine the efficacy and compare the results of the treatments. RC-160-II is a new analogue that was used for the first
time for in vivo treatment of BOP-induced tumors, although we have recently shown its significant binding to pancreatic cancers. The treatment with this acetylated somatostatin analogue RC-160-II resulted in similar inhibition of tumors as that observed after therapy with the equivalent dose of original analogue RC-160 or with [d-Trp^6]LH-RH. The number of tumorous animals, the average weight of pancreata or tumorous pancrea, the number of tumor nodules determined histologically, the number of AgNORs in tumor cell nuclei showed about the same reduction in these three groups. This means that the acetylation of the octapeptide analogue of somatostatin did not influence its inhibitory effect of BOF-induced pancreatic cancers.

As we correctly assumed, the increase in the dose of RC-160 augmented its inhibitory effect on pancreatic tumors. The use of 150 μg RC-160/day for treatment of hamsters resulted in the lowest number of animals with tumors, the lowest pancreatic and tumorous pancreas weights, the smallest number of tumor nodules among groups. The groups treated with the bombesin antagonist RC-3095 also showed inhibitory changes in the same parameters, similar to those found in the group treated with a high dose of RC-160. Moreover, the group receiving the bombesin antagonist revealed the lowest number of mitoses and smallest AgNOR count in our experiment. In summary, it can be stated that both bombesin antagonist RC-3095 and high dose of RC-160 had the most potent inhibitory effect on BOP-induced pancreatic cancers in hamsters.

No significant changes in serum gastrin were observed when levels were measured after termination of various treatments, although somatostatin analogues and bombesin/GRP antagonist can affect gastrin under certain experimental conditions. It is possible that gastrin secretion was suppressed during the continuous administration of somatostatin analogue RC-160 or a very low dose such as 20 μg/day of bombesin antagonist. Since the treatment was extended to maximum time, in view of the great expense of minipumps and microcapsules, the peptide load (content) of the microcapsules and the minipumps might have been completely exhausted when the experiment was terminated. Although serum gastrin levels were not decreased at the end of treatment in animals that received bombesin antagonist, an effect of RC-160-II on paracrine or autocrine gastrin production is also possible.

Among many possible mechanisms for tumor inhibition by agents that do not have direct cytotoxic effect, a decreased production of endocrine, paracrine, or autocrine growth factors, or the diminished responsiveness of tumor cells to these substances could play predominant roles. Insulin-like growth factor I levels in blood of hamsters treated with RC-160 were significantly reduced in our previous studies (15). The sensitivity of tumor cells for various growth factors could be decreased by down-regulation of specific receptors. EGF is involved in pancreatic cancer (7) and somatostatin and its analogue RC-160 promote the dephosphorylation of EGF receptor (46–48). The presence of bombesin receptors on BOP-induced pancreatic cancer cells shows that bombesin/GRP or related peptides may play a role in development and progression of this tumor. No down-regulation of bombesin/GRP receptors was found after treatment with bombesin antagonist in our experiment, but we had only two tumors large enough for receptor assay in the groups treated with bombesin analogue or high dose of RC-160. However, a significant down-regulation of EGF receptors found in pancreatic tumors after treatment with RC-3095 might explain at least in part the tumor growth inhibition by bombesin antagonist. In our study, high dose of RC-160 (150 μg/day) also significantly lowered the EGF binding capacity of pancreatic cancer cells, but this reduction was smaller than that caused by treatment with the bombesin/GRP antagonists.

Somatostatin analogues RC-160 and RC-121 cause the dephosphorylation of EGF receptors and inhibition of EGF-induced growth of Mia PaCa-2 human pancreatic cell lines (7, 46, 47). Somatostatin analogues may inhibit the growth of pancreatic cancers by stimulating tyrosine phosphatase (46–48). However, the mechanism of action of bombesin/GRP antagonist in inhibition of pancreatic cancer remains to be investigated. EGF binding to specific surface receptors in Swiss 3T3 cells was reported to be markedly inhibited by bombesin (49).

Our findings and previous results (11–15) suggest that, in addition to the possible use of LH-RH agonists and antagonists and somatostatin analogues for hormonal treatment of pancreatic cancers, new bombesin/GRP antagonists exemplified by RC-3095 can be also considered as potentially useful agents for treatment of tumors of the exocrine pancreas. Moreover, the possibility of the improvement in the therapeutic response by using combinations of somatostatin and/or LH-RH analogues with bombesin/GRP antagonists should be investigated.

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Inhibitory Effect of Bombesin/Gastrin-releasing Peptide Antagonist RC-3095 and High Dose of Somatostatin Analogue RC-160 on Nitrosamine-induced Pancreatic Cancers in Hamsters

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