Overexpression of Cholecystokinin Receptors in Azaserine-induced Neoplasms of the Rat Pancreas

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ABSTRACT

Cholecystokinin (CCK) is a growth factor for normal pancreas. Numerous studies also suggest that CCK promotes pancreatic carcinogenesis in the rat. Our previous studies suggested that growth of preneoplastic pancreatic foci was stimulated by CCK more than that of normal pancreas. We hypothesized that such differential growth might be due to increased numbers of CCK receptors in neoplastic tissue.

Azaserine-induced pancreatic carcinoma (DSL6) had an increased high-affinity CCK receptor binding capacity of 122 ± 23 (SD) fmol/mg protein compared to 12 ± 2 fmol/mg protein in normal pancreas (P < 0.001). The Kd of the high-affinity site was 0.33 ± 0.04 nM for carcinoma and 0.46 ± 0.08 nM for normal pancreas (P < 0.01). The amount of cholecystokinin octapeptide (CCK-8) bound to high-affinity receptor was 8.6 ± 1.9 fmol/mg protein for DSL6 compared to 0.6 ± 0.2 fmol/mg protein in normal pancreas (P < 0.001).

Azaserine-induced premalignant nodules were compared to remaining internodular pancreas. Nodules demonstrated a mean high-affinity CCK receptor binding capacity of 38 ± 9 fmol/mg protein compared to 6 ± 3 fmol/mg protein in internodular pancreas (P < 0.001). The amount of CCK-8 bound to high-affinity receptor was 3.1 ± 0.8 fmol/mg protein in nodules compared to 0.6 ± 0.3 fmol/mg protein in internodular pancreas (P < 0.001).

Overexpression of high-affinity CCK-8 receptor in premalignant and malignant azaserine-induced tumors may result in a growth advantage relative to normal pancreas.

INTRODUCTION

Adenocarcinoma of the pancreas presents a formidable clinical challenge. It has become the fifth most common cause of cancer death and remains nearly incurable, with an average survival from diagnosis until death of approximately 3 months (1). For this reason, there has been considerable interest in trying to identify factors involved in the growth of pancreatic cancer, particularly those which may be manipulable. Studies of pancreatic carcinogenesis take advantage of animal models of the disease, of which one well described example is azaserine-induced pancreatic carcinoma in the rat (2). In response to a single injection of the carcinogen azaserine (30 mg/kg body weight) at age 2 weeks, approximately 50% of Lewis rats develop acinar cell adenocarcinomas of the pancreas after a latency period of 18 months. The course of azaserine-induced carcinogenesis is characterized initially by the development of microscopic foci of atypical acinar cells (AACN), some of which become grossly visible about 6 months after exposure to azaserine. Most of these nodules, which range in size from 1 to 7 mm, remain benign; a small subset develop further dysplastic and anaplastic changes and are presumed to be the precursor lesions of invasive carcinoma. Well-differentiated nodules in the size range of 3–7 mm are classified histologically as adenomas, and frankly anaplastic nodules without evidence of invasion or metastasis are classified as carcinoma in situ.

The peptide hormone CCK, which binds to specific receptors on pancreatic acinar cells, is known to stimulate the growth of normal pancreatic tissue (3–6). This growth effect is mediated by a high-affinity CCK receptor (7). Beyond its effect on normal pancreas, several studies suggest that CCK promotes the development of pancreatic neoplasms. It was noted several years ago that long-term consumption of diets containing trypsin inhibitors in the form of raw soya flour caused the development of adenomas and carcinoma of the pancreas in rats (8). Rats fed raw soya flour also were found to be more susceptible to azaserine-induced cancers than animals on a diet of standard chow (9). Trypsin inhibitors are known to raise plasma CCK levels (10), probably because trypsin ordinarily inactivates the duodenal monitor peptide which stimulates CCK secretion (11).

Additional support for a role for CCK in experimental pancreatic carcinogenesis comes from studies of animals undergoing chronic surgical diversion of pancreaticobiliary secretion to the distal small intestine. Such diversion results in chronic elevation of plasma CCK levels (12) and to the development of adenomatous hyperplasia and in one instance carcinoma in situ of the pancreas (12, 13).

Two studies of azaserine-induced carcinogenesis in the rat pancreas strongly support a role for CCK as a relevant tumor growth factor in vivo. In each study, all rats received a single injection of the carcinogen azaserine at 2 weeks of age. Rats subsequently received s.c. injections of either CCK or control diluent for 16 weeks and were then sacrificed. In both studies, the number of AACN in the pancreas of CCK-treated rats was quantitatively measured and compared to controls. The quantitative measurement of AACN (14) is an accepted and standardized short-term bioassay for the development of carcinoma in the azaserine model. In one study (15), the administration of CCK to azaserine-treated rats led to an 8–20-fold increase in AACN as a percentage of pancreatic area. In the second study (16), the administration of CCK caused an 8-fold increase in AACN as a percentage of pancreatic area. In addition, the administration of the CCK receptor antagonist CR-1409 significantly reversed this effect, lowering AACN development to levels not significantly different from those of controls not treated with CCK.

In a previous report from this laboratory (17), feeding of the synthetic trypsin inhibitor camostate (FOY-305) increased the number and size of AACN present 4 months after treatment with azaserine. Of particular interest in that study was the demonstration by quantitative stereological techniques that camostate had a greater effect on the growth of AACN than on the intervening histologically normal pancreas. This suggested that the preneoplastic foci were more sensitive to CCK than normal pancreas with regard to stimulation of growth. This
might reflect more avid binding of CCK, either because of increased receptor number or because of increased receptor affinity for CCK. The current study was designed to test the hypothesis that premalignant and/or malignant pancreatic tumors exhibit increased binding of CCK compared to normal pancreas because of increased receptor number or affinity or both. If, in fact, pancreatic tumors possess an enhanced ability to bind CCK, this may provide a growth advantage leading to tumor promotion or progression.

MATERIALS AND METHODS

Preparation of Tissues for CCK Binding Studies. Four types of tissue were examined in this study. In the first experiment, binding of CCK to a transplantable azaserine-induced carcinoma (DSL6) was compared to normal rat pancreas. DSL6 was harvested from donor animals, minced, and injected s.c. in the intercapsular area of 1-2-month-old male Lewis rats (Charles River Breeding Laboratories, Wilmington, MA). After the implanted tumor had grown to 1-2 cm in diameter, animals were decapitated under ether anesthesia and the tumor was harvested. At the same time, the normal pancreas of the tumor-bearing animals was harvested via a midline laparotomy. Six animals bearing DSL6 tumor were studied.

In the second experiment, the objective was to compare CCK binding in premalignant pancreatic nodules to that in the remaining internodular pancreas. To that end, male Lewis rats were treated with a single i.p. injection of azaserine (30 mg/kg body weight) at age 2 weeks and followed for 12-18 months, at which time they were sacrificed and the pancreas was removed. Grossly visible pancreatic nodules in the size range 2 to 5 mm were then hand-dissected from the pancreas and pooled. The remaining internodular pancreas was prepared for comparison to the nodule pool. Nodules were harvested from 8 animals. From each animal 10-20 nodules were pooled. In one animal, the pancreas was nearly completely replaced by nodules and there was insufficient internodular tissue to harvest.

All tissues were immediately frozen on dry ice and then were transferred to a −70°C freezer. For binding assays, tissue sections (20 μm) were cut at −20°C on a cryostat microtome, mounted on gelatin-coated microscope slides, and dried for 18 h at −22°C.

Studies of Binding of 125I-CCK-8 to Tissue Sections. Binding assays were performed using the method of von Schrenck et al. (18). Tissue sections were preincubated in 50 mM MES buffer containing 0.5% albumin for 20 min at pH 6.0 and 22°C. Sections were then incubated for 4 h at 22°C, pH 6.0, unless otherwise stated, in 50 mM MES buffer containing 0.5% albumin, 0.025% bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin, 130 mM NaCl, 7.7 mM KCl, 5 mM MgCl2, 1 mM ethyleneglycoltetraacetic acid, and 25 μM 125I-BH-CCK (specific activity, 2000 Ci/mmol), obtained from Amersham Corporation, Arlington Heights, IL. Incubation volume was 4 ml for four slides. For competitive binding assays, sections were exposed in addition to graded concentrations of unlabeled CCK-8 ranging from a low of 1 nM to a high of 1 × 10−5 M, the latter concentration representing a 100,000-fold excess of unlabeled ligand. The competitive binding curve and Scatchard plot for DSL6 carcinoma is shown in Fig. 2. Analysis of binding curves for normal pancreas (n = 6) and for DSL6 (n = 6) revealed that both contain high-affinity and low-affinity receptor sites for CCK-8 (Table 1). The

![Fig. 1. Time course of saturable binding of 125I-BH-CCK to normal pancreas (C, n = 2) and pancreatic carcinoma DSL6 (D, n = 3). Values represent percentage of total added counts bound. In each experiment each value was determined in duplicate. The nonsaturable binding component (defined as binding in the presence of 1 μM CCK-8) was 19% for normal tissue and 4% for DSL6 carcinoma (not shown).](cancerres.aacrjournals.org)
CCK receptors in pancreatic carcinoma

Ordinate, percentage of maximum binding, where maximum binding with each point measured in duplicate in each experiment. B, Scatchard transformation of added unlabeled CCK. Data points are the mean ± SEM of 6 experiments, with each point measured in duplicate in each experiment. B, Scatchard transformation of the displacement data demonstrating the presence of high-affinity (Kd = 0.33 nM) and low-affinity (Kd = 82 nM) receptors.

K_d for the high-affinity site appears to be physiologically relevant since it is close to the concentration of CCK-8 required for half-maximal amylase release previously reported (0.1 nM) (18). Binding capacity (B_max) of the high-affinity receptor was 122 ± 23 fmol/mg protein for carcinoma versus 12 ± 2 fmol/mg protein for normal tissue (P < 0.001). At the tracer concentration of 25 pm used in this experiment, the combination of increased affinity and higher binding capacity for CCK-8 in DSL6 carcinoma led to a highly significant increase in actual concentration of 25 pm was predominantly to the high-affinity receptor (94.5% compared to 48.3% for internodular pancreas, P < 0.05). The increase in total binding of CCK-8 to nodules was due entirely to increased binding to the high-affinity site (3.1 ± 0.8 fmol/mg protein versus 0.6 ± 0.3 fmol/mg protein in internodular pancreas, P < 0.001) (Fig. 4). There was a corresponding reduction in amount of low-affinity site binding in nodules compared to internodular pancreas (0.18 ± 0.14 fmol/mg protein versus 0.59 ± 0.51 fmol/mg protein, P < 0.05).

Normalization of Receptor Binding Capacity to DNA Content. Although receptor binding capacity is typically expressed in relation to protein content, this presented some difficulties in this study since it might be expected that neoplastic pancreas would contain less protein in the form of secretory enzymes than normal pancreas. For this reason, binding capacity was also normalized to DNA content of the tissue slices. Normalization to DNA content would be expected to potentially underestimate receptor capacity in the transplantable carcinoma since its cells are aneuploid with hyperdiploid DNA content; nodules and adenomas have normal diploid DNA content (21). Despite this, the fundamental findings were unchanged, namely that the high-affinity receptor binding capacity of DSL6 carcinoma was significantly greater than that of normal pancreas (492 ± 115 versus 245 ± 61 fmol/mg DNA, P < 0.01). The high affinity binding capacity of nodules likewise significantly exceeded that of internodular pancreas (416 ± 83 versus 90 ± 39 fmol/mg DNA, P < 0.001).

Histological Examination of Tissue. Sections of DSL6 carcinoma revealed moderately well differentiated acinar adenocarcinoma. Sections of “nodules” consisted entirely of AACC in adenomas. Sections of internodular pancreas consisted of approximately 80–90% normal pancreatic tissue and 10–20% small nodules (<1 mm).

**DISCUSSION**

Carcinogenesis is believed to be a multistage process. The process is generally divided into an initiation phase, in which cellular DNA damage occurs, and a promotion phase during

### Table 1 CCK receptor characteristics

<table>
<thead>
<tr>
<th>Tissue</th>
<th>High-affinity</th>
<th>Low-affinity</th>
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<tbody>
<tr>
<td></td>
<td>K_d (nM)</td>
<td>B_max (fmol/mg protein)</td>
</tr>
<tr>
<td>Normal pancreas</td>
<td>0.46 ± 0.08*</td>
<td>12.4 ± 2.2</td>
</tr>
<tr>
<td>DSL6 carcinoma</td>
<td>0.33 ± 0.04</td>
<td>122.1 ± 22.9</td>
</tr>
<tr>
<td>Nodules</td>
<td>0.28 ± 0.04</td>
<td>37.6 ± 8.6</td>
</tr>
<tr>
<td>Internodular pancreas</td>
<td>0.27 ± 0.12</td>
<td>6.4 ± 2.8</td>
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*Mean ± SD.
they detected two classes of binding sites with half-maximal binding inhibition of 0.2 nm and 0.3 μM, results very similar to ours with the DSL6 carcinoma. We have also performed a limited number of competitive binding assays with AR42J and agree that there are two classes of binding sites (data not shown). Scemama’s group has also suggested that the high-affinity receptors for CCK on AR42J cells can be divided into two subsets: one binding CCK preferentially; and a second binding CCK and gastrin interchangeably. They have demonstrated that occupation of these latter receptors results in stimulation of ornithine decarboxylase activity, an event associated with cellular proliferation (24).

As noted above, there is considerable in vivo evidence of a role for CCK in pancreatic tumor promotion in rats. However, the relevance of acinar cell carcinoma in rats to carcinomas in humans is questioned since the majority of human pancreatic carcinomas have a ductal phenotype. Recently, evidence for a role of CCK in human pancreatic cancer has also emerged. CCK has been shown to stimulate the growth of several human pancreatic carcinoma cell lines in culture (25) and in xenografts into nude mice (26). In addition, in nude mice, the CCK receptor antagonist L364,718 has been shown both to inhibit the basal growth of human pancreatic carcinoma and to block the tumor-promoting effects of a high-fat diet (27, 28).

In the azaserine model in the rat, neoplastic pancreatic changes are clearly focal, with well-defined nests of acinar cells developing hyperplastic, dysplastic, or neoplastic changes while other pancreatic cells appear relatively unchanged. There may be several reasons why some cells progress through the oncogenic sequence while others show little or no change from the normal state. We believe that the current study demonstrates that overexpression of high-affinity CCK receptors characterizes the cells in the neoplastic pathway. The possibility that CCK receptor overexpression confers a growth advantage on such cells will be examined in future studies.

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