Cholecystokinin A and B Receptors Are Differentially Expressed in Normal Pancreas and Pancreatic Adenocarcinoma

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Abstract

Cholecystokinin (CCK) plays an important role in pancreatic carcinogenesis. While human CCK-A and -B receptors have been fully characterized, their relative roles in human pancreatic adenocarcinoma remain unclear. Thus, expression of CCK-A and -B receptors in normal human pancreas, pancreatic adenocarcinomas, and other human extrapancreatic tissues and malignancies was examined, using reverse transcription followed by the polymerase chain reaction (RT-PCR). mRNA isolated from 15 normal pancreas specimens, 22 pancreatic adenocarcinomas, and 58 extrapancreatic tissues and tumors was subjected to RT-PCR using primers specific for human CCK-A and -B receptors. Expression of CCK-B receptors was detected in all tissues arising from pancreas and in most extrapancreatic tissues and tumors. In contrast, CCK-A receptors exhibited a more selective pattern of expression in gall bladder, intestine, brain, ovary, spleen, and thymus. Of significance, CCK-A receptors were expressed selectively in all pancreatic adenocarcinomas, but not in any normal pancreas specimens. In situ hybridization, using receptor-specific riboprobes, localized CCK-A receptor expression to ductal cells, the presumed origin of most human pancreatic adenocarcinomas. Southern blot analysis revealed no evidence of CCK-A receptor gene amplification or rearrangement in pancreatic adenocarcinomas. Because of its selective expression, the CCK-A receptor may serve as selective biomarker for pancreatic adenocarcinoma. (J. Clin. Invest. 1997, 100:597–603.) Key words: pancreatic ductal cells • reverse transcription–polymerase chain reaction • in situ hybridization • DNA probe • tumor marker

Introduction

Pancreatic adenocarcinoma is the fifth leading cause of cancer death in the United States (1). The etiology of this malignancy is largely unknown. Advancing age, male gender, and smoking are established risk factors (2–5), while chronic pancreatitis (6) and diabetes (7) may be as well. The prognosis for patients with pancreatic cancer is poor, with reported 1-yr survival rates of 5–10% (8).

There is considerable evidence to support a central role for cholecystokinin (CCK) in human pancreatic cancer (9). The influence of endogenous hormones is well-described for several human malignancies, including breast, ovary, and prostate. Generally, the hormones implicated are important in both the health and disease of their target organ. CCK is an important mediator in the growth of the normal pancreas (10, 11). Animal studies in which exogenous CCK was administered or in which endogenous CCK levels were manipulated documented pancreatic hyperplasia, dysplasia, and the production of frank malignancies (12). Similar studies after the induction of pancreatic tumors suggest that CCK administration accelerates the growth of malignant compared to uninvolved tissue (13, 14). In human cancer cell lines and xenografted human tumors, CCK promotes the growth of pancreatic adenocarcinoma (15, 16).

The identification of a specific molecular marker for pancreatic cancer could be of substantial diagnostic and therapeutic benefit. Two CCK receptors have been characterized and cloned in animal and human studies, CCK-A and -B (17). These receptors share structural homology and can be differentiated based on their binding affinities for CCK and another related gastrointestinal hormone, gastrin (18). The CCK-A receptor has an affinity for CCK which is 1,000-fold greater than that for gastrin, while the CCK-B receptor exhibits equivalent affinities for both peptides. Studies performed in rats demonstrated that in normal pancreas, the CCK-A receptor alone is expressed, while in pancreatic malignancies, the CCK-A receptor is over-expressed and the CCK-B receptor is newly expressed (19). In limited studies with human tissues, the CCK-B receptor has been identified on normal pancreas and pancreatic adenocarcinomas, although the latter is based predominantly on data obtained from immortalized cancer cell lines (20). There are no previous reports examining the expression of CCK-A receptors by human pancreatic adenocarcinomas.

The deficiencies of animal models for human pancreatic ductal adenocarcinomas are well-described (21, 22). Further, it is well-known that the behavior of human cancer cell lines in vitro does not necessarily parallel that of tissue in vivo. Therefore, the expression of CCK-A and -B receptors by human tissues harvested at surgery was examined to evaluate the utility of these receptors as biomarkers in normal and malignant human pancreas.
Methods

Clinical specimens
Human tissues and blood were obtained under an Institutional Review Board–approved protocol from Thomas Jefferson University Hospital, the National Disease Research Interchange, and the Cooperative Human Tissue Network (Philadelphia, PA). More than 100 human tissue samples were examined for expression of CCK receptors, including 22 primary pancreatic tumors, 15 normal pancreatic specimens, 46 extrapancreatic tissue specimens, and 23 extrapancreatic tumors. Tissues were obtained from the operating room, and either processed immediately or frozen in liquid nitrogen and stored at −70°C until use. Histopathologic diagnosis of pancreatic adenocarcinoma was confirmed in all cases.

Nucleic acid extraction
Total RNA was extracted from samples using a modified version of the acid guanidium thiocyanate/phenol/chloroform method employing a single reagent (TRIzol reagent; GIBCO BRL, Gaithersburg, MD) (23, 24). Only samples exhibiting intact 28s and 18s ribosomal RNA were analyzed. mRNA was purified by oligotex poly(A)+ mRNA affinity latex beads (QIAGEN Inc., Chatsworth, CA) using the manufacturer’s protocol. Greater than 90% of the mRNA was recovered from total RNA in purified preparations. RNA preparations were stored in diethyl pyrocarbonate–treated water (RNase-free) at −80°C. To remove contaminating genomic DNA, the RNA was treated with 1 U/μl of RNase-free DNase (Promega, Madison, WI) for 15 min at 37°C, followed by a 30-min incubation at 95°C with 1 μl RNase inhibitor (PanVera Corp., Madison, WI).

Reverse transcription-PCR (RT-PCR)
RT of mRNA (≤ 1 μg) purified from tissues was performed with 0.25 U/μl of AMV reverse transcriptase XL (PanVera Corp.) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 1 mM each dATP, dCTP, dGTP, and dTTP, 1 U/μl RNase inhibitor, and 1 μM of CCK-A or CCK-B receptor–specific antisense primer (nucleotides 817–835 or 506–526, respectively) in a total volume of 20 μl (25). Thermal cycling proceeded for 1 cycle at 55°C for 2 min, 1 cycle; 94°C for 30 s, 38°C for 30 s, 72°C for 90 s, 35 cycles; 72°C for 7 min, 1 cycle. Some tissues were analyzed as described above, using sequential CCK receptor–specific PCR. In these studies, first-round amplification reactions (35 cycles) used antisense- and sense-specific primers as outlined above. Second-round amplification reactions (35 cycles) were initiated using 10 μl of the amplified cDNA solution from first-round reactions in a final volume of 100 μl under the same PCR conditions. Primers specific for human β-actin (CLONTECH, Palo Alto, CA) were used as a positive control for RT-PCR reactions (27).

Southern blotting
10 μg of genomic DNA from 15 separate histologically confirmed pancreatic adenocarcinoma specimens was digested with EcoR1 and BglII and subjected to Southern blot analysis on 0.8% agarose gels using an alkaline (0.4 N NaOH) transfer method (28). Nylon membranes (Gene Screen Plus; New England Nuclear, Waltham, MA) were hybridized with an 18-mer digoxigenin (DIG)-labeled oligonucleotide probe corresponding to a highly conserved region of the CCK-A receptor coding sequence. After high stringency washes, blots were visualized using an enhanced chemiluminescence protocol (Boehringer Mannheim Corp., Indianapolis, IN). Hybridization with a 1.3 kbp EcoR1 cDNA fragment of human β-actin was performed to normalize for DNA loading.

In situ hybridization
Probe synthesis and DIG labeling protocol. Riboprobes were synthesized (BioServ Biotechnologies, Laurel, MD) from sequences corresponding to bp 817–835 of the CCK-A receptor gene (antisense: CCA CCA UCA UCA CAA UUC C; sense: CUC UAA CAC UAC UAC CAC C), and from sequences corresponding to bp 506–526 of the CCK-B receptor gene (antisense: AUU CCC AGG AAG AGA AAG; sense: GAA AGA GAG UAA GGA CCC UUA). An oligo d(T) probe (Novacstra Laboratories Ltd., Newcastle-upon-Tyne, UK) was used as a control to evaluate the quality of the RNA. A DIG Oligonucleotide 3′-End Labeling Kit (Boehringer Mannheim) was used to label the probes. Briefly, 5 nmol/ml of probe was incubated in a solution containing 1× reaction buffer, 5 mM CaCl2, 0.5/0.5 mM DIG dUTP/dUTP tailing mixture, 5 mM dATP, 2.5 U/μl terminal transferase, and sterile water for 15 min at 37°C. Glycogen and 200 mM EDTA, pH 8.0, was then added to stop the reaction. The probe was precipitated overnight at −70°C with 4 M LiCl and 100% ethanol, centrifuged at 4°C for 15 min (13,000 g), and washed with 70% ethanol. The pellet was dried, resuspended in TE buffer (Tris-HCl EDTA) at pH 8.0, and stored at −70°C until needed. Probes were quantified using serial dilutions 0.25 pmol/μl–0.25 fmol/μl. The dilutions were spotted onto a positively charged nylon membrane (Boehringer Mannheim) and cross-linked using a UV Stratalinker (model 1800; Stratagene, La Jolla, CA). Spots were detected by rinsing the membrane in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5), incubating for 10 min in 1× blocking solution (Boehringer Mannheim), washing in maleate buffer with 3.0% (vol/vol) Tween 20, equilibrating in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5, and 50 mM MgCl2), and incubating for 1–2 h in the dark in freshly prepared NBT/X phosphate solution (Boehringer Mannheim). The yield of DIG-labeled probe was determined by comparison to a DIG-labeled control.

In situ hybridization. In situ hybridization using the DIG-labeled probes was performed as described previously (29, 30). All solutions were treated with diethyl pyrocarbonate and all glassware was baked at 240°C overnight to prevent RNA degradation. 5-μm paraffin-embedded tissues were deparaffinized in xylene, rehydrated in an ethanol series (100, 100, 95, and 70%), and washed twice for 5 min in PBS. Tissue sections were incubated in 0.1 M glycine for 5 min and 0.3% Triton X-100 for 15 min to reduce background. Slides were incubated in 30 min in proteinase K solution (10 μg/ml), postfixed for 5 min in 4% paraformaldehyde, and acetylated in 0.25% acetic anhydride for 10 min. Tissue sections were incubated for 2 h in a humidified chamber at 37°C with prehybridization solution containing 50% deionized formamide, 4× SSC, 1× Denhart’s solution, 500 μg/ml denatured salmon sperm, 250 μg/ml yeast tRNA, and 10% dextran sulfate. After prehybridization, slides were incubated overnight at 37°C in a humidified chamber with hybridization solution (prehybridization solution plus 100 pmol/ml probe). Posthybridization washes were 2× SSC for 15 min at 42°C (twice), 1× SSC for 15 min at 42°C (once), 0.5× SSC for 15 min at 42°C (twice), and 1× maleate buffer for 1 min at room temperature. Signals were detected by incubating in 2× blocking solution (Boehringer Mannheim) at room temperature followed by incubation with anti-DIG alkaline phosphatase antibody (1:500; Boehringer Mannheim) in 2× blocking solution for 4 h at 37°C. Slides were washed in maleate buffer and color detection buffer (Boehringer Mannheim) for 10 min each, then incubated in freshly prepared NBT/X phosphate (Boehringer Mannheim) color development solution overnight in a light-sealed humidified chamber. The reaction was stopped by washing slides in TE buffer, pH 8.0, and covered with Biomedica (Fisher Scientific, Pittsburgh, PA).

Results
Expression of CCK-A and -B receptors by RT-PCR. Studies examining normal human pancreas and pancreatic adenocarcinomas have been inconclusive in defining the expression of CCK-A and -B receptors. Therefore, the specificity of expres-
Cholecystokinin A Receptors Are Selective Markers for Pancreatic Carcinoma

RT-PCR of mRNA purified from indicated tissues and primers for CCK-A receptor (top), CCK-B receptor (middle), or β-actin (control; bottom), as described in Methods. Left lane, size markers. Arrows indicate size of human CCK-A and -B receptors (hCCK-A, ~250 bp; hCCK-B, ~250 bp) predicted from their defined sequences.

Table I. Tissues in which CCK-A and -B Receptors Were Detected by RT-PCR

<table>
<thead>
<tr>
<th>CCK-A</th>
<th>CCK-B</th>
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<tr>
<td>Pancreatic adenocarcinoma (22)*</td>
<td>Pancreatic adenocarcinoma (22)</td>
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<tr>
<td>Gall bladder (2)</td>
<td>Small intestine (2)</td>
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<td>Small intestine (2)</td>
<td>Colon (4)</td>
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<td>Colon (4)</td>
<td>Spleen (1)</td>
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<td>Spleen (1)</td>
<td>Ovary (2)</td>
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<td>Ovary (2)</td>
<td>Cerebellum (4)</td>
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<td>Cerebellum (4)</td>
<td>Frontal lobe (2)</td>
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<tr>
<td>Colon carcinoma metastatic to liver (1)</td>
<td>Colon carcinoma (6)</td>
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<td>Colon carcinoma (6)</td>
<td>Colon carcinoma (1)</td>
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<tr>
<td>Colon carcinoma (1)</td>
<td>Colon carcinoma metastatic to liver (1)</td>
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<td>Cerebellum (4)</td>
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<td>Cerebellum (4)</td>
<td>Frontal lobe (2)</td>
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*Number of samples evaluated.
ceptors in pancreatic adenocarcinomas (20). Compared to the wide expression of CCK-B receptors in many extrapancreatic tissues and tumors, CCK-A receptor expression appears to be limited to select tissues, including gall bladder, intestine, ovary, spleen, and brain, as demonstrated previously (32, 33, 36, 37) (Table I). Prior studies have suggested that CCK-A receptors are expressed on human stomach and kidney (32). However, these receptors could not be detected in specimens of these tissues using RT followed by two sequential rounds of PCR (Fig. 2A). Of significance, CCK-A receptors appear to be selectively expressed by pancreatic adenocarcinomas but not by other extrapancreatic tumors originating within and outside the peritoneum (Fig. 1, Table I).

**Localization of CCK-A and -B receptor expression in normal pancreas and pancreatic adenocarcinoma by in situ hybridization.** Selective expression of CCK-A and -B receptors in normal pancreas and pancreatic adenocarcinoma was examined further, using in situ hybridization to identify the cells expressing these receptors. Probes specific for CCK-B receptors specifically hybridized with mRNA in sections of normal pancreas and pancreatic adenocarcinoma. Staining was specific for CCK-B receptors since sense probes to the identical sequence did not produce specific staining. In contrast, probes specific for CCK-A receptors did not produce specific staining in normal pancreas, in close agreement with results obtained by RT-PCR. However, these probes produced specific staining of cells in sections of pancreatic adenocarcinoma, confirming the results obtained by RT-PCR (Fig. 2B). Hybridization with CCK-A receptor–specific probes occurred in ductal rather than acinar cells, consistent with the clinical observation that most human pancreatic adenocarcinomas are presumably of ductal origin (38).

**Southern blot analysis.** Southern blot analysis using an oligonucleotide probe specific for the CCK-A receptor sequence revealed the absence of CCK-A receptor gene amplification or rearrangement in pancreatic adenocarcinomas, compared to normal pancreas (Fig. 2C).

**Discussion**

The data presented are the first to evaluate expression of CCK-A receptors by RT-PCR using RNA extracted from multiple normal and malignant human pancreatic tissue specimens. They demonstrate that the human CCK-A receptor is selectively expressed by pancreatic adenocarcinoma, but not by normal pancreas. Selective tissue expression was detected by RT-PCR and in situ hybridization. This receptor appears to be expressed only by a limited number of extrapancreatic tissues, but not by other intra- and extraabdominal tumors examined. Expression in pancreatic adenocarcinomas was localized to neoplastic pancreatic ductal cells by in situ hybridization. By comparison, CCK-B receptors were widely expressed by both normal and neoplastic pancreatic tissue, and by most other normal and neoplastic extrapancreatic tissues examined. The expression of CCK-A receptors by human pancreatic adenocarcinomas, but not by normal pancreas or other extrapancreatic tumors examined, suggests that this receptor may have utility as a relatively selective biomarker for the diagnosis and staging of pancreatic tumors (39, 40).

The relative expression of CCK-A and -B receptors in normal human pancreas has been examined previously (18). Studies using Northern blotting and ligand binding revealed CCK-B but not CCK-A receptors on normal human pancreas, implying that CCK-B alone is expressed in that tissue (17). The absence of CCK-A receptors and their transcripts in normal human pancreas is consistent with the observation that CCK-induced pancreatic exocrine secretion is mediated by neurogenic mechanisms involving vagal afferent pathways, rather than by directly affecting pancreatic acinar cell function (41).

A previous study examined the expression of CCK-A and -B receptors in normal human pancreas, using PCR primers with substantially overlapping homology to both receptors and commercially obtained cDNA libraries. Amplicons for CCK-B but not CCK-A receptors were detected by ethidium bromide staining (32). Interestingly, Southern blot analysis of the electrophoretically separated products revealed a band of the appropriate size for the CCK-A receptor. However, those results must be considered carefully since application of the same Southern blotting technique failed to detect CCK-B receptor expression in a normal human brain cDNA library in which a PCR amplicon was readily detected by ethidium bromide. In addition, CCK-A receptor expression was detected by this Southern blotting technique in normal human stomach and kidney (32). In this study, expression of this receptor could not be detected in those tissues despite the use of a PCR technique which can amplify the presence of rare transcripts up to $10^{20}$ fold.

Previous reports examining the type and distribution of CCK receptors in animal pancreas have yielded results which may not be directly applicable to humans. CCK-A receptors have been observed on both normal and neoplastic rat pancreas (21, 42). In some cases of rat pancreatic adenocarcinoma,
Cholecystokinin A Receptors Are Selective Markers for Pancreatic Carcinoma

Cholecystokinin A receptors are overexpressed (19). Furthermore, in some nonhuman pancreatic cancer models, expression of CCK-A receptors has been identified on malignant tissue but not on normal pancreas (19). The relationship between different animal models of pancreatic cancer and humans remains controversial (21, 43). The majority of human pancreatic adenocarcinomas appear to be of ductal cell origin, while pancreatic malignancy in most animal models arises in acinar cells. While it is possible that among different species there is significant variation in the cell of origin for pancreatic adenocarcinoma, there is also substantial evidence to support the possibility of a pancreatic stem cell capable of differentiation into ductal or acinar cell types (44). Further, transdifferentiation from acinar to ductal cell phenotype has been described as a potential link between exocrine pancreatic cell types (21, 45). Thus, pancreatic tumor development in animals and humans could originate in the same cell type, with variable subsequent differentiation.

The role of CCK-A or -B receptors in human pancreatic carcinogenesis remains unclear. The growth of several human neoplastic cell lines derived from pancreas (15), colon (46), and lung (47) is stimulated by CCK or gastrin in vitro. Functional CCK-B receptors have been demonstrated on human biopsy specimens of small cell lung (47) and colon cancers (46), implicating a potential role for CCK or gastrin in human carcinogenesis (48). The demonstration of novel CCK-A receptor expression specifically on ductal cells in pancreatic adenocarcinomas is intriguing. This unique expression could reflect the presumed ductal origin of human pancreatic tumors. CCK-A receptors may be expressed at very low levels in ductal cells in normal human pancreas, below the level of detection by current techniques (32). Tumorigenesis may expand the population of ductal cells, increasing the expression of CCK-A receptors. In addition, CCK-A receptors on ductal cells may play a direct role in mediating the process of pancreatic tumorigenesis.

Alternatively, these studies are consistent with the hypothesis that CCK-A receptors may be a feature only of the developing human pancreas. CCK-A receptors might predominate during human fetal pancreatic development, but their expression would be unnecessary in normal adult pancreas. It has been suggested that CCK mediates normal pancreatic growth and development (10). Previous studies in the calf have demonstrated the expression of CCK-A receptors during fetal development, but with a predominance of CCK-B receptors in the normal adult bovine pancreas (49). Expression of novel antigens by pancreatic neoplastic cells, including fetal markers, has been described (50). Accordingly, expression of CCK-A receptors in pancreatic adenocarcinomas may reflect the reexpression of fetal markers during tumorigenesis. The expression...
of CCK-A receptors by human fetal pancreas is currently under investigation in this laboratory.

Pancreatic adenocarcinoma represents the fifth leading cause of cancer death in the United States (1). Although there is some evidence that early diagnosis and resection can result in improved long-term survival (51), there is a paucity of specific molecular markers to detect pancreatic adenocarcinoma or to identify patients at higher risk for cancer development (39, 40). Further study of the differential expression of CCK receptors may reveal CCK-A receptors as a useful biomarker for pancreatic adenocarcinoma diagnosis and management.

Acknowledgments

This research was supported by the W.W. Smith Charitable Trust, the Elsa U. Pardee Foundation, the American Cancer Society, and Targeted Diagnostics and Therapeutics, Inc. D.S. Weinberg is the recipient of National Institutes of Health grant CA71080.

References


