Effect of Cholecystokinin on Human Cholangiocarcinoma Xenografted into Nude Mice

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ABSTRACT

Gastrointestinal polypeptide hormones regulate growth of various normal gastrointestinal tissues as well as certain visceral cancers. Since cholecystokinin (CCK) promotes growth of normal biliary tract, we sought to determine whether CCK affects the growth and metabolism of human cholangiocarcinoma line SLU 132. Twenty-six nude mice with s.c. xenografts of this cancer received either CCK octapeptide (50 μg/kg/dose) or 0.9% NaCl solution (saline) twice a day i.p. for 14 days. Tumor volume was calculated from Vernier caliper measurements. At sacrifice on Day 15, tumors were excised, weighed, and examined histologically. DNA, RNA, and protein were measured in the xenografted carcinomas. Because this cholangiocarcinoma produces carcinoembryonic antigen (CEA), we obtained serum at sacrifice for CEA radioimmunosassay and also tumor tissue for CEA immunolabeling with murine anti-CEA monoclonal antibody. Serum CEA levels were 90% higher in the CCK-treated group. Tumor tissue in the CCK-treated group also contained more CEA than did the controls. Mean tumor volume increased significantly in the saline group during the 14-day treatment period, whereas mean tumor volume did not increase significantly in the CCK group. Exogenous high-dose CCK thus appears to increase production and release of CEA from SLU-132; it also appears to retard growth of this tumor line in the nude mouse.

INTRODUCTION

Carcinoma of the biliary tract remains a challenge to the clinician. Diagnosis is usually late, and survival statistics are unfavorable regardless of treatment (7). Surgical excision is often indicated and offers the best chance of tumor eradication. Failing this, relief of jaundice by palliative surgical bypass or prosthesis insertion is possible. However, since these patients are frequently elderly, sick, and ill-prepared for surgery, operative mortality and complications are common. This has led to a search for alternative modes of therapy. Although conventional radiation therapy has only a modest role in palliation of bile duct cancer, improved delivery techniques (particularly brachytherapy) are promising and may have curative potential (12). Chemotherapy is often used, but there is no firmly established standard regimen (22). Palliative percutaneous biliary drainage provides relief of jaundice but is associated with appreciable morbidity and mortality (4). New approaches are clearly required if treatment results are going to improve. One such approach might be hormonal manipulation, which currently has a major role in other solid tumors.

The polypeptide hormone CCK1 has profound metabolic actions, among which are gallbladder contraction, stimulation of enzyme-rich pancreatic exocrine secretion, and growth regulation of normal gastrointestinal tissues (17, 18). In addition to these, trophic effects on gastrointestinal cancers have been reported. Townsend et al. (31) demonstrated that caerulein (a CCK analogue) and secretin together stimulate growth of hamster pancreatic ductal adenocarcinoma line H2T. Lamote et al. (19) reported that acute s.c. administration of CCK increases thymidine uptake by normal murine gall bladder mucosa, and morphometric studies revealed gallbladder mucosal hyperplasia after chronic infusional administration of caerulein. This trophic action of CCK on biliary mucosa led us to investigate whether a similar effect occurs in human carcinoma of biliary origin, which might be exploitable in the management of this tumor.

An alternative approach to improving the outcome of biliary tract cancer would be early diagnosis. This might increase the likelihood of effective treatment as currently the disease is often far advanced when detected. We examined the effect of CCK administration on an aspect of tumor metabolism which might have clinical utility, namely, the production and release of CEA into the circulation. This well-known tumor marker occurs in normal, preneoplastic, and neoplastic human gallbladder epithelium (2) and is also elevated in the serum of some patients with cholangiocarcinoma (23).

MATERIALS AND METHODS

Animals. Thirty 6 to 8-week-old male nude mice (athymic nu/nu; Harlan-Sprague-Dawley, Indianapolis, IN) were used. Animals were housed in shared cages and kept in a laminar flow cabinet (Laboratory Products, Madison, WI). Husbandry was according to the National Research Council's Guide for the Care and Use of the Nude (Thymus Deficient) Mouse in Biomedical Research (14), and research procedures were according to current guidelines set by the NIH. Free access was allowed to sterilized diet (Autoclavable Rodent Chow 5010; Ralston Purina, St Louis, MO) and sterile water. Mice were identified by ear clipping.

Tumor. A specimen of tumor tissue was obtained from a liver metastasis in a 65-year-old Caucasian male with cholangiocarcinoma and successfully xenografted into nude mice. After the tumor line had undergone 3 passages in the nude mouse with stable doubling times and retention of its original histological appearances, it was designated SLU-132. The lines were passed 7 additional times prior to this study and continues to exhibit the same features. Since serum levels of CEA were not measured in the patient, we performed CEA immunofluorescence studies on slides from the original specimen and demonstrated considerable tumor tissue content of this substance. In a preliminary study, we...
measured CEA and α-fetoprotein levels in serum from mice bearing SLU-132 and found appreciable amounts of CEA but no α-fetoprotein. CEA values in non-tumor-bearing mice (0.65 ng/ml) were similar to those obtained in similar work by Schmitz et al. (28). Preliminary immunofluorescence labeling of xenografted nodules of SLU-132 taken from nude mice also showed appreciable CEA but no α-fetoprotein. This led us to also study CEA in greater detail. For the purposes of this study, the tumor line was expanded by taking nodules of SLU-132 from 10 tumor-bearing mice and finely mincing these together in a sterile Petri dish. Histology was performed on samples from the 10 nodules and confirmed that all were identical to the original tumor. Thorough mixing was carried out using a vortex mixer (S8223; American Scientific Products, McGaw Park, IL), and 0.15 ml of tumor tissue was injected s.c. into the shoulder region of each mouse with a 15-gauge 1.25-cm trocar.

**CCK.** Synthetic sulfated C-terminal octapeptide (SQ19,844, a gift from Squibb) was used. In order to ensure sterility, this was filtered through a 0.22-μm pore size; Gelman Sciences, Ann Arbor, MI into 10-ml aliquots which were stored at −70°C. An equal number of CCK and 0.9% NaCl solution (saline) vials was prepared. These were coded by a person not involved in the experimental manipulations; this blinding prevented the observer from identifying treatments when measuring tumors. Prior to each day’s injections, fresh sterile aliquots were thawed at 25°C. Animals were given injections i.p. of CCK (50 μg/kg/dose) twice daily or, in the control group, an equal volume of saline.

**Treatment Groups and Tumor Measurement.** When most tumor nodules measured 0.5 cm along the longest axis, the mice were divided into 2 groups and paired according to tumor size and body weight. Mice whose tumors had failed to grow were excluded at this time, leaving a total of 26 mice for study. This day was designated Day 0. On Days 0, 2, 5, 8, 11, and 14, animals were weighed to ±0.1 g on a triple-beam balance (Dial O Gram; Ohaus Scale Corp., Florham Park, NJ), and tumor size was measured in 2 linear dimensions using sliding-jaw Vernier calipers (PN1350-05; Ohaus Scale Corp.) accurate to 0.1 mm. The maximum longitudinal and transverse dimensions were measured. The formula

\[
\text{Volume} = \frac{(\text{length}) \times (\text{width})^2}{2}
\]

was used. This correlates well with volume determined by water displacement (11) and has been shown to be reliable when compared to other formulae (5). Since we have shown that interobserver error is significant when measuring small tumors in the nude mouse (11), one observer underwent a period of training and performed all measurements.

**Pathology.** On Day 15, animals were killed in a CO2 chamber, weighed on an electronic balance (1500D; Ohaus Scale Corp.), and exsanguinated by cardiac puncture. Blood was allowed to clot for 30 min and centrifuged. Separated serum was stored at −70°C prior to estimation of CEA. Tumors were excised, trimmed, and weighed; samples were taken for histology (hematoxylin:eosin), tissue CEA immunofluorescence staining, and RNA, DNA, and protein estimations. In order to ensure that the CCK used was biologically active, we utilized pancreas growth as a bioassay. The pancreas was excised, carefully trimmed of fat with the aid of magnifying glasses, and weighed on an electronic balance (AC100; Mettler Instrument Co., Hightstown, NJ) sensitive to 0.0001 g. Microscopic examination of small tumors, nodules, and DNA pellets was determined using the orcinol reaction (8). The precipitate was solubilized by the addition of 0.3 N potassium hydroxide. Samples were heated at 37°C for 90 min, and DNA and protein were again precipitated by the addition of 10% perchloric acid. After cooling for 45 min at 4°C, samples were centrifuged at 4000 rpm for 15 min. The RNA in the supernatant was determined using the orcinol reaction (8). The precipitate was solubilized by the addition of 10% perchloric acid and heated in a water bath at 70°C for 20 min. This was then cooled to 4°C for 45 min and centrifuged for 15 min at 4000 rpm. DNA in the supernatant was assayed using the diphenylamine reaction (8, 13). The protein in the pellets was solubilized in sodium hydroxide and measured using the biuret reaction (27).

**RESULTS**

Twenty-six animals were included in and completed the study. As a result of pairing, there were no significant differences between groups in initial tumor volume or body weight. Body weight did not change significantly during the course of the experiment (Table 1). Mean pancreas weights at necropsy (expressed as a percentage of total body weight) were 1.00 ± 0.03 in the saline group and 1.21 ± 0.02 in the CCK group, which represents a 21% increase in the CCK group (p < 0.001) and is consistent with trophic effects of CCK on this organ reported previously.

Tumors grew in both groups as discrete, multilobulated masses which were encapsulated and showed no gross invasion of surrounding tissues. No metastases were seen in any animal. The morphological features of the tumors were consistent in all animals. The tumors generally had well-differentiated glandular elements with variable amounts of mucin secretion and a fibrovascular stroma with reactive sclerosis. In foci within the tumor...
masses, the tumor cells had a more aggressive growth pattern and invaded the sclerotic stroma as small nests of cells and as single tumor cells. Vascular invasion was not seen nor was infiltration noted beyond the sclerotic margins of the tumor mass. Mean tumor volume increased from 0.19 to 0.27 cu cm in the saline group, representing a 42% increase over 14 days (p < 0.025). In the CCK group, mean tumor volume changed from 0.21 to 0.25 cu cm, representing a 19% increase over 14 days (p > 0.30) (Table 1). There were no significant differences between groups in the DNA, RNA, and protein content (not significant; p > 0.30) (Table 1). There were no significant differences between groups in the DNA, RNA, and protein content (not significant; p > 0.30) (Table 1).

DISCUSSION

Some trophic effects of gastrointestinal hormones on normal digestive tract epithelia are well known. In the Zollinger-Ellison syndrome, hypergastrinemia is associated with hyperplasia of acid-secreting mucosa. Conversely, antrectomy, with its attendant fall in gastrin, leads to mucosal atrophy of the remaining stomach (20). On the other hand, massive small bowel resection is associated with mucosal hypertrophy and hyperplasia (33). This is currently believed to be hormonally mediated. Growth-modulating effects of several gastrointestinal polypeptide hormones are also seen on certain cancers. Addition of pentagastrin to culture medium leads to increased uptake of tritiated thymidine in human gastric carcinoma cell lines (3). Administration of gastrin s.c. to nude mice, each bearing one of several gastric cancer lines, enhances growth in certain lines while retarding it in others (24).

Townsend et al. (31) demonstrated that caerulein, a homologue of CCK, when given with secretin, stimulates the growth of pancreatic ductal adenocarcinoma line H2T. Given alone, caerulein or secretin has no effect, suggesting synergism between these hormones. We know of no studies examining the effect of CCK on biliary tract cancer in animals or humans, an obvious area of study, as growth of normal biliary tract is modulated by CCK (19). Our experiments were designed to study the effect of CCK on carcinoma of the biliary tract. Since there is no suitable animal model of spontaneous or induced cholangiocarcinoma, we chose to use a human tumor transplanted into the nude mouse. This animal is widely used for the study of various exogenous agents on tumor xenografts. Since this model permits experimentation on human cancers (25), the practical and ethical dilemmas of work in humans are avoided. Clear isolation of variables is possible while retaining the advantages of an in vivo model. Tumor measurement s.c. is easy and, though animals are small, blood sampling for tumor marker substances can be performed. SLU-132, the human cholangiocarcinoma line which we used in this study, has exhibited stable growth and doubling times during repeated passages in the mouse and has retained its original histological appearances, makes appreciable substances available. This synthetic material is not contaminated with other gastrointestinal hormones which confounded some prior studies. Since its effect on pancreas size is obvious, this makes for a reliable index of hormone activity. We have demonstrated a maximum trophic effect of CCK on pancreas of the non-tumor-bearing nude mouse at a dosage of 50 µg/kg/dose twice a day (16). As the serum half-life of exogenous CCK is only a few min, we chose this high dose to minimize the chances of overlooking an
effect of this agent on the tumor.

As mentioned previously, some studies have shown that hormonal treatment stimulates growth of some cancers while inhibiting others. A familiar example is breast cancer, where estrogen therapy can cause either flare or regression. This study suggests a retardation in growth of SLU-132 by CCK. Delivery of CCK octapeptide by a different route, schedule, duration, dose, or vehicle (e.g., slow release) might affect tumor growth kinetics differently, as might using another molecular species of CCK. It may be that CCK affects the tumor by modulating the release of other endogenous substances, but the current work obviously cannot answer this question. The best way to determine that a hormone acts directly on its target cell is the in vitro demonstration of its effects, although this does not rule out involvement of intracellular second messengers. Clearly, further and independent studies along these lines are called for. It may then be possible to determine whether response is uniform among cancers with a given level of CCK receptor density.

We have no information as to whether endogenous CCK might affect growth of cholangiocarcinoma. It is possible, however, to manipulate endogenous levels of CCK, for example, by performing cholecystectomy or modifying diet (1, 32). CCK antagonists might also be useful for manipulation of CCK metabolism, and several classes of specific competitive inhibitors exist (dibutyryl cyclic GMP, proglumide, ben佐tript, and CCK 27-32-amide) (9, 15, 29). Administration of anti-CCK antibody (10) provides another attractive way to inhibit the actions of CCK. These lines of evidence suggest that it may be possible to carry out hormonal manipulation for biliary cancer in much the same way as is presently the case for breast or prostatic cancer: with surgery; drugs; diet; etc.

Although CCK has been proposed as a mediator of satiety in several species (30), we saw no such effects with our frequent high-dose i.p. injections of CCK. It may be that CCK does not cross the blood-brain barrier or that the mouse is relatively resistant to this effect of CCK. Final body weights did not differ between groups, thus suggesting the effects on the tumor were not related to dietary differences.

Many provocative tests are used in clinical practice, ranging from exercise electrocardiogram to the glucose tolerance test. Examples of valuable provocative tests in cancer diagnosis would be the calcium and secretin infusion tests for gastrinoma. Provocation of CEA release by secretin and cholecystokinin has been suggested in the diagnosis of pancreatic disease (21), but results were disappointing with pancreatic carcinoma. To our knowledge, this has not been attempted previously in biliary tract cancer. In this study, CCK increased serum CEA levels by 90%.

Tumor tissue immunolabeling showed that more CEA was concentrated along the luminal surface and within the spaces of the neoplastic glands in the CCK-treated group. It could be argued that the increase in serum CEA was due to its production by normal mucine tissue rather than tumor, but since immunofluorescence studies revealed more tumor tissue CEA in the CCK group than in the control group, we believe that CCK may have affected the biology of SLU-132. Naturally, this requires confirmation by in vitro studies in order to exclude the presence of an intermediary messenger. Perhaps, CCK challenge will be useful as a diagnostic test for high-risk patients, either in seeking early detection or in following for residual disease. We are currently pursuing these possibilities in tissue culture experiments and also in human patients with cholangiocarcinoma.

We note the apparent paradox of growth retardation concomitant with increased CEA levels in tumor and blood. It is interesting to speculate that treatment with CCK has led to a uniquely altered metabolism in favor of exportable protein (CEA) at the expense of structural protein (tumor size). The intracellular events which accompany CCK binding are incompletely understood at present, and there is much interest in being able to use CCK challenge to provide information. However, if this is a real effect, further studies are indicated to examine why this paradox occurs.

REFERENCES


Fig. 1: Tumor tissue CEA immunostaining differences for nodules of SLU-32 from CCK and saline groups. CEA was seen at the luminal surface and in the glandular spaces of all tumors. These treated with CCK generally exhibited larger, irregular spaces and heavier immunostaining for CEA (A and B), whereas saline control treated tumors were more homogeneous and CEA-rabbit antiseraum failed to stain (C). × 200.
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