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• *BASIC RESEARCH* •

Presence of CCK-A, B receptors and effect of gastrin and cholecystokinin on growth of pancreatobiliary cancer cell lines

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Abstract

AIM: To investigate the effects of gastrin and cholecystokinin (CCK) and their specific antagonists on the growth of pancreatic and biliary tract cancer cell lines.

METHODS: Five pancreatic and 6 biliary cancer cell lines with 2 control cells were used in this study. Cell proliferation study was done using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test and direct cell count method. Reverse transcription-polymerase chain reaction (RT-PCR) and slot blot hybridization were performed to examine and quantify the expression of hormonal receptors in these cell lines.

RESULTS: SNU-308 showed a growth stimulating effect by gastrin-17, as did SNU-478 by both gastrin-17 and CCK-8. The trophic effect of these two hormones was completely blocked by specific antagonists (L-365, 260 for gastrin and L-364, 718 for CCK). Other cell lines did not respond to gastrin or CCK. In RT-PCR, the presence of CCK-A receptor and CCK-B/gastrin receptor mRNA was detected in all biliary and pancreatic cancer cell lines. In slot blot hybridization, compared to the cell lines which did not respond to hormones, those that responded to hormones showed high expression of receptor mRNA.

CONCLUSION: Gastrin and CCK exert a trophic action on some of the biliary tract cancers.

Key words: Bile duct cancer; Gallbladder cancer; Pancreatic cancer; Gastrin; Cholecystokinin
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INTRODUCTION

Carcinomas of the pancreas and biliary tract remain a challenge to clinicians. The anatomical complexity and late diagnosis of such carcinomas have led to a disappointingly low resectability rate of around 10-20% especially in pancreatic cancer[1]. Moreover, even if it is possible to resect the tumor with clear margin, early recurrence and metastasis are frequently observed. The overall 5-year survival rates are reported as below 15% in pancreatic cancer and 15-50% in other biliary and periampullary cancers[2,3].

No adjuvant treatments have shown success in improving survival in periampullary cancers including pancreatic cancer until now. So with the progress of surgical treatment and early detection methods, new approaches including gene therapy and new chemotherapeutic drugs are definitely required to improve treatment results[4,5]. One such approach might be hormonal manipulation, which is currently accepted as the standard treatment modality for breast, prostate and thyroid cancers.

The gastrointestinal hormones gastrin and cholecystokinin (CCK) are structurally related. The former is known to be a stimulant of acid secretion by gastric mucosa, the latter a stimulant of enzyme secretion by the pancreas and contraction of the gallbladder. While exerting these classical actions, gastrin and CCK are also considered to act as growth stimulants for gastrointestinal malignancies such as gastric and colon cancer[6-9].

However, studies on the trophic effect of these hormones on pancreatic cancer have provided conflicting results. Although some researchers have documented that gastrin and CCK stimulate the growth of pancreatic cancer cells[10,11] and are involved in the early carcinogenesis, others have refuted these effects[12,13]. Few investigations have been performed in the bile duct cancer due to the shortage of cancer cell lines and tumor models in the biliary tract.

In the present study, we investigated the effects of gastrin and CCK on the growth of pancreatic and biliary tract cancer cell lines established at the Cancer Research Institute of Seoul National University College of Medicine,

and the expression of hormonal receptors.

MATERIALS AND METHODS

Cell lines

The human cancer cell lines in the biliary tract and pancreas used in this experiment were SNU-245, SNU-308, SNU-478, SNU-869, SNU-1079, SNU-1196, SNU-213, SNU-324, SNU-410, PANC-1, Mia-PaCa. The SNU series were established at the Korean Cell Line Bank in the Cancer Research Institute of Seoul National University College of Medicine, Seoul, Korea[14,15]. Cell lines PANC-1, Mia-PaCa, LoVo and HT-1080 were obtained from the American Type Culture Collection (Rockville, MD).

SNU-245 was established from distal common bile duct cancer. SNU-478 and SNU-869 were obtained from ampulla of Vater cancer, SNU-1079 and SNU-1196 from upper bile duct cancer, and SNU-308 from gallbladder cancer. SNU-213, SNU-324 and SNU-410 were acquired from pancreatic head cancer. The characteristics of the cell lines are summarized in Table 1.

Cell lines were grown in RPMI 1640 (GIBCO-BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37 ℃ in an atmosphere of 50 mL/L CO_2.

Cell proliferation assay

MTT assay A colorimetric assay using tetrazolium salt, 3-[4,5 dimethyl thiazole-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), was used to determine the hormonal stimulation effect on the cancer cell lines and the optimal stimulation concentration of the hormones. A single cell suspension was prepared and the cell number was calculated. MTT assay was performed as previously described[16].

An equal number of cells were inoculated into each well in 100 μL of serum free culture medium to which gastrin-17 (Sigma, St. Louis, MO) or CCK-8 (Sigma, St. Louis, MO) was added in a range from 10^{-6} mol/L to 10^{-12} mol/L. Twenty μL of gastrin-17 or CCK-8 was added daily to each well in order to maintain the determined hormonal concentration. After five days of culture, 0.1 mg MTT was added to each well and incubated at 37 ℃ for 4 more hours. Plates were centrifuged at 450 g for 5 min at room temperature and the medium was then aspirated. Dimethyl sulfoxide (150 μL) was added to each well to dissolve the crystals. The plates were read immediately at 540 nm on a scanning multi-well spectrophotometer. All experiments were performed 4 times.

We also performed another MTT assay to find the optimal inhibitory concentration of specific antagonists and to observe whether autocrine or paracrine effect of gastrin or CCK could affect the tumor cell growth by application of hormone receptor antagonists in the absence of exogenous ligand. L-364, 718 (antagonist for CCK-A receptor) and L-365, 260 (antagonist for CCK-B/gastrin receptor) were used in a range from 10^{-6} mol/L to 10^{-12} mol/L (10^{-9} mol/L), which were kindly supplied by ML laboratory in UK. Test condition, except for antagonists, was same as above.

Test for optimal antagonist concentration was performed in cell lines, which showed hormone-dependent growth stimulation. Under the optimal growth stimulation concentration of hormones, various concentrations of specific antagonists (range: 10^{-6}-10^{-12} mol/L) were added (data not shown).

Direct cell count Cell lines showing a hormonal growth-stimulating effect in the MTT assay were selected for the direct cell count test to confirm the effect of hormones and whether the hormonal trophic effect on cancer cells could be blocked by specific antagonists with the concentration determined by MTT test.

Cells (30×10^4 for SNU-308; 5×10^4 for SNU-478) were plated onto 25 cm^2 flasks (Falcon, Franklin Lake, NJ) in serum free RPMI 1640 medium. After twenty-four hours, cells were treated with hormone (CCK-8 or gastrin-17), hormonal antagonists (L-364, 718 (10^{-9} mol/L), antagonist for CCK-A receptors; L-365, 260 (10^{-9} mol/L), antagonist for CCK-B/gastrin receptors), combinations of hormones with their antagonist, or medium alone (control). Medium and reagents were added daily. Every third day, we harvested the cells and counted the cell number using a hemocytometer. Each experiment was performed in triplicate.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted according to the manufacturer's instructions (TRIzol®, GIBCO-BRL, Rockville, MD). RNA concentration was measured spectrophotometrically at 260 nm and the integrity of mRNA was controlled by analyzing the ribosomal RNA content by electrophoresis

Table 1 Characteristics of pancreatic cancer and periampullary cancer cell lines

Cell line	*In vivo*			*In vitro*			
	Origin	Degree of differentiation	Nodal status	Growth characteristics	Viability	Doubling time	Cell morphology
SNU-245	CBD	Well	0/9	Ad	85	54	P
SNU-308	GB	Moderately	-	Ad	88	48	P
SNU-478	AoV	Poorly	0/8	Ad	83	52	P/S
SNU-869	AoV	Well	5/10	Ad	91	48	P
SNU-1079	IHD	Moderately	-	Ad	89	72	Ple
SNU-1196	HDB	Moderately	0/1	Ad	94	48	Spindle to P
SNU-213	Pancreas	Moderately	1/8	Ad	89	48	P
SNU-324	Pancreas	Poorly	0/15	Ad+Fl	95	52	P/S
SNU-410	Pancreas	Poorly	-	Ad	92	72	P

CBD: common bile duct, GB: gallbladder, AoV: ampulla of Vater, Ad: adherent, Fl: floating, P: polygonal, S: spherical, Ple: pleomorphic.

on agarose gel.

After quantification of the extracted RNA, first strand complementary DNA (cDNA) of each cancer cell line was synthesized from 2 μg of total RNA using Molony murine leukemia virus reverse transcriptase (GIBCO-BRL, Rockville, MD). PCR reactions were performed in a total of 20 μg, composed of 2 μL cDNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.25 mmol/L dNTPs, 1 μmol/L of each oligonucleotide primer, and 1 U of Taq DNA polymerase (Takara, Shiga, Japan). We used the primers described by Mandair *et al*[17] for CCK-A receptor, CCK-B/ gastrin receptor, and β-actin synthesized in Bioneer Co. (Chungbuk, Korea). The forward primers were 5'-CCTAC GACACCGCCT-CCGC-3', 5'-ACCCCAACGACAGG AAAAGGT-3', and 5'-CACTGTGTTGGCGTACAGGT-3'; the reverse primers were 5'-TCCGTTCTTTCTT CTCTGCCTCCT-3', 5'-TTTGGGAAGGAAGGAG AGGGC-3', and 5'-TCATCACCATTGGCAA TGAG-3' for the CCK-A receptor, CCK-B/gastrin receptor, and β-actin respectively. The amplification reaction involved denaturation at 94 ℃ for 5 min followed by the following cycling: for CCK-A receptor, 40 cycles of denaturation at 94 ℃ for 1 min, annealing for 1 min at 55 ℃ and extension for 1 min 30 s at 97 ℃; for CCK-B/gastrin receptor, 40 cycles of denaturation at 94 ℃ for 1 min, annealing for 1 min at 58 ℃ and extension for 1 min 30 s at 97 ℃; for β-actin, 35 cycles of denaturation at 94 ℃ for 1 min, annealing for 1 min at 55 ℃ and extension for 1 min at 97 ℃.

The reaction proceeded in a DNA thermal cycler (Hybaid, Middlesex, U.K.), and the products of amplification were submitted to electrophoresis on 1.5% agarose gel and visualized with ethidium bromide staining.

Slot blot hybridization

Quantitative gene expression was determined by slot blot analysis. RNA samples were denatured at 68 ℃ for 15 min. Four different concentrations (10, 3, 1, and 0.3 μg) of RNA were loaded on per slot. Blots were prehybridized in buffer (5× SSPE, 30% formamide, 5× Denhardt's solution, 1% SDS and 100 μg/mL salmon sperm DNA) for 2 h at 42 ℃. Overnight hybridization was performed at 42 ℃ with a ^{32}P-radiolabeled probe. The complementary riboprobes were prepared by ligating PCR products for CCK-A receptor, CCK-B/ gastrin receptor and β-actin gene cDNA into the pCR®II-TOPO® vector, using a TA cloning kit (Invitrogen, Carlsbad, CA, USA). The appropriate templates for each RNA probe were generated by linearization with a restriction endonuclease *Eco*RI. Radiolabeling of the probes was performed with [α-^{32}P] deoxycytidine triphosphate using a Prime-It® II random primer labeling kit (Stratagene, La Jolla, CA, USA). After hybridization, the blots were washed twice in 2× SSPE plus 0.2% SDS at 42 ℃ for 20 min, then once with 0.1× SSPE at 42 ℃ for 15 min, and finally subjected to autoradiography with Fuji Super RX® film at -70 ℃. Hybridization with a β-actin RNA probe was used to correct RNA loading. The autoradiograms were analyzed and quantitated by densitometry (BIPS®, Biomedlab, Seoul, Korea).

Statistical analysis

Data were expressed as mean±SE. Comparison between groups was made using Mann-Whitney *U* test, $P<0.05$ was considered statistically significant.

RESULTS

MTT assay

The SNU-308 cell line originating from gallbladder cancer responded to the addition of gastrin-17, and in particular showed a maximum growth stimulating effect at the gastrin-17 concentration of 10^{-9} mol/L. At this concentration 27% of the growth stimulating effect could be seen compared to the control (Figure 1A). SNU-478 from ampulla of Vater cancer exhibited similar stimulating effects to both gastrin-17 (21%) and CCK-8 (23%) (Figure 1B). However, no significant differences between control and hormone treated cultures were observed at any concentration in any of the other cell lines. MIAPaCa-2 and PANC-1 showed similar results to those of the other pancreatic cell lines (Tables 2, 3).

Suppression of cell growth by hormonal receptor antagonists (without exogenous hormone) was not definite

Table 2 Effect of gastrin and L-365,260 on periampullary cancer cell lines

Cell line	Origin	Maximum response to hormones and hormone concentration					
		Gastrin			L-365, 260		
		Response (%)	Concentration (mol/L)	*P*-value	Response (%)	Concentration (mol/L)	*P*-value
SNU-245	CBD	108	10^{-7}	NS	98	10^{-8}	NS
SNU-308	GB	127	10^{-9}	0.038	96	10^{-9}	NS
SNU-478	AoV	121	10^{-9}	0.047	94	10^{-9}	NS
SNU-869	AoV	107	10^{-6}	NS	103	10^{-7}	NS
SNU-1079	IHD	107	10^{-9}	NS	104	10^{-11}	NS
SNU-1196	HDB	104	10^{-8}	NS	93	10^{-7}	NS
SNU-213	Pancreas	105	10^{-10}	NS	97	10^{-8}	NS
SNU-324	Pancreas	112	10^{-11}	NS	97	10^{-9}	NS
SNU-410	Pancreas	115	10^{-12}	NS	98	10^{-8}	NS
MIAPaCa-2	Pancreas	106	10^{-10}	NS	95	10^{-9}	NS
PANC-1	Pancreas	101	10^{-7}	NS	94	10^{-8}	NS

Table 3 Effect of CCK and L-364,718 on periampullary cancer cell lines

Cell line	Origin	Maximum response to hormones and hormone concentration					
		CCK			L-364, 718		
		Response (%)	Concentration (mol/L)	*P*-value	Response (%)	Concentration (mol/L)	*P*-value
SNU-245	CBD	110	10^{-12}	NS	96	10^{-10}	NS
SNU-308	GB	109	10^{-7}	NS	94	10^{-8}	NS
SNU-478	AoV	123	10^{-9}	0.043	93	10^{-9}	NS
SNU-869	AoV	107	10^{-10}	NS	97	10^{-10}	NS
SNU-1079	IHD	113	10^{-9}	NS	101	10^{-8}	NS
SNU-1196	HDB	109	10^{-8}	NS	94	10^{-8}	NS
SNU-213	Pancreas	106	10^{-7}	NS	98	10^{-6}	NS
SNU-324	Pancreas	116	10^{-12}	NS	96	10^{-10}	NS
SNU-410	Pancreas	113	10^{-9}	NS	94	10^{-8}	NS
MIAPaCa-2	Pancreas	108	10^{-10}	NS	97	10^{-9}	NS
PANC-1	Pancreas	105	10^{-8}	NS	98	10^{-8}	NS

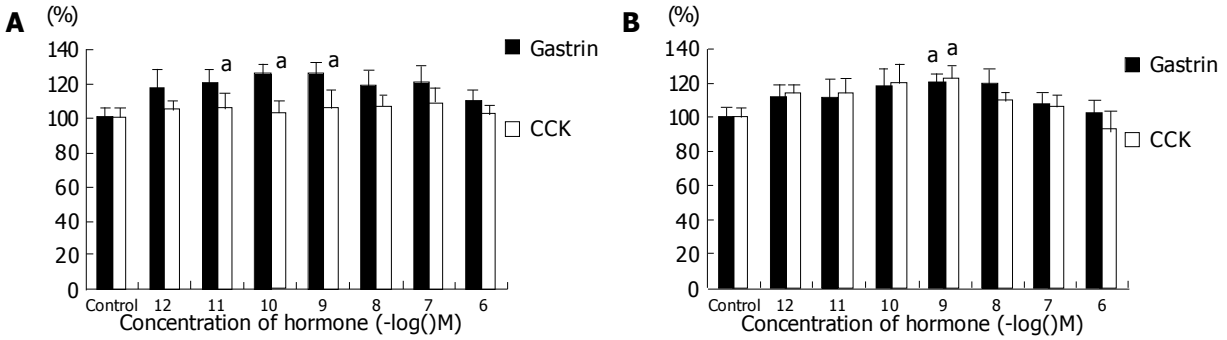

Figure 1 Effects of gastrin and CCK on SNU-308 from gallbladder cancer (A), SNU-478 from ampulla of Vater cancer (B). [a]*P*<0.05 *vs* control.

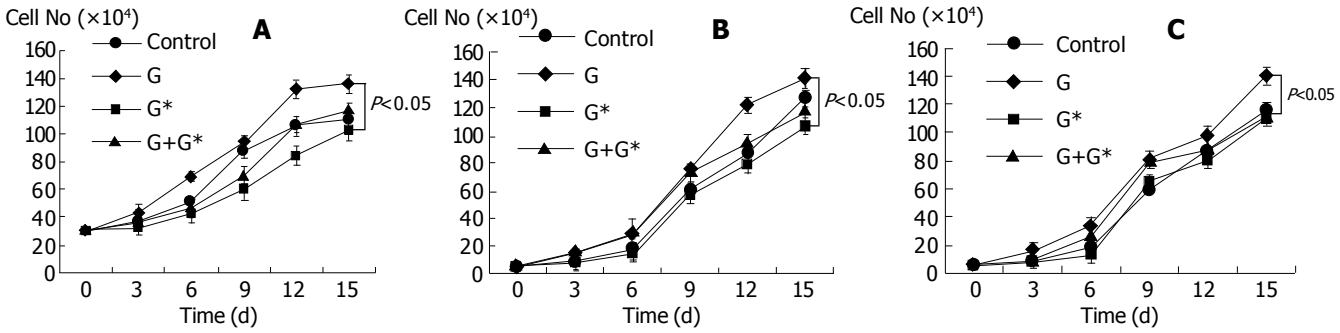

Figure 2 Growth curves for SNU-308 (A), and SNU-478 (B, C) cells grown with either hormone or hormone combined with antagonist or antagonist, or medium only. G: gastrin-17, G*: antagonist for CCK-B/gastrin receptor (L-365,260), C: CCK-8, C*: antagonist for CCK-A receptor (L-364,718).

in all pancreatobiliary cancer cell lines (Tables 2, 3).

Effects of hormonal antagonists

The stimulatory effect of gastrin-17 exposure on the growth rate of SNU-308 cells was blocked by the specific antagonist L-365, 260. The growth stimulation effects of both the hormones on SNU-478 were blocked by their respective specific antagonists (Figure 2).

RT-PCR for CCK-A and CCK-B/gastrin receptors

Amplification of cDNA yielded an approximately 340 bp fragment from the CCK-A receptor and 430 bp fragment from the CCK-B/gastrin receptor (Figure 3). Sequence analysis confirmed that each kind of PCR products was identical to that of the expected sequence[18,19]. CCK-A receptor and CCK-B/gastrin receptor mRNA were detected in all biliary and pancreatic cancer cell lines. We utilized the Mia PaCa-2 cell line as a positive control for the expression of CCK-A receptor[17] and the LoVo cell line for the CCK-B/gastrin receptor[20].

Slot blot assay for CCK-A and CCK-B/gastrin receptors

CCK-A and CCK-B/gastrin receptor mRNA levels were measured by slot blot hybridization. The autoradiographic signals were compared with those of Mia PaCa-2 (for CCK-A receptor) and LoVo (for CCK-B/gastrin receptor). Figure 4 presents the slot blot assay results. SNU-308 and SNU-478 demonstrated a high expression of receptor mRNA compared with other cell lines that did not respond to

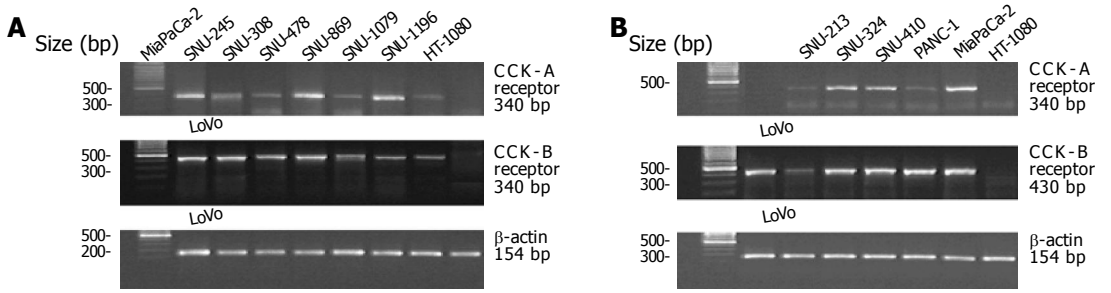

Figure 3 Amplification of CCK-A and CCK-B receptor mRNA in human biliary tract cancer cell lines (A) and pancreatic cancer cell lines (B) by RT-PCR.

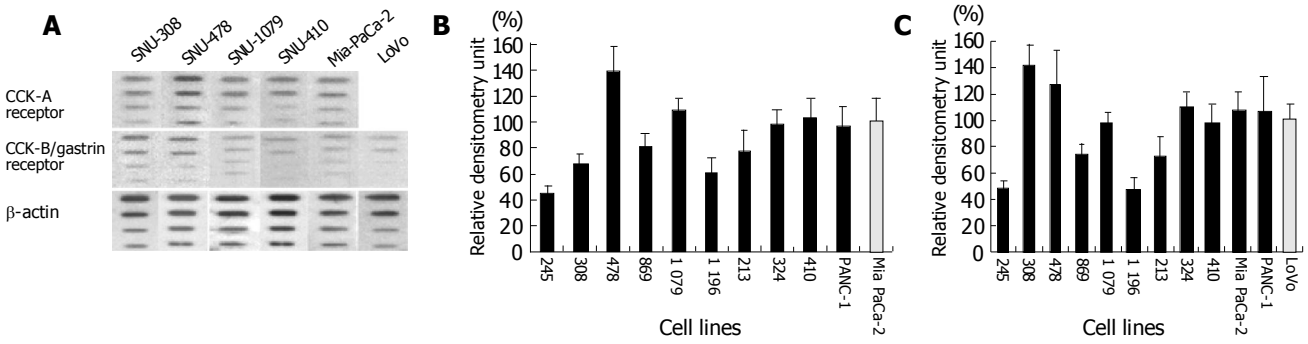

Figure 4 Representative slot blot hybridization. (A) Data from SNU-308, 478, 1079, 410, Mia PaCa-2, and LoVo cell lines. (B, C) Densitometrically quantified slot blots.

hormones.

DISCUSSION

Gastrin and CCK, which share a peptide structure including C-terminal pentapeptide amide (Gly-Trp-Met-Asp-PheNH2), exert a trophic effect on the normal gastrointestinal organs, including the stomach, gallbladder and duodenum, in addition to the stimulation of secretion in the stomach and pancreas[21-23]. Except for these classical actions, gastrin and CCK also appear to stimulate growth of gastrointestinal cancers. Chronic endogenous hypergastrinemia and exogenous pentagastrin administration are known to significantly increase tumor cell number and the concentration of DNA, RNA, and protein in colon and gastric cancers[6,24,25].

Similar trophic effects of gastrin and CCK have been reported in pancreatic cancer. Smith *et al*[10] reported that the growth responses of 6 human pancreatic cancer cell lines (SW-1990, PANC-1, MIA PaCa-2, BxPC-3, RWP-2 and CAPAN-2) are stimulated by CCK in serum-free medium, and its trophic effect can be blocked by a specific antagonist[26]. They also demonstrated that gastrin exerts a growth-stimulating effect on pancreatic cancer *in vivo* and *in vitro*[27]. Furthermore many reports about the trophic effect of CCK and gastrin on pancreatic cancer have been published[11,28-30] and some studies have demonstrated that these hormones play a role in gastrointestinal cancer carcinogenesis[31,32]. On the contrary, other studies have suggested that gastrin and CCK may have no trophic effect on pancreatic cancer, and even exert an inhibitory effect on pancreatic growth and carcinogenesis.

Liehr *et al*[12] reported that CCK cannot affect the growth of PANC-1 and MIA PaCa-2 at the concentration of 10^{-12}-10^{-6} mol/L. Robertson *et al*[13] showed that gastrin also has no trophic effect on the same pancreatic cancer cell lines. Recently, Detjen *et al*[33] demonstrated that CCK-A and CCK-B/gastrin receptors mediate growth inhibitory responses in pancreatic cancer in their experiment on the transfection of hormone receptors into the PANC-1 and MIA PaCa-2 lines.

In the experiment with cholangiocarcinoma cells (SLU 132), the growth of cancer xenografted in nude mice is significantly retarded by CCK[34]. Evers *et al*[35] demonstrated that CR-1409, a CCK receptor antagonist, prevents caerulein-mediated inhibition of cancer growth when combined with caerulein administration in SLU 132. Though a few cancer cell lines have been established in the biliary tract, there are limitations in the inhibitory effect of CCK on bile duct cancer as described above. In our study, cancer cell lines originating from the biliary tract exhibited a growth stimulating effect by gastrin or CCK. Therefore, further study using well-established cancer cells is mandatory to determine the hormonal trophic effect on biliary tract cancer.

We found that the trophic action of hormones was influenced by the degree of expression of its receptors, suggesting that biological response to peptide hormones is modulated by the amount of receptors in target cells. Some investigators explained that the trophic response of cancer to gastrin and CCK is determined by the presence of specific hormonal receptors, so that the responsiveness of tumor to hormone treatment can be predicted by the presence or absence of the receptor[7]. However, this explanation does not account for the conflicting results of stimulatory or

inhibitory effect on the same cancer cells according to the investigators. In our study, most pancreatic and biliary cancer cell lines have CCK-A and CCK-B receptors. However, only 2 cell lines demonstrated tumor growth-stimulating effect by gastrin (SNU-308, SNU-478) and only one cell line exhibited tumor growth-stimulating effect by CCK (SNU-478). PANC-1 and Mia PaCa-2, the most prevalently studied cell lines in similar experiments, did not show any growth-stimulating effects by gastrin and CCK though both CCK-A and CCK-B/gastrin receptors presented. This non-response to exogenous hormone can be explained that endogenous hormonal stimulation is enough for the trophic action. However, we could not find sufficient endogenous growth stimulation, which is contrary to some reports[36]. We think endogenous stimulation effect is minimal even if it exists.

Another possibility is the diversity or mutation of specific hormonal receptors. Peptide hormones express their biological activity by binding to specific hormone receptors. Receptors for CCK have been pharmacologically classified on the basis of their affinity for both the peptide agonists CCK and gastrin, which share the same COOH-terminal pentapeptide amide sequence but differ in sulfation at the 6th (gastrin) and 7th (CCK) tyrosyl residue, and the recently developed subtype-specific antagonists.

Although two types of CCK receptors (CCK-A and CCK-B/gastrin) are well known, the possibility of new non-A non-B receptor types has recently been reported. Imdahl *et al*[37] reported that the expression of low affinity binding gastrin/CCK-C receptor is found in 75% of human colorectal carcinomas. Smith *et al*[38] recently showed that the new CCK receptor is expressed in pancreatic cancer specimens and they therefore designated it as CCK-C (cancer) receptor.

Recent advances in molecular biology reveal a broader distribution of CCK receptors in the gastrointestinal and central nervous systems than previously recognized, thereby suggesting additional physiological roles for these receptors. Although CCK receptors feature homology in structure among different species and even intra-species, slight differences in receptor structure and distribution result in significant pharmacological and physiological differences[39,40]. Minor peptide changes, even the difference of a single amino acid by mutation or polymorphism of the CCK receptor, can profoundly affect the binding affinities of hormones and antagonists, enabling antagonists to act as agonists and vice versa[41,42]. Actually, the functional effect and clinical significance of receptor mutations have been studied in some diseases[43].

Based on the present study, it may be concluded that specific receptors for gastrin and CCK exert a trophic action on some of the biliary tract cancers. However, many cancer cell lines cannot be affected by hormones despite the presence of CCK-A and CCK-B/gastrin receptors, suggesting the possibility of hormonal manipulation in limited cases of pancreatic and biliary tract cancer. An accurate method for the identification of hormonally responsive cancers is therefore required before adjunctive hormonal or antihormonal therapy can be recommended. Therefore, further investigations are required to elucidate the mechanism of the secondary signal pathway linked to the CCK receptor family, and to determine the functional and structural differences among the receptors. Furthermore, mutation or polymorphism studies of CCK receptors may be needed to ascertain the trophic or inhibitory effect of gut hormones.

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