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Antiproliferative Effect of a Novel Cholecystokinin-B/Gastrin Receptor Antagonist, YM022

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Cholecystokinin (CCK)-B and gastrin receptors are expressed on a variety of human tumor cells. Recently, we have demonstrated that the human brain CCK-B receptors are identical to the gastrin receptors derived from the stomach mucosa, and that the brain-gut peptides, CCK-8 and gastrin I are mitogenic for mouse NIH 3T3 fibroblasts expressing human CCK-B/gastrin receptors (N-hCCKBR). In this report, we evaluated the antiproliferative potency of CCK-B/gastrin receptor antagonists by using N-hCCKBR cells. Among several antagonists, a benzodiazepine derivative, YM022 had the most potent activities in competing with [^{125}I]CCK-8 or [^{125}I]gastrin I binding, inhibition of CCK-8- or gastrin I-induced phosphoinositide hydrolysis and increasing cytoplasmic free calcium. Interestingly, a potent antagonist for rat CCK-B/gastrin receptors did not have such activities in N-hCCKBR cells. YM022 inhibited the CCK-8- or gastrin I-induced [*methyl*-^{3}H]thymidine incorporation of N-hCCKBR cells in a dose-dependent manner. In the absence of exogenous peptide ligands, YM022 also inhibited the proliferation of several human cancer cell lines expressing the genes for both gastrin and its receptor. These results suggest that YM022 could intervene in the autocrine stimulation of human tumor cell lines through CCK-B/gastrin receptors. N-hCCKBR cells are an excellent tool to screen for novel human CCK-B/gastrin receptor antagonists possessing antiproliferative activity for human cancer cells.

Key words: Cholecystokinin — Gastrin — Autocrine — Receptor antagonist — Antiproliferative effect

Cholecystokinin (CCK)-B/gastrin receptors are expressed in human tumor cell lines derived from stomach,[1] lung,[2,3] colon[1,4] or T-cell lymphoma.[5] It has been reported that some of these tumor cells were induced to proliferate with gastrin *in vitro* or *in vivo*.[6,7] Recently, we and others have cloned gastrin receptor cDNAs from the canine gastric mucosa, rat pancreas and a *Mastomys* enterochromaffin-like cell-derived carcinoid tumor, and shown that the gastrin receptor possesses the common features of G protein-coupled receptors.[8–10] Moreover, the human CCK-B receptor cloned from the brain has been shown to be identical to the gastrin receptor of the stomach mucosa.[11–13] CCK-8 or gastrin I alone has a trophic effect in Chinese hamster ovary cells and NIH 3 T3 cells transfected with the human CCK-B/gastrin receptor.[13,14] Since the CCK-B/gastrin receptor is thought to be involved in human tumor cell growth, a specific receptor antagonist could be a potent anti-cancer drug.

The affinities of CCK-B/gastrin receptors for non-peptide antagonists as well as peptide ligands are distinct among different animal species.[10,15,16] Therefore, it is necessary to screen CCK-B/gastrin antagonists by using human CCK-B/gastrin receptors. However, it is not easy to purify human cells expressing only CCK-B/gastrin receptors. The brain also expresses another CCK receptor isoform, designated the CCK-A receptor.[17] Moreover, cells expressing gastrin receptors represent only a minor population in the stomach mucosa.[18] Here, we report the establishment of a very useful system to investigate the antiproliferative effects of CCK-B/gastrin receptor antagonists by using a mouse fibroblasts cell line expressing human CCK-B/gastrin receptors. We also present data indicating possible involvement of an autocrine mechanism through CCK-B/gastrin receptors in the proliferation of several human tumor cell lines.

MATERIALS AND METHODS

Chemicals CCK-8 and gastrin I were purchased from Peninsula (Belmont, CA). Several CCK-B/gastrin receptor antagonists, YM022,[19] L365,260[20] and tetronothiodin[21] were kindly provided by Yamanouchi Pharmaceutical Co., Ltd. (Tsukuba), Merck & Co., Inc. (Rathway, NJ) and Nippon Roche (Kamakura), respectively. YM022 and L365,260 were dissolved in DMSO at 100 m*M*. Less than 0.1% DMSO (final concentration) does not interfere with the experiments.

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Cells A mouse fibroblast cell line, NIH 3T3 and its transfectant expressing human CCK-B/gastrin receptors[14] were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% calf serum (ICN Flow, Costa Mesa, CA) in a 95% air, 5% CO_2 atmosphere at 37°C. Human tumor cell lines of human small cell lung cancer (NCIH 510A), human colon cancer (COLO201, COLO320DM, WiDr, HCT116) and human gastric cancer (AGS) were obtained from ATCC (Rockville, MD). A human tumor cell line of human myeloid leukemia (U-937) was obtained from Fujisaki Cell Center (Hayashibara Biochemical Laboratories, Inc., Okayama). Each cell line was maintained in RPMI 1640 medium (Gibco) (NCIH510 A, COLO201, COLO320DM, U-937), MEM with non-essential amino acids (Gibco) (WiDr), McCoy's 5A medium (ICN Biomedicals Japan, Tokyo) (HCT116) or Ham's F-12 medium (Gibco) (AGS) supplemented with 10% fetal bovine serum, respectively.

Radioligand binding assays For binding studies, cells (4×10^4 cells/well) were inoculated in 250 μl of DMEM with 0.1% bovine serum albumin using 24-well plates. ^{125}I-Labeled CCK-8 or ^{125}I-labeled gastrin I (70 p*M*) (Amersham, Buckinghamshire, UK) was added to each well in the presence or absence of increasing concentrations of unlabeled CCK-8, gastrin I or receptor antagonists (1 p*M*–1 μ*M*) at 24°C for 60 min. The cells were then washed three times with DMEM containing 0.1% bovine serum albumin and lysed in 1 *N* sodium hydroxide. The radioactivities of lysates were determined by using a gamma counter (LKB/Pharmacia Biotech) as described.[13]

Measurement of intracellular calcium concentration $[Ca^{2+}]_i$ Intracellular calcium concentration was measured with a fluorescent Ca^{2+} indicator, fura-2-AM. Cells grown on 22 mm diameter glass coverslips in 24-well plates were incubated with 1 m*M* fura-2/acetoxymethyl ester for 30 min at 37°C. Then, cells were washed and incubated in Krebs-Ringer medium buffered with HEPES (125 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* $MgSO_4$, 1.2 m*M* KH_2PO_4, 2 m*M* $CaCl_2$, 6 m*M* glucose, 25 m*M* HEPES, pH 7.4) in the presence or absence of increasing concentrations of antagonists (100 p*M*–1 μ*M*). The cytoplasmic Ca^{2+} concentration was calculated from the fluorescence ratio at 340 nm/380 nm as described previously.[22]

Inositol phosphate determination Serum-starved cells were prelabeled for 24h with [*myo*-^{3}H]inositol (1 μCi/ml; 10–20 Ci/mmol, Amersham). Cells were washed twice with serum-free DMEM, and then stimulated with various concentrations of gastrin I or CCK-8 with or without non-peptide antagonists for 30 min at 37°C in the presence of 10 m*M* LiCl. The reaction was stopped by adding ice-cold methanol. Total inositol phosphates were separated by using a Dowex AG 1-X8 column (Bio-Rad, Richmond, CA) with 1*M* ammonium formate and 0.1 *M* formic acid.[23]

[*methyl*-^{3}H]Thymidine incorporation Serum-starved confluent N-hCCKBR or human tumor cell lines were inoculated in 96-well plates in DMEM containing insulin (5 μg/ml) and transferrin (5 μg/ml). The cells were cultured with CCK-8 or gastrin I in the presence or absence of various concentrations of antagonists for 24 h or 48 h. In the last 4 h, the cells were pulsed with [*methyl*-^{3}H]thymidine (1 μCi/ml; 25 mCi/mmol, Amersham). The radioactivity of [*methyl*-^{3}H]thymidine incorporated in 5% trichloroacetic acid-insoluble precipitates was counted by using a scintillation counter (Top Count, Packard, Meriden, CT).[23]

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNAs of human tumor cell lines were extracted by a CsCl-cushioned ultracentrifugation method.[23] The cDNA templates for RT-PCR analyses were prepared by reverse transcription of total RNA (1 μg) using the Superscript™ Preamplification System (GIBCO/BRL, Life Technologies, Inc., Gaithersburg, MD).[24] PCR reactions were performed in a reaction mixture (total 25 μl) containing 5 μl of the cDNA, 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.1 μl/μg bovine serum albumin, 200 μ*M* each deoxynucleotide triphosphate, 20 p*M* each specific primer (CCK-B/gastrin receptor sense primer: 5′-TCACCAATGCCTTCCTCCTCTCACTGGCAG-3′, antisense primer: 5′-TTGGCTGTCGCTGTCACTGTCGCCGTCAAA-3′; human gastrin sense primer; 5′-GGCCGAAGTCCATCCATCCATAGGCTTC-3′, antisense primer; 5′-TGCAGCGACTATGTGTGTATGTGCTGATCT-3′)[16,25] and 1.25 units of Taq DNA polymerase (Promega, Madison, WI). These primer pairs amplified fragments that crossed an intron, thereby distinguishing between cDNA and contaminating genomic DNA in terms of the size of the expected fragments after amplification.[26] The reaction conditions were as follows; denaturation at 94°C for 30 sec, annealing at 72°C for 1 min and extension at 72°C for 1 min. The reaction was carried out for 35 cycles in a Gene Amp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT).

Southern blot analysis To confirm amplification of the appropriate DNA fragments, the RT-PCR products (8 μl of a 25 μl reaction mixture) were electrophoresed in a 1% agarose gel and transferred onto nitrocellulose filters. The filters were hybridized at 42°C in a buffer containing 50% (wt/vol) formamide and a ^{32}P-labeled full-length cDNA probe.[13] After 16 h hybridization, the filters were washed twice for 15 min in 2×SSC, 0.1% SDS at room temperature, and twice for 15 min in 0.1×SSC, 0.1% SDS at 55°C, and then subjected to autoradiography.[14]

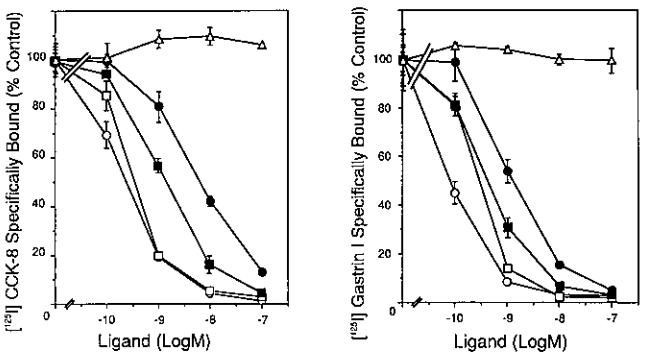

Fig. 1. Competition curves of [^{125}I]CCK-8 or [^{125}I]gastrin I binding in N-hCCKBR cells. Results are expressed as percentage of the maximal binding (mean±SD, n=3). [^{125}I]CCK-8 binding (left panel) or [^{125}I]gastrin I binding (right panel) displaced by CCK-8 (○), gastrin I (●), YM022 (□), L365,260 (■) and tetronothiodin (△).

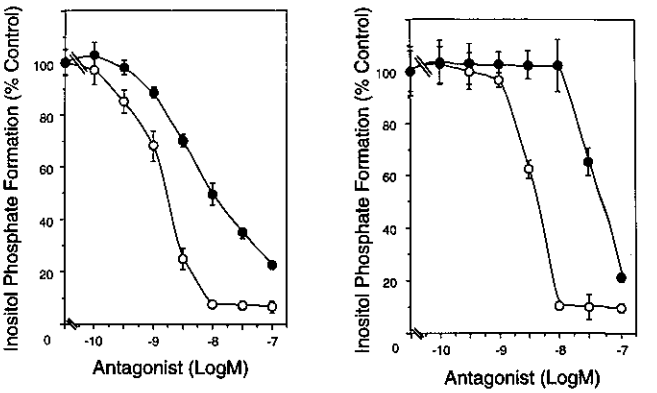

Fig. 2. Inhibition of CCK-8- and gastrin I-induced phosphoinositide hydrolysis by receptor antagonists. Results are expressed as the percentage of the maximal total inositol formation (mean±SD, n=3). N-hCCKBR cells were stimulated by gastrin I (5 n*M*) (left panel) or CCK-8 (5 n*M*) (right panel) in the presence of YM022 (○) or L365,260 (●).

RESULTS

Competitive binding of various receptor antagonists versus [^{125}I] peptide ligands in N-hCCKBR cells By using NIH 3T3 fibroblasts expressing human CCK-B/gastrin receptors, designated N-hCCKBR, we compared the competitive binding of various receptor antagonists, YM022, L-365,260 and tetronothiodin to human CCK-B/gastrin receptors. The competition curves were generated by measuring the ability of unlabeled CCK-8, gastrin I or antagonists to displace [^{125}I]CCK-8 or [^{125}I]gastrin I bound to N-hCCKBR cells (Fig. 1). The IC_{50}s of YM022 for [^{125}I]CCK-8 and [^{125}I]gastrin I were 0.4 n*M* and 0.4 n*M*, respectively. These values are greater than those obtained with a well-known CCK-B receptor antagonist, L-365,260 (the IC_{50}s of L365,260 were 1.7 n*M* and 0.6 n*M*, respectively). In contrast, tetronothiodin, which has been shown to be a potent antagonist for the rat CCK-B receptor, did not displace [^{125}I]CCK-8 or [^{125}I]gastrin binding even at 1 μM. These results seem to reflect the species specificity of CCK-B/gastrin receptors in the binding of non-peptide receptor antagonists. Miyake *et al.* reported the same results in COS-7 expressing CCK-B/gastrin receptors.[27] YM022 is the most potent antagonist specific for human CCK-B/gastrin receptors among the antagonists we examined.

Inhibition of peptide ligand-induced intracellular signaling by YM022 To examine whether YM022 could inhibit intracellular signaling through human CCK-B/gastrin receptors, we next investigated the effects of YM 022 on peptide ligand-induced phosphoinositide hydrolysis (Fig. 2). The increase of phosphoinositide hydrolysis induced by 5n*M* CCK-8 or gastrin I was inhibited by YM 022 or L365,260 in a dose-dependent manner. The IC_{50}s of YM022 for CCK-8 and gastrin I were 4.7 n*M* and 1.9 n*M*, respectively. These values were significantly better than those of L365,260 (54 n*M* and 10 n*M*).

We further investigated the effect of YM022 on the increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by the peptide ligands (Fig. 3). The $[Ca^{2+}]_i$ of N-hCCKBR cells was remarkably elevated by the addition of 10 n*M* gastrin I. In the presence of YM022 or L-365,260 the gastrin-induced $[Ca^{2+}]_i$ increase was inhibited. While 100 n*M* YM022 was enough to inhibit the $[Ca^{2+}]_i$ increase induced by 10 n*M* gastrin I, 1 μM L-365,260 was necessary for complete inhibition.

YM022 inhibits the CCK-8- or gastrin I-induced DNA synthesis of N-hCCKBR cells We previously reported that CCK-8 or gastrin I could increase the DNA synthesis and cell growth of N-hCCKBR cells in a dose-dependent manner.[14] Thus, we examined whether YM022 or L-365,260 has an inhibitory effect on the ligand-induced DNA synthesis of N-hCCKBR cells in serum-free medium. CCK-8 at 1 n*M* or gastrin I at 10 n*M* significantly increased DNA synthesis as assessed by [*methyl*-^{3}H] thymidine incorporation. YM022 or L-365,260 inhibited the peptide ligand-induced [*methyl*-^{3}H]thymidine incorporation in a dose-dependent manner (Fig. 4). The IC_{50}s of YM022 for CCK-8 and gastrin I were 6.1 n*M* and 1.4 n*M*, respectively. In contrast, the IC_{50}s of L365,260 were more than 100 n*M* and 83 n*M*, respectively. We examined the toxicity of YM022 with NIH 3T3 cells, and found that thymidine incorporation into NIH 3T3 was unaffected by YM022. The antiproliferative potencies of YM022 and L365,260 were consistent with the inhibitory effects on the ligand-induced intracellular signaling described above.

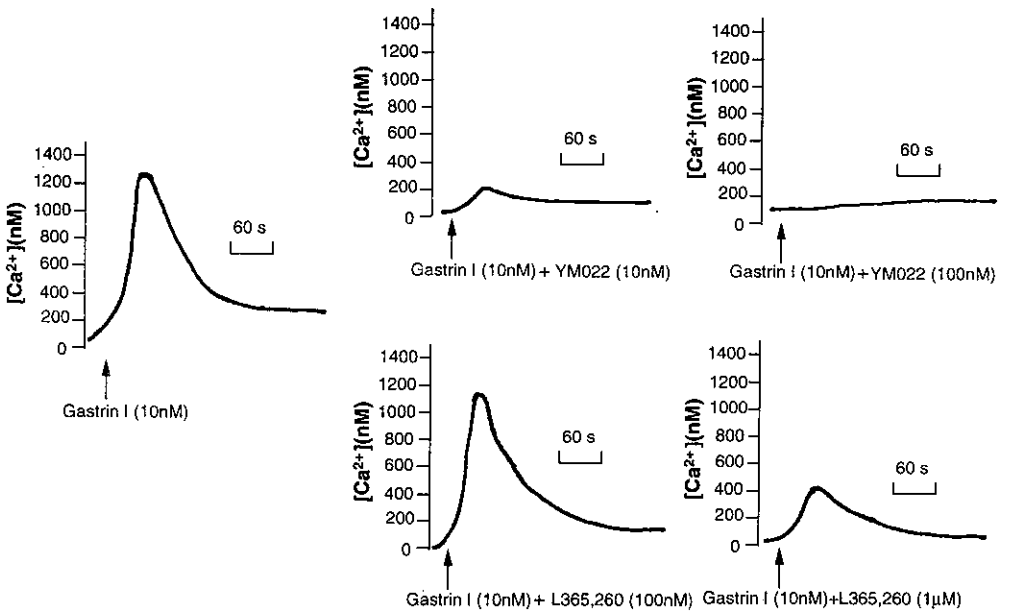

Fig. 3. Inhibition of gastrin I-induced $[Ca^{2+}]_i$ increase by receptor antagonists. N-hCCKBR cells were stimulated by gastrin I (10 n*M*) in the presence of various concentrations of receptor antagonist, YM022 or L365,260.

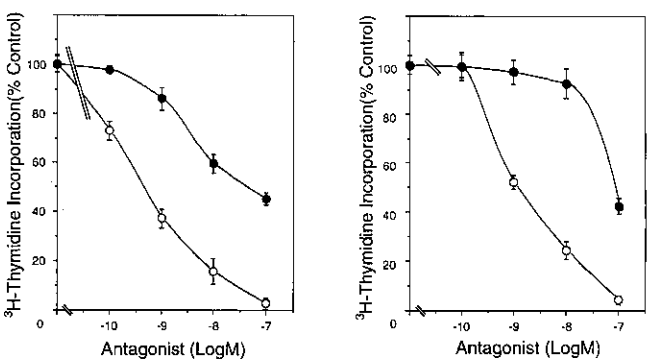

Fig. 4. Inhibition by receptor antagonists of the CCK-8- and gastrin I-induced DNA synthesis of N-hCCKBR cells. Cells were serum-starved for 6 h and then stimulated with CCK-8 (1 n*M*) (left panel) or gastrin I (10 n*M*) (right panel) for 24 h in the presence or absence of YM022 (○) or L365,260 (●). DNA synthesis was assessed in terms of the incorporation of [*methyl*-^{3}H]thymidine in the last 4h of incubation. Results are expressed as percentage of the maximal [*methyl*-^{3}H]thymidine incorporation (mean±SD, n=4).

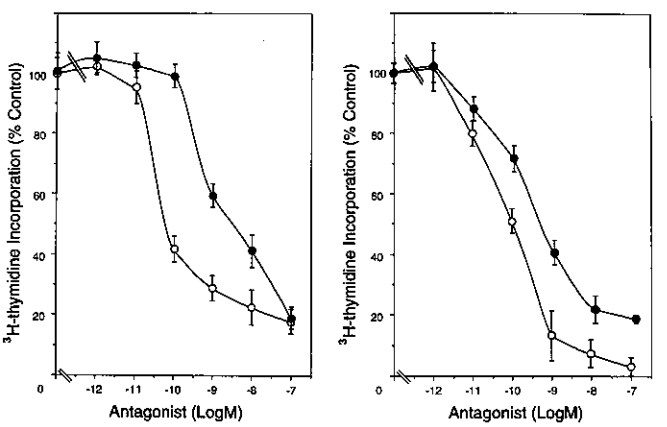

Fig. 5. YM022 inhibited the DNA synthesis of NCIH510A cells. The cells were serum-starved for 24 h and then stimulated with CCK-8 (0.1 n*M*) (left panel) or gastrin I (1 n*M*) (right panel) for 48 h in the presence or absence of YM022 (○) or L365,260 (●). Results are expressed as percentage of the maximal [*methyl*-^{3}H]thymidine incorporation (mean± SD, n=4). The maximal incorporations stimulated by CCK-8 and gastrin I were 13000±1490 cpm and 11400±835 cpm, respectively. The control incorporation was 9010±116 cpm.

A possible autocrine mechanism though CCK-B/gastrin receptors in the growth of human tumor cell lines The growth of a human small cell lung cancer cell line, NCIHG510A, was reported to be stimulated by gastrin I.[2,3] The incorporation of [*methyl*-^{3}H]thymidine into NCIH510A cells was increased by 0.1 n*M* CCK-8 as well as 1 n*M* gastrin I (Fig. 5 legend). The ligand-induced incorporation of [*methyl*-^{3}H]thymidine into NCIH510A

Table I. YM022 Inhibited the DNA Synthesis of Human Tumor Cells

Cell line	Control	YM022
AGS	2490±126	1870±103*
COLO201	19900±1670	14000±1680*
COLO320DM	8240±454	6230±434*
WiDr	2840±224	2160±36.1**
HCT116	2360±98.5	2190±36.1**
NCIH510A	13200±708	8420±716*
U-937	2150±169	2123±118

Results are expressed as [*methyl*-^{3}H]thymidine incorporation (cpm±SD) of triplicate samples in the absence (Control) or presence of YM022 (10 n*M*). * $P<0.01$, ** $P<0.02$ in *t* test.

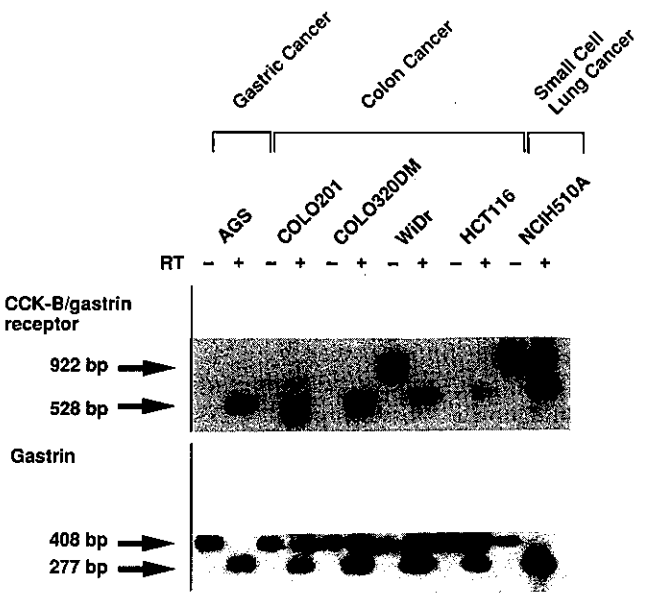

Fig. 6. Expression of gastrin and its receptor mRNAs in human tumor cell lines. Total RNAs extracted from AGS, COLO201, COLO320DM, WiDr, HCT116 and NCIH510A were subjected to RT-PCR analysis. RT-PCR generated 528 bp and 277 bp bands from mRNAs and 922 bp and 408 bp fragments from genomic DNA.

cells was also inhibited by YM022 and L365,260 in a dose-dependent manner similar to that of N-hCCKBR cells (Fig. 5).

In addition, YM022 significantly inhibited the [*methyl*-^{3}H]thymidine incorporation of several human cancer cell lines, AGS, COLO201, COLO320DM, WiDr, HCT116 and NCIH510A grown in serum-free medium without the addition of CCK-8 or gastrin I (Table I). These results suggest a possible autocrine mechanism causing stimulation of DNA synthesis of these tumor cells through CCK-B/gastrin receptors.

These observations led us to examine the expression of CCK-B/gastrin receptor and gastrin mRNA in the tumor cells. Southern blot analysis of RT-PCR products was performed to confirm the expression of CCK-B/gastrin receptor and gastrin transcripts in the tumor cells. Both CCK-B/gastrin receptor mRNA and gastrin mRNA were detectable in all these tumor cells (Fig. 6). The signals amplified from contaminating DNA seem to be stronger in the reactions without RT. This phenomenon was also noted in our previous report.[26] One possible explanation is that c-DNAs might be amplified more easily than genomic DNAs, because c-DNAs are shorter and "cleaner" than genomic DNAs.

These results support the hypothesis that gastrin is involved in an autocrine system for the growth of these tumor cells.

DISCUSSION

Acid secretion through proton pumps is regulated by gastrin, histamine (H_2) and muscarine cholinergic receptors on parietal cells in the stomach mucosa.[28,29] Antagonists for histamine receptors, as well as proton pump inhibitors, are potent anti-ulcer drugs. However, they elevate serum gastrin levels by a feedback mechanism.[30,31] Hypergastrinemia may not only induce gastric mucosal hypertrophy, but also elevate the frequency of gastric carcinoid tumor *in vivo*.[30–33]

Moreover, the gastrin receptor, which is identical to the CCK-B receptor in the brain, has been confirmed to stimulate cell growth in a ligand-dependent manner.[13] Thus, the hypergastrinemia induced by the anti-ulcer drugs might accelerate the growth of tumor cells expressing the CCK-B/gastrin receptor *in vivo*. Therefore, a specific CCK-B/gastrin receptor antagonist with antiproliferative potency would be very valuable as an anticancer drug, as well as an anti-ulcer drug. Indeed, several antagonists of CCK-B/gastrin receptors have been reported to inhibit the ligand-induced proliferation of tumor cell lines *in vitro*.[1,34–37]

Very recently, it was demonstrated that the binding affinities of peptide ligands and non-peptide antagonists for CCK-B/gastrin receptors are distinct among different species.[15,16] Between human and canine CCK-B/gastrin receptors, the different binding affinities were caused by a single amino acid change in the transmembrane domain of the receptors.[15] The species-specific binding affinity of peptide ligands was not derived from receptor isoforms generated by alternative splicing.[16] These characteristics of CCK-B/gastrin receptors led us to reevaluate the binding specificity of the receptor antagonists by using cells expressing human CCK-B/gastrin receptors, be-

cause all the CCK-B/gastrin receptor antagonists had been screened in other species. Indeed, a potent antagonist, tetronothiodin,[21] which was isolated by using rat cerebral cortex membrane, did not bind human CCK-B/gastrin receptors as demonstrated in this study.

In this report, we used a mouse fibroblast cell line transfected with a human CCK-B/gastrin receptor cDNA expression vector. The human receptors expressed on the transfectants could couple with intracellular signaling pathways in a ligand-dependent manner. Moreover, cell growth could be stimulated by CCK-8 or gastrin I in serum-free medium.[14] Therefore, the transfectant is a good tool to screen antagonists possessing antiproliferative potency.

Among the CCK-B/gastrin receptor antagonists we examined, YM022 was the most potent antagonist with respect to its binding affinity and inhibitions of peptide-ligand-induced inositol phosphate formation, $[Ca^{2+}]_i$ elevation and DNA synthesis. Thus, YM022 may serve as a novel anti-peptic as well as antiproliferative drug for humans.

It has been reported that CCK-B/gastrin receptors are expressed in cell lines derived from gastric, pancreatic, colon and lung cancers and malignant lymphomas.[1–5] Some of these were reported to be stimulated by exogenous CCK-8 or gastrin I. In this study, YM022 could inhibit not only the DNA synthesis of N-hCCKBR, but also that of a small cell lung cancer cell line, NCIH510A, stimulated with CCK-8 and gastrin I. In addition, YM022 inhibited the [*methyl*-^{3}H]thymidine incorporation of several human tumor cell lines derived from small cell lung cancer (NCIH510A), gastric cancer (AGS) and colon cancer (COLO201, COLO320DM, WiDr and HCT116) grown in the absence of exogenous CCK-8 or gastrin I. These tumor cells were demonstrated to co-express gastrin and its receptor mRNAs. These results suggest that YM022 could intervene in the autocrine stimulation of CCK-B/gastrin receptors on human tumor cell lines.

Based on results with nonselective receptor antagonists, an autocrine growth stimulation was suggested to operate in human colon and gastric cancer and Wilms' tumor cell lines.[38–40] Although the presence of a gastrin-like peptide and/or gastrin mRNA was demonstrated in these cell lines, the receptor antagonists showed a divergence between inhibition of [^{125}I]gastrin binding and cell proliferation. These results raise the possibility that a low-affinity gastrin-binding protein was the target for the non-selective antagonists to cut the autocrine loop. However, a selective antagonist, L365,260 could not inhibit cross-linking of [^{125}I]gastrin to the cloned gastrin-binding protein.[41] Thus, nonselective and selective CCK-B/gastrin receptor antagonists might inhibit tumor cell growth through different mechanisms.

Very recently, posttranslational processing intermediates of gastrin, specifically glycine-extended gastrin, as well as gastrin, have been reported to stimulate the proliferation of a rat pancreatic tumor cell line and mouse Swiss 3T3 fibroblasts.[42,43] CCK-B/gastrin receptor-selective antagonists did not inhibit the proliferation induced by glycine-extended gastrin. This implies that there could be two gastrin receptors that mediate the proliferative action of peptides derived from the gastrin gene. Here, several human cancer cell lines, in which DNA synthesis was inhibited by selective CCK-B/gastrin receptor antagonists such as YM022 and L365,260, were confirmed to express both gastrin and CCK-B/gastrin receptor mRNAs at least. It remains important to identify other gastrin receptor molecule(s). Furthermore, it is of interest to examine whether YM022 could be a useful tool for the specific delivery of cytotoxic drugs to human small cell lung cancers, because the CCK-B/gastrin receptor is a novel molecular marker for human small cell lung cancer.[26]

In conclusion, human CCK-B/gastrin receptor-selective antagonists possessing antiproliferative potency could be candidate anti-tumor agents. The N-hCCKBR cell line should provide a very valuable tool to develop such drugs.

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