Gastrin stimulates the growth of gastric pit cell precursors by inducing its own receptors

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Received 21 March 2001; accepted in final form 24 September 2001

First published September 21, 2001; 10.1152/ajpgi.00117.

Nakajima, Toshio, Yoshitaka Konda,Yoshio Izumi, Masashi Kanai, Naoki Hayashi, Tsutomu Chiba, Toshiyuki Takeuchi. Gastrin stimulates the growth of gastric pit cell precursors by inducing its own receptors. Am J Physiol Gastrointest Liver Physiol 282: G359–G366, 2002. First published September 21, 2001; 10.1152/ajpgi.00117.2001.—Gastrin/CKK-B receptors (CCKB-Rs) are present on parietal and enterochromaffin-like cells in the gastric mucosa but not on pit cells in the proliferative zone. Because serum gastrin levels are well correlated with the growth of the gastric pit, we examined whether pit precursor cells express CCKB-Rs using hypergastrinemic transgenic mice and a mouse pit precursor cell line, GSM06. In situ hybridization indicated that CCKB-R mRNA was limited to the lower one-third of the mucosa in control mice; whereas it was faintly distributed along the mid-to low glandular region in the hypergastrinemic transgenic mouse mucosa. CCKB-R-positive midglandular cells appear to have a pit cell lineage; therefore, GSM06 cells were used for an [125I]gastrin binding study. [125I]gastrin bound to the membrane fraction of the GSM06 cells when precultured with gastrin. Gastrin dose dependently induced CCKB-R expression in GSM06 cells and stimulated their growth. Thus these findings suggest that gastrin directly stimulates the growth of the pit cell lineage by inducing its own receptor in pit cell precursors.

Gastric mucosal growth; gastric surface mucous cell; gastrin/cholecystokinin-B receptor

The physiological roles of gastrin in mucosal growth were recently studied using hypergastrinemic animal models. Wang et al. (45) produced transgenic (TG) mice expressing gastrin under the control of an insulin promoter, which resulted in gastrin production in pancreatic β-cells. They reported that gastrin enhances gastric mucosal growth, whereas progastrin preferentially stimulates colonic mucosal growth. They further demonstrated that hypergastrinemic TG mice develop gastric atrophy and eventually gastric cancer (44) and postulated that the hyperplasia of the foveolar pit cell region and the decrease in the parietal cell mass are due, at least in part, to gastrin-stimulated upregulation of growth factors, including heparin binding-epidermal growth factor-like growth factor (HB-EGF) and transforming growth factor-α (TGF-α). Moreover, infection with Helicobacter felis accelerates the formation of gastric cancer in a synergistic manner with hypergastrinemia in hypergastrinemic TG mice (44).

We generated TG mice with hypergastrinemia by expressing a human gastrin transgene (19). The gastric mucosa of these mice was hypertrophic and characterized by an elongated pit with an active proliferative zone, whereas the glandular region containing parietal cells was reduced in size. The pit cells contained fewer mucous granules than those of control littermates and were not reactive to the pit cell-specific cholora toxin β-subunit (CTB) lectin. Pit cells along the foveolar region and many mucous neck cells were Alcian blue positive, suggesting the presence of sialomucin. Thus gastrin promotes the growth of gastric mucosa, especially in the pit region, whereas mucosal cells have less-differentiated features.

Gastrin, CCK, and CCK-related peptides comprise a hormone family characterized by identical COOH-terminal pentapeptide amide structures and bind to two receptor subtypes: CCK-A receptors (CCKA-Rs) and gastrin/CCK-B receptors (CCKB-Rs). CCKA-Rs exhibit a 500- to 1,000-fold higher affinity for sulfated analogs of CCK than for gastrin, whereas CCKB-Rs have approximately equal affinity for both sulfated and non-

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sulfated peptide analogs of CCK and gastrin (32, 34, 46, 47). CCKB-Rs are present in parietal, ECL, and probably somatostatin cells (2, 4, 23, 27, 28, 41). It is unknown, however, whether the pit cells express CCKB-Rs, although many investigators have examined gastrin binding on pit cells. There are specific binding sites for \([^{125}I]\)gastrin on parietal, chief, and ECL cells (2, 28). Immunohistochemical studies have also demonstrated the distribution of CCKB-R in the same cell types (41). On the other hand, Matsuda et al. (23) reported no specific binding of \([^{125}I]\)gastrin to guinea pig gastric epithelial cells in culture, most of which displayed periodic acid-Schiff (PAS)-positive granules, characteristic of pit cells. In the study by Reubi et al. (30), a high concentration of CCKB-R was detected in the midglandular region of the human fundic mucosa, although \([^{125}I]\)gastrin-bound cell types were not identified due to low magnification. Therefore, it remains to be investigated whether gastrin promotes mucosal cell growth directly by stimulating pit cell precursors in the proliferative zone or indirectly by stimulating CCKB-R-expressing parietal and ECL cells to produce HB-EGF, TGF-\(\alpha\), and regenerating gene (Reg), which then stimulate the growth of the pit cell precursors.

**MATERIALS AND METHODS**

**Animals.** We used 30- to 35-wk-old hypergastrinemic TG mice (ICR strain) in which mutated human gastrin cDNA was introduced under the control of the \(\beta\)-actin promoter as we previously reported (19). Briefly, TG mice were generated by inducing mutated human gastrin cDNA for which the peptide product contains two mutations. One mutation is a processing site at the NH\(_2\) terminus of gastrin: -Asp-Pro\(^4\)Ser\(^5\)Lys\(^2\)Arg\(^1\) (mutant). This tetrabasic site was efficiently cleaved by furin, which is distributed in many cell types (48). The other mutation is at the COOH terminus of gastrin after glycine: the progastrin sequence was terminated after the glycine position by inserting a stop codon. With these modifications, the mutated progastrin was efficiently cleaved and amidated even in nonneuroendocrine cells (11). The gastrin titer of hypergastrinemic TG mice homozygously expressing gastrin cDNA was 5- to 10-fold higher than that of control mice. Hypergastrinemic TG mice did not have elevated glycinol-extended gastrin (G-Gly) or unprocessed progastrin (19).

**Morphological studies.** Experimental procedures for removing stomachs followed the guidelines for animal experiments of Kyoto University. Briefly, stomach tissues were removed from TG mice with hypergastrinemia (>400 pg/ml) and fixed in 4\% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Small pieces of the sample underwent saccharose replacement and were then frozen for microtome sectioning. Immunostaining was performed as previously described (19). Parietal cells were identified by staining with a monoclonal antibody to the rabbit H,K-ATPase \(\beta\)-subunit (16). The proliferative zone of the mouse gastric mucosa was confirmed by staining with proliferating cell nuclear antigen (PCNA) using a monoclonal anti-PCNA antibody (DAKO Japan, Kyoto, Japan). An LSAB2/HRP staining kit (DAKO Japan) was used as the secondary antibody reaction system. Gastric pit cells were visualized using fluorescein-5-isothiocyanatelabeled CTB lectin (Sigma, St Louis, MO) binding as previously reported (6, 7).

**In situ hybridization.** In situ hybridization for CCKB-Rs was performed using the RNA Color Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). To obtain template cDNAs, total RNA was isolated from mouse gastric mucosa using Trizol reagent (GIBCO, Grand Island, NY), and first-strand cDNA was prepared by reverse transcription. Then PCR was performed using 5'-TGAGCCGCGAGCCCCAGGGC-3' and 5'-ATTCCGACCACCCGCTTCTT-3' for the mouse CCKB-R (255 bp). cDNA fragments were subcloned into the pGEM-T easy vector (Promega, Madison, WI). RNA probes were prepared in a reaction mixture containing fluorescein-labeled UTP and other NTPs from the template cDNA with SP6 RNA polymerase for an antisense strand probe and T7 RNA polymerase for a sense-strand probe. The RNA probes were heat denatured before use.

Tissue sections were mounted on a glass slide and treated with 0.02 M HCl for 10 min, followed by 0.2\% acetic acid for 5 min, and then air dried before hybridization. The sections were incubated with a hybridization solution containing sense- or antisense-strand cRNAs at a concentration of 500 ng/ml at 55°C overnight. The sections were washed several times after hybridization for the second reaction with an anti-fluorescein antibody complexed with alkaline phosphatase at a dilution of 1:1,000 for 1 h. RNA probes were visualized with an alkaline phosphatase reaction using 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride.

**Cell culture studies.** We used a gastric surface mucous cell line, GSM06, derived from the pit cells of mice expressing the temperature-sensitive SV40 large T-antigen transgene (37). The cells grew to confluence at the T-antigen-active temperature (33°C), whereas at the T-antigen-inactive temperature (39°C), the cells ceased growing and displayed differentiated features such as PAS-positive material, secretory granules, and TGF-\(\alpha\) (20). Even if this cell line is differentiated at 39°C and under high cell density conditions, its features appear to be in a prepit cell stage, according to the classification by Karam and Leblond (18). The cells were cultured in DMEM/Ham's F-12 medium (GIBCO) containing 10\% fetal bovine serum.
serum (FBS; GIBCO) at 33°C in 5% CO₂ on a collagen type I-coated plastic plate (Iwaki, Tokyo, Japan) as previously described (20).

RT-PCR. Expression of mouse CCKB-R mRNA was assessed by RT-PCR (Super Script Preamplification System, GIBCO) using 5'-CTTTGATGTGATAATGACAGCGA-3' and 5'-GCACGTAGCAGCCTCCTG-3' by amplifying the 155-bp DNA for the mRNA encoding a part of the third loop of CCKB-R (12, 46, 47). These primers also amplify the 349-bp genomic DNA for the same region including the 194-bp intron, which was used as a control. An amplified 155-bp DNA fragment underwent direct DNA sequencing for confirmation using...
the ABI PRISM Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer, Norwalk, CT).

Receptor binding study. To perform the in vitro gastrin binding experiment, we isolated the membrane fraction from GSM06 cells preincubated in the presence or absence of various peptides for 2 days at 33°C by homogenization and centrifugation at 42,000 g for 15 min as reported previously (27). The membrane fraction (1 mg) was labeled for 24 h at 24°C with 50 pM [125I]gastrin in the presence of varying concentrations (10^{-11} to 10^{-6} M) of nonlabeled gastrin, CCK-8, or G-Gly.

Cell growth study. GSM06 cells were inoculated on a 96-well plastic plate (Iwaki, Tokyo, Japan) at a seeding density of 1,000 cells/well. Cells were incubated for 24 h in DMEM/Ham’s F-12 medium containing 10% FBS, followed by 24 h culture in DMEM/Ham’s F-12 medium containing 1% FBS. Incubation was continued for 72 h with varying concentrations (10^{-11} to 10^{-6} M) of gastrin, G-Gly, or CCK-8 in the presence or absence of the CCKB-R-specific antagonist S-0509 (10^{-8}, 10^{-6}, or 10^{-4} M; kindly provided by Shionogi Pharmaceutical, Osaka, Japan). S-0509 has a specific antagonizing effect on CCKB-R (1, 10, 33, 39). Cell counting kit-8 (Dojindo Laboratory, Kumamoto, Japan) was used to count cells based on the action of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-[2H]tetrazolium monosodium salt, and the cell number was evaluated by measuring the optical density at an absorbance of 450 nm (20).

RESULTS

Characterization of the hypertrophied gastric mucosa in hypergastrinemic TG mice. The hypertrophic gastric mucosa of hypergastrinemic TG mice was recently characterized, and the pit cells in the elongated pit were found to have less-differentiated immature features (19). The present study further investigated the expression of CCKB-R. Immunostaining revealed that in control mice, PCNA-stained cells had a scattered distribution approximately one-third of the way down the gastric surface (Fig. 1A), whereas they were densely located at the base of the elongated pit of the hypergastrinemic TG mouse gastric glands (Fig. 1D). We assessed the degree of differentiation of gastric surface pit cells using gastric surface pit cell-specific binding lectin, CTB lectin. There was positive staining...
for CTB lectin along the foveolar-facing membrane of gastric pit cells in control mice (Fig. 1C). In contrast, elongated pits of hypergastrinemic TG mice were only slightly stained for CTB lectin, and the presence of lectin-positive pit cells was limited to the top of the foveolar region. The PCNA-positive elongated pits were negative for CTB lectin staining (Fig. 1F).

In situ hybridization of CCKB-R mRNA with the antisense probe revealed heavy distribution in the lower one-third of the gastric gland of control mice, whereas the sense probe did not reveal positive staining (Fig. 2, A and B). At higher magnification, the positive cells were clustered at the bottom region of the glands (Fig. 2C). In the hypergastrinemic TG mouse gastric mucosa, faintly positive cells were distributed from the middle to lower part of the gastric gland, and the cells in the midglandular region stained a little more intensely than those in the low glandular region (Fig. 2E). Because PCNA-positive cells were distributed in the midglandular region of the hypergastrinemic TG mouse glands, they appear to overlap CCKB-R-positive cells. The CCKB-R-positive cells in the midglandular region do not appear to be parietal cells but rather pit cells or their precursors, according to their size and shape (Fig. 2F), as demonstrated using electron microscopy in our previous study (19). CCKB-R-positive cells in the low glandular region, however, might represent parietal cells because of their location (Figs. 1E and 2E).

**Binding of \[^{125}I\]gastrin in GSM06 cells.** We postulated that CCKB-R-positive cells over the proliferative zone of the hypergastrinemic TG mouse mucosa are prepit cells and/or pit cell precursors. Because immature pit cells do not have characteristic features (18), it is difficult to identify these cell types as a pit cell lineage. To circumvent this difficulty, we used the mouse gastric mucous/pit cell-derived cell line GSM06 for \[^{125}I\]gastrin binding studies. Initially, \[^{125}I\]gastrin did not specifically bind to the membrane fraction at either 33°C (a growing state) or 39°C (a growth arrest state). When the cells were cultured at 33°C in the presence of 10^{-7} M gastrin for 48 h before the binding study, \[^{125}I\]gastrin bound specifically to the membrane fraction. The binding of \[^{125}I\]gastrin was competitively inhibited by the addition of unlabeled gastrin in a dose-dependent manner with a half-maximum inhibition obtained at approximately 10^{-8} M (Fig. 3). This binding was similarly inhibited by CCK-8, indicating that this receptor is shared by both gastrin and CCK. In contrast, G-Gly displaced the specific binding only at 10^{-6} M. Thus gastrin upregulated the expression of its own receptor in GSM06 cells (14).
Detection of CCKB-R mRNA by RT-PCR in GSM06 cells. The presence of CCKB-R mRNA in the cells was confirmed using RT-PCR. One band with a predicted size of 155 bp was detected, which increased in density with incubation with 10^{-7} M gastrin for up to 48 h, whereas the genomic DNA-derived 349-bp band remained constant (Fig. 4A). By analyzing the DNA sequence of the 155-bp transcript, we obtained a mouse amino acid sequence homologous, with only minor differences, to receptors of the rat, Mastomys, and human (Fig. 4B) (12). This partial sequence completely matched the mouse receptor sequence deposited in the GenBank (accession #AF019371).

Growth stimulatory effect of gastrin on GSM06 cells. We then examined the effect of gastrin on the growth of GSM06 cells. Both gastrin and CCK-8 dose dependently increased the growth of the cells (Fig. 5A). G-Gly had no effect on growth, which is consistent with the results shown in Fig. 3. The growth effect of gastrin on GSM06 cells was completely abolished by the CCKB-R-specific antagonist S0509 (Fig. 5B).

DISCUSSION

We previously demonstrated that the hypertrophic gastric mucosa of hypergastrinemic TG mice comprised an elongated pit with an active proliferative zone and that the glandular region containing parietal cells was relatively reduced in size (19). In the elongated pit, pit cells displayed less-differentiated features. For example, they showed less binding to pit cell-specific CTB lectin, although the pit region was extensively elongated. In previous studies, CCKB-Rs were localized in parietal, chief, and ECL cells (28, 41). Thus the CCKB-R-positive cells in the lower glands in control mice appear to be a mixture of these cells. In contrast, CCKB-R-positive cells were prominent in the midglandular region of the hypergastrinemic TG mouse mucosa, where PCNA-positive cells were most abundant. In our previous study, electron microscopy revealed that these cells contain various sizes of granules and enlarged endoplasmic reticulums, which actively produce secretory proteins. Moreover, morphological study with a light microscope indicated that these cells are oval in shape and smaller than parietal cells (19). Thus, although these cells were negative for staining with pit cell-specific CTB lectin, the CCKB-R-positive and PCNA-positive cells in the midglandular region of hypergastrinemic TG mice in our present study appear to be prepit cells.

To examine CCKB-R expression in the pit cell lineage, we used the GSM06 cell line as a pit cell line model in vitro. The GSM06 cell line is derived from the gastric surface mucous cells of a TG mouse that was transformed with the temperature-sensitive SV40 T antigen DNA (37). Morphologically, GSM06 cells are rounded or polygonal, as is typical of primary cultured gastric surface mucous cells (3, 8, 24, 42). Electron microscopy revealed that the cells contain a small number of secretory granules (20, 38). Immunologic studies indicate that the GSM06 cells are positive for PAS and class I concanavalin A but negative for H,K-ATPase and class III concanavalin A (37). These morphological and immunologic studies revealed that GSM06 cells display features similar to immature pit cells, perhaps corresponding to those at a prepit stage (18, 37). We initially failed to detect specific [125I]gastrin binding to the membrane fraction of GSM06 cells. Because Johnson (14) and Takeuchi et al. (40) reported that gastrin stimulation increases the expression of its own receptor, we precultured the cells with 10^{-7} M gastrin for 48 h, then performed an [125I]gastrin binding study. With this gastrin pretreatment, we detected specific binding of [125I]gastrin on GSM06 cell membranes, which was competitively inhibited by an increase in exogenous gastrin and CCK-8. Furthermore, we confirmed the increase of CCKB-R expression by gastrin stimulation using RT-PCR (Fig. 4A). Takeuchi et al. (40) suggested two possible mechanisms for the increase in CCKB-R expression by gastrin. First, gastrin might stabilize its receptors, preventing receptor degradation.

Fig. 5. A: effect of gastrin, CCK-8, and G-Gly on the growth of GSM06 cells. ○, Culture with gastrin. □, Culture with CCK-8. ▲, Culture with G-Gly. Results are expressed as means ± SE of 7 samples. B: inhibitory effect of gastrin-receptor antagonist. ○, Culture with gastrin alone (10^{-11} to 10^{-6} M). ■, Culture with gastrin (10^{-11} to 10^{-6} M) and 10^{-8} M CCKB-R antagonist S0509. ▲, Culture with gastrin (10^{-11} to 10^{-6} M) and 10^{-4} M S0509. ●, Culture with gastrin (10^{-11} to 10^{-6} M) and 10^{-4} M S0509. Results are expressed as means ± SE of 7 samples.
Second, gastrin stimulates the synthesis of its own receptors (40). Consistent with the binding studies, both gastrin and CCK-8 dose dependently increased the growth of GSM06 cells, which was abolished by a CCKB-R-specific antagonist S0509. Thus gastrin enhanced expression of its own receptors in GSM06 cells, leading to augmented cell growth by gastrin. Our in vitro data are consistent with our in vivo experiments, in which long-lasting hypergastrinemia induced CCKB-R expression in the midglandular region in association with increased proliferative activity in the hypergastrinemic TG mouse gastric mucosa. Although we did not detect CCKB-R expression in the proliferative zone of the control mice, the cells in the proliferative zone might also express a small amount of CCKB-Rs and their growth might normally be regulated by gastrin.

In our study, the growth curve induced by gastrin reached its peak at a relatively high concentration of gastrin and the displacement curve indicates a high $K_d$ value compared with previous reports (22, 29, 36). Iwase et al. (13) reported almost the same displacement curve for labeled gastrin as ours using gastric mucosal cell lines AGS and SIIA. Although the precise reason for the difference in binding characteristics remains unknown at present, it might be due to differences in cell character.

CCKB-R expression is evident in parietal cells and ECL cells; therefore, gastrin-induced mucosal cell growth is thought to be mediated by parietal cells and/or ECL cells (9, 25, 26, 43). Parietal cells produce HB-EGF (26), and recently, Miyazaki et al. (25) demonstrated that gastrin induces the expression of HB-EGF mRNA and that the secretion of a mature form of HB-EGF into the culture medium using a rat pit cell line RGM-1 when CCKB-R was expressed in this cell line. Therefore, they hypothesized that the gastrin-induced growth of gastric mucosa is mediated by parietal cells, which possess CCKB-R and produce HB-EGF. Fukui et al. (9) demonstrated that gastrin stimulates the production of Reg protein from ECL cells. Reg protein has been isolated from regenerating rat pancreatic islets (43) and stimulates the growth of cultured rat gastric epithelial cells (9). Recently, Wang et al. (44) reported that human gastrin-expressing TG mice had initial mild hypergastrinemia with an increased number of parietal cells, then later had a decreased parietal cell mass in association with increased expression of HB-EGF and TGF-α in the hypertrophied mucosa. At 20 mo of age, the mice exhibited gastric metaplasia, dysplasia, carcinoma in situ, and gastric cancer. Interestingly, Helicobacter felis infection accelerated the size and invasiveness of gastric cancer. Wang et al. (44) hypothesized that hypergastrinemia eventually induces gastric atrophy and enhances the production of HB-EGF and TGF-α, which, in turn, induce a malignant change in the atrophic gastric mucosa. These data suggest that gastrin-induced growth of gastric mucosa is mediated by gastrin-stimulated growth factors such as HB-EGF, TGF-α, and Reg protein. As demonstrated in our previous and present studies, the gastric mucosa of the TG mice with long-lasting hypergastrinemia contains a reduced number of parietal cells and ECL cells, and the CCKB-R expression in these cells is considerably reduced. Thus it is possible that long-lasting hypergastrinemia upregulates the CCKB-R expression in prepit cells and/or pit cell precursors, eventually leading to the elongation of gastric pits with less-differentiated features directly through its receptors on those cells.

Hypergastrinemia is frequently associated with both types A and B atrophic gastritis. Gastric cancer usually develops from atrophied mucosa, which leads to the hypotheses that atrophic gastritis is a precancerous lesion and that elevated gastrin induces the initiation of gastric cancer (44). This hypothesis is supported by the fact that the mucosal cells of atrophic gastritis exhibit more mitotic activity than those in healthy individuals (21). An important question is whether these mitotic cells are derived from a CCKB-R-expressing pit cell lineage.

We are grateful to E. Hamana for secretarial assistance. This work is supported by the grant-in-aid for scientific research from the Ministry of Education, Science, Culture, and Sports of Japan and the grant-in-aid for research for the future program from the Japanese Society for the Promotion of Science (JSPS-RFTF97100201).
G366 GASTRIN STIMULATES THE GROWTH OF PIT CELLS

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