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Analysis of gastrin receptor gene expression in proliferating cells in the neck zone of gastric fundic glands using laser capture microdissection

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Abstract Gastrin stimulates proliferation of progenitor cells in the neck zone of gastric fundic mucosa. However, whether it directly enhances this proliferation through its receptors remains unclear. We investigated the expression of gastrin receptors in neck zone proliferating cells in rat gastric fundic glands using a reverse transcription polymerase chain reaction (RT-PCR) coupled with laser capture microdissection and in situ RT-PCR. Gastrin receptor expression was identified in c-*fos*-expressing cells located in the neck zone, and results of the RT-PCR analysis argued against contamination by other cells, such as enterochromaffin-like, parietal or D cells. Supporting this finding, gastrin receptor gene expression was identified in the neck zone as well as base glands by in situ RT-PCR. Therefore, it is suggested that proliferating cells in the neck zone are stimulated directly by gastrin via their gastrin receptors. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Gastrin receptor; Proliferating cell; Neck zone; Laser capture microdissection; In situ reverse transcription polymerase chain reaction; Gastrin

1. Introduction

Gastric mucosa is composed of three layers: the surface epithelial layer, the neck zone and the glandular portion. At least five types of cells (parietal, chief, endocrine, mucous neck and surface epithelial) exist in a single fundic gland, while proliferating cells are located in the neck zone. Some cells migrate upwards, becoming mucous-secreting surface epithelial cells, whereas others migrate towards the base of the gland and differentiate into parietal, chief or endocrine cells [1]. The proliferation of progenitor cells in the neck zone and maintenance of the integrity and structure of gastric epithelial layers is considered to be controlled by several growth factors [2].

Gastrin is a gut–brain peptide with multiple functions in the gastrointestinal tract. Its receptors belong to the G-protein-coupled receptor superfamily, which is characterized by seven transmembrane domains [3–5]. Gastrin directly stimulates acid secretion from parietal cells [5]. In addition to this direct effect on parietal cells, it stimulates acid secretion indirectly by releasing histamine from enterochromaffin-like (ECL) cells [6]. Gastrin is also considered to be a growth-promoting factor for gastric epithelial cells by directly or indirectly stimulating the proliferation of progenitor cells in the neck zone [7–10]. Indeed, gastric mucosal thickness is increased in patients with hypergastrinemia, such as that observed in Zollinger–Ellison syndrome. The trophic effect of gastrin has also been observed during experimental chronic hypergastrinemia evoked by chronic administration of proton pump inhibitors [11,12]. Many investigators have shown the presence of gastrin receptors in the gastric mucosa of dogs, rats, guinea pigs and humans using receptor binding or molecular biological studies [3,13–27]. The strong expression of gastrin receptors, particularly in parietal and ECL cells, has been clearly shown by our previous study as well as those by other investigators [22–25,27]. However, no data are currently available that directly show the presence of gastrin receptors in proliferating cells in the neck zone, thus, a direct link between gastrin and gastric mucosal proliferation remains unelucidated. The development of laser capture microdissection (LCM) allows researchers to obtain pure cell populations and to analyze the gene expression of microdissected cells without them being contaminated by other kinds of cells [28,29]. In addition, in situ reverse transcription polymerase chain reaction (RT-PCR) has been shown to be a very powerful tool, enhancing the ability to detect minute quantities of a target gene that cannot be detected by immunohistochemistry or in situ hybridization in tissue sections [30]. The present study was designed to examine whether immature cells in the neck zone of rat gastric fundic mucosa exhibit gastrin receptor gene expression using these two novel methods.

2. Materials and methods

2.1. Effect of chronic hypergastrinemia in rat stomachs

7-week-old male Wistar rats were treated with lansoprazole (Takeda, Osaka, Japan) at a dose of 30 mg/kg body wt. diluted in a vehicle (0.5% sodium carboxymethyl cellulose, pH 7.6) via a gastric tube once a day for 28 days. Control rats received only the vehicle in the same manner. This dose was previously shown to induce hypergastrinemia in adult rats (vehicle versus lansoprazole, 82 ± 17 versus 3855 ± 1 ($P < 0.01$)) [9]. On the 28th experimental day, all rats were killed and the glandular stomachs removed for histological examination.

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Abbreviations: ECL, enterochromaffin-like; LCM, laser capture microdissection; HDC, histidine decarboxylase; VMAT, vesicular monoamine transporter; HB-EGF, heparin-binding epidermal growth factor-like growth factor; AR, amphiregulin; EGF-R, epidermal growth factor receptor

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4 μm thick sections from each specimen were mounted on glass slides and stained with hematoxylin–eosin for light microscopic examination. To identify the proliferating cells, each was also immunostained with proliferative cell nuclear antigen (PCNA). The sections were then incubated with monoclonal antibody against rat PCNA, PC10 (Novocastral Lsb, Newcastle, UK) (1:200) at 4°C overnight, which was followed by incubation with secondary biotinylated rabbit anti-mouse immunoglobulin G for 30 min. The bound antibody was detected using the avidin–biotin peroxidase method (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine (Wako, Tokyo, Japan) was used as the chromogen. The sections were counterstained with hematoxylin. PCNA-positive cells in a single fundic gland were counted in seven different fields for each section from 100 individual glands.

2.2. LCM

7-week-old male Wistar rats were used for the experiments. Freshly frozen glandular stomach tissues were cut into 5 μm sections in a cryostat and mounted on uncoated glass slides. The slides were immediately fixed in 70% ethanol for 30 s and washed in diethylpyrocarbonate (DEPC)-treated water for 30 s, after which the sections were stained rapidly with Mayer's hematoxylin for 1 min, washed with DEPC-treated water for 30 s, dehydrated through an ethanol gradient, counter-stained with eosin Y for 1 min, dehydrated with ethanol gradient, and finally cleared in xylene. Once air-dried, the sections were laser microdissected using a LCM system LM200 (Olympus, Tokyo, Japan), as reported previously [28,29]. In brief, slides with sectioned tissue were placed on the stage of a microscope and an area selected. The chosen sections were covered with LCM transfer film (CapSure TF-100; Arcturus Engineering, Mountain View, CA, USA). Under direct microscopic observation, specific portions of the tissue section, focused to the appropriate size of the desired target, were pulsed directly above the targeted cells by brief laser pulses, using a laser beam 7.5 μm in diameter and a laser power of 60 mW. The samples captured from 1000 shots on one transfer film cap were immersed in RNA extraction solution.

2.3. Extraction of RNA and RT-PCR

Total RNA from each population of laser captured cells was independently extracted by a single-step guanidium thiocyanate–phenol–chloroform method (Isogen; Nippon Gene Co., Tokyo, Japan). The transfer film and adherent cells were incubated with the extraction solution at room temperature. Chloroform was then added and the solution centrifuged for 30 min at 4°C. The aqueous layer was precipitated with a glycogen carrier (Pharma Biotechnologies, Hannover, Germany) in isopropanol. After initial recovery and resuspension of the RNA pellet, a DNase step was performed for 60 min at 37°C using DNase I (Stratagene Toyobo, Tokyo, Japan). After re-extraction of RNA, RT-PCR was performed as described previously [31]. Total RNA extracted from laser captured samples was subjected to RT, using a First Strand Synthesis Kit (Stratagene Toyobo, Tokyo, Japan) to prepare the cDNA. The first-strand cDNA was then amplified directly by the PCR method. PCR was performed by a hot start, with 35 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 2 min, and a final extension step at 72°C for 7 min. PCR was performed in a duplicate experiments and the correct amplification was confirmed by sequencing the amplified DNA. For the PCR detection of pepsinogen C, a semiquantitative multi-cycle PCR was also performed to detect quantitative information of pepsinogen C gene expression in the neck zone and glandular cells. First, H^+,K^+-ATPase gene expression was tested by RT-PCR in the neck zone cells to detect possible contamination by parietal cells in the dissected neck zone cells. Eight of the 25 microdissected samples from the neck zone did not show any expression of H^+,K^+-ATPase mRNA. The H^+,K^+-ATPase negative samples were then employed for the following experiments. To standardize the amount and quality of the total RNA, the same samples were analyzed for gastrin receptor, histidine decarboxylase (HDC), vesicular monoamine transporter 2 (VMAT2), MUC5AC, somatostatin, pepsinogen C, c-*fos*, heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin (AR), epidermal growth factor receptor (EGF-R), and β-actin. The sense and antisense primers used are summarized in Table 1 [32–43]. RNA extracted from rat total glandular stomachs was used as a positive control and laser captured samples without subjection to RT served as a negative control. The PCR products were separated on a 2% agarose gel stained with ethidium bromide.

2.4. In situ RT-PCR of gastrin receptor

In situ RT-PCR was performed as described by Thaker [44], with minor modifications. Briefly, paraffin-embedded glandular stomach tissues were cut into 5 μm sections and mounted on silanated glass slides, deparaffinized in xylane, and rehydrated by a descending ethanol series. After washing for 10 min with DEPC-treated phosphate-buffered saline (PBS), the tissue preparations were permeabilized by 5 μg/ml proteinase K digestion. To digest genomic DNA, the slides were treated overnight at 37°C in an RNase-free DNase solution at a concentration of 1 U/μl. The sections were washed with DEPC-treated PBS, dehydrated through graded ethanol, and air-dried. The in situ RT-PCR step was performed with the Omni Slide System (Hybaid, Teddington, Middlesex, UK), with cycling conditions the same as described for the tube RT reaction. After the RT of total RNA to first-strand cDNA, PCR was performed in 25 cycles under the same

Table 1

	Primer sequences	Size of amplified DNA (bp)
Gastrin receptor	5′-GGGTGTCTCCCTTCTCAACAGCAGTAG-3′ 5′-TAGGAAATGGCCTTGCAGATGACTGTG-3′	324
HDC	5′-TGCTGATGAGTCCTCTCTGAACGC-3′ 5′-TGCACCATCATCCACTTGGAAGGG-3′	405
VMAT2	5′-ATGGCCCTGAGCGATCTGGTGCTGC-3′ 5′-TCAGAAGTCCTATGAATGGGTTAGT-3′	459
H^+,K^+-ATPase	5′-GAGCTGGAGCAGAAGTACCAGACC-3′ 5′-TGACCACAACCACAGCAATGAGTG-3′	280
Somatostatin	5′-AGCTTTAAGTCTCCCTTGCC-3′ 5′-TGCCTCAGACAGCCGAGCTT-3′	515
Pepsinogen C	5′-AAGACCCACAAATATGACCCTGGC-3′ 5′-GGTACCAGGCTCATTCTCACTCAG-3′	387
MUC5AC	5′-TGCCAGCCACAGTGCAACTGGACC-3′ 5′-AACAACGGGTGGTAGAGTGAACTC-3′	433
c-*fos*	5′-AGGCCTTCACCCTGCCTCTTCTCAATG-3′ 5′-AAGGAACCAGACAGGTCCACATCTGGC-3′	184
HB-EGF	5′-CCAGCAGCCATGAAGACTTCT-3′ 5′-TCGGGCTTGAGATATTTCACA-3′	419
AR	5′-CTGAGGTCTCTACCATAAGCG-3′ 5′-TAACGCGATCTTGGATAGGTC-3′	422
EGF-R	5′-GCTGGGGAAGAGGAGAGGAGAACT-3′ 5′-GCAGGGCCCGTCACATTTTTTACA-3′	404
β-Actin	5′-CCCCTCTGAACCCTAAGGCCAACC-3′ 5′-AGGATCTTCATGAGGTAGTCTGTC-3′	256

conditions as described for the tube PCR reaction. Digoxigenin-labeled uridine triphosphate (Boehringer Mannheim, Mannheim, Germany) was added into the cocktail of the regular dNTPs and directly incorporated into the amplified DNA. For detection of the amplified PCR products, a Digoxigenin Detection Kit (Boehringer Mannheim, Germany) was used. The omission of RT, primers or AmpliTaq DNA polymerase was used as negative controls to exclude method-dependent non-specificities.

2.5. Statistical analysis

All data are expressed as mean ± S.E.M. and were analyzed using the Mann–Whitney *U*-test with $P < 0.05$ considered statistically significant.

3. Results

3.1. Effect of chronic hypergastrinemia in rat stomachs

PCNA-positive cells were identified in the neck zones of the fundic glands in both control and lansoprazole-treated groups (Fig. 1). Lansoprazole treatment was accompanied by significantly increased plasma concentrations of gastrin and significantly increased number of PCNA-positive proliferating cells in gastric glands compared with the control group (12.1 ± 1.1 PCNA-positive cells/gland in lansoprazole-treated rats versus 6.9 ± 1.0 in control. $P < 0.01$).

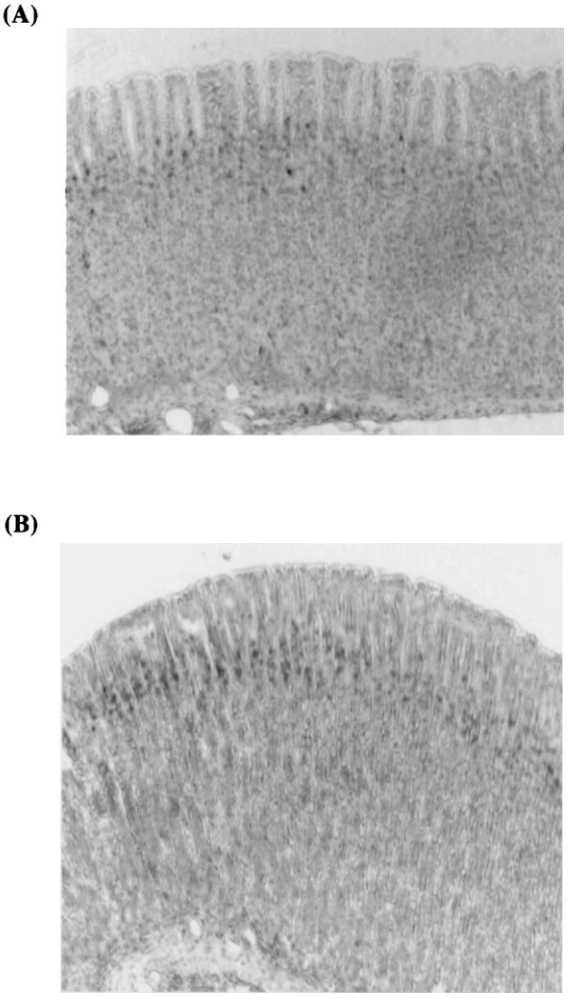

Fig. 1. Immunohistochemical staining for PCNA in (A) vehicle and (B) lansoprazole-treated hypergastrinemic rat fundic mucosa (×20). Proliferating cells in the neck zone were positively stained. Gastric mucosal thickness and the number of proliferating cells were augmented in lansoprazole-treated hypergastrinemic rats.

3.2. LCM

As shown in Fig. 2, LCM with microscopic observation allowed us to precisely sample the surface epithelial layer, the neck zone and the glandular portion of the fundic mucosa. In this manner, targeted, multiple and same cell kinds were separately obtained and bonded to the transfer film. When the film and cap were lifted from the tissue section, untargeted and unimpregnated tissues remained attached to the glass slide. As a result, the morphology of the transferred cells was preserved and could be readily visualized under a microscope (Fig. 2).

Fig. 3 shows the results of RT-PCR analyses on the LCM-dissected gastric fundic glandular cells. The expression of HDC and VMAT2 mRNA, markers of ECL cells, with gastrin receptors, was found only in the glandular cells. H^+,K^+-ATPase and somatostatin mRNA expression, markers of parietal and D cells, respectively, was also identified in the glandular cells. No expression of HDC, VMAT2, H^+,K^+-ATPase, or somatostatin mRNA was identified in either the neck zone or surface epithelial layer. The expression of pepsinogen C mRNA was identified as a faint band in the neck zone and as a strong band in the glandular cells. The expression of c-*fos* was demonstrated as strong bands in the neck zone, and faint bands in both surface epithelial and glandular cells. In addition, MUC5AC mRNA expression was identified in the surface epithelial layer as a strong band and in the neck zone as a faint band. Both HB-EGF and AR were identified only in glandular cells, whereas EGF-R was identified in the surface epithelial layer, neck zone and glandular cells. The expression of β-actin mRNA was identified in all layers of the mucosa with similarly strong bands. Notably, the expression of gastrin receptor mRNA was demonstrated not only in the glandular cells but also in the neck zone as strong bands. However, no amplified band was found after PCR amplification of the sample RNA without being subjected to RT.

3.3. In situ RT-PCR of gastrin receptor

Using in situ RT-PCR, gastrin receptor mRNA was detected in numerous cells of the fundic mucosa, though positive cells mainly showed strong cytoplasmic staining around the unstained and clearly demarcated nuclei [27]. Most of the gastrin receptor-expressing cells could be identified in the base of the glands where ECL cells and parietal cells were abundant. In the base of the fundic glands, positively stained cells could be classified into large round cells, which corresponded to parietal cells, and small cells, which corresponded to ECL cells. A small number of gastrin receptor-expressing cells could also be identified in the neck zone. The size and shape of the positively stained neck zone cells were quite different from those of the parietal and ECL cells in the base of glands (Fig. 4). In contrast, none of the negative controls showed any specific amplified signals.

4. Discussion

Gastrin is reported to be a growth-promoting factor for cells that express gastrin receptors [1]. We and others have shown that parietal, ECL and D cells are specific cell types that have gastrin receptors on their cell membranes

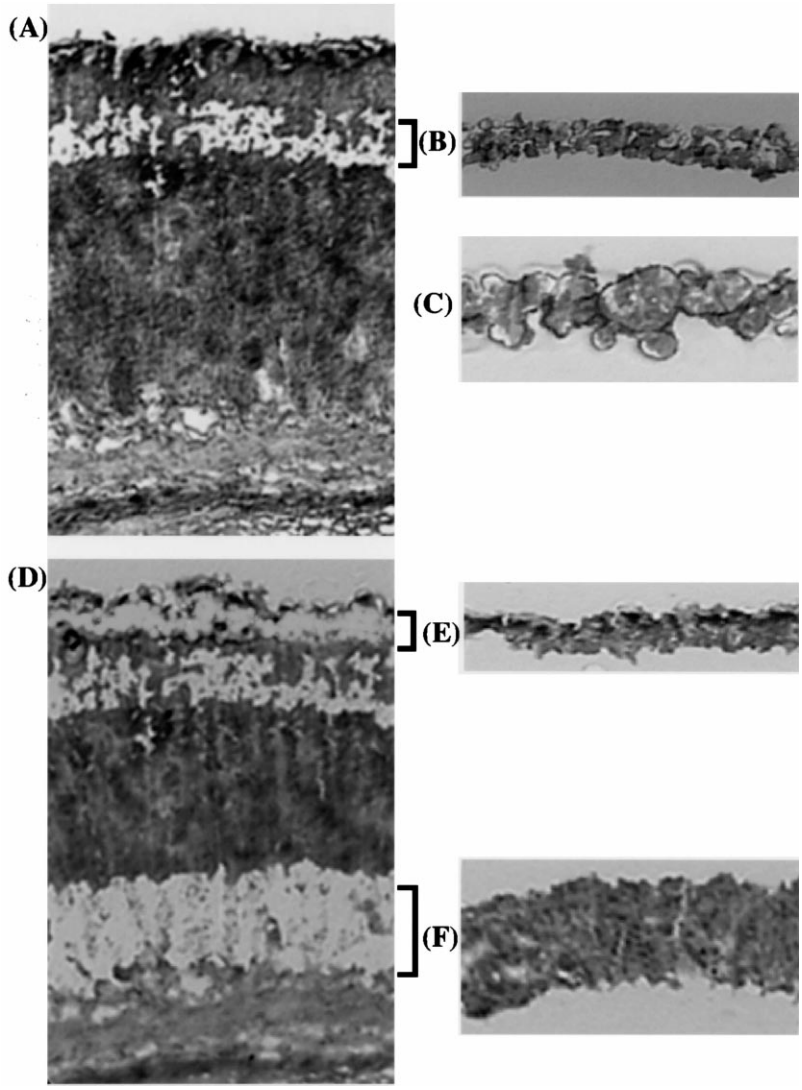

Fig. 2. LCM of freshly frozen sections of a fundic gland stained with hematoxylin–eosin (×40). (A) After removal of the targeted cells of the neck zone by LCM. (B) Captured cells of the neck zone as seen on the transfer film. (C) High-power view of captured cells of the neck zone on the transfer film (×200). (D) After removal of the surface epithelial layer, neck zone and glandular cells. (E) Captured surface epithelial layer on the transfer film. (F) Captured glandular cells on the transfer film.

[3,13,14,17,18,21–24,27]. We have also shown that ECL cells with gastrin receptors proliferate when stimulated with gastrin [9,23]. Furthermore, the proliferation of gastric mucous cells and fibroblasts transfected with gastrin receptors have been reported to be stimulated by gastrin [7,45]. For the regulation of gastric stem cell proliferation, two indirect forms of gastrin stimulation have been reported. We have found that gastrin directly stimulates Reg protein production in ECL cells and that the secreted Reg protein enhances gastric mucosal proliferation in the neck zone [9,46]. Tsutsui et al. reported that gastrin stimulates production of HB-EGF and AR in parietal cells, and that these growth factors indirectly stimulate mucosal proliferation [8,47]. In contrast, there are no definitive data available showing a direct stimulatory effect of gastrin on the proliferation of gastric mucosal stem cells or the presence of a gastrin receptor in proliferating cells in the neck zone. This lack of evidence may be due to the difficulty of collecting pure cells from the gastric glandular neck zone by conventional methods.

The development of LCM allows researchers to obtain RNA from pure cell populations using a microscope [28,29]. We performed RT-PCR with LCM to investigate gastrin receptor gene expression in neck zone proliferative cells. PCNA-positive cells had the same distribution pattern as those obtained by LCM. Gastrin receptor expression is evident in ECL, parietal and D cells [3,13,14,18,21–24,27], therefore, contamination by these cells in proliferating cells obtained by LCM may have been the major cause of the pseudopositive results for gastrin receptor gene expression seen in our experiments. Indeed, we found that only 32% of the LCM-dissected samples were free from contamination by parietal, ECL or D cells. Thus, even when using LCM, it may be difficult to isolate only proliferating cells from other differentiated characteristics of gastric fundic glandular cells. HDC and VMAT2 are both well-established ECL cell markers [48], and H^+,K^+-ATPase and somatostatin are well-established markers of parietal and D cells, respectively [33,34]. Therefore, our results showing an absence of HDC, VMAT2, H^+,K^+-ATPase, and

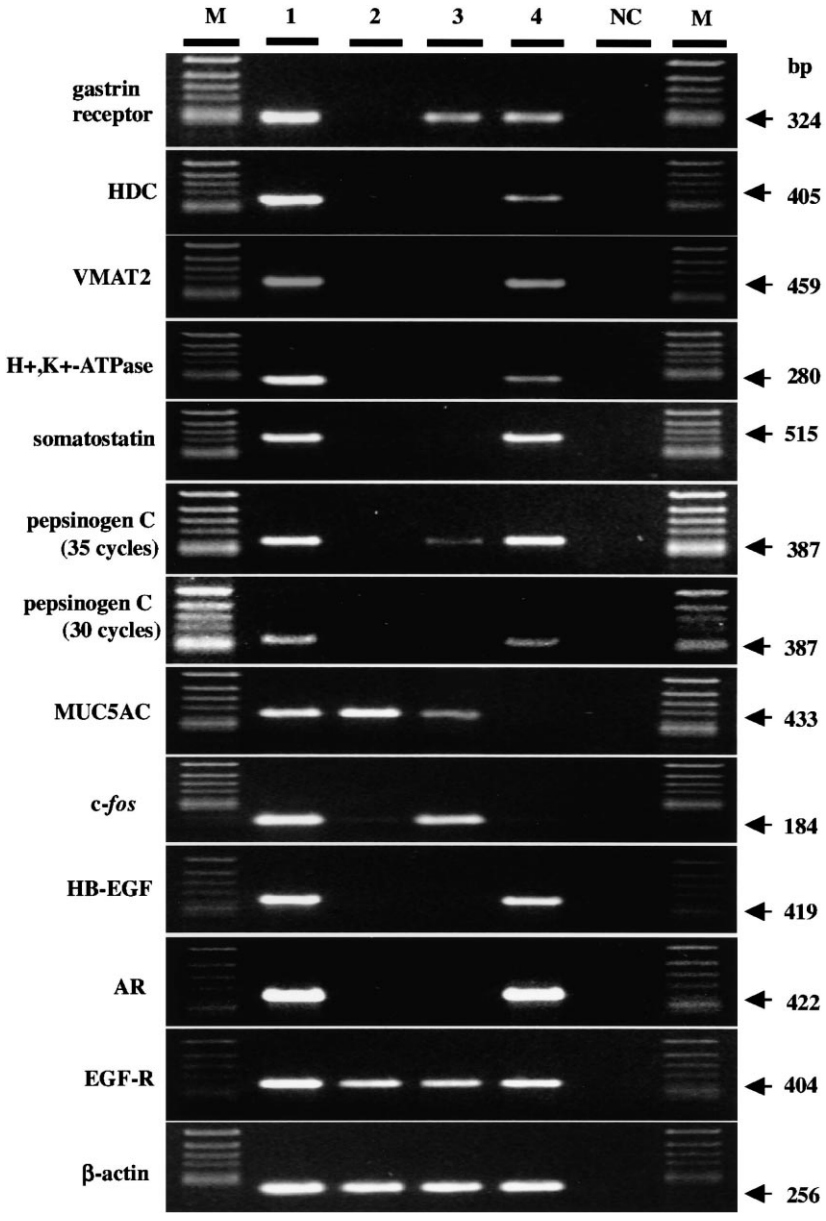

Fig. 3. RT-PCR analysis of gastrin receptor, HDC, VMAT2, H^+,K^+-ATPase, somatostatin, pepsinogen C, MUC5AC, c-*fos*, HB-EGF, AR, EGF-R, and β-actin expressions. Lane 1, whole glandular stomach; lane 2, surface epithelial layer; lane 3, proliferating cells from the neck zone; lane 4, glandular cells; NC, negative control; M, molecular size marker. The same experiments were performed in four animals, with representative data from one shown.

somatostatin mRNA in LCM-obtained neck zone cells clearly ruled out contamination by ECL, parietal or D cells. In the present study, we focused on data obtained from non-contaminated proliferating neck zone cells microdissected by LCM.

LCM-obtained neck zone cells also showed a strong gene expression of c-*fos*. C-*fos* is a nuclear transcriptional factor whose gene expression is positively regulated during the proliferation of cells [39,49]. Previously, we demonstrated that the c-*fos* gene was strongly expressed in counterflow elutriation-enriched proliferating cells in rat gastric mucosa [50]. Accordingly, the obtained neck zone cells in the present study mainly contained proliferating gastric mucosal cells without contamination by ECL, parietal or D cells. Further, HB-EGF and AR gene expressions were found in fundic glandular areas that contained parietal cells and EGF-R gene expression was detected in all layers of the fundic glands, including the neck zone area. These results confirmed previous reports which showed an indirect proliferating effect of gastrin on neck zone cells via stimulation of HB-EGF/AR production by parietal cells, as both HB-EGF and AR stimulate cell proliferation through binding to EGF-R [51,52].

In situ RT-PCR is a new molecular biological technique that takes advantage of the high sensitivity of RT-PCR to generate cDNA from small amounts of mRNA contained in individual cells [30,44]. Using in situ RT-PCR, we detected gastrin receptor mRNA in the base of glands where ECL cells and parietal cells are abundant. In addition, a low number of gastrin receptor-expressing small cells could be identified in the neck zone where PCNA-positive cells exist. Although

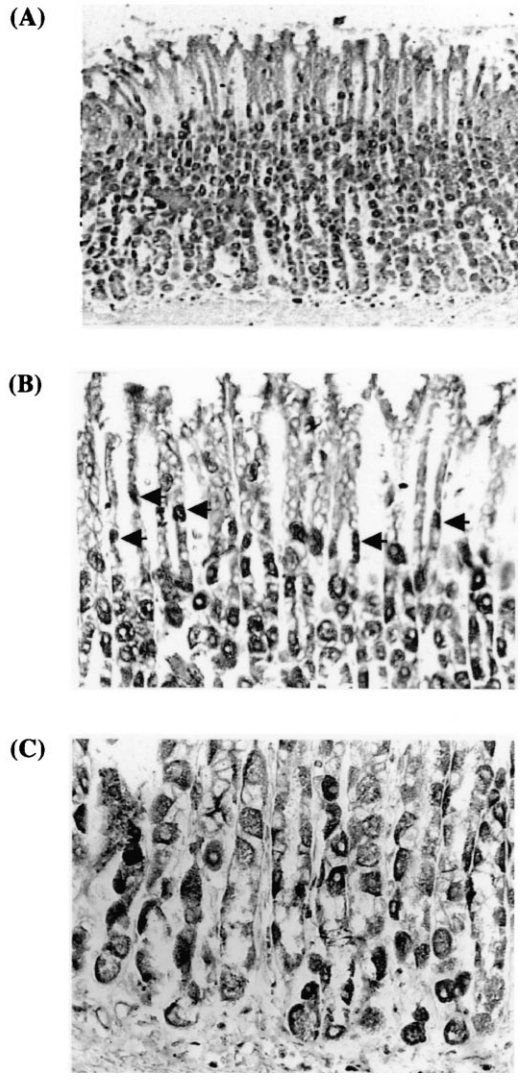

Fig. 4. (A) In situ RT-PCR of gastrin receptor expression in gastric fundic mucosa (×33). (B) Neck zone (×100). (C) Base of fundic glands (×100). In fundic glands, large round cells as well as small cells were positively stained. In the neck zone, scattered small cells were also shown to be positive for gastrin receptor gene expression (arrows).

neck zone proliferating cells were collected without contamination by parietal, ECL or D cells, pepsinogen C gene expression was detected in those neck zone cells. Since pepsinogen C is abundantly present in chief cells, the contamination of our neck zone samples by chief cells must be considered. Studies using immunohistochemistry and radioligand binding have suggested the presence of a gastrin receptor on chief cells [26,53], thus gastrin receptor gene expression detected in neck zone cells may be caused by contaminated chief cells. Pepsinogen C, however, is also well known to be expressed in proliferating mucous neck cells [54]. Accordingly, a weak gene expression of pepsinogen C does not necessarily indicate contamination by chief cells. Furthermore, the presence of gastrin receptor expression in chief cells is still controversial and we did not find gastrin receptor mRNA in chief cells in our previous in situ hybridization study [22]. As a result, it is reasonable to conclude that the gastrin receptor mRNA positive cells detected by in situ RT-PCR in the neck zone may not have been chief cells, but rather the proliferating progenitor cells usually found in gastric fundic glands.

In our experiments, gastrin receptors were detected not only in fundic glandular cells but also in neck zone cells using two novel methods. In fundic glandular cells, gastrin receptors are present in ECL, parietal and D cells. In the neck zone, however, a gene expression of the gastrin receptor may be present in proliferating cells, which are the main type seen in this zone. Therefore, our results support previous reports showing the presence of specific gastrin binding sites in the gastric mucosal neck zone [53]. Taken together, these findings indicate that gastrin may directly stimulate the proliferation of gastric epithelial cells in the neck zone along with indirect stimulation through enhanced production of Reg protein, HB-EGF and AR by ECL and parietal cells.

In conclusion, we demonstrated gastrin receptor gene expression in neck zone cells, which may indicate that gastrin-mediated direct mechanisms play a role in the generation of proliferating cells in the neck zone via their gastrin receptors.

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