Abnormal Gastric Morphology And Function In CCK-B/Gastrin Receptor-Deficient Mice

Guido Rindi\textsuperscript{a,b}, Nancy Langhans\textsuperscript{c}, Jens F. Rehfeld\textsuperscript{e}, Martin Beinborn\textsuperscript{d} and Alan S. Kopin\textsuperscript{d}

\textsuperscript{b}Department of Human Pathology, University of Pavia, Italy; \textsuperscript{c}Department of Pediatrics, \textsuperscript{d}Department of Medicine, GRASP Digestive Disease Center, Tupper Research Institute, New England Medical Center, Tufts University School of Medicine, Boston, Massachusetts; and \textsuperscript{e}Department of Clinical Biochemistry, University Hospital, Copenhagen, Denmark

Mice lacking the cholecystokinin (CCK)-B/gastrin receptor have been generated by targeted gene disruption. The roles of this receptor in controlling gastric acid secretion and gastric mucosal growth have been assessed. The analysis of homozygous mutant mice vs. wild type included measurement of basal gastric pH, plasma gastrin concentrations as well as quantification of gastric mucosal cell types by immunohistochemistry. Mutant mice exhibited a marked increase in basal gastric pH (from 3.2 to 5.2) and about a 10-fold elevation in circulating carboxyamidated gastrin compared with wild-type controls. Histologic analysis revealed a decrease in both parietal and enterochromaffin-like (ECL) cells, thus explaining the reduction in acid output. Consistent with the elevation in circulating gastrin, antral gastrin cells were increased in number while somatostatin cells were decreased. These data support the importance of the CCK-B/gastrin receptor in maintaining the normal cellular composition and function of the gastric mucosa.

INTRODUCTION

The CCK-B/gastrin receptor (CCK-BR)\textsuperscript{1} is a seven transmembrane domain protein with subnanomolar affinity for two endogenous peptides, cholecystokinin (CCK) and gastrin. In the stomach, gastrin is considered the primary physiologic stimulus for CCK-BR mediated functions [1]. Gastrin triggers acid secretion by parietal cells through the convergence of two pathways, each regulated by CCK-B/gastrin receptors: 1) an indirect mechanism in which gastrin stimulation of enterochromaffin-like (ECL) cell CCK-B/gastrin receptors results in histamine release, which in turn leads to parietal cell acid secretion via H\textsubscript{2} receptors; 2) a direct mechanism in which gastrin acts on parietal cell CCK-BRs to stimulate acid release [2]. In addition, gastrin exerts trophic effects on the mucosa of the stomach [3-5]. The gastrin-mediated increase in parietal cell mass is generally considered the result of enhanced entry of progenitor cells into the parietal cell lineage [4, 6]. In contrast, gastrin-dependent ECL cell proliferation appears to be mediated by CCK-B/gastrin receptors on fully differentiated ECL cells [7].

To better understand the mechanisms underlying gastrin-dependent secretory function and cellular proliferation within the gastric mucosa, we have generated mice lacking CCK-B/gastrin receptors.

\textsuperscript{a}Equal contributions were made by the first two authors.
\textsuperscript{b}To whom all correspondence should be addressed: Guido Rindi M.D., Ph.D., Anatomia Patologica 2, Piazza Spedali Civili, 1, I-25124 Brescia, Italy. Tel.: 39-030-3995-830/1; Fax: 39-030-3995-053; E-mail: rindi@master.cci.unibs.it.
\textsuperscript{1}Abbreviations: CCK, cholecystokinin; ECL, enterochromaffin like; IR, immunoreactive; CgA, chromogranin A.
MATERIALS AND METHODS

Generation of mutant mice

The gene targeting strategy, as well as the details pertaining to the generation of homozygous CCK-BR deficient mice, has been described previously [8]. In brief, the CCK-BR gene was isolated from mouse PCC4 genomic library (Stratagene). In the gene targeting construct, part of exon 3, all of exon 4 and part of exon 5 were replaced with a neomycin resistance gene under the control of the phosphoglycerate kinase promoter. The construct introduced a novel Sph I site into the CCK-BR locus enabling a 3’ probe to distinguish the wild-type allele (5.8 kb) from the targeted allele (5.0 kb) in Sph I digested genomic DNA. The linearized targeting construct was electroporated into D3 embryonic stem cells (gift from Richard Hynes, Massachusetts Institute of Technology, Boston, MA) [9, 10]. Embryonic stem cells were screened by genomic Southern blot analysis for the appropriate targeting event [11]. These cells, which had undergone homologous recombination, were injected into blastocysts obtained from C57B6/J mice (Taconic Farms) [10]. Embryos were transferred to pseudopregnant CBA mice (The Jackson Laboratory). Of four chimeric males, one gave germline transmission of the targeted allele and was used to produce mice homozygous for the disrupted CCK-BR gene.

Analysis of CCK-BR mutant mice

The animals used for study were between two and 9 months of age. Plasma and tissue from pairs of age/sex matched wild type and CCK-BR deficient mice were analyzed. Specific radioimmunoassays using antiserum 2604 and 7270 were utilized to measure carboxyamidated and glycine-extended gastrins, respectively [12]. Fasting gastric pH was assessed as described by Takagi et al. [13]. Gastric tissues were embedded in paraffin. Serial sections (3-5 μm) were stained with (i) hematoxylin and eosin for conventional analysis and (ii) PAS/Alcian blue to assess mucus. Immunohistochemical tests were performed using the avidin-biotin-peroxidase complex (SPA, Milano, Italy) method [14]. The following antisera were used: rabbit anti-C-terminal alpha subunit (HKαC2) of pig gastric H/K-ATPase (1:1000, A.J. Smolka, Charleston, S.C., USA) [15] for identification of parietal cells; rabbit anti-rat chromogranin A (CgA) (1:2000, H. Winkler, Innsbruck, Austria) [16] for identification of enterochromaffin-like (ECL) cells; rabbit anti-N-terminus of gastrin 34 (1:2000, AC90, Cambridge Research Biochemicals, Cambridge, UK) for gastrin (G) cells; rabbit anti-somatostatin 28 [1-14] (1:5000, CA-08-330, Cambridge Research Biochemicals, Cambridge, UK) for somatostatin (D) cells. Quantitation of immunoreactive (IR) cells was performed in samples where mucosal glands were oriented perpendicular to the gastric lumen as previously described [17]. For H/K-ATPase-IR cells, counts were performed only in oxyntic glands showing the lumen throughout their entire thickness. Quantification of parietal cells was expressed as number of H/K-ATPase positive cells per gland unit. Gland units were counted in the zymogenic region of the gastric mucosa, paying particular attention to avoid the mucosa of the oxyntic/antral junction where parietal cells tend to be more scattered. Ten gland units were counted per mouse sample. Data were expressed as mean ± standard error of the mean (SEM) and compared by non-parametric statistical analysis (Mann-Whitney). p < .05 was considered statistically significant. For ultrastructural analysis, samples of stomach wall from wild type and mutant mice were processed into Araldite. Semithin sections (0.5-1 μm) were stained with toluidine blue and areas of interest were sectioned (60-100 nm) with a Sorvall ultramicrotome. Ultra-thin sections were observed in a Zeiss 10 CR transmission electron microscope.
RESULTS

Homozygous CCK-BR deficient mice are viable and fertile. No obvious physical or behavioral attributes distinguish the knock-out mice from their wild-type littermates. Histologic analysis of the stomach reveals that the oxyntic glands of mutant animals are, to a variable extent, distinguished from corresponding wild-type mice by an increased number of hematoxylin/eosin amphophylic, PAS/AB negative, H/K-ATPase negative cells (Figure 1a and 1b). A concomitant irregular distribution of parietal and intervening cells is also evident. Preliminary electron microscopic observations (Figure 1c) suggest that this expanded population of intervening cells is made up by epithelial cell precursors as defined by the classification system of Karam and LeBlonde [18].

Basal acid production, circulating gastrin concentrations and the densities of gastric H/K-ATPase-IR parietal cells, CgA-IR ECL cells, somatostatin-IR and gastrin-IR cells are summarized in Table 1. In brief, the fasting gastric pH in mutant mice is significantly higher than in the wild-type controls 5.2 ± 0.4 versus 3.2 ± 0.4, respectively, (p < .01). This is associated with a marked decrease in the densities of H/K-ATPase-IR parietal cells.
The antral mucosa of CCK-BR deficient mice shows a decrease in the number of antral somatostatin-IR cells and increased gastrin-IR cells (Figure 3). The resulting G/D cell ratio was significantly elevated in mutant mice (Table 1). Notably, no change in the number of oxyntic somatostatin-IR cells is observed in CCK-BR deficient mice when compared with controls (Table 1).

**DISCUSSION**

This study illustrates the profound functional and morphologic effects resulting from the ablation of CCK-B/gastrin receptor gene. Gastric acid production is severely impaired in CCK-BR deficient mice. This is consequent to the reduced number of both H/K-ATPase-IR parietal cells, and CgA-IR enterochromaffin-like (ECL) cells. The increase in pH leads to high concentrations of bioactive carboxyamidated gastrin in plasma. Other animal models of hypochlorhydria also show hypergastrinemia [3, 7, 19, 20]. However, in mutant mice, (due to the absence of CCK-B/gastrin receptors) gastrin is ineffective in restoring parietal cell acid production. This contrasts with what is normally observed in the setting of hypergastrinemia where parietal cell mass and ECL cell density are invariably increased [3-5]. Therefore, our data suggest that a minimum level of CCK-BR mediated stimulation is required to maintain these cell populations at normal densities. In addition, the oxyntic mucosa of CCK-BR deficient mice shows a qualitative increase of putative precursor cells and a normal number of somatostatin-producing D cells. We speculate that the absence of CCK-B/gastrin receptors may lead to a developmental arrest or slowdown in the parietal and endocrine cell lineages resulting in a redirection of cellular differentiation into alternative pathways, which in turn may lead to the observed increase in precursor cells.

It is of note that in mutant mice the plasma concentrations of glycine-extended gastrin are comparable to those of controls. Glycine-extended gastrins are the immediate precursors of bioactive, carboxyamidated gastrin-17 and gastrin-34 [21]. The normal concentrations of circulating glycine-extended gastrin and high concentrations of carboxyamidated bioactive gastrin observed in mutant mice may reflect an elevated rate of posttranslational processing of gastrin without limitations in the G-cell capacity for amidation. Such pattern is rarely seen by increased biosynthesis of gastrin [22].

| Table 1. Comparison of gastric pH, circulating gastrins and mucosal cell populations in wild type and CCK-BR deficient mice. |
|--------------------------------------------------|
|                      | pH | Amidated | Gly-extended | Parietal | ECL | Oxyntic | Antral | G-cells | G/D |
|----------------------|----|----------|---------------|----------|-----|---------|--------|---------|-----|
| **Wild type**        |    | 3.       |               | 59       | 129 | 19      | 49     | 24      | 14  | 74  | 5.3 |
| **Knock-out**        |    | 5.2*     | 612*          | 136      | 13* | 24**    | 26     | 8**     | 113**| 15.8* |

* pmol/L; † cells/gland unit; ‡ IR-cells/mm length of mucosa; *p < .01; **p < .05.

(Figure 2a-d) and CgA-IR ECL cells (Figure 2e and 2f). Circulating bioactive carboxyamidated gastrins are elevated in CCK-BR deficient mice (612 pmol/l) versus controls (59 pmol/l) (p < .0001). In contrast, the mean concentrations of circulating glycine-extended gastrin in plasma were unchanged in CCK-BR null mice (128.9 pmol/l versus 136.4 pmol/l in controls).
An abnormal distribution of endocrine cells is observed in the antral mucosa of CCK-BR mutant mice. In addition to the hyperplasia of antral gastrin cells, mutant mice show a reduction in antral D cells. Together these abnormalities lead to a markedly increased G/D cell ratio. The elevated G/D ratio is a normal physiologic response to long term elevation of gastric pH and occurs in rodents after partial fundectomy, or as a result of chronic application of acid-inhibitory drugs [19, 20, 23].

Figure 2. Wild type (a, c, e) versus CCK-BR deficient mouse (b, d, f). Altered gastric mucosal cell populations in CCK-BR deficient mice. Comparison of immunohistochemical findings in the gastric mucosa from age/sex-matched pairs of wild-type (left) and knockout animals (right). The gastric oxyntic mucosa of CCK-BR deficient mice show a reduced number of H/K-ATPase-immunoreactive cells, which are also irregularly distributed along the gland unity (b), see (a) for comparison. At high power (c, d) H/K-ATPase-immunoreactive cells in CCK-BR deficient mice (d) are less regularly distributed and all show variable dilatation of the canaliculi as compared to wild type (c). Marked reduction of chromogranin-A immunoreactive cells in CCK-BR knockout mice oxyntic mucosa (f, arrows) as compared with a wild-type control (e). Immunoperoxidase, light hematoxylin counterstain, a, b: x160; c, d: x1000; e, f: x480.
Our data on CCK-B deficient mice support that antral D cell density is truly a function of gastric pH, independent of circulating gastrin concentrations and CCK-BR activity [19, 20, 23, 24]. Of note, despite the significant changes in antral D-cells densities, the number of D cells in the oxyntic mucosa of CCK-BR knock-out mice was similar to the number in wild type animals. The observation that an increase in gastric pH and circulating gastrin levels has no apparent effect on oxyntic D-cell densities suggests that the mechanisms which regulate the latter cell population are substantially different from those which control D-cells in the antrum.

SUMMARY

In conclusion, the CCK-BR deficient mouse model illustrates how removal of this gene product can disrupt the delicate balance that determines the distribution of endocrine and acid producing cells in the stomach. The changes observed in the stomach of CCK-BR deficient mice support the current theories on gastrin’s role as acid secretagogue and growth factor. Moreover, our results establish the relative physiologic importance of the CCK-BR in maintaining mucosal cytoarchitecture and function of the stomach. The fact that parietal and ECL cells persist in absence of the CCK-BR indicates that some degree of functional redundancy may exist and may significantly contribute to the control of the gastric acid output and cell differentiation.

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REFERENCES

1. Walsh, J.H. Gastrin. In: Walsh, J.H. and Dockray, G.J., eds. Gut Peptides. New York: Raven Press, 1994; pp. 75-121.

2. Chuang, C.-N., Chen, C.Y., and Soll, A.H. Gastrin receptors regulating acid secretory function and growth. In: Walsh, J.H., ed. Gastrin. New York: Raven Press, Ltd.; 1993. pp. 139-150.

3. Larsson, H., Carlsson, E., Mattsson, H., Lundell, L., Sundler, F., Sundell, G., Wallmark, B., Wantanabe, T., and Häkanson, R. Plasma gastrin and gastric enterochromaffinlike cell activation and proliferation. Studies with omeprazole and ranitidine in intact and antrectomized rats. Gastroenterology 90:391-399, 1986.

4. Willems, G. and Lehy, T. Radioautographic and quantitative studies on parietal and peptic cell kinetics in the mouse. A selective effect of gastrin on parietal cell proliferation. Gastroenterology 69:416-426, 1975.

5. Ryberg, B., Axelsson, J., Hakanson, R., Sundler, F., and Mattsson, H. Trophic effects of continuous infusion of [Leu]S-gastrin-17 in the rat. Gastroenterology 98:33-38, 1990.

6. Eissele, R., Patberg, H., Koop, H., Krack, W., Lorenz, W., and Arnold, R. Effect of gastrin receptor blockade on endocrine cells in rats during achlorhydria. Gastroenterology 103:1596-1601, 1992.

7. Karam, S.M. Dynamics of epithelial cells in the corpus of the mouse stomach. IV. Bidirectional migration of parietal cells ending in their gradual degeneration and loss. Anat. Rec. 236:314-332, 1993.

8. Langhans, N., Rindi, G., Chiu, M., Rehfeld, L.J., Ardman, B., Beinborn, M., and Kopin, A.S. Abnormal gastric histology and decreased acid production in cholecystokinin-B/gastrin receptor-deficient mice. Gastroenterology 112:280-286, 1997.

9. Doetschman, T.C., Eisterter, H., Katz, M., Schmidt, W., and Kemler, R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morphol. 87:2745, 1985.

10. Manjunath, N., Correa, M., Ardman, M., and Ardman, B. Negative regulation of T-cell adhesion and activation by CD43. Nature 377:535-538, 1995.

11. Ramirez-Solís, R., Rivera-Perez, J., Wallace, J.D., Wims, M., Zheng, H., Bradley, A. Genomic DNA microextraction: a method to screen numerous samples. Anal. Biochem. 201:331-335, 1992.

12. Hilsted, L. and Rehfeld, J.F. Measurement of precursors for alpha-amidated hormones by radioimmunoassay of glycine-extended peptides after trypsin-carboxypeptidase B cleavage. Anal. Biochem. 152:119-126, 1986.

13. Takagi, H., Jhappan, C., Sharp, R.I., and Merliino, G. Hypertrophic gastropathy resembling Menetrier's disease in transgenic mice overexpressing transforming growth factor alpha in the stomach. J. Clin. Invest. 90:1161-1167, 1992.

14. Hsu, S.M., Raine, L., and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. 29:577-580, 1981.

15. Smolka, A. and Swiger, K.M. Site-directed antibodies as topographical probes of the gastrin H, K-ATPase alpha-subunit. Biochem. Biophys. Acta 1108:75-85, 1992.

16. Rindi, G., Grant, S.G., Yangou, Y., Ghatel, M.A., Bloom, S.R., Bautch, V.L., and Polak, J.M. Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Heterogeneity of hormone expression. Am. J. Pathol. 136:1349-1363, 1990.

17. Annibale, B., Bonamico, M., Rindi, G., Villani, L., Ferrante, E., Vania, A., Solcia, E., and Delle Fave, G. Antral gastrin cell hyperfunction in children. A functional and immunocytochemical report. Gastroenterology 101:1547-1551, 1991.

18. Karam, S. and LeBlonde, C.P. Origin and migratory pathways of the eleven epithelial cell types present in the body of the mouse stomach. Microsc. Res. Tech. 31:193-214, 1995.

19. Creutzfeldt, W., Stockmann, F., Conlon, J.M., Folsch, U.R., Bonatz, G., and Wulfrath, M. Effect of short- and long-term feeding of omeprazole on rat gastric endocrine cells. Digestion 35(suppl 1): 84-97, 1986.

20. Lundell, L., Bishop, A.E., Bloom, S.R., Carlsson, K., Mattsson, H., Polak, J.M., and Ryberg, B. Gastrin and somatostatin in the rat antrum. The effect of removal of acid-secreting mucosa. Reg. Pept. 23:77-87, 1988.

21. Rehfeld, J.F. and Johnsen, A.H. Structure of Bioactive gastrins. In: Walsh, J.H., ed. Gastrin. New York: Raven Press, Ltd.; 1993. pp. 1-14.

22. Jensen, S., Borch, K., Hilsted, L., and Rehfeld, J.F. Progastrin processing during antral G-cell hypersecretion in man. Gastroenterology 96:1063-1070, 1989.
23. Lee, H., Hakanson, R., Karlsson, A., Mattsson, H., and Sundler, F. Lansoprazole and omeprazole have similar effects on plasma gastrin levels, enterochromaffin-like cells, gastrin cells and somatostatin cells in the rat stomach. Digestion 51:125-132, 1992.
24. Arnold, R., Hulst, M.V., Neuhof, C.H., Schwarting, H., Becker, H.D., and Creutzfeldt, W. Antral gastrin producing G-cells and somatostatin-producing D-cells in different states of gastric acid secretion. Gut 23:285-291, 1982.