Utilization of Knockout Mice to Examine the Potential Role of Gastric Histamine H₂-Receptors in Menetrier’s Disease

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Abstract. Menetrier’s disease is characterized by giant gastric folds with foveolar hyperplasia and cystic dilatation, hypoproteinemia, and enhanced mucus secretion. The etiology remains unresolved and an effective treatment has yet to be established. Here we show that histamine H₂-receptor deficient mice developed gastric pathophysiological changes resembling Menetrier’s disease for up to 17 months of observation. Mutant mice were found to have an increased stomach weight, enlarged gastric folds with cystic dilatation, hypergastrinemia, hypoalbuminemia, increased mucus secretion and overexpression of mucosal transforming growth factor (TGF) α. Both a cholecystokinin (CCK)₂-receptor antagonist and an epidermal growth factor (EGF)-receptor tyrosine kinase inhibitor significantly reduced the increase in stomach weight. It appears that lack or downregulation of histamine H₂-receptors might be involved in the pathogenesis of Menetrier’s disease.

Keywords: histamine H₂-receptor knockout mice, hyperplastic gastropathy, gastrin, transforming growth factor α, Menetrier’s disease

Introduction

The hallmark of Menetrier’s disease is marked enlargement of the gastric folds, primarily observed in the oxyntic glandular area (fundus and corpus), with foveolar hyperplasia and cystic dilatation of the glands (1 – 6). Laboratory findings in this kind of disease include hypoproteinemia, hypochlorhydria, enhanced mucus secretion, and increased expression of transforming growth factor (TGF) α (involvement of TGF α-epidermal growth factor (EGF) axis) (7 – 9). Despite the fact that more than 100 years have passed since the disease was initially reported, the etiology remains unclear and an effective treatment has yet to be established.

We have previously reported morphological and functional changes in stomachs of 16-week-old histamine H₂-receptor deficient (H₂R-KO) mice (10). The increased stomach weight and mucosal hyperplasia in mutant mice strongly suggested that H₂R are not only involved in the regulation of acid secretion, but also play a key role in normal cellular homeostasis of the gastric mucosa. The question of whether or not such gastric changes progress with time, resulting in more serious damage, remains an area of great interest. In a preliminary study, we noticed that H₂R-KO mice 6 months in age developed Menetrier’s disease-like changes in the gastric fundus (oxyntic glandular region) at a high incidence (11).

In an attempt to elucidate the underlying mechanism of mucosal hyperplasia associated with Menetrier’s disease, the present study examined the development in gastric mucosal changes in H₂R-KO mice from birth to 17 months of age.

Materials and Methods

Animals

Male H₂R-KO mice and C57BL/6 mice (Nihon SLC, Hamamatsu) were used at different times after birth. H₂R-KO mice were produced as previously described
(10). Briefly, the mouse H$_2$R gene was disrupted and chimera mice were generated. The animals were back-crossed with C57BL/6 mice 6 times, yielding heterozygous H$_2$R-KO mice. Targeted H$_2$R gene deletion was verified by RT-PCR and Southern blot analysis. Prior to experimentation, all mice were deprived of food for 22 h and water for 2 h. The maintenance of the animals and experimental procedures were conducted in accordance with the guidelines of the Ethics Committee of Kyoto Pharmaceutical University.

**Determination of intragastric pH and gross gastric examination**

Under ether anesthesia, each abdomen was opened and the stomach was removed. Subsequently, approximately 50 $\mu$l of gastric contents was collected with a Pipetman (Gilson, France) through a small hole made in the fore-stomach. The pH was directly measured using pH paper (Macherey-Nagel GmbH & Co., Düren, Germany). Each stomach was then opened, weighed (wet weight), and examined under a dissecting microscope ($\times$10).

**Determination of serum gastrin and albumin levels**

Under ether anesthesia, blood samples were withdrawn from the orbital plexus of each mouse with a capillary tube. Following centrifugation at 6,000 × g for 15 min, the serum was isolated and gastrin and albumin concentrations were determined. Gastrin concentrations were determined by radioimmunoassay using human gastrin-17 as standard (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo). Serum albumin concentrations were determined by a brom cresol green (BCG) assay (12, 13), whereby serum samples were deposited in commercially available BCG kits and placed for 10 min at room temperature. Absorbance was read at room temperature in a spectrophotometer (Pharmacia Biotech, Cambridge, UK) at 628 nm. Bovine serum albumin (Nacalai, Osaka) was used as a standard.

**Histologic examination**

Several specimens were removed from each stomach, fixed with Carnoy’s fixative overnight, and embedded in paraffin wax. Paraffin sections (4 $\mu$m) were prepared and stained with hematoxylin-eosin or examined with immunological assays. Parietal, ECL, G, and D cells were detected with a murine monoclonal anti-H$_2$ATPase antibody (MBL, Nagoya), a rabbit antibody against rat chromogranin A (Yanaihara Institute, Inc., Fujinomiya), an anti-gastrin antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a rabbit antisomatostatin antibody (Dako, Carpinteria, CA, USA), respectively. Anti-TGF-α antibody (H-50, Santa Cruz Biotechnology) was used to demonstrate TGF-α in the fundic mucosa. Each assay was visualized with the avidin-biotin-peroxidase complex method, using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and a 3,3’-diaminobenzidine tetrahydrochloride preparation (Dojindo Laboratories, Kumamoto). All sections were stained with hematoxylin.

**Pharmacological experiments**

In an earlier study, we showed that the serum gastrin and gastric histamine concentrations were elevated in H$_2$R-KO mice (10). Accordingly, the effects of a cholecystokinin (CCK); receptor antagonist (YM022 [(R)-1-[[2,3-dihydro-1-(2’-methylphenacyl)-2-oxo-5-phenyl-1H,1,4-benzodiazepin-3-yl]-3-(3-methylphenyl) urea]; Yamanouchi Pharmaceutical Industries, Tokyo) and a histamine H$_2$R antagonist (epinastine; Boehringer-Ingelheim, Ingelheim, Germany) on stomach weight and gastric mucosal changes were examined. Each drug, suspended in 0.5% hydroxypropylcellulose, was orally administered twice daily at a dose of 30 mg/kg for 5 or 10 weeks beginning 4 weeks after birth. In the clinical setting, anticholinergic drugs and acid pump inhibitors are frequently used in the treatment of Menetrier’s disease (6, 14 – 16). Accordingly, the effects of pirenzepine, omeprazole, and YM022 on stomach weight and serum albumin and gastrin levels in either wild-type or H$_2$R-KO mice were also examined with 3-month administrations beginning 6 months after birth. These drugs were dissolved in 0.5% hydroxypropylcellulose and administered either once daily (omeprazole, 30 mg/kg) or twice daily (pirenizepine, 3 mg/kg; YM022, 30 mg/kg). These mice were killed at 9 months of age and subjected to serologic and gastric examination. Typhostin A46, an EGF-receptor tyrosine kinase inhibitor, was purchased from Tocris Cookson, Inc. (Ballwin, MO, USA). After first dissolving the drug in 100% ethanol and diluting the drug in 1.5% ethanol, the drug was subcutaneously administered once daily at a dose of 0.2 mg/kg for 10 weeks, beginning 4 weeks after birth, or 3 months, beginning 6 months after birth. Control animals received vehicle alone. Epinastine and typhostin A46 doses were based on references of other investigators (17 – 20), as well as our own data. Each drug was freshly prepared and administered in a volume of 1 ml/200 g body weight.

**Statistical analyses**

All data are presented as the means ± S.E.M. Statistical analysis was performed using the two-tailed Dunnett’s multiple comparison test and the Student’s t-test, with a P-value <0.05 regarded as significant.
Results

Phenotypic changes in H₂R-KO mice

As evidenced by progression of body weight for up to 9 months, H₂R-KO mice were as viable as wild-type mice (Fig. 1a). The average stomach weight of H₂R-KO mice was significantly greater than that of wild-type mice 3, 6, and 9 months after birth. The mean intragastric pH of H₂R-KO mice was significantly elevated compared with wild-type mice 3 weeks after birth (3.4 ± 0.3 vs 1.8 ± 0.3; P<0.05, Fig. 1b). The mean intragastric pH of 3-, 6-, and 9-month-old H₂R-KO mice, however, was similar to that of corresponding wild-type mice. In wild-type mice (Fig. 2), stomach size appropriately increased with age. In contrast, stomach size of H₂R-KO mice markedly increased 3, 6, and 9 months after birth (Fig. 2). Enlarged folds were clearly observed in the fundic area of 6-, 9-, 14-, and 17-month-old mutant mice, although the antral region remained normal. The stomachs of 14- and 17-month-old mutant mice were approximately 3 times larger in size and weight than those of corresponding wild-type mice.

Histological quantification demonstrated that the thickness of the gastric fundic mucosa in ≥3-month-old H₂R-KO mice significantly increased with time. In the gastric fundus of 9-month-old H₂R-KO mice, massive hyperplasia with many cystic glands of varying sizes was observed (Fig. 3a). In 14-month-old H₂R-KO mice, a thickened fundic mucosa with various dilated cystic glands was markedly contrasted by the normal histology observed in the wild-type mice. PAS-positive mucin-secreting cells were found in the surface, neck zone and basal portions of the glands in the hyperproliferated mucosa (Fig. 3b). In approximately 30% of 17-month-old H₂R-KO mice, the thickened fundic mucosa with cystic dilatation exhibited massive diverticuli under the muscularis mucosa (Fig. 3c). In isolated cases, diverticuli penetrating the muscularis mucosa were observed.
Quantitative studies indicated a near 4-fold difference in gastric mucosal thickness of 9-month-old H$_2$R-KO mice and wild-type mice ($1.58 \pm 0.06$ vs $0.40 \pm 0.01$ mm; $P<0.05$, Fig. 4a). The number of parietal and ECL cells in H$_2$R-KO mice was significantly higher than wild-type mice at both 3 and 6 months (Fig. 4, b and c). In 9-month-old H$_2$R-KO mice, parietal and ECL cell counts were indeterminable due to deformed glandular architecture. In contrast to parietal cells, ECL cell size in H$_2$R-KO mice was similar to that observed in wild-type mice at 3, 6, and 9 months. The number of G cells in the antrum of H$_2$R-KO mice was significantly increased compared with wild-type mice at 3, 6, and 9 months (Fig. 4d). In contrast to G cells, the number of D cells in H$_2$R-KO mice was significantly reduced compared with wild-type mice at 3, 6, and 9 months (Fig. 4e). Consequently, the G/D ratio in H$_2$R-KO mice was significantly higher than that observed in wild-type mice for up to 9 months (Fig. 4f). The average parietal
cell size in H\textsubscript{2}R-KO mice was smaller than that observed in wild-type mice for up to 9 months (Fig. 5, b vs a). Similarly, in 14-month-old mice parietal cell size of H\textsubscript{2}R-KO mice was clearly smaller compared with wild-type mice (Fig. 5, j vs i). The number of ECL cells was also clearly increased, particularly near cystic glands (Fig. 5, l vs k). Interestingly, G cells were observed in the hyperplastic fundic mucosa (Fig. 5, n vs m). It should be noted that TGF \textalpha\ overexpression was noted in hyperplastic fundic regions at both 14 months (Fig. 5, p vs o) and 17 months (Fig. 6, d vs c).

\textbf{Serum gastrin and albumin levels}

The serum gastrin concentration in H\textsubscript{2}R-KO mice was slightly increased 3 weeks after birth (Fig. 1c) and significantly increased in 5-, 6-, and 9-month-old H\textsubscript{2}R-KO mice compared to wild-type mice (706.2 \pm 156.4, 778.2 \pm 302.9, and 683.3 \pm 112.3 pg/ml vs 810.0 \pm 11.4, 68.7 \pm 11.0, and 99.7 \pm 8.3 pg/ml, respectively, \(P<0.05\)).

The serum albumin level in 3-month-old H\textsubscript{2}R-KO mice did not differ from that observed in wild-type mice (5.4 \pm 0.1 vs 5.4 \pm 0.1 g/dl; Fig. 1d). The albumin level in 6- and 9-month-old H\textsubscript{2}R-KO mice, however, was significantly reduced compared with wild-type mice (4.7 \pm 0.1 and 4.2 \pm 0.1 g/dl vs 5.1 \pm 0.1 and 5.4 \pm 0.1 g/dl, respectively, \(P<0.05\)).

\textbf{Pharmacological effects on H\textsubscript{2}R-KO mice stomachs}

Continual treatment with epinastine (histamine H\textsubscript{1}R-antagonist, 30 mg/kg) twice daily for 10 weeks had no effect on the increase in average stomach weight of H\textsubscript{2}R-KO mice (Fig. 6a). In contrast, treatment with YM022 (CCK-R antagonist, 30 mg/kg) twice daily for 10 weeks significantly inhibited the increase in average stomach weight of H\textsubscript{2}R-KO mice by 19.8%. Histologic analysis of stomachs treated with YM022 exhibited a significant decrease in gastric mucosal thickness compared with control mice (0.65 \pm 0.02 vs 0.75 \pm 0.03 mm, \(P<0.005\)), parietal cell count (35.4 \pm 1.7 vs 43.5 \pm 2.6 cells/gland, \(P<0.05\)), and ECL cell count (8.1 \pm 0.5 vs 11.6 \pm 0.7 cells/gland, \(P<0.05\)).

Treatment of H\textsubscript{2}R-KO mice with tyrophostin A46 (EGF-receptor tyrosine kinase inhibitor, 0.2 mg/kg) once daily for 10 weeks beginning 4 weeks after birth also significantly prevented an increase in average stomach weight compared with control mice by 18%. Treatment of H\textsubscript{2}R-KO mice with either YM022 (30 mg/kg, twice daily) or tyrophostin A46 (0.2 mg/kg, once daily) for 3 months beginning 6 months after birth also significantly inhibited stomach weight increases by 39.1% and 14.2%, respectively. In wild-type mice treated with YM022 and tyrophostin, the stomach weight was 0.20 \pm 0.01 g (n = 4) for tyrophostin A46 and 0.20 \pm 0.01 g (n = 5) for YM022 vs 0.19 \pm 0.003 g in the control group (n = 5). The stomach weights in mice treated with drugs did not significantly differ from the control value.

Three-month administration of either omeprazole (acid pump inhibitor, 30 mg/kg, twice daily) or pirenzepine (cholinergic M\textsubscript{1}R antagonist, 3 mg/kg, twice daily) exerted no effect on average stomach weight compared with the control mice; i.e., 0.48 \pm 0.02 g for omeprazole (n = 4) and 0.46 \pm 0.02 g for pirenzepine (n = 5) vs 0.51 \pm 0.02 g for the control (n = 5)). At that time, we reconfirmed that albumin levels in H\textsubscript{2}R-KO mice were significantly reduced compared with wild-
type mice (3.90 ± 0.13 g/dl, n = 4 vs 4.60 ± 0.04 g/dl, n = 5, P<0.05). Such a reduction in albumin levels was not affected with the treatment of omeprazole and pirenzepine; i.e., 3.79 ± 0.12 g/dl for omeprazole and 4.02 ± 0.17 g/dl for pirenzepine.

Discussion

H₂R-KO (≥6-month-old) exhibited striking gastric changes confined to the fundus; i.e. sparing the antrum, resembling Menetrier’s disease. Such findings strongly suggest that Menetrier’s disease stems from a complete lack or down-regulation of gastric parietal cell H₂R. The following characterizes the similarities between the phenotypes of H₂R-KO mice and patients with Menetrier’s disease.

A histological feature of Menetrier’s disease is development of giant gastric fold hyperplasia with dilated cystic glands. Such typical morphological changes in Menetrier’s disease were clearly observed in all H₂R-KO mice, with a severity that increased with age. It is of interest that intragastric pH was significantly higher in H₂R-KO mice compared to wild-type mice at only 3 weeks after birth; thereafter, pH levels in H₂R-KO and wild-type mice were similar for up to 9 months. Since the mean intragastric pH in H₂R-KO mice was approximately 2.0 – 3.7, it is clear that parietal cells were stimulated by cholinergic nerves or circulating gastrin, as in wild-type mice. The interesting serological features of Menetrier’s disease are hypergastrinemia.

Fig. 4. Histological analysis of gastric mucosa of wild-type vs H₂R-KO mice. As expected from Fig. 2, mucosal thickness significantly increased with time (a). Parietal and ECL cell counts also significantly increased up to 6 months of age (b, c). Fundic mucosa parietal and ECL cell counts were not obtainable at 9 months of age due to deformed mucosal architecture. Although the G cell count significantly increased with age (d), the D cell count significantly decreased with age (e), resulting in a significantly increased G/D ratio (f) in H₂R-KO mice. Data are presented as the means ± 1 S.E.M (n = 8 – 13). *Significantly different from the corresponding wild type mice, P<0.05.
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and hypoproteinemia. H₂R-KO mice were also found to be hyper-gastrinemic. Histologic analysis demonstrated that although the antral G cell count in H₂R-KO mice was significantly increased 3, 6, and 9 months after birth, the D cell count was significantly reduced. The increase in G/D ratio appears to be solely responsible for the decrease of D cell number evoked by unknown mechanism.

Accordingly, the G/D ratio became quite high, suggesting that hypergastrinemia might result from reduced production of D-cell-derived somatostatin. Such an increased G/D ratio is observed in CCK₂-R-KO mice (21).

Fig. 5. Immunohistochemical staining of wild-type and H₂R-KO mice gastric mucosa. Parietal (H⁺,K⁺-ATPase) (a, b, i, j) and ECL cells (chromogranin A) (c, d, k, l) in the fundus, and G (e, f) and D cells (g, h) in the antrum of 9- and 14-month-old wild-type and H₂R-KO mice are shown. The parietal cell count was clearly increased in H₂R-KO mice compared with wild-type mice, although the average cell size was smaller. ECL and G cell counts were also increased in 9-month-old H₂R-KO mice, although the antral D cell count was decreased. In 14-month-old mutant mice, ECL cells were observed around dilated cystic glands. G-cells, not found in the fundic region of wild-type mice (m), were observed in the fundic region of H₂R-KO mice (n). Although there was little or no TGFα expression in gastric mucosa in wild-type mice (o), TGFα overexpression was observed in fundic mucosa of H₂R-KO (p). Bar indicates 50 μm (a – d, i, j) (original magnification, ×400), 0.1 mm (e – h, m – p) (original magnification, ×200), 0.1 mm (k, l) (original magnification, ×100).
Interestingly, ectopic G cells were noted in the thickened fundic mucosa similar to observations made in hypergastrinemic mice (22). Given gastrin’s trophic activity, such a finding in H2R-KO mice suggests that progenitor cells in the proliferating zone were greatly affected by enhanced serum gastrin levels (23–25).

Serum albumin levels were significantly reduced 6 and 9 months after birth; i.e., at times when enlarged gastric mucosal folds were already apparent. Such results strongly suggest that serum albumin leaked into the gastric lumen via damaged gastric mucosa; e.g., at sites of loosened tight junctions or enhanced vascular permeability. In addition, the fall in the albumin level strongly supports the idea that gastric mucosal changes observed in H2R-KO mice closely resemble Menetrier’s disease in humans.

It is well known that TGFα plays a critical role in normal cellular homeostasis and gastric mucosa functioning in humans and laboratory animals (26–33). Based on clinical and research studies, it was postulated that TGFα is intimately involved in the pathogenesis of Menetrier’s disease (28, 34, 35). Interestingly, a monoclonal antibody against EGF receptor markedly improved a Menetrier’s disease patient (36). The biochemical hallmark of Menetrier’s disease is gastric mucosal TGFα overexpression, suggesting that mucosal

![Fig. 6. Pharmacological effects on the stomach weight of H2R-KO mice vs wild-type mice.](image)

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**a**

Wild-type KO

Pre-treatment 10-wk treatment

![Graph showing stomach weight comparison](image)

**b**

Wild-type KO

Pre-treatment 3-mo treatment

![Graph showing stomach weight comparison](image)

**c**

Wild-type KO

Pre-treatment 10-wk treatment

![Histological image](image)

**d**

Wild-type KO

Pre-treatment 10-wk treatment

![Histological image](image)
hyperplasia might stem from an increase in TGF \( \alpha \) (5, 6, 37). Indeed, TGF \( \alpha \) overexpression in 14- and 17-month-old H2R-KO mice fundic mucosa was demonstrated. In TGF \( \alpha \) transgenic mice, increased gastrin levels were observed 3 months after birth (31). Accordingly, coexpression of TGF \( \alpha \) and plasma gastrin might underlie hyperplastic gastric mucosa development.

In addition, we found that epinastine had no effect on average stomach weight of H2R-KO mice, suggesting that histamine H2R has no role in mucosal changes in H2R-KO mice. In contrast, a 3 month YM022 treatment in 6-month-old H2R-KO mice significantly suppressed average stomach weight. Furthermore, long-term treatment with tyrphostin A46 for 10 weeks (administered to 4-week-old mice) or 3 months (administered to 6-month-old mice) significantly inhibited an increase in average stomach weight. Furthermore, long-term treatment with tyrphostin A46 for 10 weeks (administered to 4-week-old mice) or 3 months (administered to 6-month-old mice) significantly inhibited an increase in the average stomach weight of H2R-KO mice. Such results provide strong evidence that both the increase in average stomach weight and development of mucosal hyperplasia might be caused by both a trophic effect of gastrin and overexpressed TGF \( \alpha \) acting on EGFRI. Clinically, treatment of Menetrier’s disease with anticholinergic agents (propantheline, pirenzepine), cimetidine, and omeprazole results in some improvement (14 – 16). In our H2R-KO mice model, however, 3 month treatment with pirenzepine and omeprazole had no appreciable effect on the increase in stomach weight and mucosal thickening. Such findings suggest that blockade of cholinergic M1R or H1, K+ -ATPase has no influence on mucosal hyperplasia development.

We suggest that H2R dysfunction on gastric parietal cells represents the primary cause for development of Menetrier’s disease, as evidenced by development of a similar disease, albeit to a limited extent, with prolonged H2R antagonist treatment. Geist et al. demonstrated that a patient with a duodenal ulcer treated with a potent H2R antagonist (ranitidine) developed tremendous gastric mucosal thickening and hypoproteinemia. Such a report strongly suggests that the H2R plays an instrumental role in Menetrier’s disease pathogenesis.

Since the discovery of gastric Helicobacter pylori (\( H. pylori \)), various reports have suggested that \( H. pylori \) might represent the underlying etiology of Menetrier disease, as evidenced by disease suppression with eradication of the bacteria (39 – 43). We are currently investigating whether or not \( H. pylori \) infection suppresses the expression of gastric H2R.

In conclusion, morphological and functional changes observed in H2R-KO mice that resemble Menetrier’s disease appear to result from a combination of the trophic action of hypergastrinemia and TGF \( \alpha \) overexpression, leading to stimulation of gastric mucosal multipotent progenitor cells.

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