Alterations in gastric mucosal lineages induced by acute oxyntic atrophy in wild-type and gastrin-deficient mice

Sachiyo Nomura, Hirokazu Yamaguchi, Masako Ogawa, Timothy C. Wang, Jeffrey R. Lee, and James R. Goldenring

1Nashville VA Medical Center, and the Department of Surgery, Epithelial Biology Program, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee; 2Department of Gastrointestinal Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; 3Augusta VA Medical Center and Department of Pathology and Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia; 4Department of Medicine, University of Massachusetts School of Medicine, Worcester, Massachusetts

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Nomura, Sachiyo, Hirokazu Yamaguchi, Masako Ogawa, Timothy C. Wang, Jeffrey R. Lee, and James R. Goldenring. Alterations in gastric mucosal lineages induced by acute oxyntic atrophy in wild-type and gastrin-deficient mice. Am J Physiol Gastrointest Liver Physiol 288: G362–G375, 2005; doi:10.1152/ajpgi.00160.2004.—In addition to their role in gastric acid secretion, parietal cells secrete a number of growth factors that may influence the differentiation of other gastric lineages. Indeed, oxyntic atrophy is considered the most significant correlate with increased risk for gastric adenocarcinoma. We studied the alterations in gastric mucosal lineages elicited by acute oxyntic atrophy induced by treatment of C57BL/6 and gastrin-deficient mice with the parietal cell protonophore [S-(R*,S*)]-N-[1-(1,3-benzeniodiol-5-yl)butyl]-3,3-diethyl-2-[[4-(4-methyl-1-piperazinyl)carbonyl]phenoxyl]-4-oxo-1-azetidine carboxamide (DMP-777). In both wild-type and gastric knockout mice, DMP-777 elicited the rapid loss of parietal cells within 2 days of treatment. In wild-type mice, oxyntic atrophy was accompanied by a rapid increase in 5-bromo-2′-deoxyuridine-labeled proliferative cells and attendant increase in surface cell numbers. However, gastric knockout mice did not demonstrate significant foveolar hyperplasia and showed a blunted proliferative response. After 7 days of treatment in wild-type mice, a second proliferative population emerged at the base of fundic glands along with the development of a mucous cell metaplasia expressing TFF2/spasmolytic polypeptide (SPEM). However, in gastric knockout mice, SPEM expressing both TFF2 mRNA and protein developed after only 1 day of DMP-777 treatment. In wild-type mice, all changes induced by DMP-777 were reversed 14 days after cessation of treatment. In gastric-deficient mice, significant SPEM was still present 14 days after the cessation of treatment. The results indicate that foveolar hyperplasia requires the influence of gastrin, whereas SPEM develops in response to oxyntic atrophy independent of gastrin, likely through transdifferentiation of chief cells. In accord with this, SPEM expressing TFF2/spasmolytic polypeptide develops in wild-type mice, a second proliferative population emerges at the base of fundic glands along with the development of a mucous cell metaplasia expressing TFF2/spasmolytic polypeptide (SPEM). However, in gastric knockout mice, SPEM expressing both TFF2 mRNA and protein developed after only 1 day of DMP-777 treatment. In wild-type mice, all changes induced by DMP-777 were reversed 14 days after cessation of treatment. In gastric-deficient mice, significant SPEM was still present 14 days after the cessation of treatment. The results indicate that foveolar hyperplasia requires the influence of gastrin, whereas SPEM develops in response to oxyntic atrophy independent of gastrin, likely through transdifferentiation of chief cells.

hyperplasia: trefoil proteins; metaplasia; spasmolytic polypeptide; transdifferentiation

NORMAL GASTRIC FUNDIC MUCOSA is assembled from a diverse group of cell lineages responsible for lumenal secretion of mucins, pepsinogen, intrinsic factor (IF), and HCl. A number of investigations over the past decade has demonstrated that lineages in the normal fundic mucosa arise from a progenitor zone located in the lumenal third of the glands (18). In the gastric mucosa, the relative luminal location of the progenitor zone is maintained by the differentiation of cell lineages with differing longevity. Short-lived surface mucous cells, with a 4- to 6-day lifetime, arise from the progenitor zone and migrate toward the lumen (19). Whereas a minority of the parietal cells migrates toward the luminal surface, the majority of parietal cells arises from the progenitor zone and migrates toward the basal lamina (17). Mucous neck cells arise from preneck cells and, during migration toward the base, redifferentiate into chief cells (20). Those cells migrating toward the base have a long lifetime, ~80 days in parietal cells and 200 days in chief cells (17, 20).

The process of differentiation of specific cell lineages from the progenitor zone is regulated by both hormonal and paracrine regulators, including transforming growth factor (TGF)-α (5, 36), histamine (24, 32), and gastrin (8, 25). Metallothionein-TGF-α transgenic mice demonstrate expansion of the surface cell compartment of fundic glands along with a marked decrease in parietal cell and chief cell numbers (3, 12). Whereas histamine decarboxylase-deficient mice have relatively normal gastric lineage profiles (39), mice with targeted H2-histamine receptor knock out demonstrate foveolar hyperplasia and hypersecretion of TGF-α (24, 32).

Gastrin is the hormone most associated with lineage differentiation in the stomach (8, 15). The CCK-B/gastrin receptor is expressed in parietal and enterochromaffin-like (ECL) cells (4, 38), and gastrin stimulates the secretory function as well as the production of these cell lineages (23, 33, 40). Elevated levels of serum gastrin induce expansion of parietal cell numbers in patients with gastrinoma (30). Actin-gastrin transgenic mice, which demonstrate gastrin levels in excess of 500 pg/ml, develop foveolar hyperplasia and small-sized parietal cells without significant increases in the number of the parietal cells (26, 29). Insulin-gastrin transgenic mice are also hypergastrinemic and develop foveolar hyperplasia early in life (41). Gastrin-deficient mice have reduced fundic mucosal thickness and fewer parietal cells compared with wild-type mice (11, 25).

Recently, we have reported (13) a model for pharmacological induction of oxyntic atrophy with DMP-777. DMP-777 is a cell-permeant neutrophil elastase inhibitor, which also acts as a parietal cell-specific protonophore and specifically ablates parietal cells. Treatment of rats with DMP-777 for 3 mo
induced oxyntic atrophy and expansion of the surface cell compartment as well as the emergence from the base of fundic glands of a metaplastic mucous cell lineage expressing TFF2/spasmolytic polypeptide (SPEM) (13). We have also observed SPEM in mice infected with Helicobacter felis (42), in humans with fundal predominant H. pylori gastritis (35), and in the mucosa surrounding gastric adenocarcinoma (14, 35, 45). These results support the hypothesis that loss of parietal cells may be a primary event in the evolution of the spectrum of lineage changes. Because DMP-777 causes rapid elevations in serum gastrin secondary to hypochlorhydria, histological changes caused by the DMP-777 treatment may accrue from the influences of high serum gastrin. We have now investigated alterations in cell lineages in the stomachs of both C57BL/6 mice and gastrin-deficient mice treated acutely with DMP-777. Our results demonstrate that DMP-777 treatment of wild-type mice elicits a phenotype similar to that seen in rats with acute oxyntic atrophy, acute foveolar hyperplasia, and the emergence of SPEM after 7 days of treatment. In contrast, whereas DMP-777 treatment in gastrin-deficient mice does cause a loss of parietal cells, no foveolar hyperplasia is observed and SPEM is induced after only 1 day of treatment. The results indicate that gastrin exerts a major influence on the response of the gastric mucosa to acute oxyntic atrophy.

MATERIALS AND METHODS

Experimental design. C57BL/6 mice were obtained from Charles River Breeding Laboratories or Jackson Laboratories. Gastrin-deficient mice were constructed by targeted disruption of the gastrin gene, as described previously (25), and were maintained on a C57BL/6 background. During the experiments, the mice were maintained with regular mouse chow in a temperature-controlled room under a 12:12-h light-dark cycle.

DMP-777, formulated at a concentration of 2% as a suspension in 0.5% methylcellulose, was a gift of DuPont Pharmaceuticals. C57BL/6 mice (8 wk of age) and gastrin-deficient mice (8 wk of age) were administrated DMP-777 orally as a gavage (350 mg/kg) once daily. Groups of six mice each were killed before starting drug administration and after 1, 3, 7, 10, and 14 days of drug administration. Additionally, groups of six mice each received 14 days of DMP-777 treatment and were then killed 14 days after stopping drug administration (recovery period). For all mice, 2 h before death, 5-bromo-2’-deoxyuridine (BrdU; 200 mg/kg) in saline was injected intraperitoneally. At necropsy, the mice were perfused with 4% paraformaldehyde through the left ventricle for 10 min under anesthesia with avertin. The stomachs were excised, postfixed in fixative for 2 h, embedded in paraffin, and cut into 5-μm sections. Replicate sections were stained with hematoxylin and eosin (H&E) as well as diastase-resistant-periodic acid Schiff (DR-PAS).

Serum gastrin measurement. Blood was collected from the right ventricle of six mice treated with DMP-777 for 0, 1, 7, and 14 days, after which time the mice were killed under anesthesia. The serum was isolated following centrifugation of the blood and was kept frozen until the time of the measurement. Serum gastrin was measured using a gastrin assay kit (Gastrin RIA kit II, Abbott Japan, Tokyo, Japan).

In situ hybridization. The cRNA probe for TFF2 was constructed and labeled as previously described (31). Briefly, linearized template DNA (1 μg) was incubated for 2 h at 37°C in a buffer containing 10 mM DTT, 1 mM digoxigenin-11-NTP (Boehringer-Mannheim; Mannheim, Germany), and 40 U of T7 or Sp6 RNA polymerase. Sense cRNA probe of the same length as the antisense probe was also synthesized to determine specificity. Probe concentrations were estimated by agarose gel electrophoresis. C57BL/6 male mice and gastrin-deficient mice treated with DMP-777 or control gavage were perfusion fixed through left ventricle puncture for 10 min with 4% paraformaldehyde in PBS (pH 7.4) with RNaseFree (Ambion), and the stomachs were excised, postfixed in fixative for 2 h, embedded in optimum cutting temperature compound (Sakura Finetek, CA), and frozen on dry ice. Cryostat sections (10 μm) were cut and postfixed in fixative for 2 h. The sections were dried for 20 min and immersed in methanol at −20°C overnight. Sections were treated with an acetylation solution (1.2% triethanolamine and 0.25% acetic anhydride) to block endogenous phosphatases. After being washed with PBS, the sections were prehybridized at 60°C for 60 min with hybridization solution containing 50% formamide, 5× sodium citrate saline (SSC; pH 7.0), 25 μg/ml yeast RNA, 0.5 mg/ml sheared salmon sperm DNA, and 5× Denhardt’s solution, followed by hybridization at 60°C for 16 h with the probes (1.5 μg/ml) diluted into the hybridization solution. The sections were washed in 5× SSC at 60°C for 1 min, in 0.2% SSC at 60°C for 1 h, and then at room temperature for 5 min, and then in a solution containing 1.16% maleic acid and 0.9% NaCl, adjusted to pH 7.5 using NaOH, for 5 min. Blocking was performed with blocking solution containing 2% biotinylated detected mouse serum (Roche, Indianapolis, IN), 0.1× Tween-20, and 10% normal goat serum at room temperature for 1 h. Sections were incubated overnight at 4°C with anti-digoxigenin antibody (Roche) diluted 1:5,000 in the blocking solution. The sections were washed in PBS containing 0.1% Tween-20 for 15 min four times and three times for 5 min each in 100 mM Tris (pH 9.5) containing 50 mM MgCl2, 100 mM NaCl, 0.1% Tween-20, and 1 mM Levamisole. Chromogen was developed at room temperature for 2 h to overnight with BM Purple AP substrate (Roche). The chromogen development was terminated by washing the slides in PBS. The sections were counterstained with nuclear fast red, dehydrated, penetrated, and mounted in Cytoseal XYL. Quantitative real-time PCR. RNA was isolated from laser-capture microdissected deep fundic glandular cells from both C57BL/6 and gastrin-deficient mice treated with DMP-777 for 0, 1, or 7 days (3 animals at each treatment day). Ten thousand microdissected cells were collected from the deep cells of fundic glands from each animal, and total RNA was isolated using a Picopure RNA isolation kit (Arcturus). Reverse transcription was performed by mixing the 10.5 μl of extracted RNA with 250 ng of random hexamer primers (Promega) and incubating for 10 min at 70°C and termination on ice. The denatured RNA mixture was then mixed with 4 μl of 5× first-strand buffer, 2 μl of 2-deoxynucleotide 5’-triphosphate mix (10 mM each), 2 μl of 100 mM DTT, and 1 μl of PowerScriptRT (BD Biosciences) and incubated for 90 min at 42°C. The transcriptase was inactivated by incubation at 70°C for 15 min.

Quantitative real-time PCR was performed with a three-step method using the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Each reaction was carried out in a 50-μl mixture consisting of iQ SYBRgreen Supermix (BioRad Laboratories), additional MgCl2 for a final MgCl2 concentration of 4.0 mM, 0.2 μM of each primer, and 1 μl of template cDNA. The sense and the antisense primers were designed to cross exon-intron boundaries to avoid amplification from contaminating DNA (GAPDH sense: CCGATTGCTCTCAATGACAA; GAPDH antisense: GCCCTCTCT-TGCTCAGTGGTCC; TFF2 sense: TGCTTTGATCTGGATGGCTG; TFF2 antisense: GGAAAAAGCAGCTTGGCAG; IF sense: CTTG-GGCTCTGACCTGTATGT; IF antisense: TAGCTTGTTCAAGTGT-CAGC). All primer pairs were optimized to amplify only a single band with amplification curves in real-time PCR consistent with efficient amplification under the reaction conditions. The PCR conditions were as follows: 95°C for 3 min, followed by 50 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Quantification of cDNA template in each sample was determined using the comparative threshold cycle (CT) method. Levels requiring 36 or more cycles to achieve threshold were considered nondetectable. Real-time PCR CT data were converted to a relative fold change using the comparative method with GAPDH signal as the normalizer for
each sample. The GAPDH CT was subtracted from the CT for the marker of interest, giving the ΔCT for each marker. To compare the relative gene expression between two samples, the ΔCT for the sample of interest was subtracted from the ΔCT for the reference sample, which yielded the ΔΔCT. The fold change of expression between two samples was calculated using the formula: fold change = 2^−ΔΔCT. The SD for each ΔCT was calculated, and the range for fold change was calculated using ΔΔCT ± 1 SD. The P values corresponding to each comparison were determined using the Mann-Whitney U-test to compare the mean ΔCT between the two samples.

**Immunohistochemistry.** Murine monoclonal anti-H/K-ATPase IgG (a gift from Dr. Adam Smolka, Medical University of South Carolina, Charleston, SC), murine monoclonal anti-tTF2 IgM (a gift from Dr. Nicholas Wright, Cancer UK, London, England), and rabbit polyclonal anti-human IF (a gift from Dr. David Alpers, Washington University, St. Louis, MO) were used as markers to identify parietal cells, mucous neck cells, and chief cells in fundic glands, respectively. For immunohistochemistry of anti-H/K-ATPase and anti-tTF2, deparaffinized sections were blocked using blocking serum provided in the HistoMouse staining kit (Zymed, South San Francisco, CA). Sections were incubated with a primary antibody (1:1,000 and 1:100 for anti-H/K-ATPase and anti-tTF2, respectively) overnight at 4°C. Indirect immunohistochemical detection was then performed through incubation with biotinylated secondary antibodies and alkaline phosphatase-conjugated streptavidin (Vectastain ABC KIT, Vector Laboratories, Burlingame, CA). Chromogen was developed with Vector Red (Vector Laboratories).

For immunohistochemistry with anti-IF, deparaffinized sections were blocked with 1.5% normal goat serum and incubated with a primary antibody (1:100) overnight at 4°C followed by incubation with a biotinylated second antibody and alkaline phosphatase-conjugated streptavidin. Chromogen was developed with Vector Red (Vector Laboratories).

For immunofluorescence with anti-TFF2 and anti-IF, deparaffinized sections were blocked with blocking serum provided in the mouse on mouse (MOM) staining kit (Vector) and incubated with anti-tTF2 (1:100) and anti-human IF (1:1,000) at the same time overnight at 4°C, followed by incubation with Cy2-labeled anti-rabbit IgG and Cy3-labeled anti-mouse IgM. After being washed with PBS, sections were labeled with 50 μg/ml 4,6-diamino-2-phenylindole (DAPI) in 50 mM sodium phosphate for 10 min and mounted using Prolong antifade (Molecular Probes). Sections were viewed and photographed on a Zeiss Axiophot fluorescence microscope, with digital images captured using a SPOT digital charge-coupled device camera.

**Cell indexes.** To quantitate cell numbers in the gastric mucosa, a section on the anterior side of the stomach that showed fundic glands was chosen from each animal. Ten fundic glands were chosen from each section visualized at ×400 on a Zeiss Axiophot microscope, and PAS-positive cells, tTF2-positive cells, IF-positive cells, H/K-ATPase-positive cells, and BrdU-positive nuclei were counted and expressed as cells per gland. TFF2 and IF double immunofluorescent staining cells were counted in overlayed images using Adobe Photoshop.

**Statistics.** The differences in cell numbers were evaluated by ANOVA with post hoc comparison of significant means with Dunnett’s test.

**RESULTS**

Our previous investigation in rats demonstrated that treatment with DMP-777 caused hypergastrinemia, a loss of parietal cells, and foveolar hyperplasia, as well as emergence of SPEM (13). We now sought to evaluate the effects of DMP-777 on the gastric mucosa of normal mice and gastrin-deficient mice with a detailed evaluation of the alterations in fundic mucosal cell lineages. DMP-777 was administered to mice for 0–14 days to evaluate the onset of oxyntic atrophy, and in a second cohort, mice were treated with DMP-777 for 14 days followed by withdrawal of drug treatment for 14 days.

**Serum gastrin.** In rats, DMP-777 elicited a rapid rise in serum gastrin (13). Serum gastrin was measured to evaluate the effects of DMP-777 treatment in mice. Serum gastrin levels in gastrin knockout mice were not detectable either before or after treatment with DMP-777 (data not shown). The mean serum gastrin in wild-type mice without treatment with DMP-777 was 75.8 pg/ml (Fig. 1). After 1 day of treatment with DMP-777, the mean serum gastrin of wild-type mice rose to 350.7 pg/ml. Gastrin levels increased to 540.3 pg/ml after 7 days of treatment and remained elevated to 518.2 pg/ml after 14 days of treatment (Fig. 1).

**Parietal cells.** To identify parietal cells, we immunostained fundic sections with antibodies against the α-subunit of the H/K-ATPase (37). In wild-type mice, parietal cell numbers decreased to 28% of control mice after only 1 day of treatment and further declined to 13–15% of control after 3 days of DMP-777 treatment (Fig. 2). The remaining parietal cells often appeared vacuolated and were scattered through the middle portion of the gland (Fig. 2). Whereas parietal cell numbers appeared to recover somewhat between 7 and 14 days of treatment, the parietal cell mass was still significantly reduced compared with control. After cessation of DMP-777 treatment, oxyntic atrophy was completely reversed. Parietal cell numbers increased, and after 14 days of recovery, we observed significantly increased numbers of parietal cells (Fig. 2).

The mean parietal cell number of gastrin knockout mice without treatment with DMP-777 was 11.3 cells per gland,
50% of the parietal cell mass compared with the normal control mice (Fig. 2). In gastrin knockout mice, parietal cell numbers decreased to 70% of untreated mice after 1 day of treatment and decreased to 33.7% of levels in untreated animals after 3 days of treatment of DMP-777. In the treated gastrin knockout mice, the parietal cell numbers did not recover until the cessation of DMP-777 administration and the mean number of parietal cells was decreased to 25.0% of control gastrin knockout mice after 14 days of treatment with DMP-777. As in the wild-type mice, after the cessation of DMP-777 treatment, parietal cell numbers increased in gastrin knockout mice, and after 14 days of recovery period, the number of parietal cells was significantly elevated to 136% of that in gastrin knockout mice without treatment.

**Surface mucous cells.** To evaluate surface mucous cells, we stained sections for DR-PAS (Fig. 3). In wild-type mice, during the drug-treatment period, DR-PAS-positive cells increased rapidly with DMP-777 administration and were significantly increased compared with the control mice after only 1 day of treatment (Fig. 3). Foveolar hyperplasia reached a maximum after 3 days of DMP-777 administration with a 219% increase in DR-PAS-positive cells over control mice. The surface cell numbers remained significantly elevated throughout the 14 days of treatment (Fig. 3). Foveolar hyperplasia was completely reversible, and withdrawal of drug led to a decline in DR-PAS-positive cells to somewhat smaller numbers compared with the control mice 14 days after cessation of treatment.

In gastrin knockout mice, without treatment, the number of DR-PAS positive cells per gland was the same as that of wild-type control mice (Fig. 3). However, in contrast with the wild-type mice, in gastrin-deficient mice, treatment with DMP-
777 did not elicit any significant alterations in the number of DR-PAS-positive cells at any of the treatment times (Fig. 3B).

Labeling of S-phase proliferating cells. BrdU staining was used to assess proliferating cell lineages. In wild-type mice, BrdU-positive nuclei increased dramatically after only 1 day of DMP-777 treatment and remained significantly elevated throughout the drug-treatment period in wild-type mice (Fig. 4A). After 3 days of drug treatment, BrdU-labeled cells were mostly present in the midgland region deep to regions of foveolar hyperplasia (Fig. 4A). However, BrdU-labeled cells were also apparent in the deep portions of the mucosa (Fig. 4). By 7 days of treatment with DMP-777, the BrdU labeling index reached 612% of the level in control mice (Fig. 4B). On days 10 and 14, BrdU-labeled cells were present throughout the basal regions of the gland extending up the glands to the regions of foveolar hyperplasia (Fig. 4A). Fourteen days after cessation of DMP-777 treatment, the BrdU labeling index decreased back toward control levels, and there was a complete loss of BrdU labeling cells from the basal cells of fundic glands.

In gastrin knockout mice, the number of BrdU-labeled cells significantly increased after 7 and 10 days treatment (163 and 194%, respectively); however, the increase was less pronounced than that in the wild-type mice (Fig. 4). The BrdU-labeled cells were distributed in the lower part of the gland throughout the first 7 days of treatment, but we observed decreased BrdU labeling and a return to the middle part of the glands after 14 days of treatment (Fig. 4A). Fourteen days after cessation of DMP-777 administration, the number and the
distribution of BrdU labeled cells were similar to those in untreated mice.

IF immunoreactive cells. In the normal murine gastric mucosa, expression of IF is confined to chief cells (6). DMP-777 treatment caused a significant decrease in the number of IF-positive cells after 1, 3, and 7 days of drug administration with 62, 31, and 40% of control numbers, respectively (Fig. 5). However, by 10 days of treatment, the number of IF immunoreactive cells increased but remained significantly different from control numbers. As expected, IF-positive cells were located at the base of the mucosa in the untreated animals (Fig. 5A), and the immunoreactive cells observed at days 10 and 14 of treatment also were located at the base of the mucosa.

In gastrin knockout mice, the number of the IF-positive cells was similar to that in the wild-type control mice without treatment of DMP-777 (Fig. 5B). As with the wild-type mice, DMP-777 treatment of gastrin-deficient mice led to a decrease in IF staining cells. The nadir of the number of IF-positive cells was after 7 days of treatment with DMP-777 (38.8% of untreated animals). By 10 and 14 days of treatment, the number of IF-positive cell numbers increased after 7 days of treatment. After 10 and 14 days of treatment, the number of TFF2-immunoreactive cells had increased significantly to 189 and 234% of control levels, respectively (Fig. 6B). The TFF2 immunoreactive cells were located at the base of the glands and displayed more intense staining similar to that seen for TFF2 staining of Brunner’s gland or deep antral gland cells consistent with the SPEM cell phenotype (Fig. 6A). By 14 days of treatment, we observed glands where up to 50% of the gland length was dominated by intensely stained TFF2-expressing cells. After withdrawal of drug treatment for 14 days, both the number of TFF2 staining cells and the pattern of staining returned to the morphological appearance of normal mucous neck cells (Fig. 6).

The number and the distribution of TFF2-immunoreactive cells per gland in gastrin knockout mice without treatment with DMP-777 were similar to those in the wild-type mice (Fig. 6). However, after only 1 day of treatment of DMP-777, the number of TFF2-immunoreactive cells increased dramatically (243.3%), and the staining pattern was similar to that previously described for TFF2 staining in SPEM cells (Fig. 6A). The number and the pattern of TFF2-immunoreactive cells in gas-
trin knockout mice remained elevated throughout DMP-777 treatment. Fourteen days after cessation of DMP-777 treatment, recovery of the number and the pattern of TFF2-immunoreactive cells was incomplete in gastrin knockout mice, with TFF2-positive cell numbers still elevated to 160% of that in untreated animals (Fig. 6).

**TFF2 and IF double immunostaining cells.** We have recently noted that cells at the base of glands containing SPEM display dual expression of both IF and TFF2 (44). We therefore used dual immunofluorescence labeling to examine staining for TFF2 and IF in wild-type and gastrin-deficient mice treated with DMP-777. Figure 7 demonstrates that few cells in the normal mucosa of wild-type mice showed any dual staining. When quantitated, we observed only 0.28 dual-stained cells per gland in untreated mice (Fig. 7B). When a dual staining cell was observed, it was located at the junction between the mucous neck cell and chief cell zones (Fig. 7A). In contrast, after 7 days of DMP-777 treatment, we observed an increased number of cells with dual staining for both IF and TFF2. By days 10 and 14 of treatment, there were a significant number of dual-labeled cells per gland (3.95 and 2.5, respectively; Fig. 7). In contrast with the normal mucosa, in the 10- and 14-day-treated mice, the dual staining was present in cells located at the base of glands (Fig. 7). Dual fluorescence staining demonstrated that TFF2 and IF were in different vesicle populations in the dual-staining cells (Fig. 7A). In addition, whereas IF staining localized to the apical region of chief cells, in IF/TFF2 dual-staining cells, IF staining vesicles were more diffusely distributed throughout the cytoplasm. Most of the IF immunoreactive cells were also immunostained for TFF2. However,
cells expressing only TFF2 but maintaining the same SPEM morphology extended into the middle region of the glands. After the 14 days of recovery from treatment, dual-staining cells disappeared from the deep fundic glands and their numbers declined to control levels (Fig. 7).

In gastrin-deficient mice, similar to wild-type, few dual-staining cells were observed and always at the junction between the mucus neck and chief cell populations (Fig. 8A). In contrast, however, in gastrin knockout mice, the dual-staining cells emerged after only 1 day of DMP-777 treatment. The number of dual-staining cells per gland then declined through the 14-day treatment period, although it remained higher than the control 14 days after withdrawal of treatment (Fig. 8B). In contrast to wild-type mice, after 14 days of treatment, we observed cells at the bases of glands expressing only IF (chief cells) located deep to populations of SPEM cells (Fig. 8A).

**TFF2 and IF gene expression in SPEM.** Because immunostaining studies in gastrin-deficient mice suggested a rapid upregulation of TFF2 expression in the deep cells of the fundic mucosa following only 1 day of DMP-777, we investigated whether the changes in protein expression were associated with upregulation of mRNA transcripts. We initially investigated TFF2 mRNA expression in whole fundic stomach from C57BL/6 mice treated with DMP-777 for 0, 1, or 7 days. These quantitative PCR studies demonstrated a decrease in TFF2
levels to 89% of controls after 1 day and a further decrease in TFF2 message to 32% of controls at 7 days of treatment. The changes, however, reflect total TFF2 expression across all of the cells in the stomach normalized with GAPDH message levels. Because the foveolar hyperplasia, which develops rapidly, makes interpretation of the quantitative PCR studies in whole tissue samples difficult, we adopted laser capture microdissection to isolate specifically the lineages resident in the deep fundic glands: chief cells in normal mucosa and SPEM in the mucosa of treated animals. We examined the expression of TFF2 and intrinsic factor in SPEM cells using dual immunofluorescence staining.

Fig. 7. Dual immunofluorescence staining of SPEM for both IF and TFF2 in wild-type C57BL/6 mice. A: representative gastric fundic mucosal sections from untreated C57BL/6 mice (day 0) and from C57BL/6 mice treated for 1, 7, or 14 days with DMP-777 were triple stained with 4,6-diamino-2-phenylindole (DAPI) as well as with anti-IF and anti-TFF2. The merged triple overlays are shown below each set (blue = DAPI, green = intrinsic factor, and red = TFF2). The images at day 0 depict the middle gland region transition zone between mucous neck cells and chief cells. At the other days, the images show cells at the bases of glands. Note that whereas only a rare cell at the junction between chief and mucous neck cell populations was dual labeled in the untreated mice, the majority of the SPEM cells at the base of glands was dual labeled. Also note that intracellular patterns of IF and TFF2 immunoreactive granules were distinct in the dual-labeled SPEM cells. B: TFF2/IF dual immunostaining cell numbers were quantitated for wild-type C57BL/6 mice through 14 days of DMP-777 treatment or for mice treated for 14 days and then recovered off drug for 14 days (R14). Data are represented as means ± SE. **P < 0.01 vs. day 0.
TFF2 and IF transcripts in laser-capture microdissected deep fundic mucosal cells in wild-type and gastrin-deficient mice treated with DMP-777 for 0, 1, or 7 days. At day 0 of treatment, the microdissected deep gland cells were essentially all chief cells. Figure 9A demonstrates that although negligible levels of TFF2 transcripts were present in the deep cells of C57BL/6 mice at days 0 and 1 of DMP-777 treatment, there was a 47-fold increase in TFF2 message after 7 days. These

![Image of dual immunofluorescence staining of SPEM for both IF and TFF2 in gastrin-deficient mice.](image_url)

A: representative gastric fundic mucosal sections from untreated gastrin-deficient mice (day 0) and from gastrin-deficient mice treated for 1, 7, or 14 days with DMP-777 were triple stained with DAPI as well as with anti-IF and anti-TFF2. The merged triple overlay is shown below each set (blue = DAPI, green = IF, and red = TFF2). The images at day 0 depict the middle gland region transition zone between mucous neck cells and chief cells. At the other days, the images show cells at the bases of glands. Note that whereas only a rare cell at the junction between chief and mucous neck cells was dual labeled in the untreated mice, the majority of the SPEM cells at the base of glands was dual labeled at days 1 and 7. However, cells only labeling with IF were present at the bases of glands at 14 days of treatment. As with wild-type mice, the intracellular patterns of IF and TFF2 granules were distinct in the dual-labeled SPEM cells. B: TFF2/IF dual immunostaining cell numbers were quantitated for gastrin knockout mice through 14 days of DMP-777 treatment or for mice treated for 14 days and then recovered off drug for 14 days (R14). Data are represented as means ± SE. **P < 0.01 vs. day 0.

Fig. 8. Dual immunofluorescence staining of SPEM for both IF and TFF2 in gastrin-deficient mice. A: representative gastric fundic mucosal sections from untreated gastrin-deficient mice (day 0) and from gastrin-deficient mice treated for 1, 7, or 14 days with DMP-777 were triple stained with DAPI as well as with anti-IF and anti-TFF2. The merged triple overlay is shown below each set (blue = DAPI, green = IF, and red = TFF2). The images at day 0 depict the middle gland region transition zone between mucous neck cells and chief cells. At the other days, the images show cells at the bases of glands. Note that whereas only a rare cell at the junction between chief and mucous neck cells was dual labeled in the untreated mice, the majority of the SPEM cells at the base of glands was dual labeled at days 1 and 7. However, cells only labeling with IF were present at the bases of glands at 14 days of treatment. As with wild-type mice, the intracellular patterns of IF and TFF2 granules were distinct in the dual-labeled SPEM cells. B: TFF2/IF dual immunostaining cell numbers were quantitated for gastrin knockout mice through 14 days of DMP-777 treatment or for mice treated for 14 days and then recovered off drug for 14 days (R14). Data are represented as means ± SE. **P < 0.01 vs. day 0.
data are consistent with transdifferentiation of chief cells into TFF2-expressing SPEM after 7 days of DMP-777 treatment. Importantly, gastrin-deficient mice displayed a rapid 10-fold increase in TFF2 transcripts in deep cells of the fundus that was sustained after 7 days of treatment. In contrast to TFF2 mRNA, IF expression decreased significantly throughout the 7-day treatment period. Whereas gastrin-deficient mice demonstrated only 60% of wild-type levels of IF message before treatment, no decrease in message was observed after 1 day of treatment. IF mRNA expression did significantly decline after 7 days of DMP-777 treatment. These findings are consistent with the rapid upregulation of TFF2 expression in chief cells from gastrin-deficient mice treated with DMP-777.

To further define the changes in TFF2 expression after 1 day of treatment with DMP-777, we performed in situ hybridization and immunohistochemistry to visualize TFF2 mRNA and protein expression, respectively, in serial frozen sections (Fig. 9B). In wild-type C57BL/6 mice, we observed a distinction of in situ labeling and immunoreactive cells with in situ labeling of cells more luminal to the position of immunoreactive mucous neck cells. These findings are similar to the pattern previously reported for TFF2 mRNA expression in progenitor cells of untreated wild-type mice (12, 31). In contrast, in gastrin-deficient mice, TFF2 mRNA and protein were visualized in coincident populations of cells located at the base of fundic glands (Fig. 9B). This pattern is identical to that observed for SPEM in H. felis-infected mice (31). All of these results are consistent with the rapid upregulation of TFF2 mRNA and protein expression in transdifferentiating chief cells in gastrin-deficient mice.

**DISCUSSION**

The normal gastric mucosa is assembled from a repertoire of short- and long-lived differentiated cell lineages (17–21). All of these lineages emerge from the same primary progenitor population located in the upper half of the gland through differentiation from at least three second-order progenitor cell populations (18). The short-lived surface cells differentiate from presurface cells and migrate toward the lumen (19). Long-lived lineages populate the portions of the gland deep to the proliferative zone. The majority of parietal cells differentiates from preparietal cells and migrates toward the gland base (17). Mucous neck cells differentiate from preneck cells and migrate toward the base, eventually further differentiating into chief cells (20). Alterations in gland lineages are associated with a number of pathological conditions, but loss of parietal cells or oxyntic atrophy is most commonly associated with increased risk for gastric cancer (9). Investigations over the past decade have demonstrated that parietal cells are responsible for the secretion of a number of critical growth factors including TGF-α, amphiregulin, and heparin-binding EGF (1, 2, 28). Thus the loss of parietal cells may eliminate important agents required for appropriate differentiation of deeper gland lineages, such as mucous neck and chief cells, as well as increase the serum gastrin level as a result of hypochlorhydria. In the present investigation, to distinguish the effects of hypergastrinemia during oxyntic atrophy, we have used detailed cell-lineage analysis to clarify the effects of DMP-777 administration to C57BL/6 mice and to gastrin-deficient mice. The results demonstrate that although gastrin is required for the
induction of foveolar hyperplasia, gastrin is not required for the induction of SPEM following acute loss of parietal cells. These findings differentiate the processes of foveolar hyperplasia and mucous cell metaplasia and establish the multifactorial regulation of gastric lineage differentiation associated with oxyntic atrophy.

**Foveolar hyperplasia is caused by gastrin.** The drug-induced loss of parietal cells in C57BL/6 mice was accompanied by an immediate increase of serum gastrin associated with foveolar hyperplasia. However, in gastrin-deficient mice, the number of PAS-positive foveolar cells was not changed at all throughout the treatment and the recovery periods. These results suggest that the foveolar hyperplasia induced by DMP-777 treatment was caused by hypergastrinemia in response to hypochlorhydria. Previous investigations (26, 29, 41) have reported foveolar hyperplasia of less-differentiated pit cells in hypergastrinemic mice. TFF1, which is secreted from foveolar cells in the stomach, is under transcriptional control by gastrin (22). DMP-777 treatment caused a rapid rise in serum gastrin as well as the rapid onset of foveolar hyperplasia. This rapid response appears appropriate for the short-lived surface cells differentiating from the normal progenitor zone as the first adaptation of the mucosa to the loss of parietal cells. In humans, foveolar hyperplasia is one of the mucosal pathologies associated with oxyntic atrophy (7, 34). Foveolar hyperplasia is often associated with oxyntic atrophy following either chronic *H. pylori* infection or in Ménétrier’s disease patients (43). The general lack of hypergastrinemia in most Ménétrier’s disease patients is thought to be due to a TGF-α-induced increase in somatostatin secretion leading to suppression of gastrin release (46). Thus, whereas previous studies have associated hypergastrinemia with foveolar hyperplasia (26, 29, 41), the present investigations are the first to provide direct evidence that gastrin is responsible for the rapid emergence of foveolar hyperplasia in the setting of parietal cell loss and achlorhydria.

**Oxynitic atrophy-induced mucous cell metaplasia.** In addition to foveolar hyperplasia, a number of investigations has noted an association of goblet cell intestinal metaplasia with oxyntic atrophy in humans (9). We did not observe any goblet cell metaplasia in the stomachs of DMP-777-treated mice. Indeed, in contrast with humans, goblet cell-type intestinal metaplasia is not commonly seen in rodents. Nevertheless, as in rats (13), at the bases of gastric glands, we did observe the development of mucous cell metaplasia that displayed the characteristics of Brunner’s gland or deep antral gland cells. This SPEM lineage developed subsequent to the establishment of oxyntic atrophy after 7 days of drug administration in C57BL/6 mice. However, SPEM was present after only 1 day of DMP-777 treatment in gastrin knockout mice. The data suggest that the absence of gastrin and/or foveolar hyperplasia promote the early development of SPEM. In addition to the rapid emergence of the SPEM lineage in gastrin-deficient mice, we observed an early increase in the number of cells doubly immunoreactive for both TFF2 and IF. DMP-777 treatment also elicited a rapid induction of TFF2 mRNA expression in the deep fundic gland cells of gastrin-deficient mice treated with only one dose of DMP-777. In the wild-type gastric mucosa, TFF2 mRNA is expressed predominantly in progenitor cells, whereas protein is expressed in mucous neck cells (12, 31). In contrast, SPEM cells show high levels of both TFF2 mRNA and TFF2 protein (31). Because the emergence of SPEM was not associated with a significant increase in proliferation (BrdU-positive cells), the SPEM may originate from transdifferentiation of chief cells. Previous investigations (20) have suggested that chief cells have a long lifetime. Karam and Leblond (20) reported that chief cells were differentiated from mucous neck cells in the normal fundic gland. In untreated wild-type and gastrin-deficient mice, we observed few cells with dual staining for IF and TFF2, and TFF2 mRNA was nearly undetectable in microdissected deep glandular cells. The pattern of a loss of IF-expressing cells followed by the emergence of SPEM and dual IF/TFF2-staining cells in wild-type C57BL/6 mice also supports the notion of transdifferentiation.

Still, in wild-type C57BL/6 mice, the SPEM lineage emerged coincident with the observation of basally located BrdU-positive proliferative cells that appeared separate from the normal proliferative zone. We also observed a similar basally located BrdU-labeled cell population in rats treated with DMP-777 (13). We have previously suggested that SPEM might originate from a cryptic progenitor population located at the base of fundic glands. This basal progenitor zone position is similar to that observed in the gastric antrum. Indeed, the pattern of foveolar hyperplasia and expansion of a basally located TFF2-expressing mucous cell population is consistent with a phenotype of “antralization.” Such a cryptic progenitor cell could be a remnant of mucosal cells from development before the emergence of parietal cells. Under this model, factors secreted from parietal cells would normally suppress the proliferation of the cryptic progenitor cells. Nevertheless, we can rationalize both of the stated models if transdifferentiation of chief cells, in the absence of factors normally secreted by parietal cells, leads to a basally located cell population with proliferative capacity.

Whereas the presence of gastrin significantly altered the changes in cell lineages associated with treatment with DMP-777, it had a less prominent influence on the recovery from treatment. Previous investigations (15, 16) have suggested that gastrin levels regulate parietal cell mass. Indeed, the gastric mucosa of gastrin knockout mice does show a general attenuation of most of the mucosal cell lineages (11, 25). Still, in both wild-type and gastrin-deficient mice, the cessation of treatment was associated with a significant overshoot in the numbers of parietal cells. These results indicate that gastrin is not the only stimulator of parietal cell differentiation. In wild-type mice, other lineages recovered to levels similar to those in untreated mice. However, in gastrin-deficient mice, we still observed significant increases in SPEM and decreases in chief cell numbers 14 days after removal of drug treatment. These results indicate that a combined influence of parietal cells and gastrin are required for proper differentiation of chief cells. These findings are consistent with those of Li et al. (27), who demonstrated that loss of differentiated parietal cells in H/K-diphtheria toxin mice also cause a reduction in chief cell numbers. In addition, Franic et al. (10) have reported that H/K-ATPase β-subunit-deficient mice completely lost chief cells. The identity of key differentiation factors secreted by parietal cells remains to be determined.

**Implications of the association of SPEM with oxyntic atrophy.** Because parietal cell loss is associated with gastric cancer, an understanding of the changes in the gastric mucosa attendant with oxyntic atrophy is critical. In humans, a number of
mucosal pathologies including SPEM is associated with oxyntic atrophy. In C57BL/6 mice, chronic infection with *H. felis* leads to profound oxyntic atrophy and SPEM (31, 42). Further evidence suggests that gastric cancer in *H. felis*-infected mice develops from SPEM (41). It remains to be determined whether eradication of Helicobacter infection can reverse all of the changes of oxyntic atrophy including SPEM. Nevertheless, the results here demonstrated that the emergence of SPEM is part of the normal response to oxyntic atrophy. It is tempting to speculate that a combination of foveolar hyperplasia and SPEM are part of a coordinated response to local injury in the fundic mucosa. Under this paradigm, the conversion of this acute response to a chronic condition especially under the influence of chronic inflammation could then predispose the mucosa to pathophysiological consequences including neoplasia.

In summary, DMP-777 treatment rapidly induced parietal cell loss in mice. In wild-type mice, the gastric mucosa responded to the loss of parietal cells with rapid expansion of surface cell numbers (foveolar hyperplasia), hypergastrinemia, and the induction of a TFF2 expressing mucous cell metaplasia (SPEM) after 7 days of treatment. Oxyntic atrophy in gastrin-deficient mice was accompanied by rapid induction of SPEM after only one dose of DMP-777 without foveolar hyperplasia. The results suggest that the expression of foveolar hyperplasia following loss of parietal cells is dependent on gastrin. However, the absence of gastrin did not prevent the emergence of SPEM following DMP-777 treatment. Rather, the absence of gastrin promoted the rapid emergence of SPEM in response to oxyntic atrophy, likely from transdifferentiation of chief cells. All of these findings support the concept that dynamic hormonal and paracrine influences control the differentiation of cell lineages in the gastric fundic mucosa.

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G374 ACUTE OXYNTIC ATROPHY IN GASTRIN-DEFICIENT MICE


