Spontaneous ECL cell carcinomas in cotton rats: natural course and prevention by a gastrin receptor antagonist

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In our inbred strain of cotton rats (*Sigmodon hispidus*) 50% of the females develop spontaneous ECL cell-derived tumors in the acid-producing part of the stomach due to hypergastrinemia secondary to gastric hypoacidity. Although the mechanism behind the hypoacidity is unknown, the female cotton rat is an excellent model for studying ECL cell-related tumorigenesis. In this study we wanted to explore the malignancy potential of these tumors and the ability of a gastrin receptor antagonist (YF476) to prevent their development. First, nine hypergastrinemic female cotton rats (10 months of age) were diagnosed by laparotomy as having gastric tumors. They were killed 6 months later. Second, 18 female cotton rats (2 months of age) were dosed monthly for 6 months with YF476 (500 μmol/kg body wt) by s.c. injection, while 21 age-matched animals received vehicle. Samples from each stomach were collected for histology, immunohistochemistry and northern blot analysis. The gastric tumors harbored cells with immunohistochemical features of ECL cells. The tumors were found at times to invade and penetrate the stomach wall and to metastasize to perigastric sites. ECL-derived tumor cells were discovered in peritoneal fluid. At death only 1 out of 18 animals given YF476 displayed carcinomas (invasive growth), compared with 7 out of 21 in the vehicle dosed control group (P = 0.048). The spontaneous gastric tumors in cotton rats derived from ECL cells. The tumors were able to penetrate the stomach wall and to metastasize by intracavitary seeding. Gastrin receptor blockade lowered the incidence of such tumors. We propose that the tumors are ECL cell carcinomas and that gastrin is the driving force behind the transformation from normal to malignant ECL cells.

Introduction

The cotton rat (*Sigmodon hispidus*) was first used in poliomyelitis research in the 1930s (1). Since then it has been proven to be an excellent model for virus research in general, thus constituting one of the most important models in experiments concerning respiratory syncytial virus, adenovirus, measles and herpes simplex virus (2). In 1995 inbred cotton rats were described as developing multiple spontaneous gastric tumors, initially classified as ordinary gastric adenocarcinomas originating from non-endocrine cells (3). However, several features were atypical. First, the tumor-bearing animals displayed thickening of the entire oxyntic mucosa, with multiple local regions with different grades of dysplasia and malignant growth (3). Second, dissemination of the tumors beyond the stomach was not found, although in several cases the muscle layer was invaded (3). Third, there was a female preponderance in that ~50% of the female cotton rats displayed dysplasia or carcinoma, whereas <1% of the males were affected (3). Finally, in the tumors and the surrounding oxyntic mucosa fibroblastic proliferation (desmoplasmic reaction) gave the mucosa a scirrhous appearance. In two subsequent studies the tumors were shown to be derived from ECL cells (4,5), which are gastrin-controlled endocrine cells that are numerous in the oxyntic mucosa (6).

Gastrin stimulates gastric acid secretion, an effect mediated by gastrin (cholecystokinin-2) receptors on the ECL cells (6). The ECL cell contains histamine (7) and is the predominant endocrine cell of the oxyntic mucosa (8). When activated by gastrin, the ECL cells respond with increased production (9) and release (10) of histamine, which stimulates acid secretion from the parietal cells (11). In addition, gastrin has trophic effects on the oxyntic mucosa, most notably on the ECL cells (12). This effect has been shown to result in ECLomas in rats following long-term hypergastrinemia secondary to drug-induced inhibition of gastric acid secretion (13–16), notably in females (13–15).

A female preponderance has also been noted with respect to such tumors in humans (17). The ECL cell growth progresses gradually from linear, diffuse and micronodular hyperplasia to dysplasia. If the hypergastrinemia persists, it may lead to a foregut carcinoid of ECL cell origin, often accompanied by desmoplasia, growing invasively into the muscle layer, although rarely giving rise to metastases (18).

The analogy between spontaneously developing gastric tumors in cotton rats and the ECLoma that sometimes develop in hypergastrinemic rats prompted us to examine the gastric tumors of cotton rats with regard to a possible origin from ECL cells. In previous studies (4,5) we found gastric hypoacidity and hypergastrinemia in all cotton rats displaying thickening of the mucosa (with or without visible tumor), but not in animals with a normal appearing oxyntic mucosa. Furthermore, we found that cells of the dysplastic and neoplastic lesions possessed immunohistochemical features of ECL cells, by using antisera against the general neuroendocrine cell marker chromogranin A (CgA) and the specific ECL cell marker histidine decarboxylase (HDC) (4,5). Thus, the tumors were classified as invasive ECLomas, and we suggested that
the high incidence of such tumors in female cotton rats was due to long-standing hypergastrinemia secondary to an as yet unexplained gastric hypoacidity. In our opinion, the cotton rat constitutes an interesting model in which to study the role of gastrin in the development of invasive ECLomas induced by long-term hypergastrinemia.

During the last decade specific and efficient gastrin receptor antagonists have become available (19–21). These antagonists inhibit gastric acid secretion in rodents (19–21) and dogs (19,20) and may be potentially useful in the treatment of acid-related diseases. In experimental animals some of these drugs inhibit gastric acid secretion as effectively as proton pump inhibitors (22). Well-known side effects of the long-term hypergastrinemia that follows upon acid inhibition, such as a trophic effect on the oxyntic mucosa (notably ECL cell hyperplasia, dysplasia and neoplasia) and rebound acid hypersecretion (22), are unlikely to occur following treatment with gastrin receptor antagonists. In *Mastomys natalensis* a gastrin receptor antagonist has shown potential as an antineoplastic agent for ECL cell carcinoid development (23).

In the present study of female cotton rats we explored the malignancy potential of these gastric tumors and the ability of gastrin receptor blockade to prevent their development.

### Materials and methods

#### Test substance

The gastrin receptor antagonist YF476, (R)-1-[2,3-dihydro-2-oxo-l-pivaloyl-methyl-5-(2-pyridyl)-1H-1,4-benzo-diazepin-3-yl]-3-(3-methylaminophenyl) urea, was generously supplied by Ferring A/S (Vanløse, Denmark) and suspended in vehicle (polyethylene glycol 300; Fluka Chemie GmbH, Bucks, Switzerland) at a concentration of 300 μmol/ml.

#### Animals and animal handling

Cotton rats have been bred by Tanabe Seiyaku Co. Ltd (Toda, Japan) since 1971 and maintained by random mating. In 1982 some of these animals were found to develop spontaneous gastric tumors and were bred to create a colony (3). In the present study two to four female cotton rats were housed together in wire-top cages at 25°C with a relative humidity of 40–45% and a 12 h light/dark cycle. The Rat and Mouse Diet of B&K Universal Ltd (Hull, UK) and tap water were provided ad libitum.

We collected blood samples from the saphenous vein at monthly intervals and plasma was frozen for later determination of gastrin by radioimmunooassay (24). The normal mean plasma gastrin concentration (19.1 ± 8.5 pM) in female cotton rats is based upon 182 samples from 63 individuals (age 2–8 months) with normal appearing oxyntic mucosa. Plasma gastrin concentrations higher than the mean ± 2 SD (36.1 pM) are regarded as hypergastrinemic.

The animals were anesthetized (0.3 ml/100 g body wt with a mixture of 2.5 mg/ml fluanison, 0.05 mg/ml fentanyl and 1.25 mg/ml midazolam) before blood sampling and surgery. The animals were killed by exsanguination, the stomach weights were compared (see above). Transverse sections from the stomach wall were stained with H&E. At least three sections from each specimen were used. In each section the thickness of 10 gastric glands was measured with an ocular grid. In animals with visible gastric tumors, the mucosal thickness was measured in specimens taken at least 5 mm away from the tumors.

#### Calculation of oxyntic mucosa thickness

The specimens were taken at predetermined sites from the stomach wall (see above). Transverse sections from the stomach wall were stained with H&E. At least three sections from each specimen were used. In each section the thickness of 10 gastric glands was measured with an ocular grid. In animals with visible gastric tumors, the mucosal thickness was measured in specimens taken at least 5 mm away from the tumors.

#### Northern blot analysis

Three categories of whole wall tissue specimens (~300 mg) from the acid-producing part of the stomach were collected along the greater curvature at predetermined sites (5 mm away from the rumen–oxyntic border) and at least 5 mm outside tumors if found upon visual inspection. Separate specimens were taken from the tumors. All specimens were fixed by immersion in 10% buffered formalin. Following dehydration of the specimens and embedding in paraffin, 5 μm thick serial sections were cut perpendicular to the mucosal surface. Each section was stained with hematoxylin and eosin (H&E). The samples were classified as carcinoma (invasive growth), dysplasia (cellular atypia and/or glandular distortion) or normal appearing (neither dysplasia nor carcinoma). If the mucosa displayed areas of both dysplasia and carcinoma, it was classified as carcinoma.

Staining for argyrophil cells was performed as described by Sevier and Münger (26). This technique was chosen because it is relatively specific for ECL cells in the oxyntic mucosa (8).

#### Cytology of peritoneal fluid

Two milliliters of the fluid was centrifuged (2000 r.p.m. for 10 min) and the sediment suspended in 1 ml human serum, 0.2 ml eosin and 0.2 ml thrombostab (sterile topic 5000 units; Parke-Davis, Scarborough, Canada). The mixture was shaken to a jelly-like consistency prior to formalin fixation and paraffin embedding as described above.

#### Immunohistochemistry (IHC)

Deparaffinized sections were rehydrated and treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. The antisera used were diluted in phosphate-buffered saline containing 0.25% Triton X-100 and 0.25% bovine serum albumin (Sigma, St Louis, MO). The sections were incubated with primary antisera at 4°C overnight. The EnVision-HRP kit (K5007; Dako, Glostrup Denmark) and the AEC peroxidase kit (SK4200; Vector, Burlingame, CA) were used to visualize the immunoreaction.

A rabbit anti-porcine CgA antiserum (dilution 1:500) (Dia Sorin, Stillwater, MN) was used to detect endocrine cells in the mucosa and in the peritoneal fluid. To increase sensitivity, tyramide signal amplification (TSA) (27,28) was used on specimens from mucosa. In addition, a rabbit antiserum against rat recombinant HDC (dilution 1:3000) (Euro-Diagnostica, Malmö, Sweden) was used to detect ECL cells in peritoneal fluid. Non-immune rabbit serum served as a control (29).

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## Design of the study

### Part A

To determine whether the tumors metastasized we studied nine cotton rats (mean body weight 162 ± 5 g), each with one or several visible gastric tumors diagnosed by laparotomy at the mean age of 10 months (range 8–12). All of these cotton rats were hypergastrinemic (477 ± 99 pm) at the time of laparotomy. They were killed 6 months later. Peritoneal fluid, found in four of the nine animals, was collected for cytology. The stomach weights were compared with those found in 10 normogastrinemic (17 ± 3 pm) age- and weight-matched (165 ± 4 g) controls, having oxyntic mucosa without dysplasia or neoplasia.

### Part B

Thirty-nine female cotton rats were randomly allocated to two groups at the age of 2–3 months. One group of 18 animals (mean body weight 105 ± 4 g, not different from the control group) was given YF476 (500 μmol/kg body wt) by s.c. injection once every month up to the age of 8 months. A control group of 21 animals (mean body wt 95 ± 5 g) received vehicle. A single s.c. injection of 300 μmol/kg body wt YF476 blocks the gastrin receptors of ECL cells in rats for 8 weeks (21). The dose and frequency of dosing were increased in this study in order to compensate for possible species differences (25).

#### Histopathological techniques

Whole wall tissue specimens were rehydrated and treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. The antisera used were diluted in phosphate-buffered saline containing 0.25% Triton X-100 and 0.25% bovine serum albumin (Sigma, St Louis, MO). The sections were incubated with primary antisera at 4°C overnight. The EnVision-HRP kit (K5007; Dako, Glostrup Denmark) and the AEC peroxidase kit (SK4200; Vector, Burlingame, CA) were used to visualize the immunoreaction.

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### Northern blot analysis

Three categories of whole wall tissue specimens (~300 mg) from the acid-producing part of the stomach were analyzed: (i) YF476-treated animals having normal appearing mucosa; (ii) vehicle-dosed controls having normal appearing mucosa; (iii) vehicle-dosed controls with dysplasia. The specimens were categorized by histological examination of small pieces of tissue collected adjacent to the specimens taken for RNA extraction. The latter specimens were homogenized in a denaturing buffer (1 ml/100 mg tissue) with 4 M guanidinium isothiocyanate, 25 mM sodium acetate (pH 6.0) and 0.84% (v/v) β-mercaptoethanol. Total RNA was extracted by ultracentrifugation on a cesium chloride cushion, precipitated with ethanol and analyzed by northern blot as described previously (30).

CgA, HDC and H+ /K+ ATPase cDNA fragments were generated by reverse transcription-polymerase chain reaction (RT–PCR) and the fragments ligated into the pcR II vector (TA cloning kit; Invitrogen). The authenticity of the probes was verified by sequencing the inserted fragment. Total RNA for the RT–PCR was taken from rat brain (HDC and CgA) or oxyntic mucosa (H+/K+ ATPase), respectively. Antisense probes were generated by simultaneous transcription and 32P-labeling, using either T7 or SP6 RNA polymerase.
according to standard protocols. Membranes were prehybridized for 4 h at 68°C in 50% formamide, 5× sodium chloride, sodium phosphate, EDTA buffer (SSPE), 5× Denhardt’s solution, 0.5% SDS and 200 μg/ml sonicated salmon sperm and hybridized overnight with 2 × 10⁶ c.p.m. ³²P-labeled probe per ml hybridization fluid.

Post-hybridization washes were performed twice at room temperature for 20 min with 2× SSPE containing 0.1% SDS and once for 20 min at 65°C with 0.1× SSPE, 0.1% SDS. After washing, the membranes were exposed to a phosphor storage screen and the signal measured and analyzed using a Fujifilm BAS-1800 II bio-imaging analyzer and software. Equal loading was assessed by 18S ribosomal mRNA abundance. The membranes were stripped with boiling 0.1% SDS between hybridizations with the different probes. Due to lack of sequence information for cotton rat CgA, HDC and H⁺/K⁺ ATPase, homology evaluation could not be done. However, the northern blots revealed distinct bands, corresponding to the sizes of the three mRNA species in the rat. It was therefore assumed that sequence similarities were sufficient for the use of rat probes to analyze cotton rat HDC, CgA and H⁺/K⁺ ATPase.

Statistical methods
Unless otherwise stated, the results are expressed as means ± SEM. The Mann–Whitney U-test and the Kruskal–Wallis test were applied for comparisons of means. Fisher’s exact test was used to compare ordinal data. P < 0.05 was considered statistically significant.

Results
Part A
Nine hypergastrinemic tumor-bearing animals were compared with 10 normogastrinemic control animals of the same age. At death the body weight was the same in tumor-bearing animals (166 ± 5 g) and controls (169 ± 4 g).

Macroscopic findings. All nine tumor-bearing animals displayed a thickening of the oxyntic part of the stomach wall, which upon sectioning appeared scirrhous. The serosal surface was rugged and irregular, exposing several transparent, liquid-containing cystic structures (diameters up to 10 mm). Also, the surface of the oxyntic mucosa was irregular, displaying numerous broad-based protrusions narrowing the gastric lumen. The stomach weight was higher than in the age-matched control animals (5.5 ± 1.8 versus 0.9 ± 0.02 g, P < 0.02). In the majority of the tumor-bearing animals the stomach was found to adhere to spleen, liver and/or intestine, and clear peritoneal fluid (2–4 ml) was found in four of these animals. The two animals with the largest stomachs (18.5 and 9.2 g) suffered from cachexia and presented several small superficial tumors (up to 5 mm in diameter) on the peritoneal surface in perihepatic areas, on the liver surface and in the mesenterium.

Histopathological findings. The tumors found in perihepatic areas, on the liver surface (Figure 1) and in the mesenterium displayed a growth pattern similar to that of the gastric tumors (Figure 2). The gastric tumors were found to infiltrate the muscularis mucosa and submucosa. In five of the animals they penetrated the stomach wall. They showed a mixture of desmoplastic stromal tissue and anaplastic epithelial cells and glands (Figure 2).

The adhesions between the stomach and perigastric organs were caused by desmoplasia and not by invasive growth.

IHC and argyrophilia. Cells with CgA immunoreactivity were observed in the gastric tumors. CgA-immunoreactive cells were also detected in the tumors found in the perihepatic area, on the liver surface and in the mesenterium (TSA method, Figure 3). The peritoneal fluid was found to contain cells immunoreactive for both CgA (Figure 4A) and HDC (Figure 4B), characteristic of ECL cells.

Groups of argyrophilic cells were also found in malignant tissue and in the same pattern as CgA-immunoreactive cells.

Plasma gastrin analysis. All tumor-bearing animals were hypergastrinemic throughout the study; 477 ± 99 pM when the tumors were detected and 907 ± 263 pM 6 months later (P < 0.3). The control animals with normal mucosa were normogastrinemic throughout the same period (17 ± 2.5 pM at start, 19 ± 2.1 pM at death).

Part B
Macroscopic findings. In the YF476 group 2 out of 18 animals had visible gastric tumors in oxyntic mucosa compared with 7 out of 21 animals of the vehicle-dosed controls (Table 1). Six animals in the YF476 group (including the 2 with visible tumors) had a grossly and apparently uniformly thickened oxyntic mucosa compared with 13 (including the 7 with visible tumor) vehicle-dosed controls (Table 1). There was no infiltration of perigastric tissue in any of the animals and remote
metastases were not found. The wide range of stomach weights (0.8–3.2 g) reflected the diversity of the changes. The stomach weight was lower in the YF476 group than in the control group (1.1 ± 0.05 versus 1.6 ± 0.2 g, \( P < 0.03 \)), while the stomach weight was higher in animals with carcinoma (2.1 ± 0.3 g, \( P = 0.003 \)) or dysplasia (1.6 ± 0.1 g, \( P < 0.001 \)) than in animals with normal appearing mucosa (1.0 ± 0.4 g). The body weight gain was the same in the two groups (data not shown).

**Histopathological findings.** One (5.6%) out of 18 animals in the YF476 group and 7 (33.3%) out of 21 animals in the vehicle-dosed group had gastric carcinoma (invasive growth) (\( P = 0.048 \); Figure 5 and Table I). One of the two animals in the YF476 group that were classified as tumor-bearing upon macroscopic examination displayed desmoplasia and dysplasia rather than invasive growth (Table I). The rest of the animals harbored tumors that grew invasively (carcinoma). The mucosa around the tumors was thickened, displaying dysplasia. As in part A, the tumors consisted of a mixture of desmoplastic stromal tissue and anaplastic epithelial cells (Figure 2).

Five (27.8%) animals of the YF476 group and 6 (28.6%) of the vehicle-dosed controls displayed dysplasia without carcinoma (Table I); the dysplasia was associated with thickened oxyntic mucosa (Table I).

![Figure 3](https://example.com/image3.png)

**Fig. 3.** High power photomicrograph of a tumor found in the mesentery of a female cotton rat (18 months of age) with gastric carcinoma showing CgA immunoreactivity by use of the TSA method. Counterstaining with hematoxylin.

![Figure 4](https://example.com/image4.png)

**Fig. 4.** High power photomicrograph of sediment from peritoneal fluid showing cells with CgA (A) and HDC (B) immunoreactivity. Counterstaining with hematoxylin.

### Table I. Serum gastrin status and macroscopic findings related to dosing group and histopathological diagnosis of oxyntic mucosa

<table>
<thead>
<tr>
<th>Histopathological diagnosis</th>
<th>Control group (( n = 21 ))</th>
<th>YF476 group (( n = 18 ))</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Normal (( n = 8 ))</td>
<td>Dysplasia (( n = 6 ))</td>
</tr>
<tr>
<td>Serum gastrin (pM) at sacrifice^a</td>
<td>17.1 ± 2.8</td>
<td>1021.2 ± 148.7</td>
</tr>
<tr>
<td>Months of hypergastrinemia^a</td>
<td>0</td>
<td>3.5(2–4)</td>
</tr>
<tr>
<td>Hypergastrinemic at initiation</td>
<td>0/8</td>
<td>0/6</td>
</tr>
<tr>
<td>Hypergastrinemic at sacrifice</td>
<td>0/8</td>
<td>0/6</td>
</tr>
<tr>
<td>With macroscopic tumor</td>
<td>0/8</td>
<td>0/6</td>
</tr>
<tr>
<td>With thickened mucosa</td>
<td>0/8</td>
<td>0/6</td>
</tr>
</tbody>
</table>

^aMean ± SEM.

^aMean and range.

pM, picomolar; \( n \), number; \( ^{*} P = 0.048 \) compared to the control group; ^a^not significant compared to the control group.
Among animals with dysplasia and/or carcinoma there was a diversity of histopathological changes, ranging from different levels of dysplasia (Figure 6B) to multifocal carcinomas infiltrating the muscularis mucosae and submucosa (Figure 6C). There were no signs of atrophic gastritis and the antrum was not affected. A prominent desmoplastic reaction in the lamina propria was exclusively found in the animals with carcinoma and/or dysplasia. In the same histopathological groups (carcinoma, dysplasia and normal) there was no notable difference between YF476- and vehicle-dosed animals. The remaining 12 animals in the YF476 group (66.7%) and 8 animals in the control group (38.1%) had what seemed to be normal mucosa (Figure 6A and Table I).

IHC and argyrophilia. The dysplastic lesions were made up of CgA-immunoreactive cells, which were more numerous in thickened oxyntic mucosa than in normal appearing mucosa (Figure 7A and B). CgA-immunoreactive cells were fewer in the tumors (Figure 7C) than in the mucosa outside the tumor when using a conventional immunostaining technique. However, the more sensitive TSA method raised the number of visible CgA-immunoreactive cells in the tumors. Numerous argyrophil cells were found in dysplastic mucosa, indicating an increase in ECL cell number (Figure 8A). The malignant tissue also contained glandular cells with argyrophilia, indicating ECL cell phenotype (Figure 8B). In general, staining with the Sevier–Munger technique showed argyrophil cells in the same pattern as CgA-immunoreactive cells.

Plasma gastrin concentration. The plasma gastrin concentration in vehicle-dosed controls with normal appearing mucosa was $23 \pm 1.5$ pM at the start of the study. This level was maintained throughout the study (17.1 $\pm$ 2.8 pM at death; Figure 9 and Table I). All animals given YF476 were hypergastrinemic (Figure 9 and Table I).

In the control group all cotton rats with mucosal dysplasia or carcinoma were hypergastrinemic at the time of death (Table I), whereas cotton rats having normal appearing mucosa were normogastrinemic (Table I). Animals with carcinomas had been hypergastrinemic for a longer period of time than those having dysplasia ($5.5 \pm 0.6$ versus $3.1 \pm 0.5$ months, $P < 0.03$). At the end of the study 62% of the control animals were hypergastrinemic. The mean age when hypergastrinemia first appeared was 3.5 months (range 2–6). None of the animals developed hypergastrinemia after 6 months of age. Interestingly, two of the animals that developed carcinoma were already hypergastrinemic (250 and 207 pM) at the start of the study (Table I) at the age of 9 weeks.

In the YF476 group all cotton rats had higher plasma gastrin than vehicle-dosed controls with normal appearing oxyntic mucosa.

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**Fig. 6.** Medium power photomicrographs showing (A) normal oxyntic mucosa in a normogastrinemic cotton rat; (B) oxyntic mucosa in a hypergastrinemic cotton rat with hyperplasia, dysplasia and invasive growth. (C) High power photomicrograph showing neoplastic tissue penetrating the muscularis mucosa layer in oxyntic mucosa of a hypergastrinemic cotton rat. H&E staining.

**Fig. 5.** Percent of animals in the control group and in the YF476 group displaying carcinoma, dysplasia and normal oxyntic mucosa.
Fig. 7. Medium power photomicrographs illustrating increased density of CgA-immunoreactive cells in dysplastic/hyperplastic glands (A) compared with histopathologically normal oxyntic mucosa (B). In neoplastic tissue the density of CgA-immunoreactive cells is decreased (C). The small inset in the lower right corner of (C) shows details of immunoreactive cells. Conventional CgA immunostaining. Counterstaining with hematoxylin.

Fig. 8. High power photomicrograph from the oxyntic mucosa of spontaneous hypergastrinemic animals showing argyrophil cells stained by the Sevier–Munger procedure. There were numerous argyrophil cells in the dysplastic glands (A). Malignant tissue below the muscularis mucosa also contained glandular cells with argyrophilia (B).
The YF476-dosed animals had thinner oxyntic mucosa than the animals without carcinoma (1.1 mm; animals with carcinoma invariably had thicker mucosa than vehicle-dosed controls (0.4 mm). The other animals in the YF476 group were normogastrinemic before start of dosing (20 ± 1.7 pM). The five animals that displayed dysplasia had higher plasma gastrin during the study than the animals with normal appearing mucosa (Figure 9) (1593 ± 237 versus 719 ± 146 pM at death, P = 0.02).

**Thickness of oxyntic mucosa.** The thickness of the oxyntic mucosa was related to the histopathological findings in that animals with carcinoma invariably had thicker mucosa than animals without carcinoma (1.1 ± 0.08 versus 0.5 ± 0.04 mm, P < 0.002). Moreover, animals with dysplasia had thicker mucosa than animals with normal appearing mucosa (0.6 ± 0.07 versus 0.4 ± 0.02 mm, P = 0.005). Taking all animals into consideration (regardless of histopathological diagnosis), the YF476-dosed animals had thinner oxyntic mucosa than the vehicle-dosed controls (0.4 ± 0.04 versus 0.7 ± 0.08 mm, P = 0.005). Among animals with the same histopathological diagnosis (normal, dysplasia or carcinoma) the mucosal thickness was the same regardless of whether they were dosed with YF476 or not (data not shown).

**mRNA analysis.** The results are presented as percent of the mean mRNA expression levels in control animals with normal appearing oxyntic mucosa.

The expression of HDC mRNA in YF476-dosed animals having normal appearing oxyntic mucosa was half that found in vehicle-dosed animals with normal mucosa (P = 0.028, Figure 10A). The expression of HDC mRNA in vehicle-dosed animals with dysplastic mucosa was 12-fold that of vehicle-dosed animals with normal appearing mucosa (P < 0.009, Figure 10B). Also, the CgA mRNA abundance was well correlated with the state of the mucosa. Thus, in vehicle-dosed animals with dysplastic mucosa, the expression of CgA mRNA was about six times greater than in vehicle-dosed animals with normal mucosa (P = 0.009, Figure 10B). In animals with normal appearing mucosa we could not detect any significant difference in CgA mRNA abundance between YF476-dosed and vehicle-dosed animals (P = 0.11, Figure 10A). The expression of H⁺/K⁺ ATPase mRNA in vehicle-dosed controls with dysplasia was only 6% of the level found in control animals with normal mucosa (P = 0.028, Figure 10B). Finally, the expression of H⁺/K⁺ ATPase in YF476-dosed animals with normal appearing mucosa was only 8% of that in vehicle-dosed animals with normal mucosa (P = 0.047, Figure 10A).

**Discussion**

**The natural course of the gastric tumors in cotton rats**

The evidence that gastrin plays a key role in the development of oxyntic mucosal dysplasia and carcinoma in cotton rats is compelling. All animals displaying dysplasia or carcinoma were hypergastrinemic, whereas normogastrinemic animals had normal appearing mucosa (Table I). At times, the oxyntic mucosa exhibited a variety of dysplastic changes together with multifocal tumors in the same hypergastrinemic individual. The tumors were invasive, infiltrating beyond the muscularis mucosae and in some instances penetrating the gastric wall. In addition, several metastases were found on the peritoneal surface in perihepatic areas, on the liver surface and in the mesenterium of cachectic animals at 16 months of age. The mechanism behind the dissemination of malignant cells is probably intracavitary seeding, since cells tentatively identified as ECL cell-derived (on the basis of their content of CgA and HDC) were found in the peritoneal fluid (Figure 4). From the present findings it seems clear that the gastric tumors in cotton rats are malignant and that gastrin is a driving force behind the...
transformation from normal ECL cells to dysplastic and malignant cells.

In the initial study by Kawase and Ishikura (3) ~50% of 8-month-old female cotton rats had gastric lesions (dysplasia and/or carcinoma). In the present study 62% of the vehicle-dosed control animals had such lesions and they were all hypergastrinemic at death. No cotton rat was hypergastrinemic at the age of 2 months and none developed hypergastrinemia after the age of 6 months. The few animals which were hypergastrinemic at the start of the study were nearly 3 months old. Thus, spontaneous hypergastrinemia develops between 2 and 6 months of age. This suggests that the risk of developing gastric carcinoma can be predicted at an early age by measuring the plasma gastrin concentration. Furthermore, the degree and duration of the hypergastrinemia probably determines the severity of the lesions. This study demonstrates that 1-3 months of hypergastrinemia induces ECL cell hyperplasia and dysplasia, whereas hypergastrinemia for >3 months greatly increases the risk of developing carcinoma.

The majority of hypergastrinemic female (and probably of male (3; Fossmark et al., unpublished results)) cotton rats seem to develop ECL cell-derived tumors. In other animal models such neoplasias are often not found until after 18-24 months of hypergastrinemia, i.e. at the end of the lifespan of the animals (13-16,31-34). Thus, the cotton rat seems to be especially prone to develop ECL cell-derived tumors in response to hypergastrinemia. In Mastomys (35), dosing with potent inhibitors of gastric acid secretion (thereby raising the plasma gastrin level) shortens the tumor induction time from 12 to 4 months (35,36), which seems analogous to the situation in spontaneously hypergastrinemic cotton rats.

The spontaneous hypergastrinemia in cotton rats seems to be secondary to gastric hypoacidity induced by a gastropathy of unknown nature. Histologically there are no signs of atrophic gastritis. As discussed previously (5), the presence of parietal cells immunoreactive for H+/K+ ATPase as well as a sustained, albeit a reduced, expression of H+/K+ ATPase mRNA in the mucosa outside the tumors suggest that the mucosa remains capable of producing acid. In the present study the expression of H+/K+ ATPase mRNA in control animals with dysplasia was lower than in control animals with histopathologically normal mucosa (Figure 10B). This is in accordance with the results of an earlier study in which the H+/K+ ATPase expression in oxyntic mucosa of animals with carcinoma was low compared with cotton rats with normal mucosa (5). If this reflects a low number or a low functional activity of the parietal cells remains to be settled.

In rats (13,16) as well as in humans (17,37) there is a female preponderance of hypergastrinemia-induced gastric carcinoid tumors. To the best of our knowledge, this sex difference has never been adequately explained. We have demonstrated that female cotton rats with hypergastrinemia for more than 4 months develop ECL cell carcinoma. In view of the fact that male cotton rats with hypergastrinemia are also prone to develop such tumors (3; Fossmark et al., unpublished results) it may be argued that perhaps the female preponderance of ECL cell malignancy merely reflects a high incidence of hypergastrinemia (and the gastric hypoacidity that causes it). Thus, it seems reasonable to suggest that this female preponderance of gastric lesions in the cotton rat reflects the frequency of gastric hypoacidity.

This strain of cotton rat exhibits two remarkable features. First, more than half of the females (and a few males) become hypoacidic and hypergastrinemic spontaneously for unknown reasons. Second, all hypergastrinemic cotton rats seem to develop ECL cell hyperplasia, dysplasia, neoplasia and carcinoma.

When classifying cotton rats into those with normal mucosa, dysplasia or carcinoma, there is a correlation between the thickness of the oxyntic mucosa and the histopathological diagnosis. The mucosa in cotton rats with carcinoma is two to three times thicker than the mucosa of animals without carcinoma.

To understand the biology of the gastric lesions in cotton rats, it is crucial to identify their cellular origin. By conventional immunohistochemical and histochemical techniques we have previously (4,5), and again in this study, demonstrated an increased density of ECL cells in the oxyntic mucosa of hypergastrinemic cotton rats (Figure 7A). Moreover, there was an increase in the mRNA expression of both HDC and CgA in the mucosa of such animals (Figure 10B). Many of the tumor cells, including those invading the submucosa and the muscle layer, are argentaffins and immunoreactive for HDC and CgA, suggesting an ECL cell origin. The apparent number of such cells is lower in the tumors than in the mucosa around the tumors. However, even in classical neuroendocrine tumors, such as carcinoids and small (oat) cell lung carcinomas, only a proportion of the neoplastic neuroendocrine cells show CgA immunoreactivity (38). A loss of cell type-specific features due to dedifferentiation of tumor cells may explain this finding (39,40). In order to enhance the visibility of ECL cell-specific markers we applied TSA (27,28). With this technique many more tumor cells became immunoreactive for CgA. This observation is in line with the idea that malignant transformation involves a gradual loss of ECL cell-specific markers. Obviously, this has relevance for the classification of neuroendocrine tumors in general (27,41,42).

The visualization of argentaffin cells with the same pattern as CgA-immunoreactive cells further confirms that ECL cells constitute an important part of gastric tumors in cotton rats. On the other hand, it is of course possible that some carcinomas could originate from stem cells being overstimulated by trophic factors from the increased ECL cell mass (like REG protein) (43,44). Nevertheless, the role of gastrin in the development of ECL cell-derived tumors is crucial. A role of gastrin in the development of ECL cell-derived tumors is crucial. Interestingly, the one animal in the YF476 group that developed carcinoma was already hypergastrinemic at the start of dosing and was the oldest individual in the group. Among the five animals of the YF476 group that showed dysplasia, two were hypergastrinemic at the start of dosing (Table I). Conceivably, long-standing hypergastrinemia induces the genetic changes responsible for the neoplastic transformation, causing subsequent events to occur independently of gastrin. It cannot be ruled out that such gastrin-independent growth starts...
at the stage of dysplasia, occurring after 1–2 months of hypergastrinemia. This may explain the development of a carcinoma in one of the animals given YF476.

Alternatively, the gastrin receptor blockade could be incomplete. The following observations, however, support the view that YF476 produces an effective gastrin receptor blockade. First, the expression of H\(^+\)/K\(^+\) ATPase in YF476-dosed animals was only a fraction of that in control animals. Second, the expression of HDC mRNA was also reduced by YF476. Third, and most important, all cotton rats on YF476 had high plasma gastrin. Presumably, some of these animals became hypergastrinemic due to spontaneous gastropathy, the rest probably because of acid blockade induced by YF476. It is worth noting that the level of plasma gastrin in YF476-dosed animals with dysplasia was significantly higher than in YF476-dosed animals with normal appearing mucosa. This is probably the combined effect of spontaneous gastropathy and gastrin receptor blockade.

The level of H\(^+\)/K\(^+\) ATPase transcription in parietal cells has previously been found to be regulated by factors/substances (for instance histamine and regeneration gene protein) released from ECL cells (43–46). The functional impairment of the gastrin–ECL cell axis in YF476-dosed animals probably explains why the expression of H\(^+\)/K\(^+\) ATPase in oxyntic mucosa of such animals was lower than in control animals with normal mucosa (Figure 10A).

Gastrin promotes growth of the oxyntic mucosa. Elimination of endogenous gastrin by antrectomy (47,48) or by targeted gene disruption as in mice deficient of gastrin (49,50) or the gastrin receptor (51,52) is associated with a reduced weight and thickness of the oxyntic mucosa. In a recent study of Sprague–Dawley rats, YF476 (300 μmol/kg once a week) was found to reduce mucosal thickness by nearly 30% (53). Indeed, we found that the thickness of the oxyntic mucosa and the weight of the stomach was lower in YF476-dosed cotton rats than in vehicle-dosed controls. However, comparing cotton rats with similar mucosal classification (normal, dysplasia and carcinoma) revealed no differences in stomach weights and mucosal thickness between YF476-dosed and control animals (data not shown). Taking into consideration the fact that a few cotton rats developed dysplasia or carcinoma despite gastrin receptor blockade and that YF476 failed to reduce the thickness of the normal appearing oxyntic mucosa, it seems reasonable to assume that at the dose given the drug did not achieve maximal gastrin receptor blockade. The dose regimen was based on reports of its effectiveness in Sprague–Dawley rats (21). However, species differences in a single amino acid of the gastrin receptor may be crucial for the antagonist specificity (25). Moreover, the plasma gastrin level in cotton rats is markedly high. This could attenuate the inhibitory effect of YF476, which acts as a competitive antagonist (54). Conceivably, a persisting, albeit suppressed, gastrin effect may be sufficient to maintain a normal mucosal thickness and a maintained but delayed progression of tumorigenesis.

Concluding remarks

Fifty percent of these female cotton rats develop hypoacidity spontaneously, which leads to hypergastrinemia. The hypergastrinemia induces first diffuse and then focal ECL cell hyperplasia, and ultimately dysplasia and ECL cell carcinomas. With time, the tumors will invade and penetrate the stomach wall and metastasize to perigastric sites. The finding of cells with ECL cell features in peritoneal fluid from animals with gastric carcinomas and in metastases supports the view that neoplasms derived from ECL cells may be malignant.

The gastrin receptor antagonist YF476 lowered the incidence of spontaneous ECL cell carcinomas in female cotton rats. This finding illustrates the fact that gastrin promotes the development of ECL cell-derived malignancies and that gastrin receptor blockade may inhibit this process. The etiology and pathogenesis of the gastric hypoaclorhydria occurring spontaneously in this colony of cotton rats is not clear. Nevertheless, inbred female cotton rats represent a unique hypergastrinemia model allowing detailed studies of the spontaneous multistep development of malignant ECL cell-derived tumors.

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