Morphology of G Cells in Hypergastrinemic Cotton Rats

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Summary
In a strain of inbred cotton rats, 25-50% of females develop spontaneous gastric hypochlorhydria and hypergastrinemia. Hypergastrinemic animals develop ECL cell derived gastric carcinomas located in the oxyntic mucosa, thus being an interesting animal model for studying the role of gastrin in gastric carcinogenesis. The response to gastric hypoacidity in cotton rats as regards the level of hypergastrinemia is far more pronounced than in the more commonly used laboratory rat. It is unknown whether the pronounced hypergastrinemic response in cotton rats is due to a greater population of G cells or a greater capacity of hormone synthesis in each G cell. The aim of the study was therefore to examine G cell population and ultrastructure in normogastrinemic and hypergastrinemic cotton rats by the use of immunohistochemical methods applied on both light- and electron-microscopy. Five hypergastrinemic vs. five normogastrinemic cotton rats were compared. Cotton rats with gastric hypochlorhydria have a 55-fold increase in serum gastrin levels and a 6-fold increase in G cell number, but this is not accompanied by significant changes in G cell ultrastructure. The lack of ultrastructural changes in these activated G cells indicates that previously reported changes in chronic stimulated G cells are just one of several ways G cells are activated.

Introduction
Gastrin stimulates gastric acid secretion, an effect mediated by gastrin (cholecystochinin-2) receptors on the enterochromaffin-like (ECL) cells (Waldum et al., 2002a). The ECL cell contains histamine (Håkanson et al., 1986) and is the predominant endocrine cell of the oxyntic mucosa (Sundler et al., 1991a). When activated by gastrin, the ECL cell responds with increased production and release of histamine (Sandvik et al., 1994), which stimulates acid secretion from the parietal cell (Waldum et al., 1993). In addition, gastrin has trophic effects on the oxyntic mucosa, most notably on the ECL cell (Bakke et al., 2001). These effects have repeatedly been shown to result in ECL carcinoids and / or adenocarcinomas in animal models of long-term hypergastrinemia (Waldum et al., 2004). Zollinger Ellison syndrome (gastrinoma) and pernicious anaemia (chronic atrophic gastritis) represent models of hypergastrinemia in man. Both diseases are clearly associated with development of ECL carcinoids (Waldum et al., 2002b). In addition, pernicious anaemia also increases the risk of gastric carcinomas (Kokkola et al., 1998). However, the long-term use of potent inhibitors of gastric acid secretion is the most common cause of hypergastrinemia in humans, and there is a continuous debate concerning the safety of these drugs with regards to gastric carcinogenesis (Waldum et al., 2002b). The cotton rat (Sigmodon hispidus) was first used.
in medical research in the 1930s as it was susceptible to poliovirus (Armstrong, 1939). Since then it has proven susceptible to a wide range of human pathogens (Niewiesk & Prince, 2002) and has been useful in the search for vaccines and antiviral drugs. In a strain of inbred cotton rats, 25-50% of females, but less than 1% of males (Kawase & Ishikura, 1995), develop spontaneous gastric hypoacidity and a secondary hypergastrinemia. The mechanism behind the gastric hypoacidity is unknown. Histologically there are no signs of atrophic gastritis. Both parietal cells immunoreactive for H+/K+ ATPase and expression of H+/K+ ATPase mRNA are present, although in lower numbers/levels than normally. If this reflects a low number or a low functional activity of the parietal cells remains to be settled. If the parietal cells are normal the anacidity was previously suggested to be caused by a condition resembling Menetriers disease in man with leakage of fluid into the gastric cavity, diluting the content (Cui et al., 2000).

Hypergastrinemic cotton rats develop ECL cell-derived gastric carcinomas located in the oxyntic mucosa (Waldum et al., 1999). Hence, these animals have become an important model of hypergastrinemia and gastric carcinogenesis. The changes in the oxyntic mucosa and mechanisms of carcinoma development in these cotton rats have been thoroughly studied, and the role of gastrin as the cause of carcinogenesis is well documented (Martinsen et al., 2003a; Fossmark et al., 2004a-b). It has, for example, been demonstrated that a gastrin receptor antagonist prevents carcinoma development (Martinsen et al., 2003a) and antrectomy reverses dysplastic changes (Bakkelund et al., unpublished).

Moreover, we have shown an increased level of histidine decarboxylase mRNA in oxyntic mucosa, strongly indicating increased levels of histamine reflecting the ECL cell response to hypergastrinemia (Martinsen et al., 2003a; Fossmark et al., 2004c). The changes in the antrum and the gastrin producing G cell have not been described. Independent of sex, plasma gastrin levels increase gradually with time in hypoacidic cotton rats, reaching levels 30-50 times higher than age-matched controls with a normal gastric acidity (Martinsen et al., 2003a; Fossmark et al., 2004a). The response to gastric hypoacidity in cotton rats as regards the level of hypergastrinemia is far more pronounced than in the rat, where only close to tenfold increase has been reported (Hakanson et al., 1982; Ryberg et al., 1989; Martinsen et al., 2003b). However, the relative increase in plasma gastrin levels found in hypoacidic cotton rats is similar to the increase in patients with gastric hypoacidity due to chronic atrophic gastritis, where a proportion of the patients have plasma gastrin levels 40 times the normal value (Sipponen et al., 1985; Qvigstad et al., 2002).

At the ultrastructural level, the gastrin (G) cell displays the typical features of peptide hormone-producing cells. However, the morphological hallmark of G cells is the heterogeneous composition of granule types, from small electron-dense to large electron-lucent ones (Forssmann & Orci, 1969; Mortensen, 1980; Hakanson et al., 1982). The biological explanation for this diversity has been controversial. Electron-dense granules are found near the Golgi zone and electron-lucent granules in the periphery (Mortensen, 1980; Hakanson et al., 1982). Moreover, fasting is shown to reduce the number of electron-dense granules and generate a higher proportion of electron-lucent ones (Mortensen et al., 1979). From this it has been concluded that electron-dense granules are newly formed and immature thus containing gastrin precursors, which are converted into larger electron-lucent granules during enzymatic modifications (Mortensen, 1980; Hakanson et al., 1982). This concept is further supported by the use of non-cross reacting antibodies against gastrin-17 and progastrin, which label electron-dense and electron-lucent granules respectively (Varnell et al., 1983; Rahier et al., 1987; Dockray & Varro, 1993).

Ultrastructural signs of G cell activation due to long-term pharmacological inhibition of gastric acid secretion or surgical removal of the acid-producing part of the stomach have been described in
both humans (Nielsen & Hage, 1985) and rats (Hakanson et al., 1982; Sundler et al., 1991a; Martinsen et al., 2003b). Morphometric analyses of long-term activated G cells have shown various ultrastructural changes; most notably that electron-dense granules make up a greater proportion of the total granule population (Hakanson et al., 1982; Nielsen & Hage, 1985; Sundler et al., 1991b; Martinsen et al., 2003b). These findings further favour the view that activated G cells contain a greater proportion of immature electron-dense granules in which the conversion of gastrin-precur-sors into smaller fragments is incomplete.

It is also well documented that stimulated G cells respond with hyperplasia and to a lesser extent hypertrophy (Hakanson et al., 1982; Sundler et al., 1991b; Martinsen et al., 2003b). In addition to ultrastructural changes reflecting increased production and release of gastrin, both hypertrophy and hyperplasia are thought to contribute to increased level of serum gastrin in individuals with activated G cells. It is unknown whether the pronounced hypergastrinemic response in cotton rats is due to a greater population of G cells or a greater capacity for hormone synthesis in each G cell. It was therefore of interest to examine the G cell population in normogastrinemic and hypergastrinemic cotton rats and to identify which of the mentioned factors that contribute to the pronounced hypergastrinemia seen in these animals.

Materials and Methods

Animals

The cotton rats were originally provided by Tanabe Seiyaku Co. Ltd., Toda, Japan, in 1971 and maintained by random mating. In 1982 some of the animals were found to develop spontaneous gastric tumours and these animals were kept in a colony by sister/brother mating for more than 20 generations, thus becoming an inbred strain.

In the present study the cotton rats were housed solely in wiretop cages with aspen woodchips bedding (B&K Universal Ltd., Hull, UK). Room temperature was 24±1 °C with a relative humidity of 40–50% and a 12-h light/dark cycle. The Rat and Mouse Diet of B&K and tap water was provided ad libitum. From this inbred cotton rat strain, 5 animals were identified as hypergastrinemic after monthly plasma gastrin determination, starting at age 2 months. Plasma was frozen at -20°C for later determination of gastrin concentration by radioimmunoassay (Kleveland et al., 1985).

According to the protocol, the animals were sacrificed three months after the hypergastrinemia was detected, giving a mean age of approximately 7.5 months (range 6-8). The animals had no clinical symptoms of disease. Five age-matched randomly selected normogastrinemic female cotton rats from the same inbred strain were used as controls. The mean weight of the animals at sacrifice was similar in the two groups (hypergastrinemic animals 152±8g vs. normogastrinemic 147±6 g). Prior to blood sampling and sacrifice, the animals were anaes-thetised with a subcutaneous injection of 0.3 ml/100g bodyweight of Hypnorm/Dormicum, which is a combination of (per ml) 2.5 mg fluanison, 0.05 mg fentanyl and 1.25 mg midazolam. The injections were given during brief isoflurane inhalation anaesthesia. All blood samples (each approximately 0.5 ml) were collected from the saphenous vein.

The Animal Welfare Committee of the University Hospital of Trondheim approved the experiment.

Immunohistochemistry and G cell number

Tissue samples for analysis by light microscopy were taken from the antrum. The samples were fixed in 4% phosphate-buffered formaldehyde followed by dehydration in 80% ethanol and embedding in paraffin. Paraffin blocks were cut in 5 µm sections (Leica 2055 Autocut). The sections were deparaffinized with xylene, rehydrated, and treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Antigen retrieval was achieved by heating the slides immersed in 10mM citrate-buffer pH 6.0 using a commercial microwave oven at 160 W for 15 minutes. The sec-
tions were incubated with rabbit anti-human gastrin (A0568; DakoCytomation, Glostrup, Denmark) diluted 1:500 in phosphate-buffered saline (PBS), pH 7.3, containing 0.25% Triton X-100 (Calbiochem, CA) and 0.25% bovine serum albumin (BSA) (Sigma, St. Louis, MO) for 30 minutes at room temperature. The sections were rinsed in cold PBS mixed with 0.03% Tween 20 (DakoCytomation) between each step. The immunoreactivity was visualised using an EnVision-HRP kit (K5007, DakoCytomation) and an AEC peroxidase kit (SK4200; Vector, Burlingame, CA). As negative control, the primary antibody was replaced with non-immune antibody diluent. All sections were stained with hematoxylin.

The G cell density in the antral gastric glands was assessed per mm of sample by counting the number of nucleated cells with gastrin positive cytoplasmic granules in sections where the lumen could be seen throughout the entire length of the gland (Annibale et al., 1991; Langhans et al., 1997).

Immunoelectron microscopy

The tissue samples collected for immunanalysis using transmission electron microscopy were taken from the antrum 5 mm from the antrum-corpus border at the major curvature. Samples of 1mm³ were cut with a razor blade and immediately immersed in a mixture of 2% glutaraldehyde for 24 hours at room temperature followed by postfixation 2% OsO₄ for 1 hour. Thereafter, the specimens were dehydrated in 50%, 70%, 90%, 100% ethanol solutions and propylene oxide before they were embedded in Epoxy Resin LX 112 (Ladd Research Industries, Burlington, VT) hardening for 3 days at 56 °C. Ultrathin sections (90 nm) were cut using an RMC MTX ultramicrotome (MT-X ultramicrotome RMC, Boeckler Instruments, Inc., Tuscon, AZ) and mounted on 200 mesh nickel grids. The nickel grids with sections were placed in the gap between two microscope slides (ChemiMate Capillary Gap Microscope Slides, BioTek Solutions, Santa Barbara, CA). The slides were immersed in an alkaline solution, pH 10 (Target retrieval solution, DakoCytomation, Carpinteria, CA) before they were heated at 140 °C for 15 min. Primary and secondary antibodies and buffer were absorbed and eluted from the edge of the slides. Slides with nickel grids were incubated with rabbit anti-human gastrin (code A0568, DakoCytomation, Glostrup, Denmark) diluted 1:50 in Tris- HCl - buffered, pH 7.3, containing 0.25% Tween 20 (DakoCytomation, Glostrup, Denmark) and 1% BSA (Sigma, St. Louis, MO) at 60 °C for 30 min. Then the slides were rinsed in Tris-HCl-buffered, pH 7.6, containing 0.05% Tween 20 (DakoCytomation, Glostrup, Denmark) and 1% BSA (Sigma, St. Louis, MO) followed by incubating in secondary antibodies goat anti–rabbit IgG conjugated with 10 nm gold probe (Code 110.011, Microscopy Science, WA) diluted 1:25 in Tris-HCl-buffered, pH 7.6, containing 0.25% Tween 20 and 1% BSA at 60 °C for 30 min. After the grids were rinsed and incubated in sterile water at room temperature for 10 min, they were contrasted with uranyl acetate and lead citrate. Control grids were incubated with non-immune antibody diluent buffer instead of anti-gastrin and they showed no labelling.

Morphometric analyses of G cell ultrastructure

The ultrastructures of the G cells from each sample were studied in a transmission electron microscope (Jeol 1011x). Five or six randomly selected immunogold-labelled G cells from each sample were photographed using imaging plates and analyzed at a magnification 3-6000 x by the use of point counting technique (Weibel, 1969; Weibel & Bolender, 1973). The total cell profile area and the areas occupied by nucleus, cytoplasm, and granules were calculated from the number of point intercepts for each compartment adjusted for the magnification of the micrographs. The electron density of the granules in a G cell varies greatly (Forssmann & Orci, 1969; Mortensen, 1980; Hakanson et al., 1982; Varn dell et al., 1983). To obtain a quantitative assessment of the electron density of the granule population, each granule was counted with a set of standard granule image. The granules were separat-
ed into three groups (Mortensen, 1980; Hakanson et al., 1982; Martinsen et al., 2003b): 1) electron-dense; 2) intermediate type with either a homogeneous core of low to moderate electron density or a small dense core surrounded by a wide electron-lucent halo; 3) electron-lucent (without any electron-dense material). The G cell cytoplasm and nucleus area were measured using iTEM Analysis software (Soft Imaging System GmbH, Münster, Germany).

Statistics
The results were expressed as means ± standard error of the mean (SEM). Student’s t-test (two-tailed) was used for the analysis. P<0.05 was considered statistically significant.

Results
Plasma gastrin
At sacrifice the mean plasma gastrin levels in hypergastrinemic and normogastrinemic animals were 841±201 pM vs 15.4±1.6 pM, respectively.

G cell density
Hypergastrinemic animals had an approximately 6 times higher G cell density than normogastrinemic animals (Table 1). This marked difference is illustrated by Figure 1.

Table 1. G cell density in normogastrinemic vs. hypergastrinemic cotton rats.

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<th>Normogastrinemic</th>
<th>Hypergastrinemic</th>
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<tr>
<td>No. G cells per mm ± SEM</td>
<td>9.3 ± 1.1</td>
<td>55.4 ± 5.6*</td>
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*P < 0.005; No: number; SEM: standard error of the mean; n: number of animals.

Morphometric analyses of G cell ultrastructure
The results of the morphometric analyses of G cell ultrastructure in normogastrinemic and hypergastrinemic animals are presented in Table 2. As regards the number of the different types of granules, the intermediate granule type was the major population in both groups and the groups had an almost equal percentage distribution of the different granule types in the cytoplasm of the G cell. The area of the nucleus, cytoplasm and the total G cell was not significantly different between the two groups; however, hypergastrinemic animals tended to have larger G cells. There was a diversity of ultrastructural appearance of the G cells within each group and even in the same gland. Moreover, the variance (SEM) in the measured cytoplasmic area of the G cells in hypergastrinemic animals was higher than in the normogastrinemic animals.

Discussion
Gastrin cells were demonstrated in the antral mucosa by McGuigan in 1968 (McGuigan, 1968). The main function of the antral G cell is to synthesize and release gastrin, thereby regulating acid secretion and growth in the oxyntic mucosa of the stomach. The G cell function is primarily stimulat-
ed directly by gastric luminal content (Lloyd & Walsh, 1993; Lloyd & Debas, 1994). In addition, a complex system of paracrine and neural factors modulates G cell activity (Lloyd & Walsh, 1993; Lloyd & Debas, 1994; Sawada & Dickinson, 1997). If stimulation of G cells persists, as shown in models of surgically or drug induced gastric hypochlorhydria, the cells respond with hyperplasia already after a few days (Alumets et al., 1980; Allen et al., 1986; Creutzfeldt et al., 1987; Larsson et al., 1988; Sandler et al., 1991b). The level of hyperplasia increases gradually, leveling off at about a doubling of the G cell density after a few weeks (Larsson et al., 1988; Eissele et al., 1990). No further increase in G cell density is found after this (Sandler et al., 1991b). The proliferation labeling index of G cells increases from the third day and returns to basal levels within a month despite continuous stimulation by gastric anacidity (Eissele et al., 1990). Some unknown regulatory mechanisms seem to be activated to prevent further G cell proliferation. Consequently, despite G cell activation during the whole lifespan caused by gastric anacidity, G cell tumors do not develop.

In achlorhydric rats the plasma gastrin concentration increases approximately 10-fold compared to

Table 2. Morphometric analyses of G cell ultrastructure in normogastrinemic vs. hypergastrinemic cotton rats.

<table>
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<th>Normogastrinemic (n=5)</th>
<th>Hypergastrinemic (n=5)</th>
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<tr>
<td>No. of G cells examined</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>Electron-lucent granules: No ± SEM (% of total No)</td>
<td>11.6 ± 2.6 (7.3)</td>
<td>15.6 ± 5.9 (8.6)</td>
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<tr>
<td>Intermediate granules: No ± SEM (% of total No)</td>
<td>143.6 ± 18.3 (85.5)</td>
<td>158.6 ± 24.8 (85.4)</td>
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<tr>
<td>Electron-dense granules: No ± SEM (% of total No)</td>
<td>12.8 ± 3.3 (7.6)</td>
<td>9.7 ± 2.0 (5.2)</td>
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<tr>
<td>Total no granules granules: No ± SEM</td>
<td>167.9 ± 19.1</td>
<td>185.6 ± 29.4</td>
</tr>
<tr>
<td>Nucleus area: µm² ± SEM (% of total No)</td>
<td>16.1 ± 1.7 (19.6)</td>
<td>24.4 ± 3.8 (20.7)</td>
</tr>
<tr>
<td>Cytoplasm area: µm² ± SEM (% of total No)</td>
<td>66.4 ± 2.4 (80.4)</td>
<td>93.8 ± 16.7 (79.3)</td>
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<tr>
<td>Total area of the cell: µm² ± SEM</td>
<td>82.5 ± 3.5</td>
<td>118.2 ± 17.9</td>
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No: number; SEM: standard error of the mean; n: number of animals
In contrast, in this inbred strain of cotton rats, achlorhydria induces a plasma gastrin concentration 55 times higher (in the present study) than in animals with normal gastric acidity. This level of hypergastrinemia corresponds to the increase found in previous studies (Martinsen et al., 2003a; Fossmark et al., 2004a). The unique hypergastrinemic response in these cotton rats could principally be due to a greater population of G cells, a greater capacity of hormone synthesis in each cell or both. In the present study a high level of G cell hyperplasia was found in hypergastrinemic animals, reaching six-fold the density of G cells found in normochlorhydric animals. This level of increase in G cell density is 3 times higher than that found in rats with proton pump inhibitor induced achlorhydria (Sundler et al., 1991b). The reason for this pronounced G cell hyperplasia in cotton rats is not known.

Ultrastructural changes in stimulated G cells have previously been described in rats and humans and have been presumed to indicate an increased G cell activity (Mortensen, 1980; Hakanson et al., 1982). However, it seems that G cells react differently to acute and chronic stimulation. Acute stimulation is reported to induce an increase in number of electron-lucent granules (Forssmann & Orci, 1969; Track et al., 1978; Bastie et al., 1979), whereas in chronic stimulation models the opposite seems to occur (Hakanson et al., 1982; Sundler et al., 1991b; Martinsen et al., 2003b). Long term gastric hypoacidity and hypergastrinemia in rats induced by fundectomy (Hakanson et al., 1982) and proton pump inhibitors (Sundler et al., 1991b) (Martinsen et al., 2003b) induce quite similar ultrastructural changes in G cells. Briefly, these are: 1) hypertrophy of G cells, 2) increased proportion of the electron-dense granules, 3) reduced total number of granules per cell, 4) reduced granule volume density. Similar changes are also described in humans treated with H₂-antagonist (Nielsen & Hage, 1985).

Although the G cell size tended to be increased in hypergastrinemic cotton rats, we did not find significant changes at an ultrastructural level in the hypergastrinemic animals compared to normogastrinemic ones. In the present study the animals had been hypergastrinemic for approximately three months before sacrifice. In previous studies the hypergastrinemic periods have been shorter (4-10 weeks) (Hakanson et al., 1982; Nielsen & Hage, 1985; Sundler et al., 1991b); nevertheless this difference probably does not explain the different results. The lack of ultrastructural changes in activated G cells in cotton rats indicates that previously described changes in chronic stimulated G cells are just one of several ways G cells are activated. This assumed heterogeneity in pattern of cellular

Figure 2. Electron micrographs of cells with gastrin immunogold labelling in the antral mucosa of hypergastrinemic (A and B) and normogastrinemic (C and D) cotton rats. Inset in A demonstrates immunogold labelling of an electron dense granule (*) and an intermediate granule (arrow). Scale bars are 1 µm.
activation is also supported by a recent demonstration of ultrastructural differences in G cells activated by a proton pump inhibitor and ciprofibrate (Martinsen et al., 2003b), a peroxisome proliferator-activated receptor (PPAR) α agonist acting directly luminally on G cells (Martinsen et al., 2005).

In rats there is a 10-fold increase in plasma gastrin and a doubling of G cell density in achlorhydric animals. Consequently, each cell must produce and release five times more gastrin compared to normogastrinemic controls. The level of serum gastrin in hypergastrinemic cotton rats is approximately 50 times higher than in normogastrinemic animals. The pronounced hyperplasia with a six-fold increase in G cell density explains some of their hypergastrinemia. However, each G cell must contribute with approximately a 10-fold increase in production and release of gastrin compared to G cells in normogastrinemic animals. Nevertheless, in this study we did not find any significant changes at ultrastructural level reflecting this G cell hyperfunction. However, this study was not design to focus upon changes in the Golgi zone and rough endoplasmatic reticulum. These structures have previously been shown to enlarge in hypergastrinemic rats (Hakanson et al., 1982). The pronounced hyperplasia and hypergastrinemia indicate that the G cells in these cotton rats respond in a unique way to stimulation; hence the lack of expected ultrastructural changes in these activated G cells indicates that previously reported changes in chronic stimulated G cells are just one of the several ways G cells are activated. Taking into consideration the relatively high prevalence of hypergastrinemia in humans and the relevance of gastrin in gastric carcinogenesis, it is important to study G cell physiology. Hence, the spontaneously hypergastrinemic cotton rat represents an interesting model for studying G cell function and morphology.

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