Spontaneous enterochromaffin-like cell carcinomas in cotton rats (Sigmodon hispidus) are prevented by a somatostatin analogue

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Abstract
Among inbred female cotton rats (Sigmodon hispidus) 25–50% of the animals develop spontaneous gastric carcinomas; the corresponding figure for male cotton rats is approximately 1%. Animals with carcinomas have hypergastrinaemia and gastric hypo-anacidity and the tumours are derived from enterochromaffin-like (ECL) cells. The mechanism behind the hypo-anacidity is unknown. Carcinomas are found in all female cotton rats with hypergastrinaemia lasting more than 4 months and this represents an excellent animal model for studying gastric carcinogenesis. In this study, the somatostatin analogue octreotide was given to female cotton rats to prevent carcinoma development caused by hypergastrinaemia. Twelve female cotton rats were given monthly injections of long-acting octreotide (5 mg i.m.) for 6 months. A control group of 20 animals was not given injections. Of the 20 control animals, 13 developed hypergastrinaemia and histologically invasive carcinomas or dysplasia. Of the 12 animals in the octreotide group, five developed hypergastrinaemia. None of these five animals developed histological cancer \(P < 0.05\); whereas three had dysplasia. However, octreotide did not affect plasma gastrin concentration or antral gastrin mRNA abundance significantly. Dysplasia of the oxyntic mucosa in hypergastrinaemic animals was accompanied by a marked increase in chromogranin A-immunoreactive cells and cells positive for Sevier–Munger staining. The malignant tissue also contained groups of cells with Sevier–Munger staining. In conclusion, octreotide prevented ECL cell carcinomas in hypergastrinaemic cotton rats without lowering the gastrin concentration.

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Introduction
Among inbred female cotton rats (Sigmodon hispidus) 25–50% of the animals develop spontaneous gastric carcinomas; the corresponding figure for male cotton rats is approximately 1%. Initially, the tumours were classified as adenocarcinomas (Kawase & Ishikura 1995), but later they were reclassified as enterochromaffin-like (ECL) cell carcinomas (Waldum et al. 1999). Animals developing hypergastrinaemia also have gastric hypo-/anacidity. Female cotton rats developing hypergastrinaemia have increasing serum gastrin levels from age 2 months. Furthermore, we have found carcinomas in all female cotton rats with hypergastrinaemia lasting more than 4 months and a gastrin receptor antagonist (YF 476) prevents tumour development (Martinsen et al. 2003). Hypergastrinaemia induced by partial corpectomy also results in carcinoma in male cotton rats, indicating that gastrin plays a central role in the development of these tumours (R Fossmark et al., unpublished observations). Thus, the female predominance of tumour development is most probably related to the gastric hypocacidity and
subsequent hypergastrinaemia. However, the mechanism behind the occurrence of hypoacidity is unknown, but parietal cells are present.

The oxyntic mucosa of animals with long-standing hypergastrinaemia appears thickened. Histologically it is characterized by hyperplasia of ECL cells and dysplasia, often with multiple areas of invasive growth. Cells invading below the muscularis mucosa layer are chromogranin A (CgA)- and histidine decarboxylase (HDC)-immunoreactive and stain positively with the Sevier–Munger technique (Waldum et al. 1999, Cui et al. 2000, Martinsen et al. 2003). These are strong arguments for an ECL cell origin of the tumours. This cotton rat model contributes to the understanding of gastric carcinogenesis and should be explored and characterized further.

Somatostatin analogues are successfully used in symptomatic treatment of neuroendocrine gastroenteropancreatic tumours (Burgess et al. 1999). The presence of somatostatin receptors in these tumours represents a basis for both treatment and imaging (Scherubl et al. 1993). The somatostatin receptor type 2 (SSTR-2) is found on rat (Prinz et al. 1994) ECL cells as well as on native and malignantly transformed ECL cells in the African rodent, mastomys (Borin et al. 1996). Somatostatin analogues appear to inhibit both secretory and proliferative functions through this receptor.

The somatostatin analogue octreotide has a high affinity for the SSTR-2 (Raynor et al. 1993). Octreotide has been noted to decrease both plasma gastrin levels and affinity for the SSTR-2 (Raynor et al. 1993). Consequently we wanted to examine whether long-acting octreotide (octreotide LAR) prevents ECL cell carcinoma development caused by hypergastrinaemia in cotton rats.

Materials and methods

Animals

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. The rats were housed in pairs in wiretop cages with aspen woodchip bedding from B&K Universal Ltd, Hull, UK. Room temperature was 24±1°C with a relative humidity of 40–50% and a 12 h light:12 h darkness cycle. The Rat and Mouse Diet of B&K and tap water were provided freely. Before blood sampling, octreotide LAR injection and termination, the cotton rats were anaesthetized with s.c. injections of 0.3 ml/100 g body weight of a combination of (per ml) 2.5 mg fluanison, 0.05 mg fentanyl and 1.25 mg midazolam.

Twelve female cotton rats of age 2 months were given monthly injections of octreotide LAR (5 mg i.m.) for 6 months. A control group of 20 age-matched female animals were not given injections. The animals were killed by exsanguination under anaesthesia after removal of the stomach. The animal experiments were approved by the Animal Welfare Committee of the University Hospital of Trondheim, Trondheim, Norway.

Histopathology and immunohistochemical techniques

At termination of the study, histological samples from the gastric corpus and antrum were taken. The sample from non-tumour oxyntic mucosa was taken from the greater curvature 5 mm away from the rumen–oxyntic border and at least 5 mm outside macroscopic tumours. Other samples were taken from macroscopically visual tumours. All samples were taken from macroscopically visual tumours. All samples were immersed in 4% phosphate-buffered formaldehyde and dehydrated in 80% ethanol before paraffin embedding. Sections 4 µm thick were cut from paraffin blocks and stained with haematoxylin and eosin (H&E). The samples were classified as either carcinoma (infiltration of the submucosa), dysplasia or normal appearing (neither carcinoma nor dysplasia).

Sections for immunohistochemistry were deparaffinized with xylene, rehydrated and treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. The antisera were diluted in PBS containing 0.25% Triton X-100 (Calbiochem, San Diego, CA, USA) and 0.25% BSA (Sigma, St Louis, MO, USA). The sections were incubated with primary antiserum at 4°C overnight. An EnVision-HRP kit (K5007; DAKO, Glostrup, Denmark) and an AEC peroxidase kit (SK-4200; Vector, Burlingame, CA, USA) were used to visualize the immunoreaction. Polyclonal anti-porcine CgA was used as a general neuroendocrine cell marker (Dia Sorin, Stillwater, MN, USA) at a dilution of 1:500. Tyramide signal amplification was used as described elsewhere to increase sensitivity (Adams 1992, Qvigstad et al. 2000). Negative controls were made by replacing the primary antibody with serum (Burry 2000) and then no staining was observed. Sections were also stained using the Sevier–Munger technique to visualize ECL cells (Sevier & Munger 1965). H⁺/K⁺ATPase antibody was used at a dilution of 1:2000 as a marker of parietal cells (Affinity Bioreagents, Golden, CO, USA).

Gastrin measurement

Serum gastrin was determined by RIA as described previously (Kleveland et al. 1985). Blood samples were
collected every month, starting the day of the first injection. Blood samples were collected from either of the saphenous veins.

**Northern blot analysis**

Specimens from RNA analysis were taken from an area adjacent to the specimen for histological evaluation, but outside visible tumours. Tissue for RNA extraction was 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 caesium chloride cushion, precipitated with ethanol and Northern blots prepared with 10 μg total RNA pro-sample using standard protocols (Dimaline et al. 1993).

CgA, HDC, gastrin, somatostatin and H+K+ATPase cDNA fragments were generated by RT-PCR and the fragments ligated into the pCR II vector (TA cloning kit; Invitrogen). The authenticity of the probes was verified by sequencing of the inserted fragment. Total RNA for the RT-PCR was taken from rat brain (HDC and CgA), antrum (gastrin) or oxyntic mucosa (somatostatin and H+/K+ATPase). Antisense probes were generated by transcription and simultaneous 32P-labelling using 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-labelled probe. The membrane was finally hybridized with a rat 18S probe, which served as a gel-loading control.

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 analysed using a bio-imaging analyser (Fujifilm BAS-1800 II and software (Fuji Photo Film Co., Ltd., Tokyo, Japan)). The membranes were stripped of probe with boiling 0.1% SDS between hybridizations. Readings were adjusted for the 18S ribosomal RNA signal before estimation of mRNA abundance was done.

**Measuring oxyntic mucosa thickness**

Oxyntic mucosa thickness was measured in areas where gastric crypts were visible in their full length. Using an ocular grid, the length of five glands from three different areas of the oxyntic mucosa from each animal were measured. In animals with visible tumours, the mucosal thickness was measured in tissue samples taken outside the tumours.

**Statistics**

Stomach weights were adjusted for animal weights ((stomach weight/animal weight)×100) and are presented as median for each group. Plasma gastrin is presented as mean±S.E.M. Differences in plasma gastrin, animal weights and animal weight-adjusted stomach weights were evaluated using the Kruskal–Wallis test with Dunn’s post-test. When evaluating differences in histopathological diagnosis, the groups were considered as two binomial proportions and a standardized difference was calculated using StatExact 4.0.1 (Cytel Corp. 2000, Cambridge, MA, USA). P values (two-sided) <0.05 were considered significant.

**Results**

**Gastrin levels**

Changes in plasma gastrin levels over time are presented in Fig. 1. Of the 20 control animals, 13 developed...
hypergastrinaemia, whereas 5 out of 12 octreotide-dosed animals became hypergastrinaemic. Among animals developing hypergastrinaemia, gastrin levels at termination of the study did not differ significantly between the control and octreotide group (888 ± 148 vs 672 ± 108 pmol/l, P = 0.38). Among animals remaining normogastrinaemic, the control group did not differ significantly from the octreotide group in gastrin concentration (23 ± 8 vs 31 ± 14 pmol/l, P = 0.78).

Animal weights

Animals dosed with octreotide had significantly lower weight at termination than controls (112 ± 7.2 vs 162 ± 3.4 g, P < 0.001), but the weight at the study start was similar (89 ± 4 vs 96 ± 5 g, P > 0.30).

Macroscopic findings and stomach weights

In the control group, 3 out of 20 animals had visible tumours localized to the oxyntic mucosa compared with none of the 12 animals dosed with octreotide. The animals with visible tumours were all hypergastrinaemic. There was no perigastric involvement in any of the animals. The mucosa appeared thickened in 10 out of 13 hypergastrinaemic control animals but only in one of five hypergastrinaemic animals in the octreotide group.

Stomach weights were adjusted for animal weight as described above. Hypergastrinaemic animals dosed with octreotide had stomach weights less than half of hypergastrinaemic controls (0.67 vs 1.32 g, P = 0.003). Furthermore, normogastrinaemic octreotide-dosed animals had lower stomach weight than normogastrinaemic controls (0.67 vs 1.06 g, P = 0.026). Hypergastrinaemic control animals had higher stomach weights than normogastrinaemic controls (1.32 vs 1.06 g, P = 0.007).

Oxyntic mucosa thickness

The oxyntic mucosa thickness of the different groups is presented in Fig. 2. The oxyntic mucosa in hypergastrinaemic animals was markedly thinner in octreotide-dosed animals than controls (0.40 ± 0.06 vs 0.65 ± 0.03 mm, P = 0.006). Normogastrinaemic octreotide-dosed animals also had thinner oxyntic mucosa than normogastrinaemic controls (0.31 ± 0.03 vs 0.38 ± 0.01 mm, P = 0.04). Hypergastrinaemic octreotide-dosed animals had an oxyntic mucosal thickness similar to normogastrinaemic controls (0.40 ± 0.06 vs 0.38 ± 0.01 mm, P = 0.31).

Histopathological and immunohistochemical findings

The findings are presented in Table 1. Of the 20 control animals, 13 developed hypergastrinaemia and histologically invasive ECL cell carcinomas (n = 7) or dysplasia (n = 6). Of the 12 animals in the octreotide group, five developed hypergastrinaemia. None of these five animals had histological carcinoma, whereas three had dysplasia. The macroscopically apparent tumour in one octreotide-dosed animal consisted of hyperplastic and dysplastic changes within the mucosa. Thus, hypergastrinaemic octreotide-treated animals had significantly different histological diagnoses compared with animals with spontaneous hypergastrinaemia (P < 0.05).

In hypergastrinaemic control animals with hypertrophic and dysplastic oxyntic mucosa (Fig. 3) there was a marked increase in the number of CgA-immunoreactive cells compared with the normal oxyntic mucosa (not shown). Animals with cancer invariably had dysplasia in the remaining oxyntic mucosa. The oxyntic mucosa of hypergastrinaemic octreotide-dosed animals also contained more CgA-positive cells than found in normogastrinaemic animals, but this increase was less pronounced. In tissue with a clearly increased number of CgA-positive cells as in Fig. 3, there was a corresponding increase in Sevier–Munger-positive cells (Fig. 4A), indicating that the majority of the neuroendocrine cells in areas with

Table 1 The effect of octreotide LAR dosing in hypergastrinaemic animals

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Control</th>
<th>Octreotide LAR</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Carcinoma</td>
<td>7</td>
<td>0*</td>
<td>7</td>
</tr>
<tr>
<td>No carcinoma</td>
<td>6</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>5</td>
<td>18</td>
</tr>
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*P < 0.05 compared with the control group.
Figure 3 Photomicrographs of oxyntic mucosa from a hypergastrinaemic control animal with dysplasia above and cancer below the muscularis mucosae layer (stained with H&E). The upper and lower insets show CgA immunoreactivity in dysplastic and malignant tissue respectively. The scale bar for the main image and upper inset = 100 μm. The scale bar in the lower inset = 25 μm.
hyperplasia/dysplasia were ECL cells. Tumour cells found below the muscularis mucosae were immunoreactive for CgA as presented in Fig. 3 and positive for Sevier–Munger staining (Fig. 4B). Dysplastic oxyntic mucosa of octreotide-dosed hypergastrinaemic animals contained more CgA-positive cells than normogastrinaemic animals, as shown in Fig. 5. Sections stained for H⁺/K⁺ATPase demonstrate that animals with dysplasia also have a marked reduction in the number of parietal cells, independent of octreotide dosing (Fig. 6).

mRNA analysis

The mRNA levels are presented as per cent of levels in normogastrinaemic control animals (Fig. 7A and B). The antral expression of gastrin and CgA was significantly higher in hypergastrinaemic than in normogastrinaemic animals and the expression was not affected by octreotide dosing. The expression of CgA and HDC in oxyntic mucosa was significantly higher in hypergastrinaemic animals and independent of octreotide dosing. Finally, the expression of H⁺/K⁺ATPase was lower in hypergastrinaemic animals and independent of octreotide dosing.

Discussion

The histological results show that octreotide prevents the development of spontaneous carcinomas in hypergastrinaemic cotton rats. We have previously argued that these tumours are of ECL cell origin (Waldum et al. 1999, Cui et al. 2000, Martinsen et al. 2003). The coinciding gastric hypochloridcy, hypergastrinaemia and dysplasia of the oxyntic mucosa with a high number of
CgA-positive cells strongly suggest an ECL cell origin of these tumours. The tumour tissue consists of connective tissue and glandular structures with CgA- and HDC-immunoreactive cells (Martinsen et al. 2003), and tumour cells also stain with the Sevier–Munger technique. This present study shows that the spontaneous cancer is prevented by a somatostatin analogue, which supports our previous conclusion that the tumours are ECL cell-derived. Furthermore, ECL cell proliferation seems to be directly inhibited by octreotide, which did not reduce the
gastrin concentration. Neither did octreotide affect gastrin mRNA levels, showing that gastrin synthesis was unaffected by octreotide.

Gastrin has an established role in gastric carcinogenesis in rats (Havu 1986, Carlsson et al. 1990, Havu et al. 1990, Mattsson et al. 1991), mice (Betton et al. 1988,
Wang et al. (2000) and mastomys (Bilchik et al. 1989, Nilsson et al. 1992). Also, in humans, chronic hypergastrinaemia alone causes advanced ECL cell change and dysplasia (Peghini et al. 2002). The importance of gastrin as a gastric carcinogen in Japanese cotton rats has been illustrated in our studies referred to previously. Somatostatin inhibits gastrin-induced ECL cell proliferation, and this effect has been investigated in the search for efficient cancer therapy. Thus, both in vivo and in vitro studies have promoted somatostatin analogues as therapeutic agents with the potential of inhibiting gastrin-induced ECL cell carcinogenesis. Octreotide has a negative trophic effect on the gastric mucosa, which is reflected by a marked reduction in stomach weight compared with controls in both hypergastrinaemic and normogastrinaemic animals. Omeprazole-induced hypergastrinaemia in man is inhibited by octreotide (Meijer et al. 1993), and the hypergastrinaemia in patients with multiple endocrine neoplasia type 1 is also reduced (Burgess et al. 1999). Studies of mastomys also demonstrate that octreotide decreases plasma gastrin levels and inhibits carcinoid formation when this is due to hypoacidity (Modlin et al. 1992). In vitro studies on isolated ECL cells from

**Figure 6** H⁺/K⁺-ATPase-immunoreactive cells in the oxyntic mucosa of a normogastrinaemic control (right) and a normogastrinaemic octreotide-dosed animal (left). Scale bar = 100 µm.
mastomys have shown that a somatostatin analogue inhibits gastrin-stimulated ECL cell histamine secretion as well as DNA synthesis (Borin et al. 1996). The direct effect of somatostatin on the regulation of ECL cell proliferation appears to be mediated by the SSTR-2; however, the precise mechanism of the anti-proliferative effect of somatostatin analogues on the ECL cell remains to be defined.

Figure 7 mRNA abundance expressed as per cent of normogastrinaemic controls in antrum (A) and oxyntic mucosa (B). Values are means ± S.E.M. * Significant differences \( P < 0.05 \) between normogastrinaemic and hypergastrinaemic animals (control or octreotide-dosed). \( n = 5 \) in all groups.
A question that has been raised following previous research (Modlin et al. 1992, Borin et al. 1996) is whether somatostatin analogues inhibit tumour formation in the intact animal by inhibiting gastrin synthesis and release or by inhibiting the effects of gastrin at the target cells. This study indicates that the direct anti-proliferative effect of octreotide on the ECL cell is more important than the effect on gastrin producing cells (G-cells) in preventing ECL cell malignancy. Gastric cancer development in hypergastrinaemic cotton rats is also inhibited by a gastrin receptor antagonist (Martinsen et al. 2003), indicating that gastrin is essential in the process of carcinogenesis. However, the present study shows that the proliferative effect of gastrin on ECL cells can be inhibited by octreotide, which thus also prevents cancer.

In this respect the cotton rat model contributes to understanding the mechanism of the anti-proliferative effects of octreotide on ECL cells. In a short-term study by Tsutsui et al. (1995), where rats received an infusion of gastrin and somatostatin, the proliferative effect of gastrin was abolished. Furthermore, in a long-term rat study by Bakke et al. (2000), hypergastrinaemia was induced by the peroxisome proliferator ciprofibrate co-administered with octreotide LAR. In this study, the hypergastrinaemia was not affected by octreotide, but ECL cell function and growth were still inhibited. Also, in patients with atrophic gastritis, hypergastrinaemia and ECL cell carcinoids treated with octreotide LAR for 12 months, serum CgA was reduced while the gastrin concentration remained high (V Fykse, AK Sandvik, G Qvigstad, SE Falkmer, U Syversen & HL Waldrum, unpublished observations). These studies support the present findings, emphasizing octreotide’s direct effect on ECL cells as the most important mechanism preventing proliferation.

Hypergastrinaemic animals dosed with octreotide had more CgA-positive cells in the oxyntic mucosa than normogastrinaemic animals. This indicates that the proliferative effect of hypergastrinaemia on the ECL cells is not completely abolished by octreotide, but sufficiently reduced to prevent ECL cell neoplasia. At the mRNA level, the CgA and HDC expression was markedly higher in hypergastrinaemic animals, but unaffected by octreotide. This corresponds with the high number of CgA-immunoreactive cells in the dysplastic mucosa of hypergastrinaemic animals, both control and octreotide-treated animals. However, the mucosa of octreotide-treated animals was significantly thinner.

The cause of the spontaneous gastric hypoacidity seen in cotton rats is not known. In the present study, the number of H+ /K+ ATPase-positive cells was markedly reduced in animals with hypergastrinaemia and dysplastic oxyntic mucosa. The reduction in parietal cell density in hypergastrinaemic cotton rats seems to be caused by the process of ECL cell proliferation and dysplasia. Male cotton rats with hypergastrinaemia induced by partial corpectomy develop areas of dysplastic mucosa and ECL cell-derived cancer, and dysplastic areas in the oxyntic mucosa have very few parietal cells (R Fossmark, TC Martinsen & HL Waldrum, unpublished observations). This indicates that hyperplasia and dysplasia of other cell types cause the apparent parietal cell loss. Thus, the reduction of parietal cells is probably not a primary process, and the cause of gastric hypoacidity in Japanese cotton rats is still unknown. The fact that hypergastrinaemic male animals also develop cancer indicates that gastric hypoacidity and subsequent hypergastrinaemia cause the female predominance of spontaneous tumour development. Future studies to identify the primary cause of the female predominance should try to identify the mechanism of the gastric hypoacidity.

In conclusion, our results demonstrate that spontaneous ECL cell carcinomas in hypergastrinaemic cotton rats are prevented by a somatostatin analogue without reducing the gastrin concentration.

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