E2-Cell Derived Gastric Cancer in Male Cotton Rats Dosed with the H2-Blocker Loxtidine

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ABSTRACT

Spontaneously hypergastrinemic cotton rats (Sigmodon hispidus) develop tumors that have the phenotype of an adenocarcinoma but most likely originate from the enterochromaffin-like (ECL) cells. Among inbred animals ~50% of the females, but <1% of males develop spontaneous gastric carcinomas. Gastrin is the principal carcinogen in this model, as >4 months of hypergastrinemia results in carcinoma, but a gastrin receptor antagonist prevents carcinomas. Carcinomas can also be induced by partial corpectomy. In the present study, the insurmountable H2-receptor antagonist loxtidine (200 mg/kg/day) was given to male cotton rats for 6 months. The loxtidine-dosed animals developed hypergastrinemia, whereas control animals remained normogastrinemic. At termination, 4 of 5 cotton rats had cancer located to the oxyntic mucosa, whereas 1 animal had dysplasia. The gastric mucosa of all of the control animals was normal. In the dysplastic mucosa of loxtidine-dosed animals there was a marked increase in chromogranin A-positive cells, where numerous groups of cells also stained positive with the Sevier-Munger technique. In areas of high proliferation and cancer there were also histidine decarboxylase, chromogranin A, and Sevier-Munger-positive cells, altogether indicating an ECL cell origin of the tumors. This represents an interesting animal model where ECL cell-derived gastric cancer can be induced by pharmacological acid inhibition in 6 months.

INTRODUCTION

Among inbred cotton rats (Sigmodon hispidus) ~50% of the female animals, but <1% of males develop spontaneous gastric carcinomas (1). The tumors have later been reclassified as enterochromaffin-like (ECL) cell-derived carcinomas (2). The animals with carcinomas have hypergastrinemia and gastric hypoaecidity, and >4 months of hypergastrinemia results in carcinoma (3). The mechanism behind the gastric hypoaecidity is unknown, but parietal cells are present in areas of nondysplastic oxyntic mucosa in animals with hypergastrinemia (4, 5). Gastrin has been shown to be the principal carcinogen in this model, as carcinoma development is prevented by a gastrin receptor antagonist (YF 476) (3), and carcinomas develop after hypergastrinemia induced by partial corpectomy (6). The natural course of the spontaneous tumors is malignant; as the tumors penetrate the stomach wall, ascites occur and metastases to the liver surface have been found (3). Hypergastrinemic rats, mice, and Mastomys develop carcinoid ECL cell tumors after a varying length of time. However, spontaneously hypergastrinemic cotton rats develop tumors that have the phenotype of an adenocarcinoma but that most likely originate from the ECL cells (2–5). This cotton rat model has improved the understanding of gastric carcinogenesis and should be explored and characterized further.

The insurmountable H2-receptor antagonist loxtidine was in the mid 1980s found to cause ECL cell-derived carcinoids in rats (7) and mice (8). Some mice also had invasion of the muscle wall of the stomach or carcinoid metastasis to draining lymph nodes. Genotoxic effects of loxtidine were not found (9), and the carcinogenic effect was explained by the hypergastrinemia induced by achlorhydria (7). The strong acid inhibitory effect of loxtidine also occurs in humans, where 40 mg once daily induced a median 24-h intragastric pH >5 (10). However, the therapeutic use of loxtidine was advised against on the basis of the rodent studies (7, 8). Due to its antiserotonin effect, loxtidine has also been used for rapid induction of ECL cell carcinoids in Mastomys for studying growth regulation of the gastric mucosa (11–14). In Mastomys, there is no difference between the sexes in gastric concentration or subsequent carcinoid development after loxtidine administration.

Human gastric adenocarcinomas are classified after Laurén (15) as intestinal or diffuse type. There is evidence that a proportion of human gastric cancers with an adenocarcinoma phenotype are of neuroendocrine origin, most among those of the diffuse type (16). Furthermore, the majority of the tumors developing in hypergastrinemic patients have neuroendocrine differentiation (17). A higher proportion of differentiated neuroendocrine cells can be detected using immunohistochemical techniques with a higher sensitivity (18). This evidence, however, is considered controversial (19). In this respect the spontaneously hypergastrinemic cotton rat is a valuable model, where tumors with an adenocarcinoma phenotype develop from a mucosa with ECL cell hyperplasia and dysplasia (2, 3, 5). If such tumors could be induced by pharmacological inhibition of acid secretion and subsequent hypergastrinemia, this would underline the clinical relevance of the cotton rat model as well as support that a proportion of human adenocarcinomas could be of ECL cell origin. In the present study male cotton rats were given loxtidine for 6 months to explore the possibility of inducing ECL cell carcinomas by pharmacological inhibition of acid secretion.

MATERIALS AND METHODS

Materials. 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 tumors, and these animals were kept in a colony by sister/brother mating for >20 generations. Six male cotton rats were given loxtidine 200 mg/kg/day for 6 months mixed in the drinking water from age 4 weeks. We were restricted to use a low concentration of loxtidine to avoid side effects. After 2 weeks, the animals were anesthetized with s.c. injection of 0.3 ml/100 g body weight of Hypnorm/Dormicum, which is a combination of (per ml) 2.5 mg of fentanyl, 0.05 mg of pentazocine, and 1.25 mg of midazolam. The injection was given during brief isoflurane inhalation anesthesia. Plasma
gastrin levels were measured by a method described previously (20). Hypergastrinemia in cotton rats was defined as a plasma concentration >36 pm, as described previously (3).

Termination. Six months after study start, the animals were anaesthetized with Hypnorm/Dormicum as described above. The intragastric pH was then measured with the stomach in situ using the lowest pH obtained after searching the entire oxyntic and antral mucosa. The stomach was then removed, and the animals were killed by exsanguination. Animals having signs of poor general condition were killed before scheduled termination.

Histopathology and Immunohistochemical Techniques. At termination of the study histological samples from the gastric corpus and antrum were taken. The sample from nontumor oxyntic mucosa was taken from the greater curvature 5 mm away from the rumen-oxyntic border and at least 5 mm outside macroscopic tumors. Other samples were taken from macroscopic tumors. All of the 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 H&E. The samples were classified as carcinoma (infiltration of the submucosa), dysplasia (cellular atypia and/or glandular distortion), or normal appearing (neither carcinoma nor dysplasia). Sections for immunohistochemistry were deparaffinized with xylene, dehydrated in 80% ethanol before paraffin embedding. Sections were incubated with primary antisera at 1:20,000, respectively. The sections were incubated with primary antisera at 4°C overnight (H+K+ATPase and CgA) or for 1 h at room temperature (Ki-67 and HDC). The EnVision-HRP kit (K5007; DAKO) was used in all of the protocols. Tyramide signal amplification was used to increase sensitivity of ATPase and CgA or for 1 h at room temperature (Ki-67 and HDC). The EnVision-HRP kit (K5007; DAKO) was used in all of the protocols. Tyramide signal amplification was used to increase sensitivity of ATPase, CgA, and HDC or for 1 h at room temperature (Ki-67 and HDC). The EnVision-HRP kit (K5007; DAKO) was used in all of the protocols. Tyramide signal amplification was used to increase sensitivity of ATPase, CgA, and HDC or for 1 h at room temperature (Ki-67 and HDC). The EnVision-HRP kit (K5007; DAKO) was used in all of the protocols. Tyramide signal amplification was used to increase sensitivity of ATPase, CgA, and HDC or for 1 h at room temperature (Ki-67 and HDC). The EnVision-HRP kit (K5007; DAKO) was used in all of the protocols. Tyramide signal amplification was used to increase sensitivity of ATPase, CgA, and HDC or for 1 h at room temperature (Ki-67 and HDC). The EnVision-HRP kit (K5007; DAKO) was used in all of the protocols. Tyramide signal amplification was used to increase sensitivity of ATPase, CgA, and HDC or for 1 h at room temperature (Ki-67 and HDC).

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 stomach was measured with the stomach in situ using the lowest pH obtained after searching the entire oxyntic and antral mucosa. The stomach was then removed, and the animals were killed by exsanguination. Animals having signs of poor general condition were killed before scheduled termination.

Histopathological and Immunohistochemical Findings. In H&E-stained sections of the oxyntic mucosa we found dysplastic and/or neoplastic tissue in all of the loxtidine-dosed animals (Table 3; Fig. 2, A–C; Fig. 3A). In areas of dysplasia or cancer a desmoplastic reaction was apparent (Fig. 2B; Fig. 3A), being more dominant in the cancer tissue. The mucosa of all of the control animals was normal as in Fig. 2A. Serial sections stained with H&E and SM of an area with cancer (Fig. 2, C and D) show numerous SM-positive cells above the muscularis mucosa layer as well as positive cells in the glandular structures of the malignant tissue. Immunohistochemical examination showed an increase in CgA immunoreactivity in hyperplastic and dysplastic glands of hypergastrinemic animals and the malignant tissue contained scattered CgA immunoreactive cells (Fig. 2A, insets). In the malignant tissue there were more HDC-positive cells (Fig. 2B, inset), whereas the HDC labeling of hyperplastic glands was similar to CgA and is not shown. In hypergastrinemic animals, there was a reduction in the number of parietal cells. Almost no parietal cells (H+K+ATPase immunoreactive) were present in areas of pronounced hyperplasia or dysplasia, whereas in other areas they were present in slightly reduced numbers. The parietal cells were retained in the upper portion of the glands, whereas they were absent in dysplastic foci. Dysplasia seems to develop in the lower part of the glands, and these areas have a high rate of proliferation indicated by numerous Ki-67-positive nuclei. Serial sections of dysplastic tissue stained with H&E, H+K+ATPase, Ki-67, CgA, and HDC are presented in Fig. 3. The H+K+ATPase-positive cells had a pattern of distribution inverse to Ki-67 positive cells. There were CgA-positive cells (Fig. 3D) in the areas with numerous Ki-67-positive cells (Fig. 3C), and in these dysplastic glands there were HDC-positive cells stained with various intensity (Fig. 3D, inset). In H&E-stained glands with a marked reduction in the number of parietal cells, chief cells could be found in the bottom of some glands, whereas in other glands from such an area the cells were all CgA or SM positive (as seen in Fig. 2A, top inset, and Fig. 2D).

Invasive growth into submucosal blood vessels was seen in two of the loxtidine-dosed animals (Fig. 5), but metastasis to the liver could not be detected after examination of multiple H&E-stained sections. The antrum of all of the animals in both groups was normal (data not shown).

### Table 1

<table>
<thead>
<tr>
<th>Loxtidine</th>
<th>Control</th>
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<tbody>
<tr>
<td>Animal weight (g)</td>
<td>147 ± 19</td>
</tr>
<tr>
<td>Oxyntic mucosa thickness (mm)</td>
<td>1.05 ± 0.09</td>
</tr>
<tr>
<td>Stomach weight (g)</td>
<td>1.73 ± 0.15</td>
</tr>
<tr>
<td>Intragastric pH</td>
<td>3.1 (1.9–6.1)</td>
</tr>
</tbody>
</table>

Intragastric pH values were presented in Table 1. Median pH was higher in the loxtidine-dosed group (3.1 versus 2.0; P < 0.05).

### RESULTS

#### Survival and Animal Weight

Of the 6 animals dosed with loxtidine, 1 died after 2 months of the study. Of the control animals 14 of 16 survived until termination. In the additional presentation of results, only 5 loxtidine-dosed animals will be compared with the 14 controls. The weights of the animals at termination did not differ significantly (147 ± 19 g versus 154 ± 12 g; P < 0.05).

#### Intragastric pH

The intragastric pH values are presented in Table 1. Median pH was higher in the loxtidine-dosed group (3.1 versus 2.0; P < 0.05).

#### Plasma Gastrin Concentration

The gastrin levels are presented in Table 2. An elevated gastrin concentration was observed in the loxtidine group compared with the control group at 3 months (1550 ± 255 pm versus 17 ± 2 pm), as well as at termination at 6 months (1210 ± 328 pm versus 22 ± 3 pm). The differences between loxtidine-dosed and control animals were significant at both 3 and 6 months (P < 0.01).

#### Macroscopic Findings

A marked increase in thickness of the corpus mucosa was seen in all of the loxtidine-dosed animals. Three of 5 animals also had macroscopic tumors of 0.5–1.5 cm in size located in the oxyntic mucosa. A photograph of a tumor is presented in Fig. 1. The mucosa of control animals had a normal appearance.

#### Stomach Weight and Oxyntic Mucosal Thickness

The mean stomach weight of loxtidine-dosed animals was significantly higher than controls (1.73 ± 0.15 g versus 0.94 ± 0.06 g; P < 0.01), as was oxyntic mucosa thickness (1.05 ± 0.09 mm versus 0.38 ± 0.02; P < 0.01).
DISCUSSION

The histological changes in loxtidine-dosed animals comprised hyperplasias, dysplasia, and carcinoma. Carcinomas located to the oxyntic mucosa were found in 4 of 5 male cotton rats after 6 months of dosing with the irreversible H2-receptor antagonist loxtidine. Serum gastrin concentrations were considerably elevated in loxtidine-dosed animals, similar to the concentration in female cotton rats with spontaneous hypochlorhydria (3, 5) and males after partial corpectomy (6). Gastrin is essential in this carcinogenetic process, as a gastrin receptor antagonist prevents cancer in animals with spontaneous hypergastrinemia (3), and in the present study we assume gastrin to be essential for the induction of carcinomas after loxtidine dosing. Compared with the spontaneous carcinomas in female cotton rats, previously classified as ECL cell derived (2–5), those induced by pharmacological acid inhibition appear to be very similar. We also know that the majority of male cotton rats develop tumors after 6 months of hypergastrinemia induced by partial corpectomy (6), and these carcinomas have the same characteristics as spontaneous or loxtidine-induced carcinomas. Independent of the cause of gastric hypochlorhydria and hypergastrinemia, the oxyntic mucosa surrounding both spontaneous and induced carcinomas has a marked hyperplasia of ECL cells (SM and CgA or HDC and CgA positive). A proportion of the tumor cells was also positive for these markers. During follow-up of a patient with an ECL cell carcinoid, a gradual reduction in the number of CgA- and SM-positive cells was observed, which indicates a dedifferentiation of ECL cells over time (24). Such dedifferentiation could also explain why not all cells are CgA/HDC/SM positive in areas with high proliferation or cancer in loxtidine-dosed cotton rats. In areas of high proliferation and cancer there were more HDC-positive than CgA-positive cells. Possibly, these characteristic ECL cell proteins are lost at different stages during dedifferentiation, which parallels differences in CgA, synaptophysin, and neuron-specific endolase expression in human gastric adenocarcinomas (25). A marked desmoplastic reaction, which characterizes many neuroendocrine tumors, was also apparent in both dysplastic and malignant tissue of loxtidine-dosed animals. Altogether, this supports our hypothesis that these carcinomas are ECL cell derived and develop independently of gender in hypergastrinemic cotton rats.

In cotton rats it is evident that the number of parietal cells (H+K+-ATPase-positive cells) is reduced in animals with long-lasting hypergastrinemia and a dysplastic mucosa. In loxtidine-dosed animals we found hyperplastic areas with a reduction in the number of parietal cells, whereas in areas of pronounced dysplasia there were no parietal cells at all. When only some parietal cells remained, they were retained in the upper and middle part of the glands (Fig. 3B). The dysplastic areas without parietal cells had a marked increase in Ki-67 positivity, indicating a high rate of cell proliferation in these areas (Fig. 3C). Thus, it seems that parietal cells are replaced by rapidly proliferating cells of another type.

INS-GAS mice also have a reduced number of parietal cells after 6–7 months (26). In INS-GAS mice inoculated with Helicobacter felis, resulting in more pronounced hypergastrinemia, the parietal cell loss is enhanced. Interestingly, in patients treated with a proton pump inhibitor the degree of initial hypergastrinemia is predictive of the later development of gastric atrophy (27), which in turn could enhance the hypergastrinemia. A contribution of gastrin toward parietal cell loss and gastric atrophy is important, because it is known that gastric atrophy is associated with development of both gastric cancer (28) and ECL cell carcinoids (29) in Mongolian gerbils. This also applies to human ECL cell carcinoids (30, 31) and gastric adenocarcinomas (32). The relatively rapid loss of parietal cells in hypergastrinemic cotton rats also makes the model relevant when studying this aspect of gastric carcinogenesis.

It is well known that tumors develop in the oxyntic mucosa of rodents after prolonged hypergastrinemia, and it is interesting that these tumors have a varying morphological appearance. In rats and mice, loxtidine-induced hypergastrinemia has resulted in carcinoid tumors (7, 8), whereas omeprazole and ranitidine have induced carcinoids in rats (33, 34). In the African rodent Mastomys, multicentric gastric carcinoids develop frequently in the oxyntic mucosa of aging animals, and the development of these tumors can be significantly enhanced by drug-induced hypergastrinemia, e.g., by loxtidine (11, 12). The gastric carcinomas developing in hypergastrinemic transgenic (INS-GAS) mice have an adenocarcinoma phenotype (26). INS-GAS mice inoculated with Helicobacter felis have a more pronounced hypergastrinemic, and this results in accelerated (<8 months) development of carcinomas. Mongolian gerbils inoculated with Helicobacter pylori also become hypergastrinemic and have found to develop mainly ECL cell carcinoids, but also poorly differentiated (29) and well-differentiated gastric adenocarcinomas (28, 35). The differences in gastric phenotype among INS-GAS mice, long-term loxtidine-dosed C57BL/10ScSn mice (8), and the cotton rats are interesting. The two hypergastrinemic mouse models differ in the strains of mice used, as well as in the duration of hypergastrinemia and the nature of the acid-inhibiting drug.

Table 2 Plasma gastrin concentration (pmol/liter) ± SE in male cotton rats dosed with loxtidine

<table>
<thead>
<tr>
<th>Time</th>
<th>Loxtidine</th>
<th>Control</th>
<th>P</th>
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<tbody>
<tr>
<td>3 months</td>
<td>1550 ± 255</td>
<td>18 ± 2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>6 months</td>
<td>1210 ± 328</td>
<td>22 ± 3</td>
<td>&lt;0.01</td>
</tr>
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</table>

Table 3 The effect of loxtidine dosing in male cotton rats

The difference between the loxtidine and control group is significant (P < 0.01).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Loxtidine</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>Carcinoma</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>14</td>
</tr>
</tbody>
</table>
as in the presence of acid in the early stages. In INS-GAS mice the gastric phenotype could be related to an effect of the transgene rather than gastrin, but expression of the insulin promotor has not been found outside the pancreas (26). Increased numbers of ECL cells have been found in the loxtidine-dosed mice and in cotton rats, but not in the INS-GAS mice. The spontaneous tumors in cotton rats have an adenocarcinoma phenotype but most likely derive from ECL cells. Carcinomas with very similar characteristics can also be induced by the acid inhibiting drug loxtidine, and to the best of our knowledge, this has not been demonstrated in an animal model previously. However, patients with pernicious anemia and hypergastrinemia have an increased risk of both carcinoids and adenocarcinoma (36–38). This is also reflected by the fact that the majority of gastric adenocarcinomas associated with hypergastrinemia have neuroendocrine differentiation (18). Our findings in cotton rats additionally indicate that gastric cancer induced by
Fig. 3. Serial sections from the oxyntic mucosa of a loxtidine-dosed animal with dysplastic changes. A, a section stained with H&E. Scale bar = 100 μm. The inset shows a dysplastic gland at higher magnification. B, photomicrograph of H+K+ATPase immunoreactive cells in the top and middle part of the glands in the dysplastic area. Scale bar = 100 μm. C, the section shows Ki-67 immunoreactivity, indicating a marked increase in proliferation in the dysplastic area in the left part of the section, where there are no H+K+ATPase immunoreactive cells. Scale bar = 50 μm. D, photomicrograph of chromogranin A immunoreactive cells in the tissue with high proliferation. The inset shows histidine decarboxylase immunoreactivity in the same gland as in the A inset. Whereas some cells stain strongly histidine decarboxylase positive, the arrows indicate cell groups with less intense labeling. Both scale bars = 50 μm.
hypergastrinemia, whether of a typical carcinoid appearance or a less differentiated neoplasm, can develop from the ECL cell.

In humans, the gastrin release increases gradually with a rise in pH until a maximal gastrin is reached at a gastric pH of ~4.0 (39). Pilot studies on cotton rats showed that a dose of 200 mg/kg/day loxtidine in the drinking water was needed to reach gastric pH >4.0 and for gastrin to rise 10-fold. Gastrin concentration increased 10-fold in Mastomys receiving loxtidine 1 mg/kg/day (11), and both mice and rats developed carcinoids after administration of loxtidine 50 mg/kg/day (7, 8). Apparently the dose resulting in gastric hypoacidity and hypergastrinemia varies greatly among the species (40), whereas it is the degree of gastric hypoacidity that determines the consequences. This is also important when studying the effects of proton pump inhibitors, as mice are more resistant and require much higher doses than rats to maintain gastric hypoacidity (40).

Invasion of submucosal blood vessels is associated with malignant growth and was found in 2 of the animals dosed with loxtidine. However, we could not find distant metastasis in multiple sections of the liver. The presence of vascular invasion without distant metastasis parallels findings in tumor-bearing INS-GAS mice (26) and omeprazole-dosed rats (33), whereas vascular invasiveness was not reported in loxtidine-dosed C57BL/10ScSn mice (8).

A female preponderance of ECL cell tumors has been found after lifelong dosing of rats with omeprazole, which induced such tumors mainly in female rats (33). After lifelong administration of loxtidine to rats and mice, there was also a female preponderance (7, 8). In cotton rats the female preponderance of spontaneous tumors seems to be related to the occurrence of gastric hypoacidity and subsequent hypergastrinemia. A short-term pilot study with dosing of loxtidine to male and female cotton rats showed no difference in gastrin levels or intragastric pH after the same dose. Thus, there is neither evidence of a male-female difference in gastrin sensitivity nor of a difference in gastrin concentration after gastric hypoacidity in cotton rats.

In conclusion, this study demonstrates that gastric carcinomas that most likely derive from ECL cells develop in male cotton rats dosed with the irreversible H2-blocker loxtidine.

4 unpublished observations.
ACKNOWLEDGMENTS

We thank Anne Kristensen, Bjoen Munkvold, and Britt Schulze for their technical assistance.

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Cancer Res 2004;64:3687-3693.

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