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Helicobacter mustelae-associated Gastric Adenocarcinoma in Ferrets (Mustela putorius furo)

J. G. Fox, C. A. Dangler, W. Sager, R. Borkowski, and J. M. Gliatto

Abstract. Helicobacter pylori in humans is associated with active, chronic gastritis, peptic ulcer disease, and most recently has been linked epidemiologically to gastric adenocarcinoma. A related organism, Helicobacter mustelae, naturally infects ferrets and also causes a persistent gastritis, a precancerous lesion, and focal glandular atrophy of the proximal antrum. In this report, we document the clinical presentation and histopathologic confirmation of H. mustelae-associated gastric adenocarcinoma in two middle-aged male ferrets. The ferret appears to be well suited to study the pathogenesis of naturally occurring Helicobacter sp.-induced gastric adenocarcinoma.

Key words: Ferrets; gastric adenocarcinoma; H. mustelae.

Over 10 years have elapsed since Helicobacter pylori was isolated from the gastric mucosa of humans, and the organism if now known to cause persistent, active, chronic gastritis and duodenal ulcer disease; it has also been strongly linked to gastric adenocarcinoma. In 1985, the ferret was found to be naturally colonized with H. mustelae, a gastric pathogen that has many biochemical, molecular, and phenotypic characteristics similar to those of H. pylori. Oral
Fig. 1. Antral region of stomach, ferret, Case 1. Nests of irregular and dilated mucous epithelium-lined tubules extend downward from the mucosa and invade the superficial and deep submucosa. The infiltrating tubular nests elicit a fibrosing response. HE. Bar = 250 μm.

Inoculation of *H. mustelae* into SPF-naive ferrets included chronic, persistent gastritis similar to that observed in ferrets naturally infected with *H. mustelae.* To further define the model, the pathology of *H. mustelae* gastritis was compared to that of *H. pylori*-associated gastritis in humans. A superficial gastritis present in the body of the stomach indicates that *H. mustelae* is located on the surface of the mucosa but not in the crypts. In the distal antrum, however, inflammation occupied the full thickness of the mucosa, the so-called diffuse antral gastritis described in humans. In this location, *H. mustelae* was seen at the surface, in the pits, and on the superficial portion of the glands. In the proximal antrum and the transitional mucosa, a precancerous lesion, focal glandular atrophy, and regeneration, was present in addition to those lesions seen in the distal antrum. The type of gastritis observed in the proximal antrum and the transitional zone represent early stages of the multifocal atrophic gastritis of humans, the entity underlying the syndromes of gastric ulcer and gastric carcinoma. *H. mustelae* gastritis mimics the human disease in several ways and suggests that this model can be used to interpret the evolution of *H. pylori*-induced chronic gastritis and to study comparable pathogenic mechanisms responsible for the strong association between *H. pylori* and the development of gastric adenocarcinoma in humans. The purpose of the present study is to describe two ferrets with *H. mustelae*-associated gastric adenocarcinoma, describe the presence of *H. mustelae* in a ferret previously diagnosed with gastric adenocarcinoma, and compare these findings to that of *H. pylori*-associated gastric adenocarcinoma in humans.

Case #1: A 3.5-year-old pet male ferret presented with vomiting and lethargy. Complete blood count and serum chemistry results were within normal limits. Survey radiographs revealed gas-distended intestines and an apparent pyloric mass. During an exploratory laparotomy, a small, circular, 2-cm pyloric mass was noted. A gastrotomy indicated that the mass was protruding into and impeding the lumen of the pylorus.

Case #2: A 2-year-old pet neutered male ferret was examined because of a 3-month history of intermittent vomiting. Past pertinent history included chronic diarrhea that the owner claimed had partially responded to treatment with chloramphenicol. Upon physical exam, the ferret weighed 1,003 g, had body temperature of 39.8 C (103.1 F), and was noted to have prominent gastric contour and evidence of splenomegaly on palpation. Complete blood count and chemistry profile were unremarkable. Radiographs revealed granular material distending the stomach and splenomegaly. Abdominal ultrasound revealed a discrete, irregular, bright structure in the pyloric region of the stomach. There was also a discrete nodule 8–9 mm in diameter adjacent to the pylorus indicative of a lymph node. After ventral midline celiotomy, gastrotomy revealed a firm, gritty, infiltrative 1.5-cm mass at the pylorus that, along with the enlarged regional lymph node, was excised. A Billroth I...

Fig. 2. Antral region of gastric mucosa, ferret, Case 2. Focal fibroplasia and mineralized osseous metaplasia in the gastric mucosa adjoin a region of glandular epithelial dysplasia. HE. Bar = 250 μm.

Fig. 3. Antral region of gastric mucosa, ferret, Case 1. Metaplastic osseous spicule formed in the lamina propria. HE. Bar = 50 μm.

Fig. 4. Antral region of stomach, ferret, Case 1. Periglandular fibrosis surrounds an isolated neoplastic tubule that has invaded the deep submucosa. HE. Bar = 50 μm.

Fig. 5. Antral region of gastric mucosa, ferret, Case 2. Argyrophilic short bacterial rods are within the foveolar lumens and adhered to the mucosal epithelium. The morphology of the bacterium is compatible with *Helicobacter mustelae.* Warthin–Starry. Bar = 10 μm.

Fig. 6. Gastric adenocarcinoma, ferret, Case 1. Neoplastic cells are organized into tubules. Cytokeratin antigens are expressed by well-differentiated, neoplastic cells invading the submucosa detected as dense deposits in the apical cytoplasm.
adjacent to the basal-oriented nuclei (arrows) using an anti-pan cytokeratin monoclonal antibody. Immunoperoxidase, ABC method. Bar = 20 μm.

Fig. 7. Gastric adenocarcinoma, ferret.13 Cytokeratin antigens expressed by poorly differentiated, neoplastic cells organized into dense clusters invading the submucosa are detected as punctate perinuclear deposits in many of the neoplastic cells. The nuclei are unstained in this preparation and are identified by the pale, circular nuclear membrane (arrows) and small nucleolus. Immunoperoxidase, ABC method. Bar = 20 μm.
gastroduodenostomy was performed and routine abdominal closure was performed; postoperative recovery was unremarkable.

Resected gastric specimens of Cases 1 and 2 were preserved in buffered 10% formalin, processed using standard procedures, embedded in paraffin, sectioned at 5 μm, and stained with H&E and Warthin–Starry silver stain. In addition, paraffin blocks were obtained from the ferret previously diagnosed with gastric adenocarcinoma and slides processed in a manner identical to that described for the other two ferrets. The pyloric tissues from the two ferrets had similar histopathologic features. The tissues were characterized by multifocal segmental glandular proliferation and surface erosion, multifocal mucosal lymphoid aggregates, mixed inflammatory-cell infiltration, and coalescing, multifocal fibrosis. Superficially, the pyloric glands frequently formed nests of dilated tubules in the deep mucosa and submucosa (Figs. 1, 2). The epithelium of glands was frequently folded and comprised of well-differentiated columnar epithelium with round basal nuclei and abundant vesicular, apical cytoplasm with a mucous epithelium. Fibrosis dissected through the mucosa and submucosa and enclosed the glandular nests (Figs. 1, 2). Multiple osseous metaplastic foci had arisen in the fibrous tissue deposited in the lamina propria and superficial submucosa (Figs. 2, 3). Neoplastic tubules penetrated into the deep submucosa (Fig. 1) and, in Case 1, occasionally into the tunica muscularis. An intense fibroblastic response enveloped the invading glandular elements (Fig. 4). In Case 1, the epithelium of the infiltrating tubules was frequently disorganized, the nuclear chromatin was more hyperchromic or vesicular, and the nuclear to cytoplasmic ratio was elevated due to reduction in cytoplasmic area. The serosal surface was characterized by diffuse fibrosis, congestion of large and small caliber vessels, and acute multifocal hemorrhage. In Case 2, the infiltrating tubules were frequently dilated or cystic, occasionally containing sloughed cells, and were lined by an atrophic cuboidal epithelium. The epithelium was characterized by scant to moderate, lightly basophilic cytoplasm and by closely packed, round nuclei with a vesicular chromatin pattern. Mitotic figures were not evident in either case. In silver-stained sections of the pylorus from these two cases, there were moderate to abundant numbers of small, straight, or slightly curved bacterial rods within the superficial to middle region of the pyloric pits. The bacteria, compatible in morphology and size to H. mustelae, were present in the lumen or adherent to the apical surface of the mucous epithelium (Fig. 5).

Neoplastic tissues from Case 1 and a previously reported ferret gastric adenocarcinoma were evaluated immunohistochemically. Tissue sections from Case 2 were not available for immunohistochemical evaluation. Five-micron-thick sections of formalin-fixed, paraffin-embedded tissues were used for immunohistochemical detection of cytokeratin antigens using a pan cytokeratin monoclonal antibody reagent (Sigma Biosciences, St. Louis, MO, #C-2931). Reactions were detected using a commercial kit containing a biotinylated secondary antibody and an avidin–horseradish–peroxidase conjugate (Vector Laboratories, Burlingame, CA, #PK-6102). The immunohistochemical technique was performed as described in the product specifications for the indirect detection system. Briefly, tissue sections were deparaffinized in xylene and rehydrated through a graded alcohol series into phosphate-buffered saline (PBS). After incubation with the appropriate blocking solution, sections were incubated with a 1:400 dilution of the anti-pan cytokeratin antibody. Matching sections were incubated with a 1:400 dilution of a nonspecific monoclonal antibody of the same isotype (i.e., mouse IgG1 anti-bromodeoxyuridine) or diluted blocking serum. Subsequently, all sections were incubated sequentially with the biotinylated secondary antibody, avidin–horseradish–peroxidase conjugate, and enzyme substrate with intervening washes in PBS. Positive cells were identified on the basis of a distinctive pattern of staining, characteristic of mucosal and some glandular epithelium. This pattern was characterized by a dense punctate, perinuclear deposit, distinct from the diffuse cytoplasmic staining exhibited by hepatocytes. This pattern was easily identified in the scattered infiltrating tubules observed in Case 1 (Fig. 6) and in the more anaplastic and proliferative foci in the case described in Stauber et al. (Fig. 7). Tissue sections incubated with the nonspecific primary antibody control or no primary antibody did not exhibit notable background, indicating the absence of nonspecific binding or endogenous peroxidase.

Previous studies conducted indicated that virtually 100% of the adult ferrets examined in our laboratory are infected with H. mustelae and that ferrets become colonized with this gastric organism shortly after weaning. Oral inoculation of H. mustelae into specific pathogen-free ferrets results in the development of a gastritis similar to that observed in ferrets naturally infected with H. mustelae. The organism causes a chronic gastritis characterized by many of the same premalignant gastric lesions seen in humans infected with H. pylori living in areas of high gastric cancer risk. This report confirms the presence of argyrophilic bacteria, compatible in location and morphology to H. mustelae, within the pyloric mucosa of two male ferrets with pyloric adenocarcinoma. In addition to the two cases described here, we also examined Warthin–Starry-stained sections from a previously described case of a ferret with pyloric adenocarcinoma and identified argyrophilic bacteria consistent in morphology to H. mustelae within pyloric pits. In both cases described in the present report, the neoplasms manifested as multiple foci of tubules lined by mucous epithelium that have invaded into the deep submucosa, resulting in a marked local scirrhous response. The discrete invasive characteristics suggest the neoplasms represent early infiltration of the neoplastic cells beyond carcinoma in situ. The growth patterns of the neoplastic tissues in these two animals were similar and resemble the description of three naturally occurring pyloric adenocarcinomas in previous reports. This pattern was also seen in several gastric adenocarcinomas induced with N-methyl-N-nitro-N'-nitrosoguanidine (MNNG) in ferrets infected with H. mustelae. Consistent with our speculation that this histologic presentation is relatively early in the progression of the gastric adenocarcinoma, the ferrets in this report were young adults (i.e., 2 and 3.5 years) as were those cases published with similar invasive morphology (3–4 years). Furthermore, more confluent, highly anaplastic and transmurally invasive adenocarcinomas consistent with more protracted tu-
mor progression were reported in an older ferret. Osseous metaplasia was noted in both of the current cases, as well as in previous reports of both natural and induced pyloric tumors. Unlike the previous reports that cited poor prognosis in ferrets with gastric adenocarcinoma, both of the ferrets in this report responded well to surgical intervention and Case 1 and Case 2 remain asymptomatic postoperatively for 1 year and 6 months, respectively.

In a recent experiment on gastric carcinogenesis, 6-month-old female H. mustelae-infected ferrets were orally dosed with a single dose (50–100 mg/kg) of MNNG. Fiveagematched unmanipulated H. mustelae-infected control animals were included for comparative purposes. Nine of 10 ferrets dosed with MNNG developed gastric adenocarcinoma (29–55 months after dosing) while none of the five historic control ferrets, examined an average of 63 months after the initiation of the study, developed gastric tumors. The higher dose of MNNG (100 mg/kg) induced a clinically apparent, aggressive tumor approximately 2 years earlier than those neoplasms diagnosed histologically in the majority of ferrets receiving one-half the dose of carcinogen. However, for a single dose of MNNG, the 100% prevalence of gastric cancer is unprecedented. H. mustelae occupy the epithelial surfaces of the neck glands and cause increased gastric epithelial proliferation, presumably due to chronic inflammatory response. This is similar to the increased proliferation of gastric epithelia noted in H. pylori-infected humans. It is tempting, therefore, to suggest that the high tumor incidence reported in MNNG-treated ferrets reflected participation of H. mustelae infection in the carcinogenic process. This hypothesis is supported by a recent study completed in our laboratory where a group of H. mustelae-free SPF ferrets, dosed with a single dose of 100 mg/kg MNNG, did not develop gastric cancer 3.5 years postdosing (J. G. Fox, unpublished data).

Previously, three reports have cited a total of four female pet ferrets with gastric adenocarcinoma. Although H. mustelae was discussed as a possible factor in development of gastric tumor in two of the reports, H. mustelae was neither isolated nor depicted histologically using special silver stains in any of the ferrets. Our two cases linking H. mustelae and gastric adenocarcinoma, in addition to depicting H. mustelae in a ferret previously reported with gastric adenocarcinoma, support the hypothesis that H. mustelae, like H. pylori in humans, may be a gastric co-carcinogen in ferrets. The ferret model appears to be ideal to examine the role of H. mustelae and other cofactors in the development of Helicobacter-associated gastric carcinogenesis.

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References


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