Advances in Medical Sciences 61 (2016) 231–236

Contents lists available at ScienceDirect

Advances in Medical Sciences

journal homepage: www.elsevier.com/locate/advms

Original Research Article

CagA-positive *Helicobacter pylori* strain containing three EPIYA C phosphorylation sites produces increase of G cell and decrease of D cell in experimentally infected gerbils (*Meriones unguiculatus*)

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ARTICLE INFO

Article history:
Received 27 April 2015
Received in revised form 12 November 2015
Accepted 12 February 2016
Available online 23 February 2016

Keywords:
Helicobacter pylori
G cells
D cells
CagA
EPIYA-C motifs

ABSTRACT

Purpose: Human infection by *Helicobacter pylori* is associated with an increase in the number of gastrin-producing G cells and a concomitant decrease of somatostatin-producing D cells. However, to our knowledge, changes in G and D cell numbers in response to infection with *H. pylori* CagA-positive strains containing different number of EPIYA-C phosphorylation sites have not been analyzed to date. Therefore, the aim of this study was to perform a quantitative analysis of the number of G and D cells in Mongolian gerbils challenged with *H. pylori* strains with different numbers of EPIYA-C motifs.
Materials and methods: Mongolian gerbils were inoculated with isogenic *H. pylori* strains containing one to three phosphorylation sites. Mucosal fragments were evaluated by morphometry and immunohistochemistry using primary polyclonal rabbit anti-gastrin and anti-somatostatin antibodies. Positive cells were counted using an image analyzer.
Results: Forty-five days after infection, there was a decrease in the number of D cells and an increase in the G/D cell ratio in the group with three EPIYA-C. Six months after infection, there was a progressive and significant increase in the number of G cells and in the G/D cell ratio, with a concomitant decrease in the number of D cells, especially in the three EPIYA-C group.
Conclusions: CagA-positive *H. pylori* strains containing a large number of EPIYA-C phosphorylation sites induce a decrease in D cell number and an increase in G cell number and G/D ratio, which were correlated with the number of inflammatory cells of the lamina propria.

1. Introduction

Infection by the gram-negative spiral bacterium, *Helicobacter pylori*, causes an increase in the plasmatic and gastric concentration of gastrin [1,2]. The mechanism by which *H. pylori* enhances gastrin release remains uncertain, but studies on the regulation of gastrin secretion have shown that gastrin (G)-cells are under constant restraint by somatostatin (D)-cells in the antral mucosa. Gastrin stimulates enterochromaffin-like (ecl) cells to release histamine and, both hormones are able to bind to receptors on the surface of parietal cells regulating proton release. The high gastric acid secretion increases somatostatin release, resulting in reduced gastrin secretion, whereas low gastric acid secretion decreases somatostatin release, resulting in increased gastrin secretion. In addition to having an inhibitory effect on gastrin release, somatostatin has been suggested to inhibit antral gastrin cells at the gene transcriptional and posttranscriptional levels [3].

The changes in the gastrin concentration associated with the infection have been correlated with the degree of inflammation and activity of gastritis [4]. In addition to host factors, virulence bacterial factors participate in the inflammatory response to the infection. Among them, the Cag pathogenicity island (*cag*-PAI), a DNA locus of approximately 40 Kb in length, containing 27–31 genes, which encodes a type IV secretion system (T4SS),

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http://dx.doi.org/10.1016/j.advms.2016.02.003
1896-1126/

responsible for the translocation of the oncogenic CagA protein into gastric epithelial cells. This is followed by CagA tyrosine phosphorylation, resulting in the activation of pro-inflammatory and antiapoptotic genes and increased risk of developing pre-cancerous lesions [5,6]. Phosphorylation of CagA occurs on tyrosine residues in a repeating sequence of five amino acids located in its carboxy-terminal portion, called EPIYA (Glu, Pro, Ile, Tyr, and Ala) composed of four discrete segments named A, B, C and D based on the presence of different amino acids. *H. pylori* strains isolated in Western countries consist of EPIYA-A, EPIYA-B and a variable number of EPIYA-C, whereas strains from East Asian countries exhibit EPIYA-D site instead of EPIYA-C. Human infection by CagA-positive *H. pylori* strains with a large number of EPIYA-C phosphorylation sites is associated with more severe chronic gastritis and an increased risk of developing intestinal metaplasia and gastric cancer [7].

To our knowledge, a quantitative analysis of the changes in G and D cell numbers upon infection with different strains of CagA-positive *H. pylori* containing one or more EPIYA-C phosphorylation sites has not been performed to date. Therefore, the aim of this study was to determine the number of the G and D cells in the gastric mucosa of Mongolian gerbils infected with *H. pylori* strains containing one or three EPIYA-C sites. Biopsies of these same animals have been examined for the type and intensity of gastric lesions, which are similar to infection by *H. pylori* in human beings [8].

2. Material and methods

2.1. Animals

Forty-eight three-month-old female Mongolian gerbils, with an average weight of 80 g, were raised in an accredited animal facility at the Federal University of Minas Gerais (UFMG, Brazil). The animals were randomly assigned to study groups and the histologic assessment of gastric mucosa fragments stained with carbol-fuchsin was made. The absence of stained bacteria in the gastric mucosa of the control group, and no spiral bacteria in addition to *H. pylori* in the study groups can demonstrate the absence of cross infection. The animals were maintained with appropriate nutrition, ventilation and lighting conditions, subjected to natural day and night cycles, and handled in accordance with the guidelines outlined in the Ethical Principles in Animal Experimentation guide and was approved by the Ethics Committee on Animal Experimentation of the UFMG (272009 CEUA).

2.2. Experimental design

The animals were divided into three experimental groups: 16 uninfected animals as controls; 14 animals infected with a CagA-positive strain containing one EPIYA-C fragment and 16 animals infected with a CagA-positive strain containing three EPIYA-C fragments. Seven (one EPIYA-C group) and eight (three EPIYA-C group) animals were analyzed 45 days after infection, and seven (one EPIYA-C group) and eight (three EPIYA-C group) animals were analyzed six months post-infection. All animals were used in another study [8].

2.3. Inoculum

CagA-positive *H. pylori* strains containing one or three EPIYA-C phosphorylation sites were used to inoculate the animals. The strains were isolated from the antral mucosa of the stomach obtained during surgery from a 71 year-old patient with gastric carcinoma on Belo Horizonte medium [9]. The isolates were tested for EPIYA-C pattern by PCR according to Yamaoka et al. [10] and the results were confirmed by sequencing [6]. The presence of other genes of the *cag*-PAI involved in the T4SS (*cagL, cagI, cagX, cagY* and *cagT*) and in the induction of IL-8 (*cagE*) was also confirmed in the isolates.

We confirmed that the strains were isogenic, differing only in the number of EPIYA-C segments, by identical Random-amplified DNA (RAPD)-PCR profiles, the same *vac*A more virulent s1i1d1m1 genotype and identical *oip*A sequences with 9 CT and *vac*A i/d sequences. Furthermore, the sequences of the housekeeping genes *efp yph*C, *atp*A, *ure*I and *mut*Y [11] (total of 3439 nucleotides) were 100% identical, which indicates that isolates are clonal [8]. To inoculate the animals we used two days culture of isolates LPB 1034-14 and LPB 1034-6, containing one or three EPIYA-C phosphorylation sites.

2.4. Inoculation, necropsy and rapid urease test (RUT)

Prior to *H. pylori* inoculation, the animals were fasted for eight hours, and then, sedated slightly with isoflurane. Three samples of each bacterial isolate in suspension in sterile Brucella broth (difco) (0.8 mL) containing 10^9 colony forming units (CFU)/mL were administered by gastric gavage to each animal of the study groups at 48 h intervals. The control group received identical volumes of sterile Brucella broth alone. Four hours after each inoculation, the animals received standard rat chow (Labina®, Purina, Brazil) and water *ad libitum*. After 45 days and 6 months of infection, the animals were sacrificed by means of anesthetic overdose, the abdomen was opened, the stomach removed and opened along the greater curvature and the mucosa of the gastric body and antrum were exposed. Then, the stomach was distended and washed with saline solution.

2.5. Histopathology

Fragments of the lesser curvature of the antrum (approximate size 2.0 cm × 0.5 cm) were obtained for histological analysis. The fragments were fixed in 10% buffered formalin for 48 h, dehydrated, diaphanized, infiltrated, and embedded in paraffin. Sections (4-μm thick) were obtained for immunohistochemical and morphometric analysis. The rapid urease test (RUT) was performed by incubating one gastric antral fragment into a semi-solid agar containing phenol red 2% and urea 0.01 M at 37 °C for 24 h.

2.6. Immunohistochemical and morphometric analysis

The sections for immunohistochemical reactions were processed according to the manufacturer's guidelines. The sections were briefly washed in phosphate-buffered saline (PBS; pH 7.2). Antigens were recovered from D cells using DAKO Retrieval solution (Dako, USA) at 100 °C for 20 min. The endogenous peroxidase activity was suppressed by incubating the sections in a solution containing 3.5% H_2O_2 70:30, v/v in 200 mL PBS for 20 min, and nonspecific binding sites were blocked by the incubating sections with goat serum (dilution 1:40) for 40 min. Subsequently, the sections were incubated for 18 h at 4 °C with the following rabbit polyclonal antibodies: anti-gastrin NCL-GASP (dilution, 1:150; Novacasta Laboratories, Newcastle, UK) or anti-somatostatin (dilution, 1:500; Abcam, Cambridge, USA). The slides were then washed in PBS and incubated in biotinylated goat anti-rabbit immunoglobulin (Ig)G (Bethyl Laboratories Inc., Montgomery, USA) and peroxidase-conjugated streptavidin (dilution, 1:50; Zymed Laboratories Inc., San Francisco, USA), for 1 h at room temperature. The stain was detected using a solution containing 0.05% diaminobenzidine in 0.2% H_2O_2 60:40, v/v for 3 min. As a negative control, the primary antibody was replaced with PBS in a few antral sections from the control gerbils. Antral sections of control gerbils were used as positive controls. The sections were