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World J Gastroenterol 2003;9(3):521-524
World Journal of Gastroenterology
 ISSN 1007-9327

Overexpression of c-fos in *Helicobacter pylori*-induced gastric precancerosis of Mongolian gerbil

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Received: 2001-09-26 **Accepted:** 2001-11-06

Abstract

AIM: To explore dysregulation of *c-fos* in several human malignancies, and to further investigate the role of *c-fos* in *Helicobacter pylori* (*H. pylori*)-induced gastric precancerosis.

METHODS: Four-week-old male Mongolian gerbils were employed in the study. 0.5 mL 1×10^8 cfu· L^{-1} suspension of *H. pylori* NCTC 11 637 in Brucella broth were inoculated orally into 20 Mongolian gerbils. Another 20 gerbils were inoculated with Brucella broth as controls. 10 of the infected gerbils and 10 of the non- infected control gerbils were sacrificed at 25 and 45 weeks after infection. The stomach of each gerbil was removed and opened for macroscopic observation. The expression of *c-fos* was analyzed by RT-PCR and immunohistochemical studies in *H. pylori*-induced gastric precancerosis of Mongolian gerbil. Half of each gastric antrum mucosa was dissected for RNA isolation and RT-PCR. β-actin was used as the housekeeping gene and amplified with *c-fos* as contrast. PCR products of *c-fos* were analyzed by gel image system and the level of *c-fos* was reflected with the ratio of c-fos/β-actin. The immunostaining for *c-fos* was conducted using monoclonal antibody of *c-fos* and the StreptAvidin-Biotin-enzyme Complex kit.

RESULTS: *H. pylori* was constantly found in all infected animals in this study. After infection of *H. Pylori* for 25 weeks, ulcers were observed in the antral and the body of stomach of 60 % infected animals (6/10). Histological examination showed that all animals developed severe inflammation, especially in the area close to ulcers, and multifocal lymphoid follicles appeared in the lamina propria and submucosa. After infection of *H. Pylori* for 45 weeks, severe atrophic gastritis in all infected animals, intestinal metaplasia in 80 % infected animals (8/10) and dysplasia in 60 % infected animals (6/10) could be observed. *C-fos* mRNA levels were significantly higher after infection of *H. pylori* for 25 weeks (1.84±0.79), and for 45 weeks (1.59±0.37) than those in control-animals (0.74±0.22, $P<0.01$). *C-fos* mRNA levels were increased 2.5-fold by 25th week ($P<0.01$) and 2.1-fold by 45th week ($P<0.01$) in precancerosis induced by *H. pylori*, when compared with normal gastric epithelium of Mongolian gerbil. Immunohistochemical staining revealed exclusive nuclear staining of c-fos. Furthermore, there was a sequential increase in c-fos positive cells from normal epithelium to precancerosis.

CONCLUSION: The study suggested that overexpression of c-fos occurs relatively early in gastric tumorigenesis in this precancerosis model induced by *H. pylori*.

Yang YL, Xu B, Song YG, Zhang WD. Overexpression of c-fos in *Helicobacter pylori*-induced gastric precancerosis of Mongolian gerbil. *World J Gastroenterol* 2003; 9(3): 521-524
http://www.wjgnet.com/1007-9327/9/521.htm

INTRODUCTION

H. pylori, a gram-negative spiral bacterium first isolated in 1982 from a patient with chronic active gastritis, is responsible for a large portion of chronic gastritis and nearly all duodental ulcers, most gastric ulcers, and probably an increased risk of gastric adenocarcinoma[1-9]. More than 50 % of the adult population are infected with *H. pylori* in developing countries as well as in developed countries. Gastric cancer is a major health problem[10] and remains the second most common cancer in the world[11]. Although epidemiological studies have indicated that *H. pylori* infection plays a crucial role in human gastric carcinogenesis[12-25], there is no direct proof that *H. pylori* is actually associated with gastric carcinogenesis[26]. The purpose of this study was to elucidate the relationship between *H. pylori* infection and gastric carcinogenesis by using an animal model of long-term *H. pylori* infection, and to explore the role played by *c-fos* in gastric tumorigenesis.

MATERIALS AND METHODS

Animals treatment

Four-week-old specific pathogen-free male Mongolian gerbils weighing 20±5 g were employed in this study. They were housed in individual metabolic cages in a temperature conditioned room 23±2 ℃ with a 12 h light-dark cycle, allowed to access to standard rat chow (provided by Experimental Animal Center, First Military Medical University) and water ad labium, and acclimatized to the surrounding for 7 days prior to the experiments. *H. pylori* (NCTC 11 637) was obtained from American Type Culture Collection and cultured on Brucella agar plates containing 70 mL· L^{-1} goat blood in a microaerobic condition (volume fraction, N_2: 85 %, O_2: 5 %, CO_2: 10 %, in aerobic globe box) at 37 ℃ for 3 days. The strain was identified by morphology, Gram' s stain, urease production and so on.

Experimental protocol

0.5 mL 1×10^8 cfu· L^{-1} suspension of *H. pylori* NCTC 11 637 in Brucella broth were inoculated orally into 20 Mongolian gerbils for 14 days continuously which had been fasted overnight. Another 20 gerbils were inoculated with Brucella broth as controls. 10 of the infected gerbils and 10 of the non- infected control gerbils were sacrificed after infection for 25 and 45 weeks, respectively. The stomach of each animal was removed and opened for macroscopic observation. Half of each gastric antrum mucosa were dissected for RNA isolation. The rest of the stomach samples were used for histological examination,

which were fixed with neutral-buffered 100 mL· L^{-1} formalin and processed by standard methods that embedded in paraffin, sectioned and attained with haematoxylin for analyzing histological changes, Giemsa stain for detecting for *H. pylori* and Alcian blue (AB)/PAS stain for examining intestinal metaplasia.

RNA isolation and RT-PCR analysiss

Using Tripure isolation reagent (Boehringer Mannheim, Germany), total cellular RNA was isolated from previously frozen tissues according to the manufacturer' s instruction. All RNA samples were analyzed for integrity of 18s and 28s rRNA by ethidium bromide staining of 0.5 μg RNA resolved by electrophoresis on 12 g· L^{-1} agarose-formaldehyde gels. RT-PCR analysis was performed as follows. RNA was incubated at 60 ℃ for 10 min and chilled to 4 ℃ immediately before being reverse transcribed. 1 μg of total RNA was reversely transcripted using antisense primers in a volume of 20 μl for 40 min at 50 ℃, containing 200 U MMLV reverse transcriptase, 1×buffer RT, 1 MU· L^{-1} Rnasin, 0.5 mmol· L^{-1} dNTPs of dATP, dGTP, dCTP and dTTP and each antisense primers including *c-fos* and β-actin at 0.2 μmol· L^{-1}. The samples were heated to 99 ℃ for 5 min to terminate the reverse transcription reaction. By using a Perkin-Elmer DNA Thermocycler 4 800 (Perkin-Elmer, Norwalk, CT), 5 μl cDNA mixture obtained from the reverse transcription reaction was then amplified for c-fos and β-actin. β-actin was used as the housekeeping gene and amplified with c-fos as control. The amplification reaction mixture consisted of 10×buffer 5 μl, 0.2 mmol· L^{-1} dNTPs of dATP, dGTP, dCTP and dTTP, 2.5 U Taq DNA polymerase, and sense and antisense primers at 0.2 μmol· L^{-1} in a final volume of 50 μl. The reaction mixture was first heated at 94 ℃ for 2 min and amplification was carried out for 29 cycles at 94 ℃ for 0.5 min, 58 ℃ for 1 min, 70 ℃ for 1.5 min, followed by an incubation for 7 min at 70 ℃. The amplification cycles was previously determined to keep amplification in the linear range to avoid the "plateau effect" associated with increased PCR cycles. The PCR primers were as following: c-fos, sense 5' -CAC GAC CAT GAT GTT CTC GG-3' and antisense 5' -AGT AGA TTG GCA ATC TCG GT-3' ; β-actin, sense 5' -CCA AGG CCA ACC GCG AGA AGA TGA C-3' and antisense 5' -AGG GTA CAT GGT GGT GCC GCC AGA C-3' . PCR products of c-fos and β-actin had 348 bp and 587 bp, respectively. PCR products were run on a 15 g· L^{-1} agarose gel in 0.5×TBE buffer and then analyzed by gel image analysis system. The level of c-fos was reflected with the ratio of c-fos/β-actin.

Immunohistochemical staining

Four micrometers paraffin-embedded tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was ablated with 10 mL· L^{-1} hydrogen peroxide in methanol. The immunostaining for c-fos was conducted using the StreptAvidin-Biotin-enzyme Complex kit (Boster, Wuhan). Immunostaining by replacing primary antibody with PBS was also conducted as a negative control. The staining was evaluated semiquantitatively on the basis of the percentage of positive cells, and classified as follows[27]: diffusely positive (+++) when positive cells accounted for more than 70 % of the total cells, partially positive (++) when positive cells were 35-70 %, partially positive (+) when positive cells accounted for 5-35 %, and negative (-) when positive cells accounted for less than 5 %.

Statistical analysis

Experimental results were analyzed with Chi-square Tests and K-Related Samples Test by SPSS software. Statistical significance was determined at $P<0.05$.

RESULTS

Histopathological findings

H. pylori was detected in gastric antrum and gastric body of all infected animals in this study, and more in gastric antrum than that in gastric body. After infection of *H. Pylori* for 25 weeks, ulcers were observed in the gastric antrum and gastric body in 60 % infected animals (6/10). Histological examination showed that all infected animals developed severe inflammation, especially in the area close to ulcers; multifocal lymphoid follicles appeared in the lamina propria and submucosa; and there were mild atrophic gastritis in all infected animals. After infection of *H. Pylori* for 45 weeks, severe atrophic gastritis in all infected animals, intestinal metaplasia in 80 % infected animals (8/10) and dysplasia in 60 % infected animals (6/10) could be observed. Those metaplastic glands appeared more atypical than the surrounding nonmetaplastic and hyperplastic glands. Severe atrophic gastritis, intestinal metaplasia and dysplasia were gastric precancerosis. In the uninfected animals, there were no significant changes throughout the study.

Analysis of c-fos mRNA expression

There was c-fos mRNA expression in gastric antrum mucosa of control-animals. C-fos mRNA levels were significantly higher after infection of *H. pylori* for 25 weeks (1.84±0.79), and for 45 weeks (1.59±0.37) than that in control-animals (0.74±0.22, $P<0.01$); *C-fos* mRNA levels were increased 2.5-fold in 25 weeks ($P<0.01$) and 2.1-fold in 45 weeks ($P<0.01$) in precancerosis induced by *H. pylori*, when compared with control gastric epithelium of Mongolian gerbil (Figure 1-4).

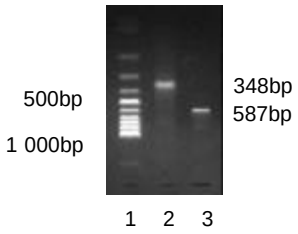

Figure 1 RT-PCR products. Lane 1 PCR marker; Lane 2 c-fos; Lane 3 β-actin.

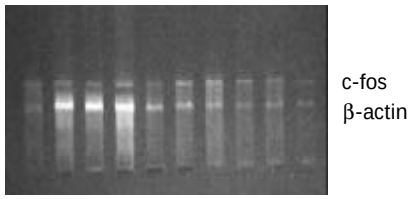

Figure 2 RT-PCR analysis of c-fos mRNA levels using β-actin as internal control. Total RNA was first reverse transcripted into cDNA and then amplified by PCR in control.

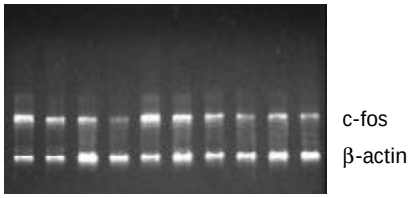

Figure 3 RT-PCR analysis of c-fos mRNA levels using β-actin as internal control after *H. pylori* infection for 25 weeks.

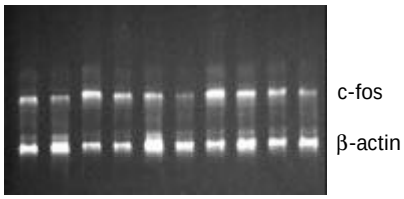

Figure 4 RT-PCR analysis of c-fos mRNA levels using β-actin as internal control after *H. pylori* infection for 45 weeks.

Immunohistochemical analysis of c-fos protein expression

Immunohistochemical analysis was performed to examine whether increased *c-fos* mRNA expression were accompanied by increased expression of c-fos protein. C-fos protein expression lied in nuclei (Figure 5). C-fos protein expression was evaluated significantly (P<0.01) in precancerosis induced by *H. pylori* for 45 weeks, when compared with control gastric epithelium of Mongolian gerbil (Table 1).

Table 1 Expression of c-fos by Immunohistochemical staining

Group	*n*	c-fos				
		-	+	++	+++	Positive/%
Control	10	10	0	0	0	0
Inf 25 weeks	10	8	2	0	0	20
Time 45 weeks	10	6	4	0	0	40[a]

[a]P<0.01, *vs* Control.

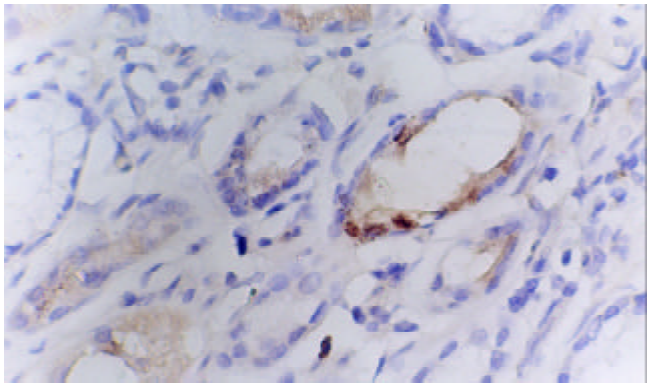

Figure 5 Expression of c-fos after *H. pylori* infection for 25 weeks, immunohistochemistry staining (×400).

DISCUSSION

H. pylori infection is now known as a major cause of acute and chronic active gastritis, peptic ulcer disease and atrophic gastritis and is also considered as a risk factor in the incidence of gastric adenocarcinoma and mucosa-associated lymphoma[1-8]. Chronic colonization of the human stomach by the gram-negative spiral bacterium *H. pylori* is associated with the development of cancer in the distal portion of the stomach. Although there is no direct proof that *H. pylori* is actually associated with gastric carcinogenesis, epidemiological studies have indicated that *H. pylori* infection plays an important role in human gastric carcinogenesis. Because of this strong epidemiological association, *H. pylori* is classified as a definite carcinogen (group I) by the International Agency for Research on Cancer (IRAC), a branch of the World Health Organization (WHO). Many animals infected with human *H. pylori* have already been studied to determine the pathogenetic background, but none of the models studied mimics human *H. pylori* infection and subsequent pathology. Recently, two exprements were conducted in Japan that demonstrated that chronic *H. pylori*-infection models of Mongolian gerbils would develope gastric carcinoma. These results will be extremely helpful to elucidate the mechanism of gastric carcinogenesis due to *H. pylori* infection[9-26]. Apoptosis, a programmed cell death, was ignored, just like *H. pylori*, only to reappear recently. However, the number of current publications dealing with apoptosis of *H. pylori* has increased exponentially. Although gastric epithelial apoptosis is a programmed physiological event in the superficial aspect of the mucosa and is important for healthy cell turnover, *H. pylori* infection reportedly promotes such a cell death sequence[27,28]. Because apoptosis regulates the cycle of cell turnover in balance with proliferation, dysregulation of apoptosis or proliferation evoked by *H. pylori* colonization would be linked to the gastric carcinogenesis[29-31].

C-fos is an immediate early response gene, and c-fos protein is an important transcription-factor of nuclei[32-39]. Oncogene *c-fos* is also a kind of effect protein of the karyomitosis signal, which can trigger and regulate the transcription of the genes related with proliferation. Besides, *c-fos* can also regulate its own gene expression with a positive feedback and promote the mitosis and proliferation of the cells. Because *c-fos* can regulate cell proliferation and cell apoptosis, its abnormal expression might induce cell turnover and carcinogenesis[40-46]. Previous studies have showed that the expression of oncogene *c-fos* is closely related to cellular multiplication and differentiation. The amplification and over-expression of c-fos gene are associated with malignancy and tumorigenecity of cells. Recently, some studies suggested that oncogene c-fos was amplified in the primary tumor DNA[47-49]. Shirin *et al* tested the hypothess that *H. pylori* might inhibit cell growth and cell cycle progression by inhibiting signaling pathways that mediate the transactivation of the serum-response element in the c-fos promoter[27].

In this study, mRNA level of *c-fos* was measured by quantitative RT-PCR analysis in Mongolian gerbil gastric antrum mucosa to explore dysregulation of c-fos in malignancies and to further investigate the role of c-fos in *H. pylori*-induced gastric precancerosis. In addition, the expression and localization of its protein product was analyzed by immunohistochemical staining. *C-fos* mRNA levels were significantly increased in precancerosis induced by *H. pylori*, when compared with normal gastric epithelium of Mongolian gerbil. Immunohistochemical staining revealed exclusive nuclear staining of c-fos. Furthermore, there was a sequential increase in c-fos positive cells from normal epithelium to precancerosis. This study indicates that the expression of c-fos mRNA and protein is increased from normal epithelium to precancerosis. The dysregulation of c-fos expression occurre relatively early in gastric tumorigenesis in this model and may participate in tumor progression. These findings suggeste that *H. pylori*- induced gastric precancerosis is associated with dysregulation of gastric epithelial cell cycle. Further studies are needed to delineate the mechanism of those alterations.

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Edited by Xu XQ