INFLUENCE OF HELICOBACTER PYLORI INFECTION PERSISTENCE ON bcl-2 EXPRESSION IN GASTRIC MUCOSA INFLAMMATORY CELLS

Karmen Brajša1, Željko Ferenčić1, Miroslava Katičić2, Berislav Bošnjak1, Vladimir Presečki3, Radan Spaventi1 and Maša Dominis2

1GlaxoSmithKline Research Centre Zagreb Limited, Department of Pharmacology and Medicinal Safety; 2Merkur University Hospital; 3Zagreb University Hospital Center, Zagreb, Croatia

SUMMARY – Chronic Helicobacter (H.) pylori infection is an etiological factor related to gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. The expression of bcl-2 protein significantly decreases as the grade of MALT lymphoma advances. The aim of this study was to evaluate bcl-2 expression in inflammatory cells in lamina propria in gastric biopsy samples collected from two groups of patients with chronic gastritis divided on the basis of the success or failure of H. pylori eradication. Sixty-five patients with chronic gastritis were divided into two groups of 45 and 20 patients according to their therapeutic response. The gastric mucosa samples were analyzed histologically in both groups of patients before and after standard therapy (for eradicated, after one therapeutic cycle; and for non-eradicated, after three therapeutic cycles) for H. pylori density, urease activity and bcl-2 expression. In the eradicated group of patients, H. pylori eradication was accompanied by significantly lower grades of bacterial colonization and lower urease activity in the corpus and antrum. Bcl-2 expression in inflammatory cells showed no statistically significant changes in either patient group at either location. There was no between-group difference in bcl-2 expression either. In conclusion, persistent long-lasting H. pylori infection is associated with higher grades of bacterial colonization and higher urease activity but not with bcl-2 expression in inflammatory cells.

Key words: Helicobacter pylori – immunity; Helicobacter pylori – complications; Helicobacter pylori – pathogenicity; Stomach neoplasms – etiology; Stomach neoplasms – microbiology

Introduction

Helicobacter (H.) pylori is a gram-negative bacterium that resides in stomach of half of all humans. The clinical consequences range from asymptomatic gastritis to peptic ulceration and gastric malignancy1. Epidemiological and pathological studies showed strong relationship between gastric adenocarcinoma, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and H. pylori infection2-4. The outcome of the infection may be related to the duration of infection and/or depend on host factors5-6. Clinical studies demonstrated an association between H. pylori infection and development of primary gastric lymphoma7. Only a minority of H. pylori positive patients with chronic gastritis developed gastric cancer3, and the exact pathogenic mechanisms responsible for the role of H. pylori in the induction of gastric carcinogenesis have not yet been identified. H. pylori induces chronic inflammatory response that fails to clear the infection and persistent infiltration of inflammatory cells is an almost invariable feature of H. pylori-infected gastric mucosa. Gene profiles in gastric mucosa biopsies during H. pylori infection showed up-regulation of inflammatory genes like pro-inflammatory cytokine receptors, chemokines and their receptors, genes involved in apoptosis process and adhesion molecules8. H. pylori and cytokines induced during infection can
stimulate the recruitment and activation of inflammatory cells including neutrophils, macrophages and lymphocytes. It is known that some human pathogens might delay apoptosis of inflammatory cells to survive\textsuperscript{20,21}. Prolongation of neutrophil, macrophage and lymphocyte life-span could contribute to the pathogenesis of \textit{H. pylori} infection. It has been shown that water-soluble surface proteins of \textit{H. pylori} could suppress neutrophil apoptosis\textsuperscript{14}, and activation of caspase-8 and 3\textsuperscript{15}. The protein product of bcl-2 gene blocks apoptosis. An aberrant bcl-2 expression was found in 68\% of chronic atrophic gastritis cases and in the majority of follicular lymphomas\textsuperscript{16}. Also, a higher bcl-2 expression in lymphocytes was observed in \textit{H. pylori}-positive biopsies, and treatment did not change the expression of this protein\textsuperscript{17}. Villuendas et al. showed an increased expression of bcl-2 protein in low grade MALT lymphomas and loss of its expression in high-grade lymphomas\textsuperscript{18}. It seems that bcl-2 protein over-expression is an early event in MALT lymphoma development. In this study, we evaluated the expression of bcl-2 protein and persistence of \textit{H. pylori} infection in relation to therapy cycles and eventual \textit{H. pylori} eradication.

**Patients and Methods**

**Patients and therapy**

Sixty-five patients (27 female and 38 male) with \textit{H. pylori}-associated gastritis who underwent endoscopies at University Department of Medicine, Merkur University Hospital in Zagreb, were divided into two groups according to eradication of \textit{H. pylori} during three years. A patient was classified as eradicated if histological test was negative, and as non-eradicated if histological test was positive. Eradicated group (E) (45 patients, 16 female and 29 male; age range 33-77; mean age 50) included patients successfully eradicated after one standard therapy cycle. Non-eradicated group (NE) (20 patients, 11 female and 9 male; age range 26-70; mean age 45) included patients with persistent infection even after three standard therapy cycles. There was no statistically significant between-group difference in the male to female ratio (Fisher exact test, p=0.05). One standard cycle of therapy consisted of omeprazole (2x20 mg/day) and amoxicillin (2 g/day) for 14 days, and metronidazole (800 mg/day) for 10 days. None of the study patients had any history of alcohol abuse or taking non-steroidal anti-inflammatory drugs.

**Gastric biopsy and specimen analysis**

Four biopsy specimens were obtained, i.e. two from the greater curvature of the antrum and two from the upper body (corpus) of the stomach according to Sydney system\textsuperscript{19}. One part of these specimens were fixed in formalin and assessed for \textit{H. pylori} density (Giemsa staining). The remaining specimens were used for rapid urease test and immunohistochemical staining for bcl-2 expression analysis.

**\textit{H. pylori} density score**

The density of \textit{H. pylori} and inflammation severity were assessed semi-quantitatively. \textit{H. pylori} density was scored as follows: 0=no organisms, 1=mild, 2=moderate, 3=marked, and 4=very high density of microorganisms\textsuperscript{19}.

**Rapid urease test (CLO test)**

Detection of \textit{H. pylori} urease was made by standard test (CLO-test, West, Bentley,Australia). Urease activity was scored semi-quantitatively as follows: yellow (grade 0), pale orange (grade 1), orange (grade 2), red (grade 3) and purplish-red, strong positive (grade 4).

**Bcl-2 immunostaining**

Tissues embedded in paraffin were cut into 3 mm-thick sections and mounted on glass slides coated with poly-L-lysine, deparaffinized in xylene and rehydrated through gradient concentrations of ethanol. After blocking endogenous peroxidase with H2O2, the sections were heated in 0.01 M citrate buffer (pH 6.0) in microwave oven for 15 minutes. The slides were allowed to cool to room temperature, and nonspecific binding was blocked with normal rabbit serum (Institute of Immunology, Zagreb, Croatia), diluted 1:5 for 20 minutes at room temperature and washed in PBS.

The primary antibody for bcl-2 (M10887, DAKO, Glostrup, Denmark) was applied to the sections in dilution 1:70 in 0.05M TRIS buffer, pH 7.6 for 60 minutes at room temperature. The sections were then incubated with biotinylated secondary antibody (EO354, DAKO, Glostrup, Denmark), diluted 1:300 for 30 minutes following avidin-biotin peroxidase reagent (K0377, DAKO, Glostrup, Denmark) for 30 minutes. After color development with diaminobenzidine-hydrogen peroxidase substrate (DAB) as chromogen, the sections were counterstained with Mayer’s hematoxylin.


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Sections that had not been incubated with primary antibody were used as a negative control and sections of follicular lymphoma as a positive control. The immunostained slides were blindly evaluated by light microscopy. Bcl-2 was examined in inflammatory cell infiltrates of gastric mucosa. Owing to heterogeneous immunoreactivity within most sections and small size of the specimen, the whole slide was scanned and graded. The bcl-2 reactivity was scored semi-quantitatively and classified as follows: no staining observed in any cell (grade 0), 10% to 20% cells positive (grade 1), 25% to 50% cells positive (grade 2), and more than 50% cells positive (grade 3).

**Statistical analysis**

All statistical tests were performed with SAS System software (SAS Institute, Cary, NC, USA) and Graphpad Software (Graphpad Software Inc, CA, USA). The unpaired t-test for age and χ²-test for sex ratios in the groups were used. For variables observed grades or scores, correlations were determined from contingency tables, using continuity adjusted χ²-test. Between-group comparisons were done by use of χ²-test for trend.

**Results**

**H. pylori density score and rapid urease test (CLO test)**

Giemsa staining confirmed the primary diagnosis of *H. pylori* infection. Grades of *H. pylori* density are presented in Figure 1. All patients from E group, i.e. 45/45 (100%) were positive for *H. pylori* infection before treatment and negative after therapy (Fisher exact test, p<0.01); and all patients from the NE group, i.e. 20/20 (100%) were positive before and stayed positive after therapy (Fisher exact test, p>0.05). Semi-quantitative evaluation of the severity of mucosal colonization was similar in the two groups before treatment (Fisher exact test, p>0.05). After treatment, density of *H. pylori* colonization did not change significantly in NE group as compared with E group (Fisher exact test, p<0.01). Comparison of CLO activity test scores between E and NE groups is presented in Figure 1. The CLO activity scores before therapy were not significantly different. After therapy, the CLO activity scores were significantly lower in group E, while showing no changes after three therapy cycles in group NE.

**Bcl-2 expression**

There was no significant difference between pretherapeutic and post-therapeutic bcl-2 expression in the gastric corpus and antrum specimens. There was no difference between the eradicated and non-eradicated group before and after treatment either (Fig. 2).

**Discussion**

Normally, the stomach is devoid of lymphoid tissue and acquires MALT only in the presence of chronic *H. pylori* infection. *H. pylori* is present in 72% to 98% of low-grade MALT lymphomas. Treatment of *H. pylori* infection has been associated with complete or partial regression of localized, low-grade gastric MALT lymphoma in patients and is now seen as a critical part of
the management of this disease. A histologic feature of *H. pylori* infection is dense infiltration of polymorphonuclear leukocytes (PMNL) in gastric mucosa. Hofman *et al.* found broth culture filtrates from *H. pylori* to cause significant delay in spontaneous polymorphonuclear cell apoptosis and this delay was independent of the VacA, cag pathogenicity island and urease status. Bcl-2 is a known inhibitor of apoptosis and previous results suggest that expression of bcl-2 protein significantly decreases as the grade of MALT lymphoma advances. Analyzing bcl-2 expression in the two groups divided on the basis of the success or failure of *H. pylori* eradication, we evaluated the prognostic value of bcl-2 expression. Results of our study showed that there was no difference in bcl-2 expression between eradicated and non-eradicated group despite completed cycles of eradication therapy. Ohara *et al.* showed that antibiotic treatment for elimination of *H. pylori* directly affected inflammatory cells to induce apoptosis and protect gastric mucosa from damage. These differences in results could be a consequence of different antibiotic class used in triple therapy because macrolide antibiotic could induce apoptosis of inflammatory cells.

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**References**


Sažetak

UTJECAJ TVRĐOKORNE INFEKCIJE BAKTERIJOM Helicobacter pylori NA IZRAŽENOST bcl-2 U UPALNIM STANICAMA ŽELUČANE SLUŽNICE

K. Brajša, Ž. Ferentič, M. Katičić, B. Bošnjak, V. Preseki, R. Spacenti i M. Dominis

Kronična infekcija bakterijom Helicobacter (H.) pylori je etiološki čimbenik želučanog adenokarcinoma i limfoma limfoidnog tkiva povezana sa služnicom (MALT limfoma). Izraženost proteina bcl-2 značajno se smanjuje sa napredovanjem stupnja MALT limfoma. Cilj ove studije bio je procijeniti izraženost bcl-2 u upalnim stanicama lamine proprije u uzorcima dobivenim želučanom biopsijom u dvjema skupinama bolesnika s kroničnim gastritismom podijeljenim prema uspješnoj ili neuspješnoj eradicaciji H. pylori. Ukupno je 65 bolesnika s kroničnim gastritismom podijeljeno u dvije skupine od po 45 i 20 bolesnika prema terapijskom odgovoru. U objema skupinama su uzorci želučane služnice analizirani histološki prije i nakon standardne terapije (kod onih s uspješnom eradicacijom nakon jednog terapijskog ciklusa, a u onih s neuspješnom eradicacijom nakon tri terapijska ciklusa) na gustoću H. pylori, aktivnost ureaze i izraženost bcl-2. Eradicacija H. pylori u skupini bolesnika s uspješnom eradicacijom bila je praćena značajno nižim stupnjem bakterijske kolonizacije i nižom aktivnošću ureaze u korpusu i antrumu. Izraženost bcl-2 nije se statistički značajno promijenila ni na jednoj lokaciji ni u jednoj skupini bolesnika. Isto tako, nije bilo nikakve razlike među dvjema skupinama bolesnika u izraženosti bcl-2. Zaključuje se kako je dugotrajna ustajra infekcija bakterijom H. pylori povezana s višim stupnjem bakterijske kolonizacije i višom aktivnošću ureaze, ali nije povezana s izraženost bcl-2 u upalnim stanicama.

Ključne riječi: Helicobacter pylori – immunologija; Helicobacter pylori – komplikacije; Helicobacter pylori – patogeničnost; Nocotuzorne želuca – etiologija; Nocotuzorne želuca – mikrobiologija


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