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

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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 – immunology; 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 malignancy[1]. Epidemiological and pathological studies showed strong relationship between gastric adenocarcinoma, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and *H. pylori* infection[2-4]. The outcome of the infection may be related to the duration of infection and/or depend on host factors[5,6]. Clinical studies demonstrated an association between *H. pylori* infection and development of primary gastric lymphoma[7]. Only a minority of *H. pylori* positive patients with chronic gastritis developed gastric cancer[8], 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 molecules[9]. *H. pylori* and cytokines induced during infection can

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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[10-13]. Prolongation of neutrophil, macrophage and lymphocyte lifespan could contribute to the pathogenesis of *H. pylori* infection. It has been shown that water-soluble surface proteins of *H. pylori* could suppress neutrophil apoptosis[14], and activation of caspase-8 and 3[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[16]. Also, a higher bcl-2 expression in lymphocytes was observed in *H. pylori*-positive biopsies, and treatment did not change the expression of this protein[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[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 *H. pylori* infection in relation to therapy cycles and eventual *H. pylori* eradication.

Patients and Methods

Patients and therapy

Sixty-five patients (27 female and 38 male) with *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 *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[19]. One part of these specimens were fixed in formalin and assessed for *H. pylori* density (Giemsa staining). The remaining specimens were used for rapid urease test and immunohistochemical staining for bcl-2 expression analysis.

H. pylori density score

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

Rapid urease test (CLO test)

Detection of *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 H_2O_2, 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 (E0354, 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 diamniobenzidine-hydrogen peroxidase substrate (DAB) as chromogen, the sections were counterstained with Mayer's hematoxylin.

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 χ^2-test for sex ratios in the groups were used. For variables showing grades or scores, correlations were determined from contingency tables, using continuity adjusted χ^2-test. Between-group comparisons were done by use of χ^2-test for trend.

Results

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

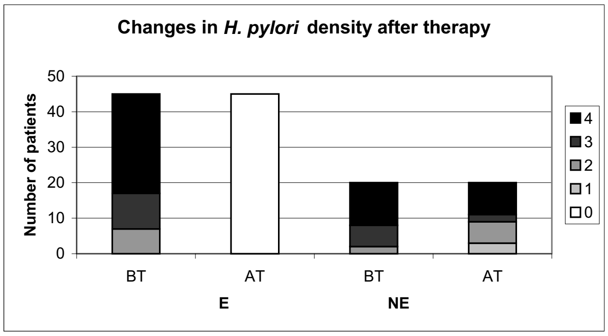
Giemsa staining confirmed the primary diagnosis of *H. pylori* infection. Grades of *H. pylori* density are presented in Figure 1. All patients from E group, i.e. 45/45 (100%) were positive for *H. pylori* infection before treatment and negative after therapy (Fisher exact test, $p<0.01$); and all patients 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

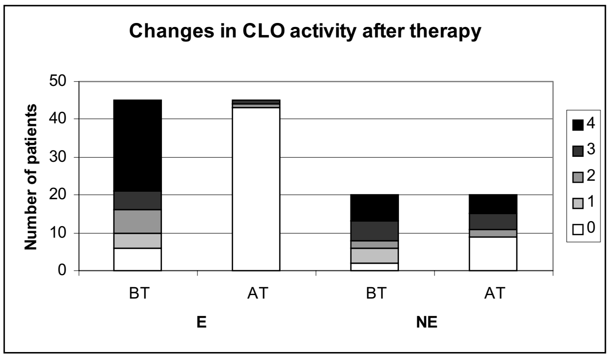
There was no significant difference between pretherapeutic and post-therapeutic bcl-2 expression in the gastric corpus and antrum specimens. There was no difference between the eradicated and non-eradicated group before and after treatment either (Fig. 2).

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 most patients and is now seen as a critical part of

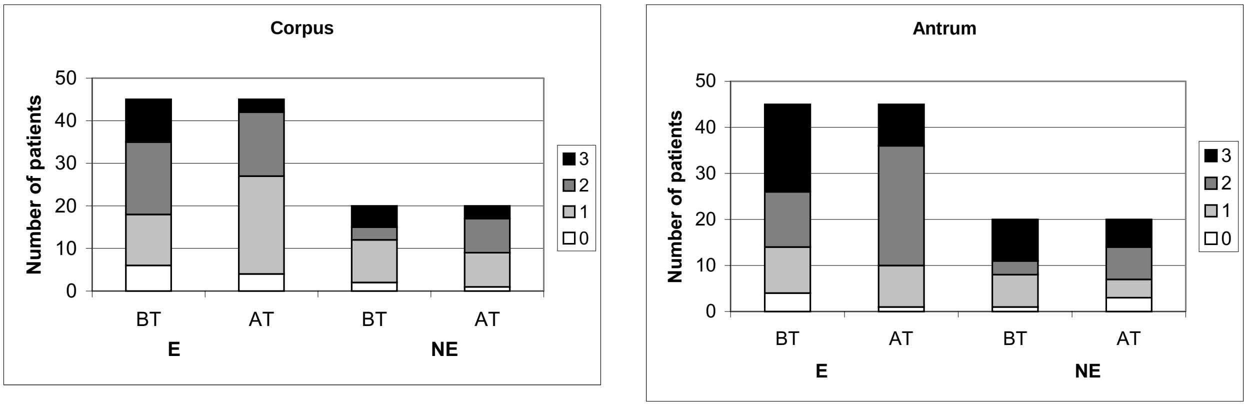


χ^2-test: E *vs.* NE (BT) $P>0.05$; E *vs.* NE (AT) $P<0.0001$



χ^2-test: E *vs.* NE (BT) $P>0.05$; E *vs.* NE (AT) $P<0.0001$

Fig. 1. Helicobacter (H.) pylori *density (left) and CLO activity (right) in eradicated (E) and non-eradicated group (NE) before (BT) and after (AT) therapy.*



χ^2-test: E *vs.* NE (BT) for corpus P>0.05; E *vs.* NE (AT) for corpus P>0.05

χ^2-test: E *vs.* NE (BT) for antrum P>0.05; E *vs.* NE (AT) for antrum P>0.05

Fig. 2. Bcl-2 expression grade in gastric corpus (left) and antrum (right) in eradicated (E) and non-eradicated group (NE) before (BT) and after therapy (AT).

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[20]. 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[21]. 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[22]. 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[23].

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Sažetak

UTJECAJ TVRDOKORNE INFEKCIJE BAKTERIJOM *Helicobacter pylori* NA IZRAŽENOST bcl-2 U UPALNIM STANICAMA ŽELUČANE SLUZNICE

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Kronična infekcija bakterijom *Helicobacter (H.) pylori* je etiološki čimbenik želučanog adenokarcinoma i limfoma limfoidnog tkiva povezanog sa sluznicom (MALT limfoma). Izraženost proteina bcl-2 značajno se smanjuje s 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 gastritisom podijeljenim prema uspješnoj ili neuspješnoj eradikaciji *H. pylori*. Ukupno je 65 bolesnika s kroničnim gastritisom podijeljeno u dvije skupine od po 45 i 20 bolesnika prema terapijskom odgovoru. U objema skupinama su uzorci želučane sluznice analizirani histološki prije i nakon standardne terapije (kod onih s uspješnom eradikacijom nakon jednog terapijskog ciklusa, a u onih s neuspješnom eradikacijom nakon tri terapijska ciklusa) na gustoću *H. pylori*, aktivnost ureaze i izraženost bcl-2. Eradikacija *H. pylori* u skupini bolesnika s uspješnom eradikacijom 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 ustrajna infekcija bakterijom *H. pylori* povezana s višim stupnjem bakterijske kolonizacije i višom aktivnošću ureaze, ali nije povezana s izraženošću bcl-2 u upalnim stanicama.

Ključne riječi: *Helicobacter pylori – imunologija; Helicobacter pylori – komplikacije; Helicobacter pylori – patogeničnost; Novotvorine želuca – etiologija; Novotvorine želuca – mikrobiologija*