Original Article

Helicobacter pylori-induced Genotoxic Damage in Human B Lymphocytes

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ABSTRACT

Background:
Helicobacter pylori (*H. pylori*) is recognized as the causative agent of peptic and duodenal ulcers, gastric adenocarcinoma, and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma. In the present study, we investigate the genotoxic damage of lysates of *H. pylori* in human B lymphocytes.

Materials and Methods:
Human B lymphocytes were treated with 0, 10, 20, and 30 µg/mL of total protein concentration of lysates obtained from *H. pylori* isolates from dyspeptic patients. Direct *H. pylori*-induced DNA damage was investigated by the in vitro cytokinesis-block micronucleus assay which detects chromosomal fragments and maldistributed whole chromosomes.

Results:
The total mean micronuclei number (tMMN) observed per 1000 binucleus B cells significantly correlated with increasing protein concentration of *H. pylori* lysates (r^2=0.994, p=0.006). The highest tMMN (3.81-fold) was observed in cells treated with 30 µg/mL of *H. pylori* lysate (12.43 ± 1.91) when compared with the control (3.26 ± 0.48).

Conclusion:
This study provides evidence of the direct effect of *H. pylori* in chromosomal breakage of human B lymphocytes, which might lead to the development of abnormal B cells. Long-term infection by *H. pylori* has been implicated in epithelial cell damage as a result of continuous induction of the immune system by bacterial antigens. However, the results of this study propose that persistent *H. pylori* infection could also directly damage B lymphocyte DNA from which gastric MALT lymphoma arises.

Keywords: *H. pylori*; Human B lymphocytes; Genotoxic damage; MALT lymphoma

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INTRODUCTION

Helicobacter pylori (H. pylori) infection is a risk factor for the development of gastric cancer(1,2) and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma in the stomach(3). Most gastric MALT lymphomas exhibit low-grade histological changes and can undergo complete or partial regression after eradication of *H. pylori* from the stomach(4-8). The results of a meta-analysis of 32 studies, including 1408 patients has shown

eradication of *H. pylori* to be an effective treatment in 75% of patients diagnosed with early stage gastric lymphoma(9). It has been concluded that there is a strong association between *H. pylori* infection and gastric MALT lymphoma. According to research, in MALT lymphoma, the intralymphomatous T cells which are continuously stimulated and activated by *H. pylori* antigens also stimulate the proliferation of neoplastic B cells(10). However, recent investigations have demonstrated the direct effect of *H. pylori* on B lymphocytes without the involvement of T cells. For example, translocation of *H. pylori* CagA into B cells induces proliferation and transformation leading to gastric MALT lymphoma, even though CagA possesses both phosphorylation-dependent and -independent activities and its biological impact depends on cell-type context(11,12). Infection with CagA-positive strains of *H. pylori* is significantly associated with gastric MALT lymphomas that have a characteristic chromosomal translocation, t (11; 18) (q21; q21) (13). The occurrence of this type of transformation and possible chromosomal breakage indicates that *H. pylori* may participate in the events which lead to DNA damage. It appears that infection by some *H. pylori* strains with particular genotypes might lead to gastric adenocarcinoma and MALT lymphoma(14). Some reports have shown an increased rate of cytogenetic damage in peripheral blood lymphocytes of subjects infected with *H. pylori*(15) and direct induction of DNA damage in gastric epithelial(16) and mouse leukemia cells(17) in vitro. It has also been proposed that low levels of *H. pylori* infections which occur in vivo not only suppress apoptosis of B lymphocytes but also induce their survival and proliferation, which is consistent with their potential to evolve into MALT lymphoma(18). These findings show that *H. pylori* might have multidirectional activities in the pathogenesis of gastric MALT lymphoma, eventually leading to the stimulation and proliferation of malignant B cells. However the direct effect of *H. pylori* in DNA breakage of human B cells has not been investigated. The present study investigates the genotoxic effect of lysates of *H. pylori* in human B lymphocytes.

MATERIALS AND METHODS

Bacterial isolates and identification

We chose seven *H. pylori* isolates from a well-characterized Cell Bank at the Digestive Diseases Research Center, Tehran University of Medical Sciences, Tehran, Iran. The isolates were previously identified and characterized(19) as follows: non-atrophic antral gastritis (SLN002, SLN004), peptic ulcer diseases (SLN007, SLN019), and gastric cancer (SLN006, SLN008, and SLN020). The isolates were cultured on selective brucella agar medium (Merck) that contained 5%-7% blood under microaerobic conditions for 3-5 days at 37°C. Bacterial colonies were identified by gram stain (gram-negative spiral forms) and positive urease, oxidase and catalase tests, as well as PCR amplification of *H. pylori* 16S rDNA(20). Amplification was performed in 35 cycles as follows: 1 min at 94°C, 1 min of annealing temperature at 56°C, 1 min at 72°C, and a final incubation at 72°C for 7 min. PCR products were electrophoresed and visualized by UV transilluminator. Amplified fragments of the *H. pylori* 16S rDNA from the seven isolates were purified and sequenced with both forward and reverse primers using BigDye technology on an ABI3700XL DNA sequencer (Applied Biosystems). The BLAST program (http://www.ncbi.nlm.nih.gov) was used to match the nucleotide sequences with sequences published in GenBank. *Escherichia coli* (*E. coli*) DH5α was the non-carcinogenic (negative) control for the in vitro cytokinesis-block micronucleus assay and tested with the *H. pylori* 16S *rDNA* primers.

The study was approved by the Ethics Committee of the Digestive Diseases Research Center, Shariati Hospital, Tehran University of Medical Sciences, based on the ethical principles of human research and experimentation as expressed in the Declaration of Helsinki.

Preparation of *H. pylori* and *E. coli* lysates

Bacterial suspensions were prepared from fresh cultures in 10 mmol tris-buffered saline (pH=7.4). Turbidity of bacterial suspensions was adjusted to that of a no. 5 McFarland standard. Bacterial lysates were prepared by sonication on ice. Total protein was determined by the Bradford method.

Cell culture

We used the human B lymphocyte cell line (ATCC CRL-1596). The cells were cultured in RPMI-1640 supplemented with 100 U/mL penicillin,100 mg/mL streptomycin, 2 mM L-glutamine, and 10%-15% heat-inactivated fetal calf serum. Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37°C. The trypan blue exclusion test was used to determine the percent of viable cells in the cell suspensions.

In vitro cytokinesis-block micronucleus assay

The exponentially growing human B lymphocytes were seeded at a density of 5×10^5 cells/mL in RPMI-1640 culture medium. Lysates of *H. pylori* and *E. coli* DH5α were added to the culture medium at final concentrations of 0, 10, 20, and 30 µg/mL of the total proteins. After 48 h, cells were centrifuged and the medium replaced, after which cytochalisin B (Fluka) was added to the culture medium at a final concentration of 3.5 µg/mL. On the fourth day, cells were harvested, exposed to hypotonic shock (0.075 M KCl) for 3-5 min and fixed in a mixture of methanol and acetic acid (Merck) at a 3:1 v/v ratio. In order to visualize nuclei and micronuclei, the slides were stained with 10% giemsa (Merck) at room temperature. Numbers of nuclei and micronuclei were scored at a magnification of 400x. We evaluated 1000 binucleus cells on each slide for the presence of micronuclei. Four slides were evaluated for each sample. Micronuclei were considered as round or oval objects separated from the nuclei that had nuclear-like staining characteristics and an area of less than a quarter of the area of a nucleus.

The in vitro cytokinesis-block micronucleus assay was performed in duplicate for the different protein concentrations. For each assay, we examined eight slides and the number of micronuleated cells per 1000 binucleus cells was recorded. The mean micronuclei number (MMN) ± SE/1000 binucleus cells was determined. Totally, 224 slides (56 slides of each concentration) that belonged to seven strains were evaluated and the total number of micronuclei was calculated for each protein concentration and expressed as the total mean micronuclei number (tMMN) ± SE/1000 cells. A total of 32 slides were also evaluated for the presence of micronuclei in the B cells treated with the control *E. coli* DH5α lysates and the results were expressed as described.

Statistical analysis

The number of micronuclei in the binucleus B cells and the protein concentrations in *H. pylori* lysates were analyzed by a bivariate correlation that used Pierson's correlation coefficient. The analysis was performed using SPSS v.14. A p-value of <0.05 was considered to be statistically significant.

RESULTS

Electrophoresis of the PCR products from the seven *H. pylori* 16S *rDNA* isolates resulted in 519 bp bands, which confirmed *H. pylori*. BLAST analysis with the sequenced fragments of each gene from the seven isolates showed that these sequences exclusively matched with the corresponding sequences in *H. pylori* J99, 26695, 60190, and Tx30a. The negative control E. coli DH5α did not produce any PCR product with the *H. pylori* 16S *rDNA* primers.

Experiments with *H. pylori* lysates of the seven samples obtained from different dyspeptic patients showed the genotoxic effects on human B lymphocytes (Figure 1). The average total number of micronuclei (tMMN) in the binucleus B cells following treatment with the different protein concentrations of 10, 20 and 30 µg/mL was 1.83 (range: 1.35-2.16), 2.59 (range: 1.88-3.30), and 3.81 (range: 3.00-4.73) times more than the controls (without *H. pylori* lysates), respectively (Table 1). A significant correlation was found between the micronuclei number and *H. pylori* lysate protein concentrations of each strain (data not shown). The tMMN of the binucleus B cells per 1000 cells significantly correlated with increasing protein concentration of *H. pylori* lysates (r^2=0.994, p=0.006). Treatment of B cells with lysates of *E. coli* as a non-carcinogenic control showed no significant increase in the number of micronuclei (r^2=0.344, p=0.656; Table 1). *E. coli* lysate did not cause cell death at higher concentrations of 40-100 µg/mL.

DISCUSSION

In this study, we have used the micronucleus assay to investigate direct DNA damage caused by *H. pylori* lysates on human B lymphocytes. This method is a potent, well-established tool to assess genotoxic damage(21,22). The results have shown a remarkable genotoxic impact of *H. pylori* lysates on cultured B lymphocytes. The highest tMMN was observed in cells treated with 30 µg/mL of *H. pylori* lysate (12.43±1.91) compared with the control (3.26±0.48). tMMN increased by 3.81-fold at this concentration. However, the frequency of produced micronuclei differed among the strains at the different protein concentrations. MMN showed a 1.35- to 4.73-fold increase, which indicated the difference between genotypes of *H. pylori* strains. At protein concentrations greater than 30 µg/mL, there was cell death and reduced numbers of micronuclei. Ning et al. observed DNA damage by *H. pylori* on the gastric epithelial cell line GES-1, by using micronucleus assay. At *H. pylori* protein concentration of 1.7 mg/L, the rate of micronucleus formation was 5.00 times higher than that of the control(23). Schmausser et al. also used the micronucleus assay to study induction of DNA

Table 1: **Average total number of micronuclei in binucleus B lymphocytes treated with different protein concentrations of *H. pylori* lysates.**

Strains (No.)	*H. pylori* protein concentration (μg/mL protein)	Total mean micronuclei number (tMMN) ± SE/1000 binucleus cells	Range
H. pylori (7)	0	3.26 ± 0.48	2.49 ± 0.21 – 4.23 ± 0.65
	10	5.95 ± 0.83	4.15 ± 0.68 – 9.12 ± 1.25
	20	8.45 ± 1.28	5.61 ± 0.78 – 13.95 ± 2.58
	30	12.43 ± 1.91	8.07 ± 1.30 – 20.00 ± 3.16
Control *E. coli* DH5α	0	3.46 ± 0.82	-
	10	3.71 ± 1.65	-
	20	3.59 ± 0.60	-
	30	3.86 ± 0.40	-

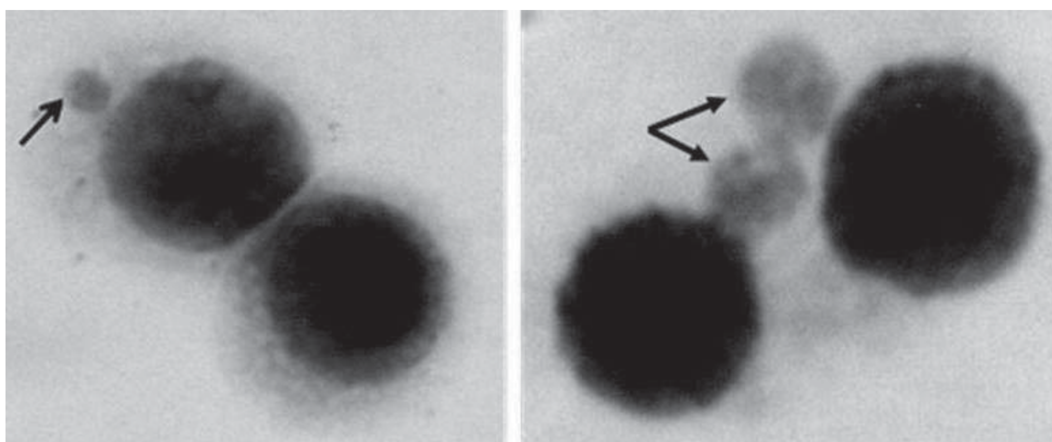

Fig 1: **Human B lymphocytes treated with** *H. pylori* **lysate isolated from a dyspeptic patient. The micronuclei produced in the binucleus B cells are shown by arrows (400x).**

damage on L5178Y mouse lymphoma cells treated with the lysate of the cagA-positive *H. pylori* strain 6650. The results showed that *H. pylori* lysate without the involvement of carcinogen materials created chromosomal breakage. When the protein concentration was 20 μg/mL, the frequency of micronuclei in the binucleus cells was 3.00 to 3.8-times more than controls; higher concentrations of protein caused cell death without the formation of micronuclei(17). A case-control study was also performed on peripheral blood lymphocytes of patients infected with *H. pylori*. The overall frequency of binucleated micronucleated cells per 1000 cells was higher (17.65 ± 1.55) than in the controls (7.39 ± 0.66), which has shown a significant association between *H. pylori* infection and the rate of cytogenetic damage in the cells from infected hosts(15). The difference in micronuclei formation among the recent studies, including our study, could be due to differences in study design, genotype of *H. pylori* isolates, and cell-types at the intra-/interspecific levels. It has been demonstrated that the biological impact of CagA is highly influenced by cell-type(11) and its translocation into human B lymphoid cells might lead to the upregulation of Bcl-2 and Bcl-X(L), which prevents apoptosis(12).

Micronucleation has been implicated in the genomic plasticity of tumor cells and apoptosis. It has been suggested that cells bearing micronuclei often undergo abnormal mitoses and produce daughter cells with numerous disorders. These disorders are delivered to descendent cells and typically amplified(22). In this study, observation of micronuclei in the binucleated B cells has indicated that *H. pylori* lysates could strongly cause chromosomal breakage in human B lymphocytes. It could be proposed that the formation of micronuclei

may itself be involved in the development of MALT lymphoma; e.g., if an important gene such as p53(24) is surrounded and removed during micronuclei formation, a transformed cell might develop. These speculations need to be taken into consideration when designing further studies.

The nature of a possible agent(s) in *H. pylori* extracts that causes chromosomal damage in non-human or human cells, particularly human B lymphocytes, has not been distinguished. However, it has been suggested that production of some components by *H. pylori* and their injection into human cells may promote certain biochemical events in these cells(11,12,25). In this regard, Chitcholtan et al. have shown that the small outer membrane vesicles (OMVs) which are continually shed from *H. pylori* may have a direct and potentially carcinogenic effect on epithelial cells. They found that OMVs could influence micronuclei formation, glutathione (GSH) levels, morphology and proliferation of gastric epithelial cells. These researchers have also shown that OMVs isolated from *H. pylori* strain 60190 increased micronuclei formation(25). The role of *H. pylori* in increasing the rate of epithelial turnover in inflamed stomach tissue has been implicated in tumor pathogenesis in *H. pylori*-infected mucosa. When cell proliferation occurs, the frequency of the mutation may be increased by errors in gene replication(26). It has been reported that the rate of cell proliferation of the gastric mucosa is significantly higher in patients with *H. pylori*-positive gastritis compared with those that have *H. pylori*-negative gastritis(27).

Results of the present study provide an evidence of the direct effect of *H. pylori* in genotoxic damage of human B lymphocytes, which might play an important role in the development of abnormal B cells. It is proposed that persistent infection by *H. pylori* in gastric epithelial cells could not only continuously stimulate the immune cells by bacterial antigens, but also directly cause DNA damage in human B lymphocytes from which gastric MALT lymphoma arises. The nature of trapped genes in the micronuclei of human B lymphocytes treated with *H. pylori* lysates is not evident. Thus, isolation and molecular characterization of micronuclei contents could be considered in future studies. Furthermore, identification of genotoxic agent(s) of *H. pylori* will reveal more information regarding the role of bacterium in tumor pathogenesis.

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REFERENCES

1. Suerbaum S, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002;347:1175-86.
2. Watanabe T, Tada M, Nagai H, Sasaki S, Nakao M. Helicobacter pylori infection induces gastric cancer in mongolian gerbils. *Gastroenterology* 1998;115:642-8.
3. Parsonnet J, Hansen S, Rodriguez L, Gelb AB, Warnke RA, Jellum E, et al. Helicobacter pylori infection and gastric lymphoma. *N Engl J Med* 1994;330:1267-71.
4. Fischbach W, Goebeler-Kolve ME, Dragosics B, Greiner A, Stolte M. Long term outcome of patients with gastric marginal zone B cell lymphoma of mucosa associated lymphoid tissue (MALT) following exclusive Helicobacter pylori eradication therapy: experience from a large prospective series. *Gut* 2004;53:34-7.
5. Hong SS, Jung HY, Choi KD, Song HJ, Lee GH, Oh TH, et al. A prospective analysis of low-grade gastric malt lymphoma after Helicobacter pylori eradication. *Helicobacter* 2006;11:569-73.
6. Nakamura S, Matsumoto T, Ye H, Suekane H, Matsumoto H, Yao T, et al. Helicobacter pylori-negative gastric mucosa-associated lymphoid tissue lymphoma: a clinicopathologic and molecular study with reference to antibiotic treatment. *Cancer* 2006;107:2770-8.
7. Nakamura T, Seto M, Tajika M, Kawai H, Yokoi T, Yatabe Y, et al. Clinical features and prognosis of gastric MALT lymphoma with special reference to responsiveness to *H. pylori* eradication and API2-MALT1 status. *Am J Gastroenterol* 2008;103:62-70.
8. Todorovic M, Balint B, Jevtic M, Suvajdzic N, Ceric A, Stamatovic D, et al. Primary gastric mucosa associated lymphoid tissue lymphoma: clinical data predicted treatment outcome. *World J Gastroenterol* 2008;14:2388-93.
9. Zullo A, Hassan C, Cristofari F, Andriani A, De Francesco V, Ierardi E, et al. Effects of Helicobacter pylori eradication on early stage gastric mucosa-associated lymphoid tissue lymphoma. *Clin Gastroenterol Hepatol* 2009;8:105-10.
10. Koulis A, Diss T, Isaacson PG, Dogan A. Characterization of tumor-infiltrating T lymphocytes in B-cell lymphomas of mucosa-associated lymphoid tissue. *Am J Pathol 1*997;151:1353-60.
11. Umehara S, Higashi H, Ohnishi N, Asaka M, Hatakeyama M. Effects of Helicobacter pylori CagA protein on the growth and survival of B lymphocytes, the origin of MALT lymphoma. *Oncogene* 2003;22:8337-42.
12. Lin WC, Tsai HF, Kuo SH, Wu MS, Lin CW, Hsu PI, et al. Translocation of Helicobacter pylori CagA into Human B

lymphocytes, the origin of mucosa-associated lymphoid tissue lymphoma. *Cancer Res* 2010;70:5740-8.

13. Ye H, Liu H, Attygalle A, Wotherspoon AC, Nicholson AG, Charlotte F, et al. Variable frequencies of t(11;18)(q21;q21) in MALT lymphomas of different sites: significant association with CagA strains of *H pylori* in gastric MALT lymphoma. *Blood* 2003;102:1012-8.
14. Koehler CI, Mues MB, Dienes HP, Kriegsmann J, Schirmacher P, Odenthal M. Helicobacter pylori genotyping in gastric adenocarcinoma and MALT lymphoma by multiplex PCR analyses of paraffin wax embedded tissues. *Mol Pathol* 2003;56:36-42.
15. Suarez S, Sueiro RA, Araujo M, Pardo F, Menendez MD, Pardinas MC, et al. Increased frequency of micronuclei in peripheral blood lymphocytes of subjects infected with Helicobacter pylori. *Mutat Res* 2007;626:162-70.
16. Obst B, Wagner S, Sewing KF, Beil W. Helicobacter pylori causes DNA damage in gastric epithelial cells. *Carcinogenesis* 2000;21:1111-5.
17. Schmausser B, Mueller SO, Eck M, Moller M, Muller-Hermelink H, Stopper H. Helicobacter pylori induces DNA damage in vitro. *Cancer Lett* 2000;152:145-9.
18. Bussiere FI, Chaturvedi R, Asim M, Hoek KL, Cheng Y, Gainor J, et al. Low multiplicity of infection of Helicobacter pylori suppresses apoptosis of B lymphocytes. *Cancer Res* 2006;66:6834-42.
19. Latifi-Navid S, Ghorashi SA, Siavoshi F, Linz B, Massarrat S, Khegay T, et al. Ethnic and Geographic Differentiation of Helicobacter pylori within Iran. *PLoS One* 2010;5: e9645.
20. Lu Y, Redlinger TE, Avitia R, Galindo A, Goodman K. Isolation and genotyping of Helicobacter pylori from untreated municipal wastewater. *Appl Environ Microbiol* 2002;68:1436-9.
21. Stopper H, Muller SO. Micronuclei as a biological endpoint for genotoxicity: A minireview. *Toxicol In Vitro* 1997;11:661-7.
22. Utani K, Kohno Y, Okamoto A, Shimizu N. Emergence of micronuclei and their effects on the fate of cells under replication stress. *PLoS One* 2010;5:e10089.
23. Ning T, Ma H, Zhou J. Micronucleus effect induced by Helicobacter pylori on human gastric mucous epithelial membrane. *Zhonghua Yu Fang Yi Xue Za Zhi* 1996;30:139-40.
24. Molchadsky A, Rivlin N, Brosh R, Rotter V, Sarig R. p53 is balancing development, differentiation and de-differentiation to assure cancer prevention. *Carcinogenesis* 2010;31:1501-8.
25. Chitcholtan K, Hampton MB, Keenan JI. Outer membrane vesicles enhance the carcinogenic potential of Helicobacter pylori. *Carcinogenesis* 2008;29:2400-5.
26. Butterworth BE, Goldsworthy TL. The role of cell proliferation in multistage carcinogenesis. *Proc Soc Exp Biol Med* 1991;198:683-7.
27. Cahill RJ, Sant S, Beattie S, Hamilton H, O'Morain C. Helicobacter pylori and increased epithelial cell proliferation: a risk factor for cancer. *Eur J Gastroenterol* 1994;6:1123–7.