Expression of cell-cycle related proteins in Helicobacter pylori gastritis and association with gastric carcinoma

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Helicobacter pylori (H. pylori) infection is associated with changes in epithelial turnover, through their significance of these in gastric carcinogenesis is still controversial. The purpose of this study was to determine the influence of H. pylori infection on cell proliferation and the relation with the cell-cycle regulators, and finally to provide insights into the mechanism by which H. pylori may lead to gastric carcinogenesis.

We investigated Ki-67, p53, p21Wafl/Cip1, cyclin D1 expression in 55 patients with H. pylori gastritis, and compared the results with patients those of non-H. pylori gastritis patients (n=21), gastric adenocarcinoma patients (n=8) and samples with normal gastric mucosa (n=12). Gastric biopsies were histologically evaluated for inflammatory reaction, intestinal metaplasia and atrophy according to the Sydney system. Overexpression of Ki-67, p53, p21Wafl/Cip1 and cyclin D1 was found in H. pylori gastritis patients (32.7%, 10.9%, 20.0% and 7.3%, respectively), whereas only scattered expression in cells in the neck region of the crypts, but no overexpression was found in gastric antral epithelial cells in biopsy specimens from patients with non-H. pylori gastritis and noninflamed mucosa. A significant relationship was found between the grade of H. pylori colonization and Ki-67, p53, p21Wafl/Cip1 and cyclin D1 expression. Expression was significantly higher in patients with intestinal metaplasia with atrophy, whereas no overexpression was found in patients without intestinal metaplasia with atrophy (p=0.05).

H. pylori infection is associated with increased cell proliferation, increased epithelial DNA damage, and atrophy, which might contribute to the development of gastric cancer. Even if the exact mechanism has not been elucidated yet, our results suggest that H. pylori infection acts as a cofactor in gastric carcinogenesis.

Key words: Helicobacter pylori, p53, p21Wafl/Cip1, Ki 67, cyclin D1.

Helicobacter pylori (H. pylori) was first isolated in 1983, and its pathogenic significance has become increasingly recognised since that time. It has been categorized as a group 1 carcinogen in humans by the International Agency for Research on Cancer (IARC) and the World Health Organisation (WHO) [14, 17, 22]. Numerous studies have shown that patients with chronic colonization of the stomach by the H. pylori have up to 6 times higher risk of developing cancer in the distal portion of the stomach. However, many aspects of its mechanism of carcinogenic action still need to be clarified [15, 18].

Multiple cyclins and cyclin-dependent kinases (CDKs) are positive regulators of the progress of the cell cycle. At the same time, there are several distinct protein inhibitors for of CDK, such as p21Wafl/Cip1 and p27. p21Wafl/Cip1 is a critical downstream effector in the p53-specific pathway of growth control in mammalian cells. p53 acts as a molecular policeman, preventing the propagation of genetically damaged cells. Accumulated p53 binds to DNA and stimulates the transcription of several genes that mediate the two major effects of p53, cell-cycle arrest and apoptosis. p53-induced cell-cycle arrest occurs late in the G1 phase and is caused by p53 p53-dependent transcription of the CDK inhibitor p21Wafl/Cip1. The p21Wafl/Cip1 gene inhibits the cyclin/CDK complex and thus prevents the phosphorylation of pRb necessary for cells to enter the S phase. Such a pause in cell cycling is welcome because it gives the cell time to repair DNA damage inflicted by the mutagenic agent. p21Wafl/Cip1 inhibits the activity of cyclin E-CDK2 and cyclin D-CDK4 and prevents cells from entering the S phase. Defects in these regulator mechanisms of cell proliferation are crucial in cell transformation and tumor progression [4,
pression of cyclin D1 has been implicated in the pathogenesis of several types of neoplasias [3].

Chronic H. pylori infection impairs the gastric barrier function and stimulates gastric cell proliferation, which leads to mucosal repair but can also induce cellular DNA damage, the most frequent epiphenomenon of which is the alteration of onco-suppressor genes. The role of these genes has been studied in colon carcinogenesis and, to a lesser extent in gastric carcinogenesis, but their interrelation with H. pylori infection has yet to be determined [18].

We hypothesized that hyperproliferation and DNA damage in gastric epithelial cells may occur in response to infection with H. pylori. Therefore we investigated the role of the tumor suppressor p53 protein during infection with H. pylori. The p53 protein mediates cell cycle arrest by transcriptionally activating the CDK inhibitor p21Wafl/Cip1. Cyclin D1 positively regulates proliferation at the G1 phase of the cell cycle. With this aim, we investigated the immunohistochemical expression of cell cycle regulator proteins, including p21Wafl/Cip1, p53, cyclin D1 and Ki-67, in H. pylori-induced gastritis and compared the results with those of non-H. pylori gastritis patients, gastric adenocarcinoma patients and samples with normal gastric mucosa.

Material and methods

Tissue samples. A total of 96 gastric biopsy specimens were obtained from the antrum of patients, who underwent diagnostic upper gastrointestinal tract endoscopy. They were then subdivided into four groups according to histological features and H. pylori status: 12 controls suffering from functional dyspepsia with normal gastric histology; 21 patients with H. pylori-negative chronic gastritis; 55 patients with H. pylori-positive chronic gastritis; and 8 patients with gastric adenocarcinoma (4 intestinal type and 4 diffuse type). H. pylori status was evaluated by histology and the urea breath test. The scoring system for all antibodies tested was as follows: 0–5%, negative; 5–25%, low positivity; 25–50%, moderate positivity; >50%, high positivity.

Histopathologic diagnosis. Hematoxylin and eosin stains were used for histopathologic diagnosis. Modified Giemsa stain was used for H. pylori identification and double staining with periodic acid-Schiff Alcian Blue at pH 2.5, was performed in order to detecting intestinal metaplasia. The inflammatory reaction, glandular atrophy, and intestinal metaplasia in each biopsy sample were diagnosed and classified according to the updated Sydney system [9]. Colonization was expressed in grades. Grade 1: few bacteria not grouped in foci; grade 2: grouping of bacteria; grade 3: grouping of bacteria and deep colonization of the foveolar gastricae.

Immunohistochemistry. Serial sections from paraaffin-embedded antral biopsy specimens were deparaffinized in xylene and dehydrated through graded concentrations of ethanol. After the blocking of endogenous peroxidase activity with H2O2, the sections were heated in 0.01 mol/l citrate buffer in a microwave pressure cooker for 20 minutes. The slides were allowed to cool to room temperature, and non-specific binding was blocked with normal horse serum for 20 minutes at room temperature. The MIB-1 monoclonal antibody was used for detection of nuclear Ki-67, a marker of proliferating cells (dilution 1:40, DAKO, Hamburg, Germany). For p53 detection, monoclonal antibody which recognizes both wild type and mutant p53 was used (dilution 1:50, DAKO, Hamburg, Germany). For detection of p21, WAF1/(Ab-1) antibody was used (dilution 1:40, DAKO, Hamburg, Germany). Cyclin D1 monoclonal antibody used with 1/40 dilution (DAKO, Hamburg, Germany). The antibodies were incubated overnight at 4 °C in a humidified chamber. The sections were then stained using the avidin-biotin complex (ABC) immunoperoxidase technique employing commercially available reagent (ABC kit, Labvision, Fremont, USA). The sections were counterstained with Mayer’s hematoxylin and mounted with paraform. Human tonsil sections served as positive controls for MIB-1, p53 and cyclin D1. Colonic tissue sections served as a positive control for p21Wafl/Cip1.

The degree of immunopositivity was evaluated semiquantitatively. A total of 400 cells were counted in random fields from representative areas of the lesion, and the immunoreactive cells were assessed and expressed as a percentage. For all markers, only nuclear staining was considered positive. The scoring system for all antibodies tested was as follows: 0–5%, negative; 5–25%, low positivity; 25–50%, moderate positivity; >50%, high positivity.

Statistical analysis. Statistical analyses were performed with the SPSS 9.00 package program for windows. The categorical variables (grade of H. pylori colonization, atrophy, intestinal metaplasia, inflammatory reaction and overexpression of Ki-67, p53, p21Wafl/Cip1, and cyclin D1) were analysed with the χ2 test. The nonparametric Kruskal-Wallis test was used to compare the staining of Ki-67, p53, p21Wafl/Cip1, and cyclin D1. Differences with p values of <0.05 were considered to be statistically significant.

Results

Tables 1, 2 and 3 show the immunohistochemical variables of the population studied. In gastric antral sections
Table 1. Frequency of overexpression of Ki-67, p53, p21\textsuperscript{Waf1/Cip1}, and cyclin D1 in the population studied

<table>
<thead>
<tr>
<th>No of (subjects)</th>
<th>Controls</th>
<th>Non-H. pylori G</th>
<th>H. pylori G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Ki-67</td>
<td>12</td>
<td>21</td>
<td>55</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21\textsuperscript{Waf1/Cip1}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 (32.7%)</td>
<td>6 (10.9%)</td>
<td>11 (20.0%)</td>
<td>4 (7.3%)</td>
</tr>
</tbody>
</table>

Table 2. Staining characteristics of Ki-67, p53, p21\textsuperscript{Waf1/Cip1}, and cyclin D1 in H. pylori gastritis

<table>
<thead>
<tr>
<th>% of D1 positive cells</th>
<th>Ki-67</th>
<th>p53</th>
<th>p21\textsuperscript{Waf1/Cip1}</th>
<th>Cyclin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (0-5%)</td>
<td>37</td>
<td>67.3</td>
<td>49</td>
<td>88.1</td>
</tr>
<tr>
<td>1 (5-25%)</td>
<td>14</td>
<td>25.4</td>
<td>5</td>
<td>9.1</td>
</tr>
<tr>
<td>2 (25-50%)</td>
<td>4</td>
<td>7.3</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>3 (&gt;50%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. Staining characteristics of Ki-67, p53, p21\textsuperscript{Waf1/Cip1}, and cyclin D1 in gastric carcinoma

<table>
<thead>
<tr>
<th>% of D1 positive cells</th>
<th>Ki-67</th>
<th>p53</th>
<th>p21\textsuperscript{Waf1/Cip1}</th>
<th>Cyclin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (0-5%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 (5-25%)</td>
<td>2</td>
<td>25.5</td>
<td>1</td>
<td>12.5</td>
</tr>
<tr>
<td>2 (25-50%)</td>
<td>2</td>
<td>25.0</td>
<td>2</td>
<td>25.0</td>
</tr>
<tr>
<td>3 (&gt;50%)</td>
<td>4</td>
<td>50.0</td>
<td>3</td>
<td>37.5</td>
</tr>
</tbody>
</table>

Overexpression of p53 was found in 10.9% (6 of 55) of patients with H. pylori gastritis. A significant relationship was found between the overexpression of p53 and the degree of chronic inflammation and intestinal metaplasia (p=0.005, p=0.003), but no significant relationship was found between the overexpression of p53 and the grade of H. pylori colonization and the degree of acute inflammation and atrophy (p=0.295, p=0.688, p=0.307). p53 was expressed in 75% of gastric adenocarcinomas. H. pylori density in patients with p53 overexpression (n=6) were as follows: grade 1 colonization in 1 patient, grade 2 colonization in 4 patients, and grade 3 colonization in 1 patient (Fig. 2).

In H. pylori gastritis, overexpression of p21\textsuperscript{Waf1/Cip1} was found in 20.0% (11 of 55) of the patients. A significant relationship was found between the overexpression of p21\textsuperscript{Waf1/Cip1} and the grade of H. pylori colonization (p=0.004). No significant relationship was found between the overexpression of p21\textsuperscript{Waf1/Cip1} and the degree of acute and chronic inflammation, atrophy and intestinal metaplasia (p=0.295, p=0.627, p=0.687, p=0.556). p21\textsuperscript{Waf1/Cip1} was overexpressed in 62.5% of gastric adenocarcinomas. H. pylori densities in patients with p21\textsuperscript{Waf1/Cip1} overexpression (11 patients) were as follows: grade 1 colonization in 1 patient, grade 2 colonization in 10 patients.

Overexpression of cyclin D1 was found in 7.3% (4 of 55)
Figure 2. H. pylori positive chronic gastritis. Moderate nuclear positivity for p53 (double arrows), with H. pylori colonization (arrows). Original magnifications x 200.

Figure 3. Increased nuclear immunoreactivity for cyclin D1 (double arrows) in gastric mucosa, with H. pylori colonization (arrows) in the foveolar spaces. Original magnifications x 200.

Discussion

Helicobacter pylori, by means of receptors on its surface, attaches to the gastric epithelium, and can survive in this state for years [13]. Although H. pylori's role in the development of gastric cancer has not yet been resolved, it is agreed that it is not directly mutagenic. There are various hypotheses concerning gastric cancer caused by H. pylori, among which are that H. pylori's metabolic products lead to the direct transformation in the gastric mucosa, or that increased epithelial turnover related to mucosal damage occurring in association with H. pylori, increasing the risk of DNA damage with the effect of endogenic mutagens and resulting in transformation in the gastric mucosa [18, 21].

In the present study, Ki-67, p53, p21\textsuperscript{Waf1/Cip1}, and cyclin D1 showed no expression in normal gastric epithelium and non-H. pylori gastritis, other than at physiologic levels. Similar results have been obtained in most immunohistochemical studies carried out on this subject [5, 7, 12, 15, 16]. Cell proliferation is an important stage in carcinogen-
expression. In the present study, a significant relationship was found between Ki-67 immunoreactivity and H. pylori density in H. pylori gastritis (p=0.019). The higher sensitivity of proliferated cells to mutagenic factors and the high risk of DNA damage explain the p53 overexpression in our H. pylori gastritis patients. DNA damage is a prognostic indicator for cancer. However, we observed the p53 overexpression in our patients to be independent of H. pylori density. In other studies, it has been asserted that proliferation in the epithelium is a result of chronic gastric damage independent of H. pylori [3, 15, 18]. In non-H. pylori gastritis, while inflammatory infiltration in the lamina propria is mostly lymphomonoctary, in H. pylori gastritis the dominant cells are neutrophilic leucocytes. There is a hypothesis that DNA damage occurring in H. pylori gastritis is the result of mucosal damage occurring due to increased neutrophilic activity [18].

It has been argued that neutrophils are concentrated in areas colonized by H. pylori and, as a result, the increased iNOS and reactive oxygen products in the infiltrated neutrophils may be responsible for oxidative DNA damage in the epithelium and the pathogenesis in the H. pylori-associated lesions [15]. However, in the present study, we found no significant relationship between acute inflammation and p53 overexpression (p=0.933). This contrary result may be related to the Cag strain of H. pylori, sex, dietary habits and genetic factors of patients. Because the number of patients studied for the Cag A gene in this study was very low, we are unable to support this hypothesis directly, but studies carried out on this subject have indicated that preneoplastic and neoplastic gastric lesions tend to be associated with CagA-positive strains. In CagA-positive H. pylori gastritis, increased risk of cancer, atrophy and metaplasia has been found [2, 18, 21]. It has been reported that H. pylori increases the risk of DNA mutation by stimulating growth factors involved in cell proliferation, and that it plays a role in the progression of chronic gastritis to gastric carcinoma [20].

The tumor suppressor protein p53 begins to accumulate in the cell nucleus when there is DNA damage. The p53-associated cell cycle arrest is dependent on the transcriptional activation of p21*Wafl/Cip1* [15]. Perhaps the most important function of p21*Wafl/Cip1* is its arrest of the cell cycle in the G1 phase by inhibiting cyclin-dependent kinases. Defects in p21*Wafl/Cip1* functions cause the impairment of the cycle process [3, 4, 25]. In the present study, p21*Wafl/Cip1* overexpression was observed in 60% (3/5) of patients with p53 overexpression. In contrast, p21*Wafl/Cip1* overexpression was determined in 16.3% (8/49) of patients without p53 overexpression. In the present study, we found p53 and p21*Wafl/Cip1* overexpressions in H. pylori gastritis to be significantly higher in cases with intestinal metaplasia and atrophy. Perhaps the chance of genomic instability increases along with H. pylori gastritis and atrophy [18]. It has been reported that p53 overexpression was found only in patients with intestinal metaplasia and dysplasia [15, 26]. On the other hand, in general we observed a negative relationship between p21*Wafl/Cip1* and Ki-67 expression, although it was not statistically significant (p=0.095). It has been shown that atrophy in the gastric epithelium and intestinal metaplasia disappeared generally after H. pylori therapy, but that the DNA damage occurring in the atrophic epithelium did not disappear. This explains why atrophic gastritis is a risk factor in gastric carcinogenesis [18, 21, 26].

In the present study, cyclin D1 overexpression was found to be 7.3% in H. pylori gastritis and, in contrast, 62.3% in gastric adenocarcinoma. Cyclin D1, positively regulates the proliferation. Because its derangement can lead to the tumor characteristic finding of uncontrolled cell proliferation, cyclin D1 is considered to be a protooncogene [19]. Cyclin D1 overexpression has been reported to occur at a rate of 22% in gastric cancers [3].

Despite the large number of hypotheses, the mechanisms of the progression from chronic H. pylori gastritis to cancer have not been established. However, H. pylori’s colonization of the antrum is closely related to the location of 50–80% of gastric cancers in the antral and pyloric regions [13]. Recent research has been concentrated on host response to H. pylori in the development of H. pylori gastritis and gastric carcinogenesis [1, 21]. Further searches on bacterial genus of H. pylori and the host response to H. pylori may provide insights into the role of H. pylori in gastric carcinogenesis.

References

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