Alterations in the Proliferating Compartment of Gastric Mucosa during *Helicobacter Pylori* Infection: The Putative Role of Epithelial Cells Expressing p27^{Kip1}

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The proliferating zone contains stem cells that give rise to all epithelial cells of the gastric mucosa. In the present study, we investigated the turnover of gastric epithelial cells in the proliferating zone of *Helicobacter pylori*-infected mucosa, with or without intestinal metaplasia, before and after eradication of the microorganism. In addition, we studied the topographical distribution of the cyclin dependent kinase inhibitor p27^{Kip1}, which plays a critical role in cell cycle progression and differentiation programs. Twenty-eight patients (22 male), aged 32–78 years and with dyspeptic symptoms, were endoscoped, and gastric biopsies were obtained from antrum and corpus for histopathological examination and the Campylobacter-like organisms test; eradication therapy was given to infected patients, and all patients were re-endoscoped after 105 ± 33 days (mean ± SD). The kinetics of gastric epithelial cells and p27^{Kip1} status was assessed by means of immunohistochemistry and TUNEL (Tdt-mediated dUTP-biotin nick end labeling) assay. Twenty-one (21) of 28 patients were *H. pylori* positive, and 7 were found *H. pylori* negative and served as controls. In antrum, intestinal metaplasia was detected in 7/21 (33.3%). In *H. pylori* gastritis, Ki67 expression was found increased in the proliferating

zone, compared with normal ($P = .03$); analogous results were obtained with the other proliferation markers, namely retinoblastoma protein and topoisomerase II α . An inverse relationship between proliferation index and atrophy was disclosed ($P = .02$). A reduction in the proliferation index was observed after eradication, albeit not significant. Apoptotic epithelial cells were found significantly increased ($P < .01$) in *H. pylori* gastritis, and a significant reduction was observed after eradication ($P < .01$). In addition, apoptotic index was found to correlate with *H. pylori* density. The topographical study of p27^{Kip1} revealed a p27^{Kip1}-positive epithelial cell population that resided deep in the proliferating zone; these cells were considered to be stem cells and were found significantly increased in areas with intestinal metaplasia ($P < .05$); in *H. pylori* gastritis, there was also an increase that did not reach statistical significance. *H. pylori* infection induces apoptosis and increases proliferation in the proliferating zone. The increased cellular turnover, together with the increased number of putative p27^{Kip1}-positive stem cells in the context of intestinal metaplasia, provides further evidence for the role of *H. pylori* infection in gastric carcinogenesis.

KEY WORDS: Apoptosis, Gastritis, *Helicobacter pylori*, p27^{Kip1}, Proliferation.

Mod Pathol 2003;16(11):1076–1085

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VOL. 16, NO. 11, P. 1076, 2003 Printed in the U.S.A.

Date of acceptance: July 21, 2003.

Partly supported by grant 70-3-2945/95ED-1909 PENED/KA from the Ministry of Industry, Energy and Technology, Greece.

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DOI: 10.1097/01.MP.0000093626.15701.76

The discovery of *Helicobacter pylori* has revolutionized our thinking on the etiology of peptic ulcer disease (1), MALT lymphoma of the stomach (2), and gastric adenocarcinoma (3).

H. pylori colonization of the human gastric mucosa induces a specific inflammatory tissue reaction described as *chronic active gastritis* (4), the

common pathophysiologic denominator of all the above-mentioned diseases. Chronic active gastritis, under the influence of environmental and intrinsic factors, may lead to chronic atrophic gastritis and intestinal metaplasia, which are considered precancerous lesions according to the multistep model of gastric carcinogenesis (5). Although the sequence of events that leads to these lesions is not completely understood, intestinal metaplasia can be viewed as the product of an altered differentiation program that is adopted by the epithelial cells to gain a survival advantage over the original epithelium (6). This modified differentiation program is more likely to be adopted and executed by the gastric epithelial stem cells in an attempt to react to the adverse environment induced by the infection (7). Stem cells are poorly characterized, multipotent cells that are located in the isthmus region of the gastric pit and give rise, through intermediate daughter cells, to the whole epithelial cell population of the gastric mucosa. Divergent behavior of these cells, as expressed mainly by alterations in their turnover rate, probably has a great impact on gastric histopathology (8).

Increased cellular turnover has been linked to cancer development in many epithelial tissues in diseases in which inflammation plays a pivotal role, such as inflammatory bowel disease and colon carcinomas (9, 10), autoimmune (Hashimoto's) thyroiditis and papillary thyroid carcinomas (11), chronic pancreatitis and pancreatic cancer (12, 13), chronic viral hepatitis, and hepatocellular carcinoma (14, 15). Previous studies have shown increased turnover of gastric epithelial cells during *H. pylori* infection; both proliferation (16) and apoptosis (17) were found to be increased in the infected mucosa and connected to the development of atrophy (18, 19) and intestinal metaplasia (20). However, detailed analysis of gastric epithelial cell turnover in the discrete gastric pit zones (Zone 1 = foveolar (surface) epithelium, Zone 2 = isthmus (neck), and Zone 3 = glands) has not been addressed so far.

In an attempt to shed more light on *H. pylori*-related gastric epithelial alterations, we investigated the turnover of gastric epithelial cells in each discrete gastric pit zone of *H. pylori*-infected mucosa, with or without intestinal metaplasia, before and after eradication of the microorganism. We particularly focused our analysis on the proliferating compartment of the antral mucosa for two reasons: first, it has been demonstrated that *H. pylori* induces more intense and active inflammation in this part of the stomach, and second, the infection has been mainly linked to the development of distal (antral) adenocarcinoma (21). In addition, we studied the topographical distribution of the cyclin dependent kinase inhibitor p27^{Kip1}, which plays a crit-

ical role in cell cycle progression and differentiation programs.

MATERIALS AND METHODS

Study Design

A total of 130 patients with dyspeptic symptoms were screened for possible enrollment in this prospective study, and 80 of them were found to be eligible. We finally studied gastric mucosa tissue specimens from 28 patients (22 males, 6 females), aged between 32 and 78 years (53.3 ± 13.9 years (mean $\pm$ SD), who attended for a follow-up endoscopy approximately 3 months after their initial visit.

Patients were included if they were tested *H. pylori* positive or negative by both used methods, namely the CLO (Campylobacter-like organisms) test and histologic identification of the microorganism. Patients with peptic ulcer disease who tested *H. pylori* negative and those who reported any eradication therapy or any previous gastric surgery were excluded. Patients who reported any anti-ulcer therapy or any antibiotic or bismuth salt therapy during the previous 4 weeks were also excluded. Nonsteroid anti-inflammatory drug or aspirin users, patients on corticosteroids or any immunosuppressive therapy, pregnant women, women of reproductive age who did not take efficient contraception, and patients suffering from severe disease of any kind were not included in the study.

The study was performed in accordance with the declaration of Helsinki. The protocol was fully explained to the patients, and consent was obtained from all of them.

All patients underwent upper gastrointestinal endoscopy, and six biopsies were taken as follows: two biopsies, one from the antrum and one from the corpus for CLO test; two from the antrum (anterior and posterior wall); and two from the corpus (anterior and posterior wall) that were immediately fixed in formalin 10% for histologic and immunohistochemical evaluation. The patients who tested positive for *H. pylori* were given triple eradication therapy consisting of omeprazole (20 mg bid) plus amoxicillin (1 g bid) plus clarithromycin (500 mg bid) for 7 days. Omeprazole (20 mg qid) was given for an additional 3 weeks after eradication therapy. No anti-ulcer therapy was given to the patients who tested negative for *H. pylori*. The second endoscopy was performed on all patients 105 ± 33 days after the initial examination. Tissue specimens were obtained again and processed in the same way. All biopsy specimens from the initial and follow-up endoscopies were assessed simultaneously for the immunohistochemical parameters to avoid dis-

crepancies in evaluation due to different laboratory conditions.

Proliferation was assessed with three cell cycle-specific markers, namely Ki67, retinoblastoma protein (pRb), and topoisomerase II α , to better define the width of the proliferation zone, whereas apoptosis was evaluated with the TUNEL assay. After evaluating gastric epithelial cell turnover, we investigated p27^{Kip1} topographical distribution.

Histopathology

Formalin-fixed, paraffin-embedded tissue samples were routinely cut at 3–4 μ m and stained with hematoxylin and eosin, Alcian blue (pH = 2.5), and Giemsa. Only well-oriented mucosal specimens were evaluated, and specimens were classified independently by three pathologists (PGF, VGG, and PD) who were unaware of the corresponding clinical and endoscopic findings. Re-examination and consensus resolved differences in their independent reports.

Gastritis parameters (chronic inflammation, activity, intestinal metaplasia, gland atrophy, and *H. pylori* density) were graded semiquantitatively on a scale of 0 to 3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe), using a standard visual analogue scale, according to previously reported criteria (updated Sydney System) (22).

Immunohistochemistry

Antibodies

For immunohistochemical analysis the following antibodies were used:

Anti-Ki67 (mouse monoclonal, IgG1, Clone MIB-1; Oncogene Science, 1/50 dilution), anti-pRb (mouse monoclonal, IgG1, Clone LM95.1; Calbiochem, 1/50 dilution), anti-topoisomerase II α (mouse monoclonal, IgG1, Clone SWT3D1; DAKO, 1/250 dilution), and anti-p27^{Kip1} (mouse monoclonal, IgG1, Clone SX53G8; DAKO, 1/100 dilution).

Immunohistochemistry was performed on paraffin sections on poly-L-lysine (Sigma Chemical Co., St. Louis, MO)-pretreated slides, by applying the indirect streptavidin-biotin-alkaline phosphatase method, as we have described elsewhere (23). Unmasking of the studied proteins was carried out with the heat-mediated antigen retrieval method (microwave treatment in citrate buffer at pH 6 for 20 minutes), before blocking serum application.

For negative control, the same immunohistochemical procedure was followed, replacing the primary antibody either with Tris buffered saline or with an irrelevant antibody of the same animal and isotype.

Evaluation and quantification

For scoring, only nuclear immunopositivity for all the tested proteins was considered as evidence of expression of the molecule. There was some variation in nuclear staining intensity, and therefore all positively stained nuclei, regardless of color intensity, were scored as positive. For counting, the gastric mucosa was divided into three zones: Zone 1, surface and upper one third of gastric pit; Zone 2, the other two thirds of gastric pit; and Zone 3, gastric glands. In each zone, ≥ 500 cells were counted; the number of positively stained nuclei was expressed as the percentage of positive nuclei to the total number of counted nuclei and was defined as labeling index. From this point onward, the Ki-67 labeling index will be referred to as the *proliferation index*.

Inter- and intraobserver differences were $< 5\%$, and only in some discordant cases, re-evaluation was performed with a multiheaded microscope.

Tdt-Mediated dUTP-Biotin Nick End Labeling

Method

Double-strand DNA breaks were detected by TUNEL according to Gavrieli *et al* (24). Briefly, 5- μ m paraffin sections were mounted on poly-L-lysine-coated slides, dewaxed, and rehydrated in alcohol gradient. Pretreatment was carried out by incubating the sections with Proteinase K (Sigma, Greece; 20 μ g/mL) for 15 minutes at 37° C. The labeling step was performed with TdT (terminal deoxytransferase; 15 U per slide; New England Biolabs, Bioline, Greece) for 1 hour at 37° C in 25 mM Tris-HCl, pH 7.2, 200 mM potassium cocodylate, 0.25 mM CoCl₂, 250 mg/mL of BSA, and 24 μ M biotin-dATP (Life Technologies, AntiSel, Greece). Reaction was stopped by rinsing the sections in 20 mM EDTA. This was followed by 30 minutes' incubation in StreptABComplex/AP (DAKO, Kalifronas, Greece). For color development, we used Fast Red (Sigma) as chromogen and hematoxylin Mayer as counterstain.

Human tonsil tissue and tissue sections incubated with DNAase I before treatment with TdT were used as positive controls; sections incubated in TdT buffer omitting the terminal deoxytransferase served as negative controls. Apoptotic events in germinal centers of secondary lymphoid follicles, in some cases of gastritis, were also used as internal positive control.

Evaluation and quantification

Cells were considered to undergo apoptosis when apoptotic bodies' staining, without cytoplasmic background, was observed. As in the evaluation and quantification of immunohistochemical results, ap-

optotic index was calculated per zone, and the results are expressed as the percentage of TUNEL-positive apoptotic bodies to the total counted cells (≥ 500 were evaluated in each zone). Two TUNEL-positive apoptotic bodies were considered as belonging to different cells if their distance was more than the diameter of an epithelial cell. Slide examination was performed by three independent observers (PGF, VGG, and PD), and interobserver variability was minimal ($< 5\%$).

Statistical Analysis

The nonparametric Wilcoxon tests for paired and unpaired measurements and Spearman's rank correlation coefficient test were used as appropriate. In addition, the Kruskal-Wallis test was used for intergroup comparisons (more than two groups), followed by Dunn's multiple comparisons test (computer program: Graphpad Prism, Version 2.01; Graphpad Software Inc. CA).

RESULTS

Of the 28 patients, 21 were *H. pylori* positive. Of them, 17 had duodenal ulcer; 3, duodenal and gastric ulcer; and 1 had only gastric ulcer. Seven patients were *H. pylori* negative and served as controls. Patients and controls did not differ in age. *H. pylori* was successfully eradicated in 15/21 patients.

Histopathology Findings

Infected patients showed significantly greater chronic inflammation and *H. pylori* density in the gastric antrum than corpus (Wilcoxon: $P = .008$, $P = .01$, respectively). Activity was also more pronounced in the antrum but did not reach statistical significance in this group of patients ($P = .06$). However, significant correlation between the activity of gastritis and *H. pylori* density was found (Spearman, antrum: $P = .008$, corpus: $P = .0006$).

In the antrum, atrophy was detected in 8/21 (38.1%, all cases were graded as mild) and intestinal metaplasia in 7/21 (33.3%). In the corpus, only two

patients had mild atrophy, and one had intestinal metaplasia.

After eradication, significant reduction of chronic inflammation both in antrum and corpus was observed (Wilcoxon: $P < .001$, $P < .001$, respectively). Activity was also significantly decreased (Wilcoxon: antrum, $P = .002$; corpus, no activity after eradication). No changes in prevalence of atrophy and intestinal metaplasia were observed during the follow-up period.

Gastric Epithelial Cell Kinetics

Proliferation status

In normal mucosa, Ki67-positive cells were restricted in Zone 2 (Table 1) without any stained nucleus in Zones 1 and 3. In *H. pylori*-inflamed mucosa, there is a significant increase of positively stained nuclei in Zone 2 (Wilcoxon, $P = .03$) with an increase in the width of the zone, which is defined by Ki67-positive cells, due to an upward extension of the proliferation compartment (Fig. 1A). After eradication, the proliferation index was reduced but not significantly (Wilcoxon, $P = .09$; Fig. 1B). The proliferation index in Zone 2, in antrum, was found inversely related to atrophy; this was confirmed by both proliferation markers used, namely Ki-67 (Wilcoxon, $P = .02$) and pRb (Wilcoxon, $P = .067$; Fig. 2). No correlation with the other gastritis parameters was disclosed.

In normal mucosa, pRb(+) cells were located in Zone 2; the staining was almost continuous, starting from the lower border of Zone 2 and upwards. Furthermore, a significant correlation between the pRb- and Ki67-labeling indices was found (Spearman, $r_s = 0.81$, $P = .01$). In *H. pylori* gastritis, the area of pRb-stained cells was enlarged toward the surface; the number of pRb-positive cells was also markedly increased. After eradication of the microorganism, a reduction, although not a statistically significant one, was observed (Table 1).

To further investigate the extent of the proliferation zone in gastric mucosa and better verify its borders, we studied the expression of topo-

TABLE 1. Measurements Refer to the Proliferation Compartment (zone 2) of the Antrum: Gastric Epithelial Cell Kinetics (PI% and AI%) and the Percentage (LI%) of pRb and p27^{Kip1} Positive Cells in Normal and *Helicobacter pylori*-Infected Mucosa, Before and After Eradication of the Microorganism

	Ki67 (PI%)	TUNEL (AI%)	pRb (LI%)	p27 ^{Kip1} (LI%)
Normal mucosa	5.16 $\pm$ 0.79	0.01 $\pm$ 0.04	8.99 $\pm$ 2.6	0.82 $\pm$ 0.03
<i>H. pylori</i> gastritis	13.78 $\pm$ 6.5	0.86 $\pm$ 0.745	31.02 $\pm$ 12.7	1.68 $\pm$ 0.97
After eradication	8.74 $\pm$ 5.12	0.185 $\pm$ 0.221	22.02 $\pm$ 14.57	0.99 $\pm$ 0.58
	$p_A^* = 0.03$	$p_A = < 0.01$	$p_A = < 0.01$	$p_A = \text{NS}$
	$p_B^{**} = \text{NS}$	$p_B = < 0.01$	$p_B = \text{NS}$	$p_B = \text{NS}$

* p_A values refer to comparisons between normal and *Helicobacter pylori*-infected mucosa.

** p_B values refer to comparisons in *Helicobacter pylori*-infected patients before and after eradication.

PI, proliferation index; AI, apoptotic index; LI, labeling index; NS, not significant.

FIGURE 1. Ki67 expression in *Helicobacter pylori*-infected mucosa (A) and after eradication of the microorganism (B; 100 $\times$; from the same patient).

FIGURE 2. An inverse relationship between atrophy and the proliferation index of the antral proliferating zone was observed. As shown, Ki-67 was found significantly reduced in the atrophic mucosa (*Wilcoxon, $P = .02$); pRb had the same trend, although the difference in staining between nonatrophic and atrophic mucosa did not reach statistical significance (**Wilcoxon, $P = .067$). Results are expressed as mean $\pm$ SE.

isomerase II α , a clear S to G2/M phase marker, in some of the biopsies. The width of the topoisomerase II α -positive compartment was similar to that of the Ki67-positive compartment, although fewer cells were stained positive with the topoisomerase II α .

Apoptotic status

In normal mucosa, TUNEL-positive apoptotic bodies were observed only in superficial cells (Zone 1), and no apoptosis was detected in foveolar and glandular epithelium (Zones 2 and 3). In *H. pylori* gastritis, the apoptotic index was increased in Zone 1, both in antrum (Wilcoxon, $P = .0005$) and corpus compared with normal; however, in corpus, the increase was not statistically significant (Wilcoxon, $P = .15$). Apoptosis was

also observed in Zone 2 and correlated significantly with *H. pylori* density, both in antrum and corpus (Fig. 3). After *H. pylori* eradication, apoptotic index was reduced in Zone 2 (Wilcoxon; antrum: $P = .002$, corpus: $P = .03$), and the degree of apoptosis approached that of normal mucosa (Table 1).

p27^{Kip1} expression

In normal mucosa, nuclear p27^{Kip1} immunoreactivity was restricted in two discrete epithelial cell populations. One population that was invariably p27^{Kip1} positive was the terminally differentiated cells in the superficial epithelium (Zone 1). The staining intensity was weak in these cells compared with in few p27^{Kip1}-positive mature lymphocytes of the lamina propria, which served as internal positive control. The second p27^{Kip1}-positive population was localized in the most

FIGURE 3. Apoptotic index in the proliferation compartment was found to be significantly related to *Helicobacter pylori* density; this was confirmed in both antral (Kruskal-Wallis, $P = .002$; Dunn's, $P = .01$) and corpus (Kruskal-Wallis, $P = .02$; Dunn's, $P < .05$) mucosa.

deep portion (neck zone) of Zone 2, confined in the area of Ki67-positive cells (proliferating zone). The staining was more intense than in superficial cells and equal to lamina propria lymphocytes. No staining was observed by glandular (Zone 3) epithelial cells.

In *H. pylori* gastritis, there was an increase, but not one statistically significant, of p27^{Kip1}-positive cells in Zone 2 (Fig. 4A). No differences were disclosed in the staining pattern of Zones 1 and 3. After *H. pylori* eradication, p27^{Kip1} expression in nonmetaplastic mucosa looks quantitatively and spatially almost like normal mucosa (Fig. 4B), restricted in the the most deep area of

Ki67-positive proliferation compartment. Interestingly, in gastric pits with intestinal metaplasia, a significant increase of p27^{Kip1}-positive cells in the proliferating zone was observed (Fig. 4C, C*, D, and D*) as compared with either normal (Wilcoxon, $P < .05$) or *H. pylori*-infected mucosa without intestinal metaplasia (Wilcoxon, $P < .05$). Moreover, the epithelial cells in the proliferating zone exhibited a mutually exclusive pattern of staining between Ki-67 and p27^{Kip1}, both in *H. pylori* gastritis and metaplastic mucosa (compare Fig. 4C* and D*). In areas of intestinal metaplasia, the staining pattern remained the same during the follow-up period.

FIGURE 4. p27^{Kip1} expression in gastric mucosa. **A**, p27^{Kip1} expression in the neck region epithelial cells in a case of *Helicobacter pylori* gastritis (p27^{Kip1}, PreHP; 400×). **B**, p27^{Kip1} expression in the antral neck area, after *H. pylori* eradication (p27^{Kip1}, PostHP; 400×). **C** and **C***: p27^{Kip1} expression in a case with intestinal metaplasia (p27^{Kip1}, IM; **C**, 100×; **C***, 400×). **D** and **D***: Ki67 expression in serial sections of the same case with intestinal metaplasia (Ki67, IM; **D**, 100×; **D***, 400×). (The dotted squares in **C** and **D** denote the area that is amplified in **C*** and **D***, respectively). Note the mutually exclusive staining pattern for p27^{Kip1} and Ki67 of metaplastic epithelial cells in the most deep epithelial compartments, comparing **C*** and **D***.

DISCUSSION

We report here that *H. pylori* infection increases the gastric epithelial cell turnover in the proliferation compartment of the gastric mucosa as well as the population of p27^{Kip1}-positive epithelial cells in the proliferation compartment, in areas of *H. pylori*-related intestinal metaplasia.

We observed an increase in epithelial cell apoptosis in *H. pylori* gastritis that likely represents a cellular self-defense mechanism (25) against the hostile milieu generated by the infection. It seems to be the result of *H. pylori*-epithelial interactions because we found a significant relationship between the apoptotic index and the density of *H. pylori*, both in antrum and corpus (Fig. 3). This finding, together with the observed significant reduction of apoptosis in gastric epithelial cells after eradication of the microorganism, suggests that the bacterium itself play an important role in inducing apoptosis in gastric mucosa. Several *in vitro* studies using different *H. pylori* strains have also shown that bacterial factors can directly mediate epithelial apoptosis (26–28). Moreover, *in vivo* studies propose that there is no increase in apoptosis in gastritis of different etiology than *H. pylori* infection (16), suggesting that inflammation *per se* does not cause apoptosis in gastric mucosa.

On the other hand, proliferation, although increased during the infection (Table 1), was not found to be statistically decreased after eradication, in agreement with a recent study by El-Zimaity *et al.* (29), who reported that hyperproliferation of gastric mucosa persists for 3 years after *H. pylori* eradication. Although the reason for the sustained hyperproliferation is not clear, it may represent a counterbalance to the increased apoptotic rate, as suggested by our finding of the inverse relationship between the proliferation index in the proliferating zone and atrophy (Fig. 2). Nevertheless, we must admit the limitations of this analysis that are caused by the known difficulties in estimating gastric atrophy, especially in the context of inflammation (30).

For assessment of proliferation, we performed immunohistochemistry with three different antibodies, namely anti-Ki67, anti-pRb, and anti-topoisomerase II α (31), and we found that all three antibodies are reliable in estimating proliferation in the gastric mucosa.

Given that gastric epithelial cells' turnover is associated with chronic infection and that the latter induces intestinal metaplasia (32), a possible alteration in the gastric epithelial cell differentiation and maturation program may exist in *H. pylori* gastritis. One popular theory suggests that intestinal metaplasia may be the result of an altered differentiation program adopted by stem cells to survive in a hos-

tile environment (6). This is probably achieved through modifications in their gene expression program that favor differentiation along an alternative, but close to "normal" route (6, 7). Based on evidence showing the important function of p27^{Kip1} in many cellular processes, including proliferation, differentiation, and apoptosis (33, 34), we looked for p27^{Kip1} expression in an attempt to investigate the role of this molecule in *H. pylori*-gastritis and in *H. pylori*-associated intestinal metaplasia. Previous studies have shown that loss of p27^{Kip1} expression is associated with aggressive tumor behavior in gastric cancer (35) as well as other epithelial malignancies (36, 37).

We found that expression of p27^{Kip1} was faint on surface epithelial cells and quantitatively invariable between normal and inflamed mucosa; it seemed to be independent of the underlying histologic lesions. This is in agreement with the role of p27^{Kip1} in tissue development and expression by senescent, terminally differentiated cells (38, 39). In the proliferation compartment, we defined two cellular populations: one that is p27^{Kip1} negative and one that is p27^{Kip1} positive. The phenotype of these cells was similar, but they differed in topography. The p27^{Kip1}-positive epithelial cells were located in the deepest area (neck zone) of Zone 2, and their number was found increased, although not significantly, in inflamed mucosa (Fig. 4A). Moreover, we observed a mutually exclusive staining pattern between p27^{Kip1} and Ki67 on those cells. Based on the topographical distribution of p27^{Kip1} in our study and other reports of its expression in various tissues (40–42), we speculate that the p27^{Kip1}-positive population in the neck area represents stem cells, whereas the more superficial Ki67-positive, p27^{Kip1}-negative cells belong to transiently proliferating cells. The constitutive expression of a universal cyclin-dependent kinase inhibitor, like p27^{Kip1}, by neck zone cells is compatible with the main property of stem cells, which is long cell cycle periods in order to control replication quality. Walsh *et al.* (41) have reached a similar conclusion in inflamed colonic mucosa, in which they observed increased p27^{Kip1} immunopositivity in the lowest on third of crypts, suggesting that enhanced expression of p27^{Kip1} by this putative stem cell compartment represents a protective reaction. In accordance with the above observations and suggestions are also the findings of De Marzo and coworkers in the prostate (42).

Although we did not investigate p27^{Kip1} expression in gastritis of other etiologies, we believe that our findings are specific to *H. pylori* infection based on evidence from two recent reports. The first, a study on a transgenic mouse model, has demonstrated that a main effect of *H. pylori* infection was the amplification of the presumptive gastric epithe-

lial stem cells and their immediate committed daughters (43). The second, an *in vitro* study (44), suggests that *H. pylori* secrete a soluble factor or factors of approximately 40 kDa that up-regulate p27^{Kip1} expression in epithelial cells. Those investigators speculate that because bacterial-cell attachment is not required, the factor or factors may exert their effects in noncolonized distant epithelial cells, such as the cells in the proliferating compartment.

An interesting finding of our study was the demonstration of an increased number of p27^{Kip1}-positive cells in areas of intestinal metaplasia. In the absence of a reliable marker for the secure identification of stem cells, one may argue that the observed increase of p27^{Kip1}-positive cells reflects induction of expression of the molecule by transiently proliferating cells. We believe, based on topography (schematic representation in Fig. 5) and the mutually exclusive pattern of staining between p27^{Kip1} and Ki67 in serial sections (Fig. 4C*, D*), that they represent an increase in the number of stem cells that probably accounts for the known enhanced proliferative capacity of the metaplastic epithelium (45). Alternatively, increased expression of p27^{Kip1} may contribute to the altered differenti-

ation program engaged by epithelial cells in the area of metaplasia.

To the best of our knowledge, only two reports have studied p27^{Kip1} expression in normal mucosa and *H. pylori* gastritis. Our results are not fully compatible with the observations reported by Shirin *et al.* (46), who found decreased expression of p27^{Kip1} in the infected mucosa compared with normal. Except from the different antibody used in that study, we can offer two possible explanations for this discrepancy: first, Shirin *et al.* assessed the staining semiquantitatively, and second, they did not perform detailed analysis by dividing the epithelial compartment into zones as we did in our study. In another recently published study (47), p27^{Kip1} was also found to be decreased in *H. pylori* gastritis and in areas of intestinal metaplasia, compared with normal. However, in this study, a polyclonal antibody was used, both nuclear and cytoplasmic staining were counted as expression, and the analysis was not focused in the proliferating compartment.

Taken together (Fig. 5), our data suggest that *H. pylori* induces apoptosis in epithelial cells *in vivo*. Hyperproliferation is probably a compensatory re-

FIGURE 5. Schematic representation of the observed staining pattern for TUNEL assay, Ki67 and p27^{Kip1} antibodies in normal mucosa, *H. pylori* gastritis, and intestinal metaplasia. 1 = Zone 1 (surface epithelium and upper one third of the gastric pit); 2 = Zone 2 (proliferating compartment, the remaining two thirds of gastric pit); 3 = Zone 3 (gastric glands).

sponse of the mucosa, and its relative failure may lead to gastric atrophy. Furthermore, we propose that p27^{Kip1} cells in the proliferation compartment are putative stem cells, and the increased number of these cells in *H. pylori*-associated intestinal metaplasia is in accordance with its characterization as a precancerous lesion. We believe that the metaplastic epithelium, occupied by an increased number of progenitors, is more susceptible to noxious environmental stimuli and that the overall chance of mutation acquisition with oncogenic potential is amplified.

Acknowledgments: The authors thank Prof. H.M. Moutsopoulos, Head of the Department of Pathophysiology, University of Athens, Medical School, for his suggestions and comments. They also thank Professor D.A. Antonioli at Beth Israel Deaconess Medical Center, Harvard Medical School, for critical review of the manuscript. The authors are grateful to Ms. Maria Koi and Ms. Peggie Andriopoulou for their valuable technical assistance and to Ms. Pinelopi Tsiara, who produced all the photomicrographs in this article.

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