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EFFECTS OF *H. PYLORI* INFECTION OF GASTRIC EPITHELIAL CELLS ON CELL CYCLE CONTROL

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1. ABSTRACT

Chronic infection of the gastric mucosa by the bacterium *H. pylori* results in an intense inflammatory response which can last for decades. An associated host response is a chronic hyperproliferative state, in which there is increased cell turnover and also increased apoptosis of the gastric epithelial cells. Recent studies have also demonstrated abnormalities in the expression of cell cycle control proteins. This review describes these events, emphasizing recent studies on the effects of *H. pylori* infection on cell cycle progression and the expression of cell cycle regulatory proteins. The systems that have been studied include *in vivo* studies in humans and in experimental animals, and *in vitro* studies in which gastric epithelial cells were co-cultivated with *H. pylori.* The earliest event following *H. pylori*'s interaction with epithelial cells appears to be growth inhibition and apoptosis. The hyperproliferative response observed in the gastric mucosa is secondary to this initial insult and is associated with increased expression of cyclin D1, the cyclin dependent kinase inhibitor $p16^{ink4a}$ and of p53 and decreased expression of the cyclin dependent kinase inhibitor $p27^{kip1}$. Dysregulation of the hyperproliferative response may, ultimately, be responsible for the ability of *H. pylori* to enhance the development of gastric cancer.

2. GENERAL INTRODUCTION

Chronic infection of the stomach by *H. pylori* can result in a wide spectrum of clinical outcomes, from completely asymptomatic events to fatal malignancy. This diversity of clinical outcomes is mirrored by a broad range of histological changes - including infiltration of the

mucosa with inflammatory cells without any obvious changes in epithelial morphology (chronic gastritis), loss of mucosal mass (ulcers, atrophy), and pre-neoplastic and neoplastic changes in the gastric epithelium and in infiltrating lymphocytes associated with the development of masses (hyperplastic polyps, adenomas, carcinomas and lymphomas). The consequences of infection in any one individual are dependent on both *H. pylori* virulence characteristics and the genetic and environmental makeup of the individual infected. With such a wide range of possible outcomes it is perhaps not surprising that the interaction of *H. pylori* with the gastric epithelium can result in the activation of numerous pathways. In investigating the effect of *H. pylori* on the gastric epithelium it is clear that *H. pylori* can influence the replication of epithelial cells (proliferation) and also the death of these cells (by apoptosis). However, most of the lifespan of the gastric epithelial cell is spent traversing the cell cycle, which is normally highly controlled by the activity of specific cell cycle regulators. There has been considerable efforts in recent years to investigate the effect of *H. pylori* on epithelial proliferation and apoptosis, but in trying to understand malignancy, it may be equally important (if not more so) to focus on abnormal cycling, unrelated to cell birth or death. We shall therefore review the effects of *H. pylori* on not only proliferation and on the large body of work which has accumulated regarding the effects of *H. pylori* on apoptosis, but also the relatively uncharted waters of the effects of *H. pylori* on the gastric epithelial cell cycle.

3. CELL CYCLE CONTROL

The process of multistage carcinogenesis is accompanied by the progressive accumulation of mutations together with underlying deregulation of the normal cell cycle. In addition to abnormal expression of cellular oncogenes and tumor suppressor genes during cancer development, many tumors display abormalities in expression and function of specific cell cycle regulatory proteins, suggesting that these proteins are critical targets during carcinogenesis (1). We will first review how the cell cycle is normally regulated, and then consider the effects of *H. pylori* on cell cycle regulation and the relationship of these abnormalities to cell proliferation, apoptosis and the development of gastric cancer.

3.1. The Cell Cycle

The cell cycle is the set of events that is responsible for the duplication of the cell. It is typically subdivided into four phases: G1, a phase during which the cell prepares to synthesize DNA; S, a period of DNA synthesis; G2, a period in which preparations are made for cell division; and M, the mitotic phase itself. Progression through the cell cycle is regulated by several families of cyclin-dependent kinases (CDKs) which are the engine of the cell cycle machinery. At specific stages of the cell cycle CDK activity is regulated positively by cyclins that bind to CDKs to form active cyclin-CDK complexes, which lead eventually to DNA replication and cell division. In normal cells the proliferative effects of these cyclins are opposed by another family of proteins called cyclin dependent kinase inhibitors (CDIs), thus providing a homeostatic control mechanism (2).

The transitions between different phases of the cell cycle are regulated at specific checkpoints. The three major cell cycle control checkpoints are the G1/S, the G2/M and the spindle checkpoint. One of the important checkpoints, where most cancer-related defects occur, is the restriction point in late G1, at which the cell commits itself to another round of DNA replication and at which both positive and negative external signals are integrated into the cell cycle. When hypophosphorylated, the product of the retinoblastoma (Rb) gene blocks the G1/S transition by binding to the members of the transcription factor family, E2F, thereby inactivating them (3) (4). Hyperphosphorylation of the Rb protein and the other members of this “pocket” family of proteins (p107 and p130) during the G1 phase, by cyclin/CDK complexes, inactivates this inhibitory function, thus permitting progression through the cell cycle (5). The deregulation of this checkpoint can result either from overexpression of the positive regulators, such as the cyclins and CDKs, or from the loss of expression of the negative regulators, including the Rb protein and specific CDIs. Indeed, there is accumulating data for mutations and abnormalities in the expression of various cyclins, CDKs and CDIs in several types of human cancers (6).

3.2. Cyclin-Dependent Kinases

Mammalian cells contain multiple CDK genes that are a family of serine/threonine kinases. They are numbered CDK1 (also termed Cdc2 in yeast) through CDK8. Specific cyclin-CDK complexes are assembled and activated to regulate the progression through different phases of the cell cycle. Current evidence indicates that cyclin D (D1, D2, or D3) complexed to CDK4 or CDK6 regulates the transition in mid to late G1. The cyclin E-CDK2 complex regulates the G1-S transition, and the cyclin A-CDK2 complex is needed for progression through S phase. Cyclin A-CDK1 is required for the G2-M transition, but cyclin B-CDK1 is the main effector at this step (1). Figure 1 shows the major cyclin-CDK complexes that act at each phase of the cell cycle.

3.3. Cyclins

Based on their patterns of expression and functional roles during the cell cycle in mammalian cells, cyclins can be categorized as:

3.3.1. G1 cyclins

The major G1 cyclins are D1, and E. The three D-type cyclins (D1, D2, and D3) and cyclin E are rate-limiting controllers of the G1 phase progression in mammalian cells (7). Cyclin E assembles with CDK2 and peaks sharply at the G1/S transition, suggesting that it controls the ability of cells to enter the S phase. The human cyclin D1 gene, probably the most important G1 phase cyclin, was first cloned through its ability to complement a *Saccharomyces cerevisiae* strain that was mutant in all three of the known yeast G1 cyclins (8). Cyclin D1 antibodies arrest cells in G1, thus directly demonstrating the role of of this gene in proliferation. Evidence for the

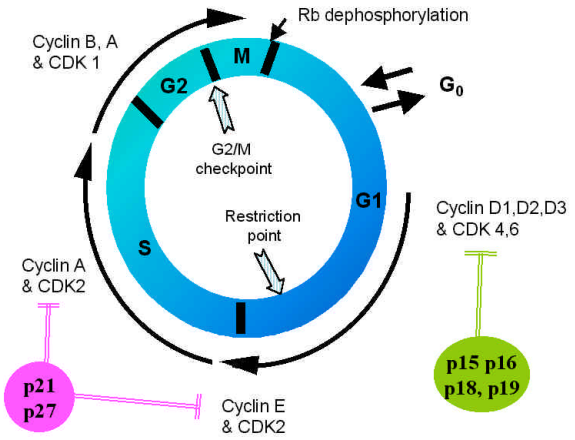

Figure 1. The normal cell cycle, indicating the roles of specific cyclins, CDKS, and CDIs, the restriction point at the G1 transition which is controlled by Rb phosphorylation and the G2/M checkpoint which is controlled by CDC25. For additional details see text.

oncogenic properties of deregulated cyclin D1 expression is provided by the analysis of several model systems, in which increased expression of cyclin D1 enhances malignant cell transformation (9). In continously proliferating cells the D cyclins are expressed throughout the cell cycle and their levels oscillate only minimally. However, when growth factor deprived cells are restimulated to enter the cell cycle, D-type cyclins peak in mid to late G1, earlier than cyclin E. Cyclin D1 associates with several CDKs, including CDKs 1, 2, 4, 5, and 6, but thus far only the complexes of cyclin D1-CDK4 and cyclin D1-CDK6 display enzymatic activities. Although cyclin D synthesis begins during the G0 to G1 transition, the associated kinase activity is not manifested until mid-G1 and increases as cells approach the G1/S boundary. Interestingly, in several cancer cell lines and in some human cancers cyclin D overexpression is accompanied by relatively high levels of the growth inhibitory proteins $p27^{kip1}$ and Rb. This finding supports the existence of a homeostatic feedback loop between cyclin D1 and $p27^{kip}$ (10). However, it should also be emphasized that although the cyclins are typically characterized as proliferative cell cycle regulatory proteins, in certain cell lines increased expression of cyclin D can also inhibit growth and induce apoptosis (11).

3.3.2. S-phase cyclins

The synthesis of cyclin A is initiated during late G1 and its kinase activity is first detected in the S phase. Cyclin A binds to at least two CDKs depending on the phase of the cell cycle: a cyclin A-CDK1 complex is involved in the G2/M transition, and a cyclin A-CDK2 complex is involved in the G1/S transition and S phase. Cyclin F also functions to regulate the S-phase.

3.3.3. G2/M phase cyclins

Cyclin A and cyclins B1 and B2 regulate the G2/M transition. The B-type cyclins associate with the CDK1 subunit, and the complexes show maximal protein kinase activity during metaphase. Removal of the inhibitory phosphates from cyclin B-associated CDK1 at the G2/M transition by CDC25 phosphatase activates the kinase and triggers entry into mitosis.

3.4. Cyclin-Dependent Kinase Inhibitors

Since their discovery in the early 1990's, the CDIs have proved to play a critical role in the normal regulation of cell proliferation and also to be important targets during carcinogenesis (12). The mammalian CDIs have been classified into two groups, based on their structural homology and functional similarity. One group includes $p21^{cip1}$ (also called $p21^{waf/cip1}$), $p27^{kip1}$, and $p57^{kip2}$ (13-16). These Cip/Kip inhibitors have broad specificity. They bind to and inhibit the kinase activity of the G1-phase cyclin D-CDK4/6 and cyclin E-CDK2; the S-phase cyclin A-CDK2; and, to a lesser extent, the mitotic cyclin B-CDK1 complexes. The second category of mammalian CDIs includes $p16^{ink4a}$, $p15^{ink4b}$ $p18^{ink4c}$ and $p19^{ink4d}$ (17-20). Ink4 inhibitors are specific for CDK4 and CDK6, compete with cyclin D for binding to these kinases and show no similarity to the Cip/Kip family. Because the CDIs bind to and inhibit the active kinase complexes and exert antiproliferative effects it has been suggested that they also function as tumor suppressor genes and that their loss plays an important role in the development of a variety of human cancers (21). This hypothesis is supported by accumulating evidence from mutant mice with germline inactivating mutations in specific CDIs, and from the decreased expression of CDIs in clinicopathological studies of human tumors. For example, a tumor suppressor role for $p27^{kip1}$ has been demonstrated in hemizygous and homozygous $p27^{kip1}$ deleted mutant mice, which are predisposed to tumors induced by exogenous carcinogens (22). Furthermore, decreased or loss of expression of the $p27^{kip1}$ protein has been found to be a poor prognostic factor in many cancers, including gastric cancer (23,24). $p16^{Ink4a}$ deficient mice are also cancer-prone. They develop spontaneous tumors at an early age, and are highly sensitive to carcinogens (25). Loss of $p16^{ink4a}$ and $p15^{ink4b}$ expression, due to gene mutations, deletion, and/or DNA methylation (26,27) has been also seen in a variety of human tumors, including gastric cancer (28).

3.5. p53

p53 is a multifuctional protein involved in coordinating cellular reponse to injury. The p53 protein is normally maintained at low levels, through an interaction with the Mdm-2 protein. Phosphorylation of either p53 or Mdm-2 prevents the interaction between these two proteins and activates the role of p53 as a transcription factor (29). Activation of p53 results in two well described cell cycle events - cell cycle arrest and apoptosis. Although a significant part of the growth inhibition induced by p53 is mediated through the induction of the CDI $p21^{cip1}$, alternative $p21^{cip1}$-independent pathways have also been described (30).

4.CELL TURNOVER IN THE NORMAL STOMACH

The functional sub-unit of the stomach is the gastric gland, which comprises a straight tubular section

opening into the gastric lumen, and a convoluted and branching lower part at the base of the gland. Cells move away from the proliferative zone, which is located about one third of the way down the gland, in the isthmus, and they differentiate as they migrate up towards the lumen or down towards the base of the gland. Labeling cells with tritiated thymidine and then following their migration over several days has proven that there is normally a bi-directional migration of cells, up to the lumen over about 3-5 days in the mouse (and a little longer in humans) and also down to the base of the gastric gland over 50 days in the mouse and 2-3 months for human parietal cells (31-33). In hyperproliferative states the proliferative zone is expanded longitudinally up and down the gastric gland. Apoptotic cells at the end of their lifespan can be readily observed at the lumen and occasionally at the base of the gastric gland.

The putative stem cell is located in the isthmus and is believed to give rise to all the lineages found within the gastric gland (at least eleven differentiated cell types including both epithelial cells and enteroendocrine cells) as well as renewing its own population (33). The gastric stem cell has still not been precisely identified or characterized further other than by its ultrastructural features as a "granule-free cell" (33). The gastric gland was, until fairly recently, thought to comprise a monoclonal population of cells arising from a single stem cell. However, this idea has lately been challenged by the evidence in both humans and mice that a proportion of gastric glands have the characteristics of polyclonality, particularly in early embryonic life, although monoclonal glands generally populate the gastric mucosa later in life (34,35). The stimuli responsible for activating differentiation and apoptosis as a terminal differentiation event are not precisely known. They probably include time-related gene activation, the interactions of one cell type with its neighbors and signals from the extracellular matrix, as well as circulating locally-acting peptides. It is likely that as the cell reaches the lumen, the relative lack of contact from the extracellular matrix and other cells is partially responsible for activating the apoptotic program (sometimes referred to as anoikis).

5. *H. PYLORI* AND GASTRIC EPITHELIAL PROLIFERATION

5.1. Human Studies

It has long been recognized that chronic inflammation of epithelia leads to a state of hyperproliferation and is often associated with an increased risk of cancer (36). It is hypothesized that the resulting increased cell turnover leads to an increased number of cells carrying possible mutations, and the increased rate of cell turnover may also result in decreased time for cells to undergo surveillance and the elimination of abnormal and potentially neoplastic clones. Prior to the recognition of *H. pylori* as the responsible agent for most cases of chronic gastritis it was well recognized that chronic active gastritis and even chronic atrophic gastritis were hyperproliferative conditions (32,37). These findings have been re-evaluated over the last 10 years by multiple investigators who have documented that stomachs colonized by *H. pylori* have increased numbers and percentages of gastric epithelial cells that express markers of proliferation, when examined by a variety of immunohistochemical techniques. That *H. pylori* is responsible for hyperproliferation has also been shown by a reduction in proliferative indices following successful eradication therapy (reviewed in reference 38). Most studies of this type have examined proliferation in the gastric antrum, although patients with *H. pylori*-associated corpus gastritis have a similar, approximately two-fold, increase in the numbers and percentages of proliferating epithelial cells (39).

It is of interest that although most investigators report a normalization in proliferation within weeks following eradication of *H. pylori*, one study found that whereas increased proliferation returned to normal at about a year following the eradication of *H. pylori* in the corpus, a hyperproliferative state persisted in the antrum even up to three years later (40). Conceivably, the population evaluated in this study may have had other reasons for persistent hyperproliferation, such as treatment with a proton pump inhibitor (41). It is also possible that following an initial reduction in proliferation after *H. pylori* eradication, epithelial turnover remains abnormal. Other studies to date have not followed the long term consequences of *H. pylori* eradication on gastric epithelial cell kinetics.

What are the causes of increased proliferation associated with *H. pylori*? There are several possible contributory factors. These include direct proliferative stimuli from the bacteria and from the inflammatory cells and mediators associated with gastric *H. pylori* colonization. In general, most studies have shown a positive correlation between parameters of epithelial proliferation and gastric inflammation scored by histological criteria, suggesting that inflammation provokes increased proliferation (39, 42-45). Since *H. pylori* strains carrying the *cag* pathogenicity island are known to be associated with a more intense mucosal inflammatory response (46), it would be expected that patients infected with *cag*-positive strains have higher proliferative indices than patients infected predominately by *cag*-negative strains. Indeed, there have been two reports that proliferation is higher in association with *cag*-positive than *cag*-negative colonization (43) (47). However, in a recent study we could not confirm this difference (39). The cause of these discrepant results is not clear. It may be explained by differences between the patient populations studied, including the ethnicities of the study populations. Furthermore, in the first two studies, many patients had either an active peptic ulcer or a history of peptic ulcer disease whereas these patients were specifically excluded in the latter study. The presence of an ulcer might alter the epithelial response - either by local means or through an association with more indirect mechanisms, for example, gastrin (see below). The evidence from *in vitro* models is similarly inconclusive regarding the role of *cag*-associated genes in epithelial cell turnover (see section 6, below). Thus, despite increased understanding of the functions of genes within the *cag* pathogenicity island in the translocation of *H. pylori* products into epithelial cells and

the subsequent alteration of signal transduction and downstream pathways (48), the effect of *cag*-related genes on cell cycle – related events is unclear. Further studies of larger patient groups with different clinical outcomes may be necessary to fully determine whether genes within the *cag* pathogenicity island influence the epithelial proliferative response *in vivo*.

What is the natural history of the hyperproliferative response? An important study of *H. pylori*-infected children (mean age of 15 years) demonstrated that these teenagers had a doubling of their proliferative index, which was associated with increased expression of p53 by immunohistochemistry (49). Thus increased proliferation appears to occur relatively early in the course of an infection which, typically, can last many decades.

5.2. **Animal Models**

Further insights into the natural history and consequences of *Helicobacter* infection on gastric epithelial cell turnover have been recently gained using a variety of animal models. Colonization of C57BL/6 mice with the mouse-adapted "Sydney strain" of *H. pylori* led to hyperproliferation in both the antrum and corpus. This was most severe in mice fed a high salt diet – thus showing the potential importance of dietary co-factors on cell cycle changes (50). The mouse model is particularly attractive for investigating the pathogenesis of *H. pylori* with respect to the functions of specific host genes. For example, loss of function of the APC gene product, which is known to predispose mice to intestinal carcinogenesis, somewhat surprisingly, was associated with an attenuated proliferative response to *Helicobacter felis*, suggesting that APC may paradoxically have pro-proliferative effects in the gastric mucosa. In contrast, heterozygosity at the p53 locus is associated with slightly increased proliferation at 12 months (51). However, at 15 months in this same model a very unexpected "protective" effect of p53 heterozygosity against gastric cancer was recently reported. Thus, whereas some wild type mice developed gastric cancer at this time point, none of the p53 +/- animals did so (52). In a further interesting model, mice lacking functional Fas developed inflammation but did not exhibit increased apoptosis or increased proliferation after inoculation with *H. felis*, thus implicating Fas-mediated pathways in initiating increased cell turnover (*53*).

There has recently been considerable interest in the Mongolian gerbil, as an animal model of gastric carcinogenesis associated with *H. pylori* infection. Initial reports documented that *H. pylori* alone, without any additional co-carcinogen, induced a high incidence of gastric adenocarcinoma in these animals (54) (55). We have had the opportunity to examine the gastric epithelial cell cycle response to *H. pylori* infection in Mongolian gerbils (56). Proliferation increased at about 4 months after experimental infection and then decreased again sharply. Interestingly, this delayed and transient increase in proliferation seen at 4-5 months post infection occurred after an earlier burst of apoptosis, thus supporting the hypothesis that the hyperproliferative response seen in chronic *H. pylori* infection is a secondary and compensatory response to increased programmed cell death (57). Of interest, the proliferative increase observed in Mongolian gerbils was associated with increased serum gastrin concentrations in these animals, most likely secondary to hypochlorhydria (58). Thus, increased gastrin release, as occurs in humans during *H. pylori* infection (59), is a possible mechanisms by which *H. pylori* can stimulate a hyperproliferative response, because raised plasma gastrin levels are known to be trophic to the gastric mucosa and to be associated with gastric mucosal hyperproliferation, in both man and experimental animals. (60). Further evidence supporting an effect of gastrin on proliferation comes from experiments in which rats treated with omeprazole developed markedly increased plasma gastrin and gastric mucosal hyperproliferation (41). Whether or not the moderate hypergastrinemia due to *H. pylori* in humans is responsible for increased epithelial cell proliferation, the combination of *H. pylori*-associated hyperproliferation and hypergastrinemia may be a particularly harmful combination of factors for promoting gastric adenocarcinoma. In another model of experimental *Helicobacter* infection, Wang and co-workers (61) infected *H. felis* into FVB/N mice carrying the rat human gastrin gene under control of the rat insulin promotor. These uninfected mice have increased plasma gastrin levels (initially about twice normal) which rises with age, probably secondary to acid hyposecretion. When infected with *H. felis*, they developed cancers in old age, suggesting a synergism between the effects of a high plasma gastrin concentration and *Helicobacter* infection. Although proliferation was not specifically reported, there were clearly changes in epithelial cell populations including parietal cell loss and expansion of the number of enterochromaffin-like cells, as well as changes in mucosal growth factors which are often associated with epithelial hyperproliferation. However, it should be emphasized that the link between *H. pylori*, gastrin and hyperproliferation may not necessarily be unidirectional. Although it is known that *H. pylori* increases gastrin concentrations, it has also been shown that gastrin can increase the growth of *H. pylori in vitro*, a specific effect not found with cholecystokinin, pentagastrin or epidermal growth factor (62). If gastrin were to act in a similar manner in the gastric lumen, this could set up a positive feedback between increased gastrin release and the infection density of *H. pylori*.

Animal models, such as those described above, may give important clues to the relevance of hyperproliferation to gastric carcinogenesis, and also shed light upon cell cycle changes associated with *H. pylori* infection. *H. pylori* not only infuences the number of cycling epithelial cells, but also alters the relative sub-populations of epithelial cells in the gastric mucosa and, consequently, the topography of *H. pylori* infection itself. For example, in a mouse model, selective ablation of parietal cells induced the overexpression of adhesins for *H. pylori* that are expressed on non-parietal epithelial cell lineages (63). The increased expression of adhesins for *H. pylori* then resulted in more *H. pylori* colonizing the stomach, and a more pronounced mucosal inflammatory response.

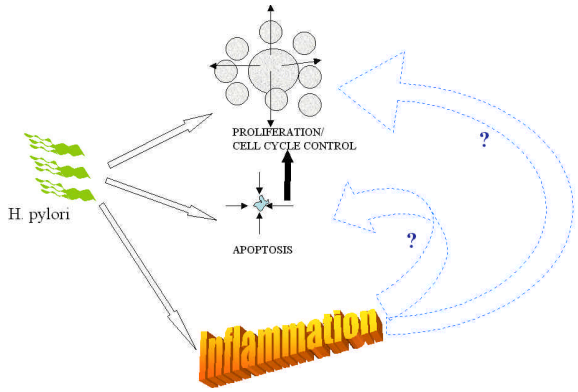

Figure 2. Interrelationship between *H. pylori* bacteria, the associated inflammatory response, cell proliferation, cell cycle control, and apoptosis. The large solid arrow designates a secondary hyperproliferative response.

5.3. *In vitro* Models

In contrast to the association of *H. pylori* infection with increased proliferation in gastric biopsies and in experimental animal models, the addition of *H. pylori* to epithelial cells in culture usually results in reduced growth (see section 6, below). This is due to the predominant induction of apoptosis and the inhibition of cell cycle progression (64-66). While such co-culture models have been used extensively to investigate the mechanisms of apoptosis associated with *H. pylori* infection, they may not be suitable for investigating the molecular mechanisms of the proliferative response. However, although almost all investigators have found that *H. pylori* inhibits proliferation of gastric cells *in vitro*, Fan *et. al.* (67) reported that live *H. pylori* bacteria or sonicates increased the proportion of AGS gastric epithelial cells which stained positively for the proliferation-associated antigen Ki-67. Furthermore, the differential response of gastric cells *in vitro* may be dose dependent, since DNA synthesis has been found, by some investigators, to be increased in response to a low number of *H. pylori* bacteria and decreased with higher number of *H. pylori* (68). Thus, it is conceivable that *H. pylori* may have a dual proliferative/anti-proliferative effect, which is dose-related. However, the majority of evidence to date suggests that *H. pylori* does not directly stimulate epithelial proliferation. Instead, a more indirect effect is likely, for example through the initial induction of apoptosis followed by a hyperproliferative response (consistent with the sequence of changes shown in the Mongolian gerbil (56), or though alterations in local growth factor expression and secretion (69). Since *in vivo* there is a fairly consistent relationship between inflammatory and epithelial proliferative responses to *H. pylori*, it is also conceivable that it is the inflammatory cells and/or mediators associated with *H. pylori* infection, rather than the bacteria themselves, which stimulate epithelial hyperproliferation. This could occur through epithelial cell death and a subsequent hyperproliferative response, or via direct epithelial cell proliferative stimuli (see **figure 2**). There is experimental evidence that links inflammatory cells and cytokines with increased proliferation in many cell systems (70), but whether the inflammatory response associated with *H. pylori* does so has not been extensively investigated.

6. *H. PYLORI* AND GASTRIC EPITHELIAL APOPTOSIS

6.1. Human studies

Numerous studies have demonstrated that *H. pylori* increases the apoptosis of gastric epithelial cells *in vivo* (39, 43,47, 49, 71-79) and only two reports are at variance with these findings (45,80). Increased apoptosis associated with *H. pylori* infection has been shown in a variety of different patient groups, including children (49) and in adults both with and without peptic ulcer disease. Although almost all of these studies examined apoptosis in the gastric antrum, some of the studies also investigated biopsies from the corpus, and again apoptosis was clearly increased in association with *H. pylori* infection (39, 79). In general, apoptosis is increased about two-fold compared with the control (uninfected) mucosa and no clear association has been demonstrated between the amount of apoptosis and either *H. pylori* infection density or the extent and severity of gastritis, as assessed histologically. It should also be noted that all these studies have assessed apoptosis by the TUNEL assay since this appears to be the only satisfactory way to measure apoptosis in inflamed gastric biopsies (81). In our early description of increased apoptosis in association with *H. pylori* we examined duodenal ulcer patients before and after eradication therapy (71). We speculated that the increased apoptosis we observed was due to *H. pylori* virulence determinants, including products of the *cag* pathogenicity island or *vacA*. We suspected that the patients in the study carried *vacA*, *cagA* positive strains since they all had ulcer disease. However, it still remains controversial whether genes within the *cag* pathogenicity island are associated with increased apoptosis. Two of three cross-sectional studies examining apoptosis in patients stratified for infection by *cag*-positive or *cag*-negative strains have reported, somewhat suprisingly, that increased apoptosis was more associated with *cag*-negative strains than *cag*-positive strains (43, 47). However, our recent study failed to confirm this and indeed showed the converse, i.e., that apoptotic index was greater in patients colonized by *cag*-positive rather than the *cag*- negative strains (39).

6.2.Animal Models

Infection of mice, rats, or the Mongolian gerbil with *H. pylori* strains has now also shown that *H. pylori* induces apoptosis in the gastric epithelium. In all cases, it is notable that increased apoptosis precedes the onset of hyperproliferation by several weeks, implying that the proliferative response is secondary to the increased apoptosis. However, this increased apoptosis tends to then decrease with time, suggesting an adaptive response of the epithelium to prolonged cell death stimuli (56, 82, 83). Studies on mice with defined abnormalities in the gastric mucosa demonstrated that the Fas antigen appears to be involved in the apoptotic response to *H. pylori* (53), and that the apoptotic and proliferative response is strain-dependent (84), perhaps, as speculated by the authors,

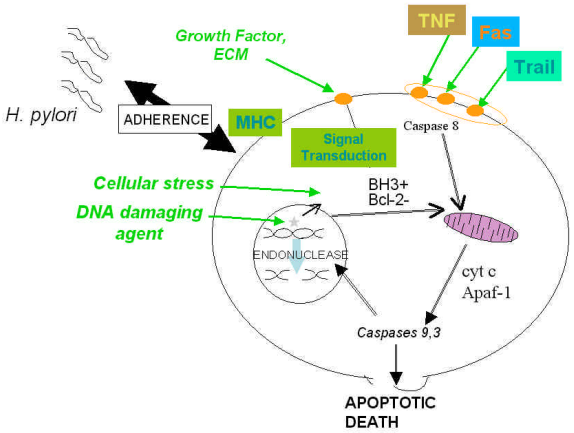

Figure 3. Pathways to apoptosis associated with *H. pylori* infection. Multiple stimuli converge on the gastric epithelial cell to influence apoptosis. Most, such as *H. pylori*, Fas ligand and tumor necrosis factor (TNF) are pro-apoptotic. Growth factors and the extracellular matrix (ECM) generally exert a negative influence.

because of the absence of secretory phospholipase A2 in the C57 BL/6 strain. In attempting to dissect bacterial-related pro-apoptotic factors in the gerbil model, apoptotic responses have been correlated *in vivo* with two strains of *H. pylori*, defined at the molecular level using whole genome microarrays (85). In this study, we found that a gerbil-adapted strain of *H. pylori* established from a patient with gastric ulcers was associated with more apoptosis *in vivo* than a strain from a patient with a duodenal ulcer. This could be related to the fact that the duodenal ulcer strain had a non-functional *cag* island. However, disruption of the *cag*-island in the gastric ulcer strain did not alter the ability of this *H. pylori* strain to induce apoptosis *in vitro*, although IL-8 induction was impaired in the deleted mutant, thus again arguing against a role for *cag* genes in apoptosis.

6.3. *In vitro* Models

Although it is abundantly clear that *H. pylori* induces apoptosis *in vivo* in humans and experimental animals, the pathways and mechanisms remain poorly defined. It is easy to demonstrate the induction of apoptosis by *H. pylori in vitro*, and there is a burgeoning literature in this area (for review, see reference 86). Some of the putative pathways involved are shown in figure 3. Studies of apoptosis *in vitro* using either malignant or normal (primary) gastric epithelial cell cultures have implicated a number of *H. pylori* related-stimuli as being responsible for initiating apoptosis. However, only a few of these mechanisms have been examined or confirmed *in vivo*. A limitation of the *in vitro* studies is that they examine isolated interactions between bacteria or bacteria-related products and either epithelial cells or inflammatory cells. Obviously *in vivo* the epithelium is in close proximity with inflammatory cells and their products as well as the tissue matrix and other tissue elements, which may have important pro- or anti-apoptotic effects. Humoral factors may also be relevant, including gastrin (discussed in section 4), and circulating antibodies. In favor of the latter hypothesis, some investigators have implicated anti-parietal cell antibodies in an autoimmune-related apoptotic attack on the epithelium (87), although we could not confirm this (39). Studies on the effects of *H. pylori* on apoptosis during short-term culture *in vitro* may provide results different from the long-term *in vivo* effects of the interactions between *H. pylori* and gastric epithelial cells. It is likely that with the passage of time *in vivo* there is adaptation in both the *H. pylori* population and the epithelial cell population, thus leading to a balance between apoptosis and increased proliferation - very different from the situation in short term culture *in vitro*. Furthermore, evidence from a long-term co-culture model supports the concept of an adaptive downregulation of the epithelial response to apoptosis occurs, which results in resistance to other apoptotic stimuli too (88).

As mentioned previously, the controversy regarding the role of *vac* and *cag* related genes to the induction of apoptosis has not been resolved despite the multitude of *in vitro* studies in this area. A number of groups have used defined clinical strains and isogenic mutant strains in which specific *cag* or *vac* related genes have been disrupted in an attempt to determine whether these genes are involved in epithelial apoptosis. Most studies suggest that live *H. pylori* bacteria and direct bacterial epithelial cell adherence are important in initiating apoptosis. However, it is unclear whether genes within the *cag* pathogenicity island and/or specific *vac* polymorphisms have an effect on apoptosis (64, 65, 74, 89, 90). Using a different approach, Galmiche et. al. (91) recently reported that transfection of *H. pylori* DNA encoding specific regions of the vacuolating cytotoxin into HeLa cells resulted in apoptosis and mitochondrial dysfunction, as determined by several parameters including caspase 3 activation and cytochrome c release. However, whether it is valid to extrapolate from this artificial situation to *in vivo* effects of Vac A remains to be determined. Very recently, Kuck *et al* (92) have observed that depleting VacA from *H. pylori* supernatants abolishes *H. pylori*-induced apoptosis. However, since in this study recombinant vacA at high concentrations induced apoptosis without vacuolation, there is some question about the biological relevance of this observation, since vacuolation is an obviously important marker of biologically active *vacA*.

7. *H. PYLORI* & OTHER CELL CYCLE CHANGES

Chronic gastric colonization by *H. pylori* is related to a diverse group of clinical outcomes, including a subclinical chronic gastritis, relatively benign gastric and duodenal pathologies (gastric ulcer, duodenal ulcer, gastric hyperplastic polyps) and malignancy, including gastric lymphoma and adenocarcinoma. The molecular events underlying the pathological progression from *H. pylori*-associated chronic gastritis through intermediate preneoplastic stages to gastric adenocarcinoma are still unknown. One of the proposed mechanisms is modulation of gastric epithelial cell cycle kinetics by alteration of regulators of the cell cycle (93). The information reported thus far on the effects of *H. pylori* on the different aspects

of the cell cycle is limited, although there is some evidence that mutations and/or altered expression of genes that regulate the cell cycle do occur in gastric carcinogenesis. Studies on cell cycle changes associated with *H. pylori* infection have been of 2 types:

1. Assays for the direct effects of *H. pylori* on the cell cycle in gastric epithelial cells. These studies are mechanistic, but since they are usually performed in cell culture, they do not assess the influence of inflammatory cytokines, growth factors, or other factors that may be important *in vivo*.

2. Assays for the expression of specific cell cycle proteins in the gastric epithelial cells of experimental animals, or patients, chronically infected by *H. pylori*. Although descriptive, these studies may better reflect the combined effects of *H. pylori* infection, the associated inflammatory response, and other host responses.

7.1. *H. pylori* and gastric epithelial cell cycle kinetics

Co-culturing gastric epithelial cells with *H. pylori* can clues to possible disturbances in cell cycle events that might occur in the gastric mucosa. However, it should be emphasized that there are several limitations with these types of *in vitro* studies that may also explain certain discrepancies with *in vivo* studies. Thus,

a) Almost all of the *in vitro* studies involve the examination of gastric epithelial cell lines derived from gastric cancers, rather than normal cells.

b)There may be significant variations between different gastric cancer cell lines, and therefore only studies performed with the same cell line may be strictly comparable.

c)Cell lines tend to change their characteristics during prolonged passage.

d)Almost all the studies have been performed over a short period of time, which may not reflect the consequences of chronic colonization of the gastric mucosa by *H. pylori*.

Of the available gastric cancer cell lines for *in vitro* studies, AGS is probably the best model for normal gastric epithelial cells (81). In performing these studies, we have considered it important to induce cell cycle synchronization by serum deprivation prior to the addition of *H. pylori*, because without synchronization it is difficult to interpret cell cycle phase changes and changes in expression of cell cycle regulators in heterogenous, non-synchronized populations (66).

One of the major findings in most of the *in vitro* studies, in contrast to the proliferative effect of *H. pylori in vivo*, is that *H. pylori* inhibits cell cycle progression. In our studies (66) and in studies reported by Ahmed *et al* (94), live *H. pylori* inhibited cell cycle progression at G1-S, and the deletion of the *cagA* gene in an isogenic mutant strain did not alter this effect (66). On the other hand, treatment with a cytosolic *H. pylori* extract did not change the cell cycle phase distribution of AGS or HM02 cells (68). Peek *et al* incubated AGS cells with 21 clinical *H. pylori* isolates (10 *cagA* positive and 11 *cagA* negative) or a laboratory *H. pylori* strain, in order to examine whether the apoptosis induced by *H. pylori* was associated with changes in specific phases of the cell cycle (65). They reported that although the majority of the *cagA* positive strains affected cell cycle events, causing movement from G1 to G2/M and transient accumulation in G2/M, some of the *cagA* positive strains and all the *cagA* negative strains did not do so. Thus it remains to be resolved whether the inhibition caused by *H. pylori* is predominantly at G1/S or at G2/M and also whether or not *cag*-related genes are involved.

In contrast to the accumulation of cells in G1/S that we observed in short-term *H. pylori* co-culture studies (66), we found that chronic co-culture of AGS cells with progressively increasing doses of *H. pylori* (either wild type or its isogenic *cagA*-negative mutant) resulted in the accumulation of some cells in G2/M, with a reciprocal reduction of cells in G1/S (88). Thus the effects of *H. pylori* on cell cycle events can change with time.

The cellular mechanisms responsible for the cell cycle inhibition observed in the *in vitro* studies remain to be determined. We found that G1/S inhibition induced by *H. pylori* in AGS cells is accompanied by decreased expression of $p27^{kip1}$, with little or no change in $p21^{cip1}$ or p53 expression (66), in agreement with the findings of Peek *et al* (65). In contrast, using a similar model, Ahmed *et al* have reported that the G1/S cell cycle arrest induced by *H. pylori in vitro* is associated with increased p53 and $p21^{cip1}$ expression (94). These opposite findings are difficult to reconcile.

7.2. Cell cycle proteins in gastric epithelial cells

7.2.1. Cyclins

In vitro, the addition of serum plus *H. pylori* to serum-deprived AGS cells induces increased expression of cyclin D1 when compared with cells refed with serum alone (66). This effect is mediated through a mitogen-activated protein kinase pathway (95). Consistent with this observation, Murakami *et al* (96) found increased expression of cyclin D1 in patients with hyperplastic polyps associated with *H. pylori* infection. Cyclin D1 overexpression has also been observed in human gastric cancer, (97) although according to Takano *et al*, cyclin D2, but not cyclin D1 overexpression correlates with progression and prognosis (98). In a further report by this same group, increased CDK4 expression was associated with a poor prognosis, whereas an increase in cyclin E correlated with a favorable prognosis. However, Blok *et al* did not find any difference in cyclin D1 expression between the *H. pylori*-positive and *H. pylori*-negative early gastric carcinoma (99), suggesting that these changes in cyclin D1 expression in gastric cancer are independent of *H. pylori*. Of interest, increased expression of cyclin D1, CDK4 and the cyclin D1/CDK4 complex was found in the livers of mice with chronic active hepatitis and liver tumors induced by the *H. pylori*-related bacterium *Helicobacter hepaticus* (100). Thus, other *Helicobacter* species may also modulate the expression of D-type cyclins. This is not surprising given the fact that increased cyclin D expression commonly occurs in hyperproliferative states (9).

7.2.2. Cyclin Dependent Kinase Inhibitors

In human gastric cancer there is accumulating evidence for mutations or abnormalities in the expression of various CDIs. Recently Shin *et al* (101) reported that the formation of inactive chromatin through histone deacetylation appears to be responsible for the loss of function of the the p21 and the p57 genes in gastric cancer cells, and that the p57 gene promoter can also be hypermethylated as an alternative pathway to loss of function of p57 (101). We have recently shown that reduced levels of the CDI $p27^{kip1}$ can be an early event in *H. pylori* infection of gastric epithelial cells both *in vivo* and *in vitro* (66, 88).

Although the decreased expression of $p27^{kip1}$ is well established as a poor prognostic factor in gastric cancer (23), the data regarding $p21^{cip1}$ is controversial. The downstream target of p53, $p21^{cip1}$, is frequently reduced in gastric carcinoma, sometimes despite normal p53 expression. However, loss of $p21^{cip1}$ expression is correlated with a poor prognosis in some studies (102-104) but not others (105, 106). It may be that a combined analysis of both p53 and p21 has a more significant prognostic value than each of these factors alone – tumors with disruption of both p53 and p21 pathways (p53+/p21-), as assessed by immunohistochemistry, have the poorest survival (107, 108).

There is currently conflicting data regarding changes in $p16^{Ink4a}$ in gastric cancer. Decreased expression of $p16^{Ink4a}$ and mutation of the p16 gene have been reported in about 10-20% of gastric cancer cell lines and gastric tumors (28, 109, 110), consistent with data from other gastrointestinal tumors, including carcinoma of the pancreas and esophagus, and also in *H. pylori* associated high grade mucosa associated lymphoid tissue (MALT) lymphoma (111-113). However, in contrast to these studies showing decreased $p16^{Ink4a}$ expression in gastric cancer, Tsujie *et al*. recently reported paradoxical over expression of $p16^{ink4a}$ in 65% of patients with gastric carcinoma, when compared to adjacent non-neoplastic mucosa (114). Since the *H. pylori* status of these patients was not included, it is not possible to conclude if any of the above phenomena described are associated with or due to *H. pylori*. Therefore, we recently evaluated $p16^{ink4a}$ expression in patients with *H. pylori*-associated chronic gastritis compared with uninfected controls and found a significant positive correlation between increased $p16^{ink4a}$ immunostaining in the foveolar and neck regions of the glands of the gastric mucosa and increased epithelial apoptosis, in *H. pylori* infected patients (115). These findings suggest a potential role for $p16^{ink4a}$ in the cell-cycle regulatory response to chronic *H. pylori* infection.

7.3.3. p53 and *H. pylori*

p53 mutations are among the most common mutations in cancer, including both intestinal and diffuse-type gastric cancer (116). In general, increased levels of the p53 protein, by immunohistochemistry, implies p53 mutation, although this correlation is not perfect. It has been reported that the risk of p53 mutations is greater with *cagA* positive *H. pylori* associated gastric cancers (117), and that these mutations are principally located at CpG sites (118). However, comparison of p53 alterations in these two types of cancers found that these changes occur earlier in the intestinal type than the diffuse type. Thus, p53 mutation may be associated with tumor progression only in diffuse type gastric carcinoma (110, 119). Increased levels of p53 and p53 mutations have also been found in the early precancerous stages of *H. pylori* infection, atrophic gastritis and intestinal metaplasia (120-122). In monkeys, Kodama *et al* demonstrated positive immunostaining for p53 in gastric epithelial cells at only three years after inoculation of the animals with *H. pylori* (123). Several other groups have reported overexpression of p53 in patients, including children, with *H. pylori* associated chronic gastritis - particularly in the neck region of the gastric gland (49,124,125). Furthermore, eradication of *H. pylori* can result in a decrease in p53 expression (122). However, it is not known whether these alterations in p53 are a direct effect of the bacteria or secondary to mediators of gastric inflammation. As mentioned above, there is only one report that direct bacterial-epithelial cell co-culture increases p53 expression (94), and long-term *in vitro* co-culture does not induce p53 mutation (88).

8. CONCLUSIONS & FUTURE DIRECTIONS

H. pylori and its associated inflammatory response are associated *in vivo* with increased p53 expression, alterations in expression of some cell cycle control regulators, such as cyclin D1 and $p27^{kip1}$, and $p16^{ink4a}$ and marked and persistent abnormalities in epithelial cell proliferation, cell cycle progression and apoptosis. It remains to be determined which of these events represent primary responses and which are compensatory to restore epithelial homeostasis in the context of ongoing cellular damage. Furthermore, the contribution of the inflammatory infiltrate and specific *H. pylori* virulence determinants to these epithelial cell cycle events needs to be addressed by more sophisticated *in vitro* co-culture systems and specific animal models.

Since *H. pylori* is a persistence inhabitant throughout most of the lifespan of most people in the developing world, it is likely that there may be some evolutionary gain for some of these changes, dependent on specific environmental factors. However, in a small percentage of individuals, abnormalities of cell cycling may contribute to the development of gastric neoplasia with age. It is believed that alterations in cell cycle events precede and may be predictive of gastric carcinogenesis ; such a hypothesis is worthy of formal testing. Further studies on changes in epithelial cell cycling events throughout an individuals' lifespan are likely to provide further insight into how this symbiotic relationship between humans and their gastric bacteria may occasionally become detrimental and result in malignant transformation.

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