Carcinogenesis, apoptosis and cell proliferation

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Biological agents, especially viruses, have been linked to the carcinogenesis process in major human cancers, especially lymphomas (retroviruses), hepatocarcinomas (hepatitis viruses) and carcinomas of the female genital organs (papilloma viruses). Chronic infection and inflammation have long been suspected to play a role in human carcinogenesis. *Helicobacter pylori* is the first bacterial infection recognized as a human carcinogen, essentially on the basis of epidemiological evidence of causality. Contrary to most other recognized human carcinogens, experimental evidence of carcinogenesis is lacking. As a consequence, mechanistic explanations of *H. pylori* carcinogenesis at this point in time are hypothetical.

Infection of the gastric mucosa by *H. pylori* is practically always chronic and in most patients lasts for decades. By inference, the host reaction appears incapable by itself of curing the infection without the help of bactericidal or antibiotic drugs. What is not resolved are the consequences of this persistent host response. It is not clear if the carcinogenic influences of the infection are due to bacterial, host or a combination of factors. Clear inter-strain differences in virulence factors have been identified, but no credible evidence has been provided for a direct mutagenic effect of the bacteria. At least 3 types of host-bacterial interactions are well recognized. Although probably interrelated, they are usually described independently for reasons of convenience:

1. *H. pylori* infection impairs mucin secretion by the gastric epithelium. Mucus is assumed to play a protective role by creating a barrier between the gastric epithelium and acid secretions and toxins present in the gastric lumen. The degree of damage to mucus varies greatly between individuals and is a function of both bacterial strains and host characteristics. A set of genes clustered in the ‘pathogenicity island’ of the bacterial genome has been identified. The *cagA* gene, although probably not directly responsible for cell damage, is a marker of the presence of such an island. A vacuolating cytotoxin produced by the bacteria is capable of damaging cells. Although the *vacA* gene is present in all bacteria, the toxin is not expressed in all...
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strains. Host factors of importance appear to be related to the so-called blood group antigens, especially determined by the secretor gene and the Lewis b antigen.

2 *H. pylori* attracts inflammatory cells to the gastric mucosa, the so-called mucosa associated lymphoid tissue or MALT, described in other chapters. In some patients, the resulting gastritis is antrum predominant, not atrophic (no gland loss) and may be associated with duodenal ulcer. Such patients are not at high risk for gastric cancer. In other patients, the type of gastritis associated with the infection leads to focal gland loss (multifocal atrophic gastritis or MAG) and intestinal metaplasia. Such patients do carry an elevated risk of gastric carcinoma. The determinants and the mechanisms involved in such divergent pathways are mostly unknown and constitute a major scientific challenge. They may eventually explain why some populations with high prevalence of infection have much lower risk of cancer than other populations with a similar prevalence.

3 The third major mechanism of carcinogenesis in *H. pylori* infection deals with the alterations of the cell cycle. Recently, accumulated evidence strongly suggests that such alterations play a major role in the outcome of the infection, including eventual tumor development. Researchers have concentrated their attention on two aspects of the cell cycle: cell replication and apoptosis.

We will review the available literature on the subject. Additionally, we will comment on other cell alterations of importance in the carcinogenesis process, namely DNA damage and mutations which if immortalized in repeated cell cycles may result in invasive neoplasms. Finally, we will examine the possible role of oxidative stress since it appears to be the favorite scientific hypothetical explanation of the cell cycle alterations observed in *H. pylori* infection of relevance in the gastric carcinogenic process.

**Cell proliferation**

The gastric mucosa has a characteristic pattern of cell replication and differentiation. The glands, although different in antrum and corpus, are located in the deeper compartment of the mucosa. The epithelial lining of the glands continues towards the surface forming first a relatively narrow passage called glandular 'neck', which in turn are continued by 'foveolar' of 'surface' epithelial cells which line the pits (foveolae) and the gastric folds. The glands secrete mucus in the antrum and HCl and pepsinogens in the corpus, but their cells do not replicate. The foveolar and surface cells do not normally replicate; they secrete mucus. The cells
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**Fig. 1** Diagrammatic representation of cell replication in the gastric mucosa. Cells in black represent S phase labeling with MIB-1 antibody. Zone 2 represent the gland necks where replication takes place. Cells lost in the other compartments are replaced by migration of new cells from the neck region. (From Harvard et al. with permission)

lining the ‘necks’ do not actively secrete but control cell replication. They are considered stem cells which differentiate as they migrate to either the surface or to the glands, replacing cells lost in those compartments.

Prior to the discovery of *H. pylori* and its recognition as a cause of gastritis, studies of cell replication in the gastric mucosa had identified the ‘neck’ region as the normal replication zone of the gastric mucosa. The prevailing technique then was autoradiography after incubation of gastric biopsies with tritiated thymidine. The same techniques were applied to biopsy specimens with atrophic gastritis. They showed an expansion of the replication zone and a migration of replicating cells towards the surface and the glands. Figure 1, from Havard et al., illustrates the topography of the gastric cell replication. These changes were characterized as ‘hyperproliferation’ by Winawer and Lipkin. One such study was carried out in subjects from Nariño, Colombia. Later studies in the same population have reported a prevalence of *H. pylori* infection higher than 90%. More recent studies have utilized relatively simpler labeling techniques which do not require radioactive materials and prolonged incubation.

Most studies have clearly shown that *H. pylori* infection increases epithelial cell replication and results in expansion of the replication zone. These findings have been supported using bromo-deoxyuridine.
(BrdU) labeling after incubation as a marker of the S phase of the cell cycle as well as the proliferative cell nuclear antigen (PCNA) which is positive in G₁, S and G₂ phases of the cycle. Since successful treatment of the infection results in clearance of the bacteria as well as the inflammatory cells, it has been difficult to decide which event accounts for the excessive proliferation. Bechi et al, utilizing tritiated thymidine, reported excessive replication in corpus biopsies showing *H. pylori* but no inflammation, pointing towards a direct effect of the bacteria on cell replication. Fraser et al, on the other hand, reported that, after bismuth/antibiotic therapy, all patients showed a reduction of replication rates, apparently indicating that decreasing the inflammatory infiltrate resulted in a decrease in cell replication, independent of the presence of *H. pylori*. Lynch et al reported the same initial result of decreased cell replication in response to decreased inflammation independent of *H. pylori* presence. However, when the same patients were studied 18 months later, only *H. pylori* negative patients maintained lower rates of proliferation. These findings indicate that the cause of excessive replication is *H. pylori* infection and that the effect may be linked to the bacterium itself, to the inflammatory response or to a combination of both factors. Anti-*Helicobacter* drugs, such as bismuth, may decrease cell proliferation directly or through a decrease in the inflammatory response. Patients with 'chemical' gastritis, also called 'type C', 'reflux' or 'reactive' gastritis, have not shown excess replication and may reflect the effects of non-steroidal anti-inflammatory drugs (NSAIDs).

Several reports have examined cell replication parameters in the different postulated stages of the gastric precancerous process. Cahill et al reported a trend towards increased cell replication from normal mucosa to atrophic gastritis, to intestinal metaplasia to carcinoma. Panella et al reported similar findings and were able to separate intestinal metaplasia into the 'complete' (type I or small intestinal type) and the 'incomplete' (type III or colonic). The latter type showed higher rates of replication, which is significant given its recognized premalignant connotations.

The effect of *H. pylori* on cell replication has also been explored in gastric cancer cell lines, even though it is not clear how these findings relate to the replication of the intact (not neoplastic) gastric mucosa. Fan et al used the AGS cell line and the Ki67 (MIB) antibody, followed by flow cytometry, to select tagged cells in S phase. They reported increased cell replication directly associated with exposure to *H. pylori* but not to *Campylobacter jejuni* or *Escherichia coli*. Additionally, the same effect was shown when cell cultures were exposed to cytokines from lymphocytes. These experiments support the notion that the bacterium and its resultant inflammation stimulate (independently?) cell replication. Ricci et al, using the MKN 28 cell line, reported inhibition
Carcinogenesis, apoptosis and cell proliferation by vacA expressing H. pylori strains and no effect of cagA positive strains.

Studies of cell proliferation in experimental animals have reported excessive replication in atrophic gastritis in rats exposed to the potent gastric carcinogen N-methyl-N-nitroso-N-nitrosoguanidine (MNNG). Infection with Helicobacter mustelae results in excessive cell replication in ferrets. Helicobacter urease results in ammonia production in the gastric microenvironment. Ammonia itself induces increased cell replication in the gastric mucosa.

Studies using ornithine decarboxylase (ODC), as a marker of cell replication, have reported increased proliferation in patients with intestinal metaplasia. H. pylori infection has been associated with metaplasia. ODC levels can be reduced in gastritis patients after β-carotene supplementation.

DNA damage

It is well appreciated that free radicals, oxidants and reactive nitrogen species cause DNA damage. These genotoxic species contribute to the high rate of lung cancer in smokers where the genotoxic species are inhaled, or in the case of asbestos exposure, where these genotoxic species are generated by the host response. DNA damage can be quantified by assay of the stable oxidative deamination product, 8-oxo deoxyguanine (8-OH guanine). Recently, peroxynitrite, a potent oxidant generated from the interaction of two free radicals nitric oxide and superoxide, has been demonstrated to form nitro-guanine adducts. These adducts are the smoking guns of DNA damage in states of inflammation.

Cells respond to the burden of DNA adducts, generated at the natural rate or at the heightened level associated with inflammation, by two mechanisms: DNA repair or induction of apoptosis (programmed cell death). This process removes cells with damaged DNA from the pool of replicating cells in order to avoid the introduction of mutations into the genome and the associated heightened risk for carcinoma.

Repairing damaged DNA is of primary importance. However, nitric oxide can impair this repair process by compromising the activity of Fpg, a DNA repair protein. Thus, NO can cause DNA damage and impair repair mechanisms designed to prevent the formation of genetic mutations. If DNA is not repaired before a cell replicates, then damage persists in the form of a mutation. In the case of NO and oxidants, this usually involves a point mutation, the most common one being G:C → A:T. This is the most common type of transformation in cancer and has the greatest link to carcinogenicity.

Cells have several defenses against the mutagenic effects of oxidants and reactive nitrogen species. The heterogeneous class of chemicals...
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Antioxidants → Oxidants, nitric oxide, peroxynitrite

DNA Damage

INFLAMMATION, NO GLAND LOSS

NORMAL → PROLIFERATION → MUTATIONS

Apoptosis

Fig. 2 Diagrammatic representation of cell cycle alterations induced in the gastric mucosa by H. pylori infection and the oxidants-antioxidants forces.

called anti-oxidants can either destroy the oxidants themselves\textsuperscript{34,35}, or the consequences of their actions, e.g. inhibition of lipid peroxidation by β-carotene. Figure 2 illustrates the consequences of sustained exposure to genotoxic species in H. pylori gastritis. While some DNA damage is apparent in the normal mucosa, this does not lead to pathological outcomes. With increasing production of oxidants and in combination with depletion of antioxidant defenses, this burden increases\textsuperscript{36}. Cells may be lost to desquamation at the surface which will be balanced by an increase in the replicative rate of the progenitor zone in the neck region of the mucosa. With continued exposure and/or increasing burden of oxidants, gland loss may occur and not replaced as a result of apoptotic cell death. The accumulation of mutations may then lead to metaplasia and dysplasia, particularly if these mutations occur at a site that regulates the cell cycle, e.g. p53.

Apoptosis

As noted above, the cell proliferation rate is enhanced in H. pylori infection. This increased cellular replication must be also associated with an increased rate of cell death or otherwise the mucosal thickness would be increased and that clearly does not occur. It is very likely that the increased mitotic index is a response to the increased loss of epithelial cells in order to maintain a viable, functional mucosa. The possibility that enhanced cell replication in an environment of DNA
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damage enhances the possibility that mutations will be generated with the logical extension to carcinoma.

If epithelial cells are dying at an enhanced rate in *H. pylori* gastritis, how are they dying? Cell loss may result from the extrusion of cells from the surface epithelium, as occurs under physiological circumstances, and indeed this is apparent. Cells may lose their attachment from the basement membrane as a result of oxidants and proteases released during the inflammatory process. In this case, cells are dying from a process called ‘anoikis’, or ‘homelessness’ which is a form of apoptosis (programmed cell death or delayed cell death). Apoptosis is apparent in some cells because of a loss of growth factors or withdrawal of glucocorticoid support but, in gastritis, apoptosis is likely to be due to excessive cell activation. Cells that cannot repair damage done to organelles (membranes, mitochondria) or DNA due to the release of inflammatory mediators will undergo suicide rather than pose a risk for cancer, as would be the case if they did not repair damaged DNA (Fig. 2). Indeed, we and others have noted an increased rate of apoptosis in gastritis.

As noted above, atrophic gastritis is the phenotypic response to *H. pylori* infection that is regarded as the precursor to gastric cancer. We have proposed that atrophic gastritis is the result of sustained, large scale apoptosis of the gastric mucosa. In particular, apoptosis of the neck region of the mucosal glands is a critical site as this region is the site of epithelial replication and regeneration of mucosal glands and surface epithelia. The depletion of the mucosal glands (the definition of atrophic gastritis) may be due to a failure to replace gland cells steadily over time via apoptosis as a consequence of a persistent inflammation. There is evidence that this proposal may have validity; the neck region, the site of surface and gland cellular production, is the site of greatest inflammation during *H. pylori* infection and exaggerated levels of apoptosis. Whether, there is a quantifiable difference between non-atrophic and atrophic forms of gastritis remains to be determined. Peek et al. recently noted that the degree of apoptosis did not match the replicative rate in patients infected with the virulent *cagA* vacA s1a strain. It is possible that cell loss via desquamation was greater in those patients with the more toxic strain. Certainly, when compared to normal gastric mucosa, where the apoptosis is limited to the gastric mucosal surface, the degree of apoptosis in *H. pylori* gastritis is considerably greater and located in multiple sites, but importantly includes the neck region.

If apoptosis is markedly exaggerated in *H. pylori* gastritis, what are the mechanisms underlying this process? Currently, four major pathways have been proposed: (i) loss of attachment to the matrix; (ii) *H. pylori*-epithelial cell interactions; (iii) cytokines; and (iv) reactive nitrogen and oxygen species. Recently, apoptosis due to *Fas* activation secondary to *H. pylori* attachment to epithelial cells has been noted by...
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several groups. This process, which has great similarities to TNFα (tumor necrosis factor), historically has been linked to the death of immune cells in immune privileged sites. However, the degree of apoptosis in gastritis cannot be fully explained by Fas mechanisms or other sites of attachment, as H. pylori generally remains in the mucus layer above the epithelial cells, and the degree of apoptosis is remarkable. Thus additional mechanisms must be operating.

Immune activation is involved in apoptosis through the release of cytokines (IL-8, TNFα) in the inflammatory process. TNFα is a prime candidate as it can initiate apoptosis through several mechanisms, including a Fas-like mechanism and the formation of oxidants. Endotoxin derived from H. pylori can also induce apoptosis. The mechanism for this effect is likely to involve a combination of cytokine actions, like TNFα, as well as oxidants/nitric oxide.

Oxidative stress

Gastritis, by virtue of the inflammatory process, is associated with an increased production of oxidants and reactive nitrogen intermediates (nitric oxide and its oxidative by-products). Gastritis is associated with increased expression of the inducible isoform of nitric oxide synthase, the isoform associated with host defense and inflammatory conditions. This isoform is characterized by a continuous production of large amounts of NO. The resultant burden of nitric oxide can induce epithelial and inflammatory cells to undergo apoptosis. Figure 3 outlines a potential cascade of events that may be involved in the gastritis initiated by H. pylori infection. Another oxidant that may be involved is peroxynitrite, a product of two free radicals, nitric oxide and superoxide. Peroxynitrite is a potent oxidant that can be tracked in vivo by its ability to nitrate tyrosine residues. Normally, little nitrotyrosine is present in tissues but it is increased in inflammation, including bowel inflammation, atherosclerosis, lung injury, arthritis, fetal morbidity and, relevant to this discussion, gastritis. Anti-oxidants which destroy peroxynitrite negate the apoptosis of epithelial cells and macrophages in vitro. In patients with H. pylori gastritis, treatment with anti-oxidants attenuated the degree of apoptosis and nitrotyrosine staining, suggesting that the cellular loss associated with infection may indeed involve oxidants, nitric oxide and nitrating species (peroxynitrite is the leading candidate).

In addition to an increased rate of oxidant production, patients with atrophic gastritis may have diminished anti-oxidant defenses. In particular, the luminal concentration of ascorbic acid is reduced. This is not necessarily due to a reduced intake, but rather a defect in a pump
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Fig. 3 Diagrammatic representation of hypothetical events in the formation of oxidant species in the gastric microenvironment. On the left side of the diagram are represented events triggered by the induction of nitric oxide synthase (iNOS) in macrophages and polymorphonuclear cells attracted by *H. pylori* to the gastric mucosa, leading to the formation of peroxynitrite (ONOOH) from nitric oxide (NO) and its possible damage to epithelial cells. Blocking effects of anti-oxidants are illustrated. On the right side of the diagram are shown possible pathways leading to oxidants in the gastric lumen. Nitrite from saliva from bacterial nitrate reductases acting on food-borne nitrate are shown as possible sources of $\text{N}_2\text{O}_3$ and nitroso compounds.

mechanism which transports ascorbate from the blood to the lumen. The mere existence of a mechanism to pump ascorbic acid, a vitamin that is not produced endogenously but rather must come solely from dietary sources, is meaningful. While the purpose of this pump may remain partially obscure, the ability to protect the mucosa from reactive nitrogen species may be an important part of the answer.

Nitric oxide can also be generated in the gastric lumen from non-enzymatic sources. The acidification of nitrite to NO is demonstrable
but the major reactive nitrogen species generated from this reaction is dinitrogen trioxide (N$_2$O$_3$, see Fig. 3). This reactive nitrogen species is a potent nitrosating agent, i.e. can form nitrosothiols and nitrosamines, and is a potent antimicrobial agent. N$_2$O$_3$ may well account for the large scale death of ingested bacteria, before a meal is passed onto the duodenum. This mechanism is augmented by the active concentration of nitrite in the saliva, and the utilization of bacterial nitrate reductase to convert the innocuous nitrate to nitrite (Fig. 3). Thus, ingested bacteria may contribute to their own demise. How *H. pylori* survives this milieu of oxidants is not yet clear.

The final component that suggests that this aspect of the host defence response is important for the progression of infection to cancer is that the known gastric carcinogens are nitrosated species, *e.g.* N-methyl-N’-nitro-N-nitrosoguanidine$^{59,60}$. The formation of nitrosamines from endogenous nitric oxide has been demonstrated in neutrophils, and nitrosamine formation can be negated by the addition of ascorbic acid and other antioxidants$^{61}$. It seems more than a coincidence that dietary nitrite, nitrosamines and *H. pylori*-induced gastritis share so much chemistry and the association with cancer$^{62}$. These links are expressed in Figure 3, where the pathways involving nitrosative chemistry and cell damage, apoptosis, mutations and cancer are delineated. It would seem reasonable that this chemistry is critical for establishing a milieu favourable for the development of gastric cancer. All that is needed is additional inputs (viral, mutations) directed at disturbing cell cycle regulation. As this process is chronic, the opportunity for random hits to the genome to occur at critical sites increases dramatically.

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