Inducible Nitric Oxide Synthase, Nitrotyrosine, and Apoptosis in Helicobacter pylori Gastritis: Effect of Antibiotics and Antioxidants

Elizabeth E. Mannick, Luis E. Bravo, Guillermo Zarama, J. Luis Realpe, Xiao-Jing Zhang, Bernardo Ruiz, Elizabeth T. H. Fontham, Robertino Mera, Mark J. S. Miller, and Pelayo Correa


ABSTRACT

Helicobacter pylori infection is a known risk factor for gastric cancer. We hypothesized that H. pylori infection would lead to the sustained production of the reactive nitrogen species nitric oxide and peroxynitrite as part of the host immune response. We further hypothesized that H. pylori infection would lead to increased apoptosis of gastric epithelial cells, possibly in response to free radical-mediated DNA damage. Using immunohistochemistry, we stained and scored gastric antral biopsies from 84 Colombian patients with nonatrophic gastritis before and after treatment for H. pylori infection. We examined expression of inducible nitric oxide synthase (iNOS); nitrotyrosine, a marker for peroxynitrite; and DNA fragmentation, a marker for apoptosis. Patients were treated with triple therapy (amoxicillin, 500 mg three times a day for 2 weeks; metronidazole, 400 mg three times a day for 2 weeks; and bismuth subsalicylate, 262 mg four times a day for 2 weeks, followed by 262 mg every day for 4–12 months). Eradication of H. pylori infection resulted in a significant reduction in iNOS and nitrotyrosine staining and a marginally significant reduction in apoptosis. Dietary supplementation with β-carotene (30 mg every day for 4–12 months) resulted in a significant decrease in iNOS staining. Supplementation with ascorbic acid (1 g twice a day for 4–12 months) led to a significant reduction in nitrotyrosine staining. In patients supplemented with either ascorbic acid or β-carotene, there was a trend toward a reduction in apoptosis, but this was not statistically significant. We conclude that H. pylori infection is accompanied by the formation of endogenous reactive nitrogen intermediates, which may contribute to DNA damage and apoptosis. In addition to antimicrobial therapy, dietary supplementation with β-carotene and ascorbic acid may prevent the formation of these potential carcinogens.

INTRODUCTION

The role of exogenous N-nitroso compounds in gastric carcinogenesis has been extensively studied (1–5). Recently, a role for endogenous reactive nitrogen species in cancers related to chronic inflammation has been proposed (6–8). These reactive nitrogen species are derived from the synthesis of nitric oxide, stimulated by iNOS in a variety of cell types, including activated neutrophils and macrophages. Increased iNOS activity has been observed in patients with chronic gastritis (9). Although nitric oxide is known to be mutagenic in vitro (10–12), a link between nitric oxide and gastric cancer has not been shown to exist to date. Helicobacter pylori infection is considered a risk factor for gastric cancer (13–16), but the mechanisms underlying its carcinogenic potential are still unclear. We hypothesized that H. pylori infection would lead to the expression of iNOS and the sustained production of nitric oxide by host macrophages and polymorphonuclear leukocytes infiltrating the gastric mucosa as part of the host response to this infection. In the presence of inflammation-associated oxygen free radicals, nitric oxide can form potentially genotoxic nitrating species such as peroxynitrite and nitrating species such as the nitrosonium ion (17, 18). Nitrotyrosine, a stable end product of the nitrination of tyrosine residues, can be used as a marker for the more ephemeral peroxynitrite and other nitrating species (19). Therefore, we postulated that H. pylori infection would lead to the formation of nitrotyrosine in the gastric mucosa. In addition, we hypothesized that through the generation of nitrating and oxidizing agents, H. pylori infection would lead to increased rates of DNA damage and, as a consequence, apoptosis in the gastric mucosa. In tissue sections, apoptosis may be assessed by staining fragmented DNA using an end-labeling technique known as TUNEL (20). Finally, we sought to determine whether successful treatment of H. pylori-infected patients would reduce staining for iNOS, nitrotyrosine, and apoptosis. In addition to antimicrobial therapy, we examined the role of dietary ascorbic acid and β-carotene supplementation, because these antioxidants are believed to reduce the risk of gastric cancer by interfering with either the formation or the action of reactive oxygen and nitrogen species (21).

One hundred three patients from a Colombian population at very high risk of gastric cancer were entered in a clinical trial with a 2³ factorial design testing the effect of anti-Helicobacter therapy as well as ascorbic acid and β-carotene dietary supplementation on the histopathology of chronic nonatrophic gastritis. Three biomarkers, iNOS, nitrotyrosine, and apoptosis, were studied in gastric biopsies obtained before and after intervention.

MATERIALS AND METHODS

Study Population

Patients were recruited from Narino, Colombia, a rural, mountainous region known to have high rates both of H. pylori infection (90%) and gastric carcinoma (150 per 100,000; Ref. 22). Briefly, patients were agricultural or blue collar workers of Spanish-Indian ("mestizo") extraction. Demographic characteristics of the patient population have been described previously (23). Endoscopic evaluations of patients from the community who volunteered to participate in the study were performed in the Hospital Departamental (Pasto, Colombia) after obtaining informed consent from subjects and approval by the local Human Subjects Committee and the Louisiana State University Institutional Review Board. Patients eligible for enrollment in the study had historically confirmed H. pylori infection, detected by the Steiner modification of the Warthin-Starry staining method, and nonatrophic gastritis at the time of entry. Uninfected subjects endoscoped in the recruitment phase of the study were not enrolled as controls for follow-up endoscopy due to ethical concerns. Of the 103 infected patients who entered the study, 84 returned for repeat endoscopy following a mean interval of 12.5 (range, 9–15) months. At gastroscopy, three biopsies from the lesser curve of the antrum, one from the greater curve of the antrum, and one from the midpoint of the anterior wall of the corpus were obtained. Eradication of H. pylori infection was defined as the absence of characteristic organisms in any of the repeat biopsies, as assessed by light microscopy using the Steiner modification of the Warthin-Starry staining method.

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Study Design

Patients were randomized into treatment groups using a 2^3 factorial design, depicted in Table 1. The anti-<em>Helicobacter</em> treatment consisted of a 2-week course of amoxicillin (500 mg three times a day), metronidazole (400 mg three times a day), and bismuth subsalicylate (262 mg four times a day). Bismuth subsalicylate (262 mg once a day) was continued until 2 weeks prior to the second gastroscopy procedure (range, 4 months–1 year). Ascorbic acid was provided as 1-g tablets, taken twice a day. β-Carotene was given in a 30-ml capsule once a day. Matched placebos for β-carotene and ascorbic acid were provided by Hoffman-La Roche Inc. Compliance was assessed by triweekly pill counts as well as measurements of serum antioxidant levels at the time of second endoscopy. Compliance was consistently greater than 90%.

Immunohistochemistry

iNOS. One biopsy from the antral lesser curvature was fixed in 95% ethanol and embedded in paraffin. Specimens from only 64 of the original 84 patients were able to be evaluated for iNOS due to tissue shrinkage and distortion from ethanol fixation in 20 specimens. Fixed tissues were deparaffinized and rehydrated. Protein-digesting enzyme 54508 (20 µg/ml; Oncor, Gaithersburg, MD) was applied to the specimen for 10 min at room temperature. Nonspecific binding was blocked with 0.5 M Tris buffer containing 3% normal goat serum and 1% Triton X-100 for 30 min. The sections were rinsed with Tris buffer and incubated with anti-human iNOS antiserum generated in mice (Transduction, Inc., Lexington, KY) at a dilution of 1:500 for 60 min at room temperature in a humidity chamber. A goat biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) was then applied for 40 minutes. Endogenous peroxidase activity was blocked with periodic acid (Zimed Laboratories, San Francisco, CA) for 45 s, and then avidin-biotin complex reagent (Vector) was added for 30 min. Sections were developed with the chromogen DAB (Vector) and then counterstained with Mayer’s hematoxylin, dehydrated, and mounted. Sections were rinsed with Tris buffer for 3 min twice between incubation steps. Details of this staining technique for iNOS have been reported (24).

In Situ Apoptosis as Detected by TUNEL. The TUNEL method for detecting apoptosis involves the labeling of 3'-OH ends of single- or double-stranded DNA with digoxigenin nucleotide (dUTP) complexes using the enzyme terminal deoxynucleotidyl transferase. Increased numbers of 3'-OH ends can be found in nuclei undergoing fragmentation and the characteristic morphological changes of apoptosis. The digoxigenin-labeled DNA ends can then be detected using anti-digoxigenin peroxidase (20).

Briefly, 82 gastric antral biopsies were fixed in 10% formalin and embedded in paraffin. Fixed sections were deparaffinized and treated for 15 minutes with proteinase K (20 µg/ml; Oncor). Endogenous peroxidase was quenched with 2% H<sub>2</sub>O<sub>2</sub> in PBS for 5 min. Equilibration buffer (Oncor) was applied for 10–15 s. Fifty-four µl working-strength terminal deoxynucleotidyl transferase (Oncor) were pipetted onto the section, and the section was incubated at 37°C for 1 h. Stop wash buffer (Oncor) was added, and the section was incubated an additional 30 min at 37°C. The section was washed three times in PBS and incubated for 30 min with two drops of anti-digoxigenin peroxidase (Oncor).

After washing three times with PBS, sections were developed with DAB. Slides were counterstained with hematoxylin, dehydrated in xylene, and mounted.

Nitrotyrosine. Eighty-three antral biopsies fixed in 10% buffered formalin were embedded in paraffin. Sections were deparaffinized and rehydrated to distilled water. Endogenous peroxidase was quenched using 3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min, followed by rinsing with water and Tris buffer. Nonspecific binding was blocked with Tris buffer containing 1.5% normal goat serum for 30 min. Specimens were incubated for 40 min in rabbit antisera against nitrotyrosine (a kind gift of Dr. Joe Beckman, University of Alabama, Birmingham, AL) diluted 1:100 at room temperature in a humidity chamber. Negative control specimens were incubated with antisera and an excess of free nitrotyrosine. Antibody binding was amplified using a goat biotinylated secondary antibody for 30 min (Vector), detected by the addition of avidin-biotin complex reagent (Vector) for 30 min, and visualized with DAB. Sections were rinsed with Tris between incubation steps. Slides were counterstained with hematoxylin, dehydrated, and mounted (25).
deletions and insertions at homocopolymer tracts (repeats of pu

In conclusion, we analyzed a deletion/insertion event in p53 in a

DISCUSSION

The current study shows that Helicobacter pylori infection of the
gastric antrum in humans is associated with the expression of iNOS in
tissue neutrophils and mononuclear cells. Treatment with antimicrob-
ial therapy and eradication of the infection resulted in a significant
reduction in iNOS expression determined by immunohistochemistry.
The combination of ascorbic acid and antibiotics resulted in the
greatest reduction in iNOS as well as the highest cure rates (100%).
Eradication of the infection also resulted in a decrease in nitrotyrosine
staining and a trend toward a reduction in apoptosis. β-Carotene
supplementation significantly attenuated iNOS staining, whereas
ascorbic acid significantly reduced nitrotyrosine staining.

These results add H. pylori to a growing list of chronic infectious
agents, including hepatitis B and C viruses, cytomegalovirus, and liver
flukes, that are associated with iNOS expression and/or the production
of endogenous nitrates (26–28). Our results demonstrate a clear cor-
relation between H. pylori and iNOS staining. Although there has
been difficulty in demonstrating iNOS expression in blood-borne
human neutrophils, the current results indicate that the local milieu of
gastritis is conducive for iNOS expression in neutrophils and other

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<th>Table 3 Reduction in pretreatment and posttreatment scores by Helicobacter clearance</th>
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β-carotene. Patients who were treated with antibiotics experienced a
significant reduction in iNOS scores. Patients who were not treated
with antibiotics did not. There was a marginally significant reduction
in iNOS seen with ascorbic acid treatment (Wilcoxon signed rank test,
\( P = 0.06 \)).

Effects of Different Treatments on Nitrotyrosine. Ascorbic acid
treatment was associated with a significant reduction in nitrotyrosine

(Wilcoxon signed rank test, \( P < 0.001 \)). Patients treated with ascorbic acid
experienced a significantly larger reduction in nitrotyrosine com-
pared with those not treated with ascorbate (Fig. 3). There was a trend
toward a reduction in nitrotyrosine in patients treated with antibiotics.
β-Carotene supplementation had no protective effect.

| Effects of Different Treatments on Apoptosis. A trend toward a
reduction in TUNEL staining in patients receiving either β-carotene
alone (11.9-point score reduction) or ascorbic acid alone (8-point
score reduction) was noted (Fig. 4), but this did not achieve statistical
significance. As seen in Fig. 4, there was a significant negative
interaction of β-carotene and ascorbic acid, with either one alone
protecting against apoptosis, but not both together (\( P < 0.05 \), repeated
measures ANOVA).

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mononuclear cells. Rachmilewitz et al. (9) reported an association between chronic gastritis and iNOS activity in duodenal ulcer patients; in contrast to our findings, however, there was no clear association with infection (9).

In addition to the marked effect of antibiotic treatment on iNOS, the antioxidant β-carotene also diminished iNOS staining independent of antibiotic treatment. This is the first report to our knowledge of a diminution of iNOS activity by β-carotene in vivo, although in vitro protection has been shown to occur (11). The mechanisms by which β-carotene supplementation leads to a reduction in iNOS are unknown.

Nitrotyrosine staining, a marker for peroxynitrite and other nitrating species, could be demonstrated in H. pylori-infected patients, but its presence did not correlate as clearly with infection as did iNOS. In fact, following treatment with antibiotics, nitrotyrosine staining declined both in patients who cleared the infection and in those who did not. The reduction in nitrotyrosine staining in persistently infected patients may reflect decreased inflammation due to partial treatment of the infection in antibiotic-treated patients. However, because the majority of patients who failed to clear their infection did not receive antibiotics but did receive antioxidants, an alternative explanation of our findings is that, in addition to infection itself, oxidants govern the formation of nitrating species in the gastric mucosa. In support of this hypothesis, patients supplemented with ascorbic acid experienced a significant reduction in nitrotyrosine and, presumably, peroxynitrite as compared with those who did not receive ascorbic acid. These findings are consistent with findings of others of a protective effect of ascorbic acid on the formation of related N-nitroso compounds in vivo and in vitro (29–32). In contrast to ascorbic acid, β-carotene did not protect against the formation of nitrotyrosine, although it did result in a significant reduction in iNOS. One possible explanation for this counterintuitive result is that β-carotene may be inhibiting iNOS (e.g., by inhibiting nuclear factor-κ B) but not superoxide formation (e.g., by not inhibiting NADPH oxidase or xanthine oxidase). Our data do not prove that iNOS-related nitric oxide contributes to the formation of nitrotyrosine but only demonstrate coincidence of these two species. It is possible that dietary nitrites and/or H. pylori-related intraluminal ammonia production are also sources of reactive nitrogen intermediates (33). However,
TM domains, it is hard to speculate on the possible function(s) of the markers D4S2462 and D4S1557, with the GeneBridge 4 radiation brown/scarlet groups. However, given the sparse knowledge of subas well as the yeast ADPJ and two other human white-related genes Drosophila white protein, and it represents a member of an ABC gene consists of an ATP-binding domain at the NH2 terminus and six predicted TM segments in the COOH-terminal portion of the mole where two equally strong transcripts are present. Both transcripts segments underlined.

The ABCP gene was mapped to human chromosome 4q22, between clusters are significantly related (data not shown). ABC8, human; Abc8, mouse; ABC8L, human ABC8-like; Y0L075, yeast open reading frame; bfrlC, COOH-terminal half of bfrl. neighbor-joining analysis. Bootstap analysis of both neighbor-joining and maximum sequence was trimmed to a 673-residue, minimally overlapping segment and used for and several related genes was generated using PILEUP (Genetics Computing Group). The proteins transport very specific molecules, so it is likely that the chromosome 4, but to a region that would appear to exclude ABCP for some specific substrates, such as glucose (21).

In our study population, apoptosis, particularly of epithelial cells in the gastric neck glands, was associated with H. pylori infection, and clearance of the organism resulted in a trend (P = 0.08) toward a reduction in apoptosis. Although this association needs to be confirmed in larger studies, the presence of widespread apoptosis in the context of chronic infection is interesting in light of initial views of apoptosis as mediating physiological processes but not pathological states (35). Our findings again add H. pylori to a growing list of chronic infections associated with apoptosis, including HIV, Shigella flexneri and Escherichia coli (36–38). The association of H. pylori with apoptosis of epithelial cells is of further potential significance, because Helicobacter has been linked to atrophic gastritis (39); we hypothesize that an excess of apoptosis may contribute to atrophy of gastric glands and a reduction in acid-pepsin secretion that favors carcinogenesis.

A possible beneficial effect of antioxidants in preventing epithelial cell apoptosis found in this study is interesting in light of recent reports in a number of cell lines of protection against apoptosis by both retinoids (metabolites of β-carotene) and ascorbic acid (40–42). In one series of experiments reported by Delia et al. (43), however, retinoids and ascorbate had opposing effects on apoptosis, with ascorbic acid protecting against apoptosis induced by the retinoid N-(4-hydroxyphenyl)-all-trans-retinamide (43). These findings may help explain our otherwise puzzling observation of a negative interaction between ascorbic acid and β-carotene in the diminution of apoptosis.

Prevention of DNA damage may represent one common pathway by which either ascorbic acid or β-carotene prevents cancer and apoptosis. In vivo and in vitro studies have shown that β-carotene and ascorbic acid each diminish DNA damage, as measured by DNA adducts, micronuclei, and DNA single-strand breaks (44–47). Arroyo et al. (11) demonstrated that the mutagenicity of nitric oxide in Salmonella typhimurium TA1535 was effectively inhibited by β-carotene (11). It is conceivable that the chemopreventive effects of β-carotene could involve the inhibition of iNOS, because in our study, β-carotene led to a reduction of iNOS. Likewise, the reduction by ascorbic acid of nitrotyrosine (and, presumably, peroxynitrite) formation may help explain the protective effects of ascorbate. However, because both nitric oxide and peroxynitrite may be important elements in host tumor defense, their suppression by antioxidants could be undesirable in late stages of carcinogenesis.

This report shows that there is a spatial and temporal colocalization of H. pylori infection, expression of inducible nitric oxide, nitration of tyrosine residues, and epithelial cell apoptosis. Immunohistochemical expression of iNOS and nitrotyrosine is diminished by successful treatment with antimicrobial therapy, and this effect is potentiated by dietary supplementation with β-carotene and ascorbic acid. We speculate that these events may play a role in the pathogenesis and prevention of gastric cancer.

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The location of the ABCP gene on human chromosome 6. These markers define a short syntenic interval that is flanked in the human by SPPI some 4q22 and Abcp on mouse chromosome 6, some 6 segment is flanked by Hoxa (homeo box resistance. These pumps (PDR5, SNQ2, CDR1, and CDR2) confer treatment of acute leukemias and has shown promise in the treatment cell lines (22) resistant to the chemotherapeutic drugs mitoxantrone overexpressed and amplified in certain human breast and colon cancer is a transporter for some chemotherapeutic compounds and that overexpression of a number of full transporters that are involved in yeast multidrug transport genes. Cancer Res. (in press).

Acknowledgments

Further understanding of the function and regulation of ABCP may be important to effective chemotherapy.

References


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