Regulation of Pro-Inflammatory Phenotypes by Carcinogenic and Non-Carcinogenic *Helicobacter pylori* strains

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BRIEF. In order to quantify *H. pylori* virulence, a comparison of concentration of IL-8 secreted by AGS gastric epithelial cells infected with *H. pylori* strains varying in phenotype showed no correlation between strain virulence and IL-8 levels.

ABSTRACT. *Helicobacter pylori* is a Gram-negative bacterium that infects over 50% of the world's population and is strongly associated with the development of gastric cancer. However, only a fraction of infected persons develop this malignancy. Different variables affect *H. pylori* virulence including the production of inflammatory chemokines. This study focuses on differences in the ability of *H. pylori* strains isolated from patients with gastric cancer or gastritis alone to induce the pro-inflammatory chemokine, Interleukin-8 (IL-8). Strains isolated from Honduran biopsies were grown on TSA blood plates and then in liquid culture for infection. Cells were grown , plated , and incubated for 24 hours. Cells were infected at a multiplicity of infection (MOI) of 100. Six hours later, the supernatants were harvested, and used to measure IL-8 concentrations by ELISA. It was expected that *H. pylori* strains isolated from patients with gastric cancer would induce different levels of IL-8 compared to that of patients with gastritis alone. The results show no significant difference in the levels of IL-8 production. This suggests that IL-8 is not the only protein that could be quantified to indicate *H. pylori* virulence and emphasizes the importance of other factors.

INTRODUCTION.

Helicobacter pylori is a Gram-negative bacterium that is the strongest known risk factor for the development of gastric adenocarcinoma [1]. Consequently, *H. pylori* was named a Type I carcinogen by the World Health Organization. In 2012, gastric adenocarcinoma accounted for more than 700,000 deaths worldwide [1]. It is estimated that more than 50% of the world's population is infected with *H. pylori*, however, only 2-3% develop gastric adenocarcinoma [2]. This suggests that there is a combination of different aspects, such as bacterial factors, host genetics, and host dietary conditions, which influence the risk for disease [2]. Several bacterial virulence factors have been shown to contribute to increased risk for disease, and one of the most studied is the *cag* pathogenicity island. The *cag* island encodes a type IV secretion system that allows for delivery of the bacterial oncoprotein CagA into the host cell. Once inside the host cell, CagA induces numerous host cell changes that promote oncogenic effects [1]. Hence, presence of the *cag* pathogenicity island (*cag*PAI) factors could drastically affect the risk of disease [3].

Certain regions of the world have a higher incidence of gastric adenocarcinoma than others. They include East Asia, Eastern Europe, and Central America. This could be due to a multitude of variable ranging from diet to topography. It could also be due to the genetic variability amongst *H. pylori* strains, which can consequently determine virulence. To address, how strains may differ within high-risk populations, this study focused on strains isolated from patient biopsies from Honduras, a country in the Central American region. In a study done by Morgan *et al.*, analyzing a population in Western Honduras demonstrated the prevalence of certain polymorphisms in cytokines that made an individual more likely to acquire gastric adenocarcinoma [4]. It was found that the population in Western Honduras was extremely susceptible to gastric adenocarcinoma as about 17% of the surveyed population was homozygous for at-risk alleles and 89% of the population carried at least 1 at-risk allele [4]. This results in Honduras having a high incidence of gastric cancer, >15.4% for men and >8.2% for women, and a high mortality rate, >13.0% for men and >6.6% for women [2].

H. pylori induces gastritis in 90% of infected patients and does not typically progress further. However, a small percentage of patients go on to develop gastric adenocarcinoma, which occurs late in life between the ages of 60-80 years [5]. Gastric adenocarcinoma develops in a stepwise fashion starting with chronic gastritis [5]. From chronic gastritis, the disease evolves into multifocal atrophy, which causes gland loss, followed by intestinal metaplasia [5]. Finally, the disease proceeds into dysplasia, the abnormal growth of tissue and cells, which leads to gastric adenocarcinoma [5]. Both of the diseases observed in this study, gastritis and gastric adenocarcinoma, are associated with inflammation, which is induced by the same inflammatory chemokine, Interleukin-8.

Interleukin-8 is a proinflammatory chemokine secreted by different cells within the gastric epithelium. Il-8 induces a recruitment of neutrophils at the site of infection in order to control it. A study by Aihara *et al.* demonstrated that IL-8 is secreted by gastric epithelial cells following infection with *H. pylori* [6]. It also showed that *H. pylori* induces an upregulation of *IL-8* transcript levels within the host cell. Upregulation of *IL-8* mRNA and subsequent increases in IL-8 protein secretion lead to the recruitment of immune cells and the development of gastric inflammation [6]. Monitoring the production of this proinflammatory protein could define a relationship between strain virulence and inflammation.

This study sought to investigate the differences between strains isolated from Honduran patients with pre-malignant lesions, such as gastritis, in comparison to those with advanced malignant lesions, like gastric adenocarcinoma. Specifically, the concentration of IL-8 secreted by AGS gastric epithelial cells grown in cell culture following a co-culture with *H. pylori* strains isolated from patients with gastritis or gastric adenocarcinoma as measured. Previous findings in the laboratory suggested that *H. pylori* strains isolated from animals that developed adenocarcinoma showed attenuation in their ability to activate NF-κβ *in vitro* compared with the parental strain (Data not published). NF-κβ is a regulatory protein of proinflammatory chemokines and also regulates induction of apoptosis and cell proliferation. Therefore, it was hypothesized that *H. pylori* strains isolated from patients with gastritis as compared to *H. pylori* strains isolated from patients with gastric adenocarcinoma would induce different levels of IL-8 following co-culture with gastric epithelial cells.

MATERIALS AND METHODS.

Bacterial Culture.

Prior to the study, *H. pylori* bacterial strains were isolated from gastric biopsies acquired from Honduran patients diagnosed with either gastritis only or gastric adenocarcinoma. Eighteen strains were grown on TSA blood plates for 24-48 hours. Bacteria were then cultured in Brucella broth with 10% fetal bovine serum (FBS) for 16-18 hours at 37°C and 5% CO_2 with continuous shaking.

Cell Culture.

Human gastric epithelial cells (AGS) were grown in RPMI medium with 10% FBS at 37°C and 5% CO_2. Once cells reached 80-100% confluence, they were trypsinized, centrifuged, and resuspended in fresh media. Cells were then counted using a Neubauer chamber, plated into a six-well plate at $5x10^5$ per well and incubated for 24 hours.

After 16-18 hours in liquid culture, the concentration of *H. pylori* was measured by optical density (OD). The target OD range after 16-18 hours was from 1.0-

2.0. *H. pylori* cultures were then centrifuged and washed in 5 ml of phosphate-buffered saline (PBS). Pellets were suspended in PBS to a concentration of $1x10^9$ colony forming units (CFU)/ml. AGS cells were co-cultured with *H. pylori* at MOI of 100. One well was left with no bacteria as a negative control. Co-cultures were incubated at 37°C and 5% CO_2 for 6 hours and supernatants were harvested and stored at -20°C until IL-8 analysis was performed. Each experiment was done in duplicate.

Table 1. Information regarding patient demographics was collected when the biopsies were taken. The mean age for males was 42 years old while the mean age for females was 52 years old. All strains were cagA positive.

Gastritis Only			Gastric Adenocarcinoma		
Strain	Age	Sex	Strain	Age	Sex
135	40	F	108	49	F
141	37	M	112	77	M
145	52	F	116	35	M
151	30	F	199	34	M
152	51	F	209	61	F
165	37	M	235	40	M
188	42	F	236	74	M
189	73	F	238	64	F
213	61	M	242	61	F
229	45	M			

ELISA.

IL-8 was quantified in co-culture supernatants by Enzyme-Linked Immunosorbent Assay (ELISA) using the Human IL-8 ELISA Kit II from R&D Systems. Harvested supernatants were incubated for 2 hours in a 96-well plate precoated with a monoclonal antibody specific for IL-8. Wells were then washed. Next, streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IL-8 was added. The reaction was stopped after 30 minutes by adding sulfuric acid 2N, which acted as stop solution. Photometric intensity was then measured in a plate reader at 450 nm wavelength. IL-8 concentrations were determined using a standard curve made from known concentrations of IL-8.

Statistical Analysis.

Levels of IL-8 protein were expressed as fold over uninfected negative control. All experiments were performed on at least 2 independent occasions. Statistical analyses were performed using ANOVA with a Bonferroni post-test for specific comparisons within Prism GraphPad Software. The ANOVA was used to compare the three groups, uninfected, gastritis only and adenocarcinoma, and the Student's t-test was used to obtain a P-value from two group comparisons. P<0.05 was considered statistically significant.

RESULTS.

Patient Demographics and H. pylori cagA *Genotyping.*

During the patient biopsies, demographic data, such as patient sex and age, was acquired shown in Table 1. The median age for males was 56 years old and the median age for females was 52 years old. After isolating the strain, the cagA status for each was determined by genotyping. All strains were cagA positive.

IL-8 Production by H. pylori *Strains.*

H. pylori strains were isolated from Honduran patients with either gastritis alone or gastric adenocarcinoma. In this project, these strains were co-cultured with AGS gastric epithelial cells and levels of the proinflammatory chemokine, IL-8, were assessed by ELISA. Each data point represents an individual strain and data are represented as fold increase over uninfected group, the negative control. *H. pylori* isolated from patients with either gastritis alone or gastric adenocarcinoma induced significantly increased levels of IL-8 compared to the negative control, P<0.001. However, there was no significant difference (P=0.102) in levels of IL-8 induced by *H. pylori* strains harvested from patients with gastritis alone versus gastric adenocarcinoma (Figure 1). Demographic comparisons were performed as well. IL-8 levels between were compared . The strains were divided by virulence and then subdivided into strains that were acquired from males and females (Figure 2). The strains were also compared by subject age. The strains were divided and compared by virulence, but this time they were subdivided into strains acquired from subjects that were less than 50 years old and those that were over 50 years old (Figure 3). In both cases comparing by gender or age the data were not significant.

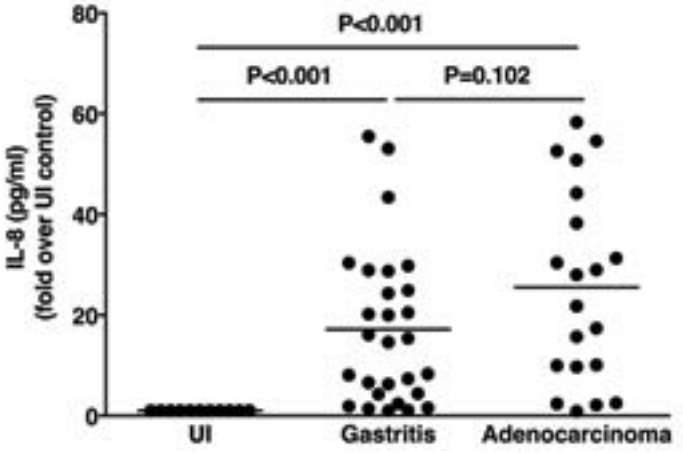

Figure 1. *H. pylori* induces significantly increased levels of IL-8 during co-culture with gastric epithelial cells. Each data point represents an individual strain of H. pylori isolated from patients with either gastritis alone or gastric adenocarcinoma. Data are represented as fold over uninfected control and ANOVAs were used to determine statistical significance. P<0.05 is considered statistically significant.

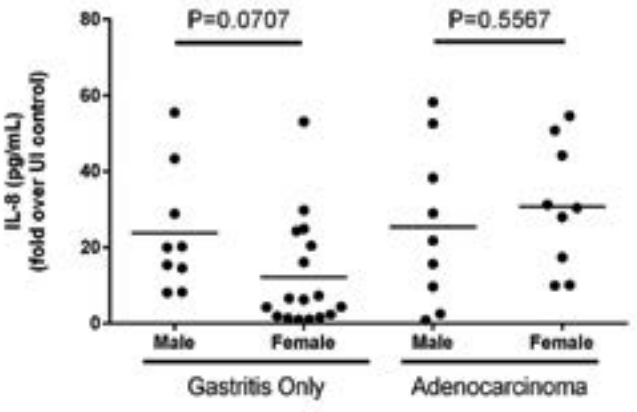

Figure 2. Representation of IL-8 production in reference to gender of patients. The demographic data specified the gender of the patient. Using that information, each virulence group was subdivided into groups regarding sex. The data showed that there is no significant difference.

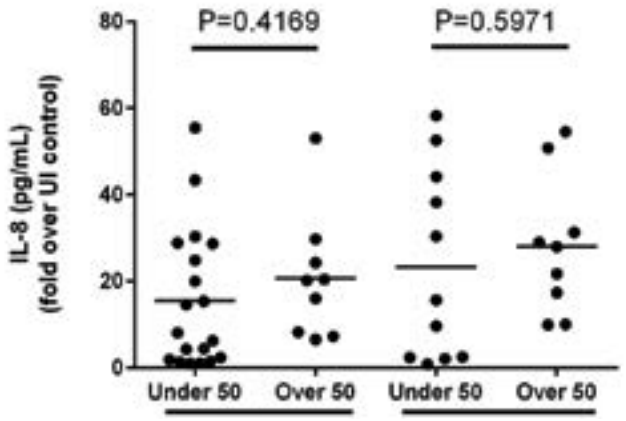

Figure 3. Representation of IL-8 production in reference to age of patients. The data was looked at in the context of age. The ages were divided at 50. Although there is no significant difference between IL-8 levels in patients under 50 when compared to patients over 50, in both cases, patients over 50 have a higher mean of IL-8 production.

DISCUSSION.

The original hypothesis was that *H. pylori* strains isolated from patients with gastritis alone would induce different levels of IL-8 following co-culture with gastric epithelial cells than *H. pylori* strains isolated from patients with gastric adenocarcinoma. It was found that *H. pylori* isolates from patients with either gastritis only or gastric adenocarcinoma induced significantly increased levels of IL-8 compared to negative group. However, there was no significant difference in levels of IL-8 induced by *H. pylori* strains harvested from patients with gastritis alone versus gastric adenocarcinoma. The same findings are upheld when comparing gender and age within the demographics of the isolates' subjects. These data demonstrate *H. pylori* strains isolated from Honduran patients with different disease states do not differ in their ability to induce IL-8. It is likely that other factors such as strain-specific virulence determinants affect the ability of these clinical isolates to induce different disease states. While there may be a relationship between *H. pylori* virulence and inflammation and disease progression, focusing on the presence of *cagA* gene and the ability of these strains to induce IL-8 production has proven to be negligible with respect to disease progression in this population. Alternatively, it is possible that the *H. pylori* strains isolated from patients with gastritis only had the potential to develop gastric adenocarcinoma, making the strains isolated from patients with gastritis only versus adenocarcinoma just as capable of producing the similar levels of the proinflammatory chemokine, IL-8, *in vitro*. In addition to the *cagA* status and virulence of *H. pylori* strains, another possibility that was not addressed in this study is that there are other factors that have been shown to contribute to increased risk for the development of gastric adenocarcinoma, such as other bacterial virulence factors, host genetics, and various environmental factors that can contribute to enhanced risk for disease. It has been shown in a previous study using Thai strains that there is a significant correlation between high levels of IL-8 and the risk of gastric adenocarcinoma [7]. However, this relationship seems to be different in the subset of Honduran strains tested for IL-8 in this study. It could also be due to the short time period in which the study was done. To assess risk, a longer time span is needed to get realistic, accurate data.

There are a couple of ways to refine this study even further to more accurately describe the relationship between *H. pylori* virulence and IL-8 production, as it has been previously demonstrated that CagA translocation is directly proportional to the level of IL-8 induction [8]. This way a relationship can be established between the translocation of CagA and how it could possibly relate to production of IL-8 between the two groups of strains. Another signaling cascade that is important in inflammation is NF-kβ activation. When virulence factors, such as CagA, are translocated into the cell, they interfere with host cell signaling that promote a proinflammatory response [9]. One result of *H. pylori* infection is the activation of NF-kβ, which in turn induces the production of IL-8 and subsequently a recruitment of immune cells [9]. Assessing NF-κβ activation by these strains may give a clearer picture of the relationship between virulence and IL-8 production. Since the development of gastric cancer is multifactorial, it would also be imperative to address not only the *H. pylori* strains isolated from these patients, but also evaluate how host genetics differ in the patient population and how environmental elements may also contribute to disease risk [1]. Cumulatively, the data demonstrate that strains isolated from Honduran patients with various disease outcomes do not differ in their ability to induce IL-8 *in vitro*.

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