*Helicobacter pylori* infection stimulates plasminogen activator inhibitor-1 (PAI-1) production by gastric epithelial cells.


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**Abbreviations:** *cag*, cytotoxin associated genes; ERK, extracellular-regulated kinases; JNK, Jun N-terminal kinase; MAP kinases, mitogen-activated protein kinases; PAI-1, plasminogen activator inhibitor-1; MOI, Multiplicity of infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAG, non-atrophic gastritis, PCR, polymerase chain reaction; PBS, phosphate buffered saline; uPA, Urokinase-type plasminogen activator; uPAR, Urokinase-type plasminogen activator receptor.
Abstract

Chronic infection with the gastric pathogen Helicobacter pylori significantly increases the risk of developing atrophic gastritis, peptic ulcer disease and gastric adenocarcinoma. H. pylori strains that possess the cag island, which translocates CagA into the host cells, augments these risks. The aim of this study was to determine the molecular mechanisms through which H. pylori upregulates the expression of plasminogen activator inhibitor-1 (PAI-1), a member of the urokinase activator system that is involved in tumor metastasis and angiogenesis. PAI-1 mRNA and protein levels were examined in tissues from H. pylori infected patients and in vitro using AGS gastric epithelial cells. In vitro, cells were infected with cag (+) toxigenic or cag (-) nontoxigenic strains of H. pylori or isogenic mutants. PAI-1 secretion was measured by ELISA and mRNA levels were determined using real-time PCR. The regulation of PAI-1 was examined using the ERK1/2 inhibitor and siRNA. Analysis of human biopsies revealed an increase in both PAI-1 mRNA and protein levels in patients with H. pylori gastritis compared to uninfected controls. Infection of AGS cells with H. pylori significantly increased PAI-1 mRNA expression and the secretion of PAI-1 protein. Moreover, PAI-1 mRNA and protein production was more pronounced when AGS cells were infected by H. pylori strains carrying a functional cag secretion system compared to those where it was absent. PAI-1 secretion was also reduced when cells were infected with either cagE- or cagA- mutants. Ectopic overexpression of CagA significantly increased PAI-1 mRNA and protein, whereas blockade of the ERK1/2 pathway inhibited H. pylori-mediated PAI-1 upregulation. These findings suggest that upregulation of PAI-1 in H. pylori-infected gastric epithelial cells may contribute to the carcinogenic process.
Introduction

*Helicobacter pylori* is a type I carcinogen and infection with this pathogenic bacterium is the leading cause of gastric cancer worldwide (28). It has been postulated that infection by *H. pylori* may contribute to this disease in two distinct ways. One way is by activating a chronic inflammatory response which causes a cascade of molecular and morphologic changes in the inflamed epithelium leading to mucosal atrophy, metaplasia, dysplasia and eventually gastric cancer. The other is that this bacterium may directly modify epithelial cell function and promote carcinogenesis by interfering with genes such as those regulating apoptosis, cell cycle control, tumor suppression and cell-to-cell contacts (25).

Plasminogen Activator Inhibitor-1 (PAI-1) is a 50 kDa protein belonging to a family of serine protease inhibitors known as SERPINS. PAI-1 is synthesized by a variety of cells and is induced by growth factors, cytokines, hormones and other stimuli and high PAI-1 levels are present in plasma from patients with acute or chronic inflammatory conditions. The main function of PAI-1 in tissue is to inhibit the action of urokinase-type plasminogen activator (uPA), a serine protease involved in tissue remodeling and cell migration (6).

Recent studies have shown that PAI-1 levels are elevated in tumors and the extent of increase correlates with increased potential for spread of the malignancy (15). It has also been noted that cancer patients with high PAI-1 levels have a poor prognosis for survival (3, 11, 15). High PAI-1 levels may promote the degradation of extracellular
matrix by decreasing the adhesive strength of cells for their substratum and detaching
cells from the extracellular matrices by inactivating integrins (8).

Previous reports have shown that PAI-1 levels are increased in *H. pylori*
associated gastric carcinoma and intestinal metaplasia (4, 33). The aims of this study
were 1) to determine whether PAI-1 is upregulated in *H pylori* gastritis; 2) to determine
whether infection of AGS gastric epithelial cells with *H. pylori* can directly result in
increased PAI-1 mRNA and protein; 3) to then identify the signal transduction pathways
that are required to regulate this process; and 4) to examine which pathogenic bacterial
factors influence the upregulation of PAI-1.
Material and methods

Cell culture and reagents. AGS gastric epithelial cells (American Type Culture Collection, Rockville MD) were maintained in RPMI with 10% fetal calf serum. Cell culture experiments were carried out using 6, 12, and 24 well polypropylene tissue culture plates (Corning Costar, Cambridge, MA). All experiments were carried out using 90% confluent monolayers unless otherwise stated. ERK1/2 inhibitor PD98059 was obtained from Calbiochem (La Jolla, CA.).

H. pylori strains, clinical isolates and isogenic mutants. H. pylori were cultured and prepared as previously described (19). Unless otherwise stated, experiments were performed using the cag+ H. pylori strain 43504 (American Type Culture Collection, Rockville MD). Isogenic H. pylori mutants lacking the cagE or cagA gene were studied together with the parental cag+, toxigenic wild type strains 60190 and J166. Mutants and clinical isolates were obtained from the Vanderbilt University Campylobacter and Helicobacter Laboratory (Nashville, TN). RNA isolated from isogenic H. pylori mutants was analyzed by RT-PCR to confirm that transcription of adjacent upstream and downstream cag genes (cagD, cagF and cagL) were intact. The bacterial strains used in this study are outlined in Table 1. H. pylori were heat-killed by boiling for 10 minutes, pelleted by centrifugation and resuspended in fresh media before being added to cells.

Patients and biopsy samples. Biopsies were obtained from patients who were recruited from routine endoscopies performed at Beth Israel Deaconess Medical Center, Boston. Full ethical approval for the study was granted by the Beth Israel Deaconess Medical Center Committee for Clinical Investigations. The eight patients used for the study...
comprised five males and three females, with an average age of fifty years old, and
comprised five Caucasians and three persons of Asian descent. Gastric biopsies for PAI-1
protein analysis were taken from the antrum, snap frozen in liquid nitrogen and stored at -
80ºC until needed for further processing. Biopsies were also collected for *H. pylori*
culture. For analysis of *H. pylori* infection, adjacent biopsies were placed in formalin and
stained with H & E. Biopsies with equivocal stains were analyzed further by Warthin-
Starry silver stain. The slides were examined microscopically for *H. pylori* gastritis using
the modified Sydney system (31). *H. pylori* infection was confirmed by culturing on
selection media (Skirrow agar) testing for urease and identified by colony morphology
(pinhead-sized translucent colonies) and microscopy (gram-negative curved organisms).

**PAI-1 ELISA.** PAI-1 protein levels were determined using a commercially available
human PAI-1 ELISA kit that detects the latent and active forms of PAI-1, as well as
uPA:PAI-1 complexes (Oncogene Science). This was used in accordance with the
manufacturer’s instructions.

**Immunohistochemical staining for PAI-1.** Immunohistochemical studies were
performed on archival formalin-fixed, paraffin embedded gastric biopsies, obtained from
Vanderbilt University Medical Center, Nashville and the Department of Pathology,
Technische Universität München, Munich, Germany. Sections were stained using PAI-1
mAb TJA6 (Abcam) according to the manufacturers instructions. In brief 4µm, sections
were deparaffinized and subjected to antigen retrieval by boiling in 10mM Sodium
Citrate buffer pH 6.0 for 10 minutes and placed on ice for 20 minutes, blocked in 3%
H₂O₂ for 10 minutes. The slides were washed twice in water, twice in TBST (TBS with 0.1% Tween-20) and twice in TBS.

Sections were blocked with the Avidin/Biotin Blocking kit (Vector Lab), and then treated with 5% normal donkey serum (Jackson Immuno Lab) in TBS for an hour at room temperature. Sections were then incubated with anti-PAI-1 mouse monoclonal antibody (1:20) at 4°C overnight. After washing slides twice in TBST and once in TBS, biotin conjugated donkey anti-mouse secondary antibody (1:200) was added to slides and incubated for an hour at room temperature. Signal was enhanced with Vectastatin ABC kit (Vector Lab). Peroxidase substrate was added for 4 minutes with DAB kit (Vector Lab). Slides were counterstained for 3 seconds in Hematoxylin (Fisher Scientific), then dehydrated and mounted using Permount (Fisher Scientific).

**Human gastric biopsy samples.** Gastric biopsies were homogenized on ice in 0.1M Tris pH 7.6 containing 0.1% Tween-80. Homogenates were centrifuged for 15 minutes at 4°C at 14,000g. Protein concentrations of the supernatant were then determined using the Bicinchoninic Acid (BCA) Protein Assay (Pierce).

**Western blotting.** Cell lysates were loaded onto a 7.5 % SDS-PAGE gel and transferred to nitrocellulose membranes (BIO-RAD, Hercules, CA.). Western blotting analysis was carried out using CagA antibodies (Austral Biologicals, San Ramon, California), ERK1/2 antibodies (Cell Signaling Technology Inc. Danvers, MA), PAI-1 (Abcam) and an actin antibody (Sigma-Aldrich, St. Louis, MO). Secondary antibodies were obtained from Santa Cruz Biotechnology.
Analysis of PAI-1 and GAPDH mRNA levels using real-time RT-PCR (Taqman assay). Total RNA and cDNA were prepared as described previously (16). PAI-1 and GAPDH mRNA levels were determined by real-time PCR using an ABI PRISM 7700 sequence detection system (ABI/Perkin-Elmer). cDNA was incubated for 2 minutes at 50°C, denatured for 10 minutes at 95°C, and then subjected to 40 cycles of annealing at 55°C for 20 seconds, extension at 60°C for one minute followed by denaturation at 95°C for 15 seconds. The gene specific primers used were PAI-1 sense primer, 5’ AAGGGTCTGCTGTGCACCAT 3’; PAI-1 antisense primer, 5’ AAACACCCTCACCCCAAGT 3’; GAPDH sense primer, 5’ GACCACGTCCATGCCATCA 3’; and GAPDH antisense primer, 5’ CATCACGCCACAGTTTCCC 3’. To detect amplicons generated using gene specific primers, dual labeled fluorogenic (real-time) probes containing FAM (at the 5’ end) and TAMRA (at the 3’ end) were synthesized (Sigma-Genosys, The Woodlands, Texas, USA). The real-time probes used were: PAI-1, 5’ CCCATCCTACGTGGC 3’ and GAPDH, 5’ ACCCAGAAGACTGTGGATGGCCCC 3’. PAI-1 levels in each sample were normalized to GAPDH expression, and the relative change in mRNA level was expressed as fold induction compared to untreated cells using the –ΔΔ CT method (22).

Plasmids and transfection. Transfection of AGS cells was carried out by using JetPEI (Polyplus transfection, NY) according to the manufacturer's recommendations; 1µg DNA for 24 well plates and 3µg DNA for 6 well plates. Cells were transfected with GFP-CagA expression vector containing the full length CagA sequence from H. pylori strain G27 (2), which was kindly provided by Dr. Manuel Amieva, Stanford University.
Small Interference RNA (siRNA) Transfection. Validated siRNA targeting MAPK1 mRNA, were obtained from Qiagen (Valencia, CA), and prepared according to manufacturer's instructions. Non-silencing siRNA duplexes (Qiagen) were used as negative controls. The day prior to transfection AGS cells were seeded at a density of $8 \times 10^4$/ml in 24 well plates. Cells were transiently transfected with 100nM MAPK siRNA or with the negative control using HiPerfect transfection reagent (Qiagen) according to the manufacturer’s instructions. Cells were used forty-eight hours post transfection and lysates were analyzed by Western blotting to assess gene knockdown.

Statistical Analyses. Statistical analyses were performed using Sigma-Stat for Windows version 2.0 (Jandel Scientific Software, San Rafael, CA). Analysis of variance followed by protected t tests were used for intergroup comparisons, except where otherwise stated.
Results

**PAI-1 protein and mRNA levels are increased in patients with *H. pylori* gastritis.**

Gastric biopsies were taken from uninfected patients with normal pathology and from patients with *H. pylori* gastritis. In order to determine PAI-1 protein levels, antral biopsies were homogenized and then lysates assayed by PAI-1 ELISA. The ELISA used measures free, total, active or complexed PAI-1 and is therefore able to measure total PAI-1 rather than just enzymatically active forms, which was the case in previously available kits. The data presented in Fig. 1a, show the total concentration of PAI-1 (ng/mg protein) in each biopsy. Patients with *H. pylori* gastritis were found to have a mean 2 fold higher level of PAI-1 compared to normal uninfected controls (n=4 for each group). PAI-1 mRNA levels were also analyzed using cDNA from control and *H. pylori*-infected subjects. Fig. 1b shows the results of the real time PCR analysis in which PAI-1 mRNA levels were found to be increased 5.6-fold (p<0.01) in gastric biopsies from *H. pylori*-infected patients compared to biopsies from uninfected controls (n=4 for each group).

To ascertain the cellular source of PAI-1 production in gastric tissue we performed an immunohistochemical analysis of PAI-1 in gastric biopsies from patients with different disease conditions (Fig. 1c). These specimens represented uninfected control tissue (panel A), uninfected non-atrophic gastritis (NAG) (panel B), *cag+ H. pylori*-infected NAG (panel C), *cag- H. pylori* infected NAG (panel D), intestinal metaplasia (panel E) and gastric cancer (panel F). Normal antral biopsy specimens showed little or no immunoreactivity for PAI-1; however biopsies from patients with gastritis (panels B, C and D) showed a marked increase in PAI-1 levels. Increased staining was observed in...
both epithelial and lamina propria inflammatory cells, in all the NAG samples; however, increased epithelial PAI-1 expression was most pronounced in the NAG specimens of those infected with \textit{cag+ H. pylori} (panel C). Intense PAI-1 staining of epithelial cells was evident in both intestinal metaplasia (panel E) and gastric cancer (panel F), which confirms previous findings (4, 33).

\textit{Cag+ H. pylori} selectively upregulate PAI-1 mRNA and protein secretion in AGS gastric epithelial cells. To confirm that gastric epithelial cells could be a source of increased PAI-1 during infection with \textit{H. pylori} we next investigated whether this bacterium could directly induce PAI-1 production by AGS cells. One aspect that may affect disease outcome is \textit{H. pylori} strain variation. Studies have demonstrated that strains harboring a 40 kb region of the \textit{H pylori} genome known as the \textit{cag} pathogenicity island, that encodes for a type IV secretion system, are more likely to induce symptomatic disease than those that do not carry this particular genetic element (29). Therefore to address this issue AGS gastric epithelial cells were infected with either a \textit{cag+} strain (J166) or a \textit{cag-} strain (J68) of \textit{H. pylori} at a MOI 20:1 (bacteria: cell). Uninfected cells were used as a control. Media was harvested over a 24-hour time course and the level of PAI-1 in the conditioned media at 4, 8, 12 and 24 hours was determined by ELISA. As shown in Fig. 2a, infection with the \textit{cag+} strain resulted in a significant increase in secreted PAI-1 levels compared to uninfected control AGS cells or AGS cells infected with the \textit{cag-} strain. PAI-1 secretion was also found to be dependent upon \textit{H. pylori} concentrations; \textit{H. pylori} at an MOI of 100:1 increased PAI-1 protein production by approximately 28\% compared to \textit{H. pylori} at an MOI of 20:1. In contrast, PAI-1 protein production was only increased by 7\% when AGS cells were treated with \textit{H. pylori} at an
MOI of 200:1; however this treatment was also associated with a marked increase in cell death (data not shown). To determine whether PAI-1 mRNA expression was increased in *H. pylori*-infected cells, AGS cells were infected with *cag*+ (J166) or *cag*- (J68) strains (MOI 20:1), RNA was isolated, reverse transcribed and the resulting cDNA was subjected to real-time PCR to quantify PAI-1 mRNA levels. In Fig. 2b infection of AGS cells with the *cag*+ strain resulted in increased in PAI-1 mRNA levels; 5-fold at 2 hours and 25-fold at 4 hours. In contrast, infection with the *cag*- strain produced only a 3-fold increase in PAI-1 mRNA levels at 4 hours. Data are expressed as fold induction above uninfected control cells as normalized to GAPDH mRNA levels.

Contact with live *H. pylori* is required to induce PAI-1 production by AGS cells. Our next experiment was to determine if PAI-1 upregulation was mediated by soluble secreted factors such as urease or vacuolating toxin, or through direct contact with the epithelial cells. AGS cells were either directly infected with live *H. pylori* (*cag*+ ATCC strain 43504) or co-cultured with the *H. pylori* using 0.1 μm filters to separate bacteria from cells. Heat–killed bacteria were also added to the cells. Only contact with live *H. pylori* was able to cause an increase in AGS cell PAI-1 secretion (Fig. 2c).

PAI-1 upregulation is dependent on CagA and the type IV secretion system. *H. pylori* has a number of virulence factors aside from soluble factors. The *cag* pathogenicity island, encodes for a type IV secretion system that has been demonstrated to activate an inflammatory response in gastric epithelial cells (5). In addition to its role in generating an inflammatory response, the type IV secretion system functions as a molecular syringe to deliver the immunodominant 120kDa CagA protein into the cytosol...
of the cell (1, 26, 37). CagA interacts with several host cell proteins including Grb2, SHP-2 and src kinases and is thought to be important in tumorigenesis (13, 23, 36). We set out to determine whether the cag pathogenicity island genes and/or the CagA protein regulate PAI-1 production by H. pylori-infected AGS cells. In order to do this we used H. pylori isogenic mutants. The mutants selected were the cagE- mutant, which contains a dysfunctional secretion system and thereby prevents translocation of the CagA protein into the eukaryotic cell, and the cagA- mutant, which although it has a functional type IV secretion system is not capable of expressing the CagA protein (1, 26). AGS cells were infected with the parent strain 60190, or cagE- or cagA- isogenic mutants (MOI 20:1) for a 24-hour period, conditioned media was harvested and assayed for PAI-1 by ELISA. As shown in Fig. 3a, wild type H. pylori stimulated PAI-1 protein production ~ 2.5 fold. This induction was significantly decreased following co-culture with either cagE- or cagA-mutant. There was no statistical difference between results obtained from the cagE- and cagA-mutants in terms of PAI-1 protein secretion, suggesting that the CagA protein is required for PAI-1 protein production by H. pylori-infected gastric epithelial cells. We also examined whether PAI-1 mRNA levels were reduced when cells were infected with cagE- or cagA- mutants when compared to the parental wild type strain. As shown in Fig. 3b, infection with the H. pylori cagE- mutant caused a significant reduction in PAI-1 mRNA levels at 2 and 4 hours compared to the wild type strain; however, with the cagA-mutant, decreased PAI-1 mRNA levels were only observed at the 4 hour time point (Fig. 3c). Additional experiments using an independent H. pylori cag+ strain (J166) and its cagE- and cagA- isogenic mutants produced similar results those obtained with strain
60190. The effects of the mutants on PAI-1 regulation were not a result of polar effects
induced by gene inactivation, since RT-PCR analysis of isogenic mutants revealed no
decrease in the expression of other cag genes adjacent to or downstream of cagA and
cagE (data not shown).

Overexpression of CagA results in increases in PAI-1 mRNA levels. To investigate
whether CagA per se was sufficient to induce upregulation of PAI-1, we next
overexpressed CagA in AGS cells. Forty-eight hours after transfection, PAI-1 mRNA
levels were determined using real time PCR. As shown in Fig. 4a, overexpression of
CagA was able to induce a 2-3 fold increase in PAI-1 mRNA levels. To determine
whether CagA overexpression could result in increased levels of PAI-1 protein, AGS
cells were transfected for up to 72 hours, and lysates collected at 24, 48 and 72 hours.
CagA expression and PAI-1 levels were then determined by Western blotting. As shown
in Fig. 4b, CagA was detected at all three time points. In cells expressing CagA, PAI-1
protein levels increased approximately 2.5 fold (by densitometry) 24, 48 and 72 hours
after transfection compared with those transfected with the control plasmid.

PAI-1 production is dependent on the activation of ERK1/2. We next explored the
signal transduction pathways involved in H. pylori-mediated PAI-1 upregulation. The
involvement of the MAPK signaling pathway in PAI-1 upregulation in epithelial and
other cell types has been well documented (9, 32). In previous studies we have found that
certain strains of H. pylori are able to induce epidermal growth factor receptor
transactivation and through this increased ERK1/2 signaling (17, 19). In our next series
of experiments we examined the contribution of the MAPK signaling pathway to the
regulation of PAI-1 in response to *H. pylori* infection. AGS cells were pretreated for one hour with different concentrations of the ERK1/2 inhibitor PD98059 (1µM, 5µM, and 10µM). Cells were then infected with *H. pylori* cag+ strain ATCC 43504 for 24 hours. Conditioned media was collected, and assayed for PAI-1 protein by ELISA. The results of this experiment shown in Fig. 5a, demonstrate that *H. pylori*-mediated PAI-1 production was inhibited in a dose dependent manner by the ERK1/2 inhibitor PD98059. Real-Time PCR analysis also revealed that PD98059 at 10µM reduced *H. pylori* induced PAI-1 mRNA levels to baseline (data not shown).

To confirm that ERK proteins participate in the signal transduction process we also used siRNA directed towards MAPK1 (ERK2). Fig. 5b shows a reduction in MAPK1 protein as a result of treatment of the cells with siRNA. Compared to cells transfected with the control siRNA we found that PAI-1 mRNA levels were reduced by approximately 50% in AGS cells treated with MAPK siRNA (Fig. 5c). A significant decrease in PAI-1 protein was also observed in *H. pylori*-infected AGS cells transfected with the MAPK1 siRNA (Fig. 5d).
Discussion

Plasminogen activator inhibitor type 1 (PAI-1) plays a key role in tumor progression and is believed to an important regulator of invasion, metastasis and angiogenesis (6). In this report, we have shown that infection of gastric epithelial cells by the gastric pathogen \textit{H. pylori} upregulates PAI-1, which if dysregulated may contribute to the process of oncogenic transformation.

A number of studies have shown that PAI-1 is upregulated in gastric cancer (15, 33). In a study by Sakakibara \textit{et al}, PAI-1 expression scores markedly increased with tumor stage and there was a significant increase in PAI-1 expression scores in metastasis-positive gastric cancers compared to metastasis-negative cancers. It was also determined in this study that overexpression of PAI-1 is a strong and independent prognostic factor for gastric cancer (33).

In the current study, we demonstrated that patients with \textit{H. pylori} gastritis have elevated PAI-1 mRNA and protein levels, compared with those who are uninfected. We have also shown that AGS gastric epithelial cells infected with \textit{H. pylori} respond by upregulating PAI-1 mRNA and protein production. While other investigators have examined the upregulation of different members of the urokinase plasminogen system and found increased levels of both uPA and uPAR (14), this is, to our knowledge, the first report of PAI-1 upregulation by \textit{H. pylori}-infected gastric epithelial cells. Iwamoto, \textit{et al.} found a significant upregulation of both uPA and uPAR in MKN45 and KATO-III cells, and also report that this effect was limited to \textit{H. pylori} carrying the \textit{cag} pathogenicity island genes, that parallels our findings with PAI-1 (14).
Another study by Varro et al examined the upregulation of PAI-2 by *H. pylori* in AGS cells. PAI-2 is another SERPIN closely related to PAI-1, and in that study, the authors were able to demonstrate that induction of PAI-2 by *H. pylori* was mediated by release of interleukin-8 and activation of cyclooxygenase-2.

In our study we determined that one of the bacterial factors required for generating the PAI-1 response was the type IV cag secretion system. Metalloproteinases (MMP’s) are similar to PAI-1 functionally in that they play a role in the processes of tumor growth and metastasis (35). Interestingly, the type IV secretion system also appears to be required for the upregulation of a number of epithelial cell MMP’s by *H. pylori*. Oliveira et al, have reported that *H. pylori* induced increases in MMP-2 and MMP-9 activity are dependent on the presence of a functional bacterial type IV secretion system (27). Krueger et al have shown that the type IV secretion system is also required for MMP-1 upregulation (21). Similar findings have been reported for MMP-7 by Crawford et al (7).

Once CagA is translocated into host cells, it activates various signal transduction pathways resulting in pathological cellular responses such as increased cell proliferation, motility and apoptosis, all of which can potentially contribute to the carcinogenic process (12). Recently investigators have shown that CagA can upregulate MMP-1 (30), activate β-catenin (10) and also cause disruption of tight junctions and loss of apical-basolateral polarity (2). In this study, we found that infection of AGS cells with *cagA*- mutants resulted in a decreased PAI-1 response when compared with the wild type parental strains, both at the level of protein and RNA. However, the reduction in PAI-1 mRNA
data with the cagA- mutants was not as low as that observed with the cagE- mutants. One possible explanation for this apparent discrepancy is that the bacteria may be regulating PAI-1 production at both the transcriptional and post transcriptional level and, therefore, mRNA data and protein production may not necessarily correlate (24). However, overexpression of CagA in the absence of infection produced a modest but statistically significant increase in PAI-1 mRNA and over a 72 hour period resulted in increased PAI-1 protein levels, demonstrating that CagA is required for maximal upregulation of PAI-1 by H. pylori.

We and others have previously reported that the MAP kinases are differentially regulated by cag+ and cag- strains of H. pylori, with cag- bacteria inducing weak or no activation of the various MAP kinases in infected epithelial cells (18). In this present study we found that ERK1/2 activation is required in part for H. pylori-mediated PAI-1 upregulation. This may offer one potential explanation as to why there is a decreased PAI-1 response to infection with both the cag- H. pylori strains and mutants. We also observed that some cag+ H. pylori strains were able to induce a more robust upregulation of PAI-1 than others; this is currently being investigated in greater detail by our laboratory.

In summary we have shown that PAI-1 is elevated both in H. pylori gastritis and in AGS cells infected with H. pylori. PAI-1 is upregulated by a number of known carcinogenic agents (20, 34, 38) and plays a pivotal role in invasion, metastasis and angiogenesis. Our findings suggest that increased PAI-1 production during H. pylori infection may contribute to H. pylori associated carcinogenesis.
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Table legends.

Table 1. Shows *H. pylori* strains and mutants used in the study.

Figure legends.

Fig. 1a. PAI-1 protein levels are elevated in gastric biopsies in patients with *H. pylori*-gastritis compared with controls. PAI-1 levels in gastric biopsies were measured by ELISA and expressed as mean concentration of PAI-1 ng/mg protein. Graph shows mean PAI-1 concentration ±SD for each group. *p*<0.01 *H. pylori*-infected patients compared to biopsies from uninfected controls (n=4).

Fig. 1b. PAI-1 mRNA levels are elevated in gastric biopsies obtained from patients infected with *H. pylori* compared with healthy controls. Data are normalized by GAPDH. n=4 for each group.

Fig. 1c. Histochemical staining of formalin-fixed paraffin embedded gastric biopsies with PAI-1 mAb. (A) Normal uninfected control tissue, (B) non-atrophic gastritis-uninfected, (C) non-atrophic gastritis-infected with *cag+ H. pylori*, (D) non-atrophic gastritis-infected with *cag- H. pylori*, (E) intestinal metaplasia and (F) gastric cancer. Magnification 40X.

Fig. 2a. Time course of PAI-1 secretion by *cag+ H. pylori*-infected AGS cells. Graph shows secretion of PAI-1 by AGS over 4, 8, 12 and 24 hours in response to infected with control (media alone), *cag+ strain J166*, *cag- strain J68*. At each time point, media was collected and PAI-1 levels measured by ELISA. Data are presented as means ± SD of
triplicates. *** p< 0.0001 control levels of PAI-1 vs. cag+ H. pylori. Results are representative of 3 independent experiments.

**Fig. 2b.** Infection of AGS cells with cag+ H. pylori upregulates PAI-1 mRNA expression. AGS cells infected with either cag+ H. pylori strain J166, or cag- H. pylori strain J68. RNA was isolated from infected cells at 2 and 4 hours, reverse transcribed and then analyzed by Real-Time PCR. Data are presented as fold induction above control and normalized by GAPDH.

**Fig. 2c.** PAI-1 secretion by H. pylori-infected AGS is not mediated by soluble factors. AGS cells were infected with live H. pylori cag+ strain ATCC 43504, live bacteria separated by a 0.1µm filter or heat-killed bacteria. Media was harvested at 24 hours and PAI-1 protein levels were determined by ELISA. Data are presented as means ± SD of triplicates. p< 0.0001 PAI-1 live H. pylori infection vs. filter and p< 0.0001 live H. pylori vs. heat-killed. Results are representative of 3 independent experiments.

**Fig. 3a.** Infection of AGS cells with cagE- and cagA- H. pylori mutants generated a weaker PAI-1 secretory response than infection with their wild type counterpart. AGS cells were infected with wild type strain 60190 or its cagE- or cagA- isogenic mutants for 24 hours. Media was collected and assayed for PAI-1 protein by ELISA. Data are presented as means ± SD of triplicates. p< 0.0001 PAI-1 wild type H. pylori infection vs. cagE- and p< 0.0001 wild type H. pylori vs. cagA-. Results are representative of 3 independent experiments.
Fig. 3b. Infection of AGS cells with the cagE- mutant resulted in an attenuation in the PAI-1 mRNA response compared with infection with its wild type counterpart. AGS cells were infected with wild type strain 60190 or its cagE- isogenic mutant for 2 and 4 hours. RNA was isolated, reverse transcribed and PAI-1 mRNA analyzed by real time PCR. Data are presented as fold induction above control and corrected for GAPDH.

Fig. 3c. Infection of AGS cells with the cagA- mutant resulted in a decrease in the PAI-1 mRNA response compared with infection with its wild type counterpart. AGS cells were infected with wild type strain 60190 or its cagA- isogenic mutant for 2 and 4 hours. RNA was isolated, reverse transcribed and PAI-1 mRNA analyzed by real time PCR. Data are presented as fold induction above control and corrected for GAPDH.

Fig. 4a. Transfection of CagA into AGS cells results in increased PAI-1 mRNA levels. Graph shows PAI-1 mRNA levels in AGS cells 48 hours after being transfected with either a CagA or control plasmid. Samples were analyzed by real time PCR and data are presented as fold induction above control and corrected for GAPDH. p< 0.0001 PAI-1 mRNA control vs. CagA transfection (n=3). Results are representative of 4 independent experiments.

Fig. 4b. Western blot demonstrating the time course of CagA and PAI-1 expression in AGS cells following transfection over a 72 hour period. Blots were stripped and reprobed for actin which acts as a loading control. Results are representative of 2 independent experiments.
**Fig. 5a.** The ERK1/2 inhibitor PD98059 inhibits *H. pylori*-mediated PAI-1 protein production. AGS cells were pretreated for one hour with 1µM, 5µM or 10µM PD98059, then infected with *H. pylori* cag+ strain ATCC 43504. Media was harvested at 24 hours and PAI-1 protein levels were determined by ELISA. Data are presented as means ± SD of triplicates. Results are representative of 3 independent experiments.

**Fig. 5b.** Representative Western blot showing ERK1/2 expression in AGS cells following transfection with control siRNA and MAPK1 siRNA. Blots were stripped and reprobed with actin to act as a loading control.

**Fig. 5c.** Transfection of MAPK1 siRNA into AGS cells results in decreased PAI-1 mRNA levels. Graph shows PAI-1 mRNA levels in AGS cells 48 hours after being transfected with either control or MAPK siRNA. Samples were analyzed by real time PCR and data are presented as fold induction above control and corrected for GAPDH. p< 0.005 PAI-1 mRNA control siRNA vs. MAPK1 siRNA (n=3). Results are representative of 3 independent experiments.

**Fig. 5d.** MAPK1 siRNA inhibits *H. pylori*-mediated PAI-1 protein production. Graph shows level of PAI-1 secreted by AGS cells transfected with control or MAPK siRNA. Data are presented as means ± SD. (n=3). Results are representative of 3 independent experiments.
Table 1.

<table>
<thead>
<tr>
<th>Strain of H. pylori</th>
<th>Virulence factors</th>
<th>mutants</th>
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<tbody>
<tr>
<td>ATCC 43504</td>
<td>cag+</td>
<td></td>
</tr>
<tr>
<td>J68</td>
<td>cag-</td>
<td></td>
</tr>
<tr>
<td>J166</td>
<td>cag+</td>
<td>cagE-, cagA-</td>
</tr>
<tr>
<td>60190</td>
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</table>
Fig. 2a.

Fig. 2b.

Fig. 2c.
Fig. 5a.

![Graph showing PAI-1 levels with different conditions.](image)

*** p<0.001

Fig. 5b.

![Western blot images of ERK1/2 and Actin in Control, MAPK1 siRNA, and MAPK1 siRNA conditions.](image)

Fig. 5c.

![Bar graph showing PKC-1 mRNA levels with control and H. pylori conditions with MAPK1 siRNA.](image)

P < 0.005

Fig. 5d.

![Graph showing PAI-1 levels with control and MAPK1 siRNA conditions.](image)

P < 0.001