Epithelial and bacterial metalloproteinases and their inhibitors in *H. pylori* infection of human gastric cells

MONIKA GÖÖZ, PAL GÖÖZ, AND ADAM J. SMOLKA

*Divisions of *1Gastroenterology and Hepatology and *2Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29425

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Gööz, Monika, Pal Gööz, and Adam J. Smolka. Epithelial and bacterial metalloproteinases and their inhibitors in *H. pylori* infection of human gastric cells. *Am J Physiol Gastrointest Liver Physiol* 281: G823–G832, 2001.—To test the hypothesis that *Helicobacter pylori* regulates gastric cell secretion of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), culture media from infected and uninfected human gastric adenocarcinoma (AGS) cells were analyzed by zymography, MMP activity assays, and immunoblotting. AGS cells secreted gelatinolytic (prominently 90 kDa) and caseinolytic (110 kDa) enzyme activity, and TIMP-3 immunoreactivity. *H. pylori* infected AGS cells secreted gelatinolytic activity (60 kDa), MMP-3-like enzyme activity, and TIMP-3 immunoreactivity. *H. pylori* infection increased the 110-kDa caseinolytic activity and induced new gelatinolytic (~35 kDa) and caseinolytic (22 kDa) activities. Infection also increased both basal secretion and activation of MMP-1 and MMP-3, enhanced TIMP-3 secretion, and increased the formation of MMP-3/TIMP-3 complexes. TIMP-1 and TIMP-2 secretion were unchanged. Normal AGS cells showed a pancellular distribution of TIMP-3, with redistribution of immunoreactivity toward sites of bacterial attachment after *H. pylori* infection. The data indicate that MMP and TIMP secretion by AGS cells is modulated by *H. pylori* infection and that host MMP-3 and a TIMP-3 homolog expressed by *H. pylori* mediate at least part of the host cell response to infection.

AGS cells; *Helicobacter pylori*; host response; metalloproteinase; tissue inhibitor of metalloproteinases

*Helicobacter pylori* is a spiral, microaerophilic, neutralophilic gram-negative bacterium that colonizes the gastric mucosa in 25–50% of the population in developed countries and 70–90% in developing countries (15). *H. pylori* is a causative agent of peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (4). Gastrroduodenal diseases caused by *H. pylori* are associated with infiltration of the gastric mucosa by neutrophils, lymphocytes, monocytes, and plasma cells. Mobilization of inflammatory cells is a host-response mechanism induced by *H. pylori*-stimulated gastric epithelial cell secretion of an array of cytokines, most prominently interleukin-1β and -8 (9). Another host-response mechanism may involve secretion of the antibiotic peptide β-defensin. mRNA coding for β-defensin was reported to be induced by in vitro *H. pylori* infection of human gastric epithelial cells (18). An analogous host-response mechanism was recently identified in mouse small intestine, where *Escherichia coli* infection stimulated generation of the antibiotic peptide α-defensin by host metalloproteinase-catalyzed hydrolysis of an inactive α-defensin proform (19).

The identities and mechanisms of action of virulence factors secreted by *H. pylori* that induce such host responses are poorly understood. Genomic DNA of virulent *H. pylori* strains includes a 40-kb segment of DNA (pathogenicity island) containing up to 29 open reading frames, two of which encode a vacuolating toxin (VacA) and cytotoxin-associated immunodominant protein (CagA) (29). Other pathogenicity island genes encode membrane-associated proteins similar to those comprising bacterial type IV secretion systems (20). Most recently, *H. pylori* type IV secretion system proteins have been shown to promote migration of CagA protein into gastric epithelial cells, where it is tyrosine phosphorylated and subsequently modulates phosphorylation of host proteins (10).

Inflammatory cell mucosal infiltration and antibiotic peptide activation both involve metalloproteinase activity. Matrix metalloproteinases (MMPs) are zinc-containing endopeptidases that degrade extracellular matrix proteins during tissue morphogenesis and remodeling in wound healing and are associated with tumor angiogenesis, invasion, and metastasis, arthritis, and atherosclerosis (8, 13, 23). MMP expression is regulated transcriptionally by growth factors, hormones, cytokines, and cell-cell matrix interactions. Cell surface (membrane-type) and extracellular MMPs secreted by epithelial and stromal cells are activated by proteolytic cleavage of their NH2 terminal domains and are inhibited by noncovalent 1:1 stoichiometric interaction with tissue inhibitors of metalloproteinases (TIMPs). These constitute a family of four homologous 20- to 30-kDa proteins (TIMP-1, -2, -3, and -4) with 125-amino acid NH2 terminal domains and 65-amino
acid COOH terminal domains, each stabilized by three disulfide bonds (1).

Recent studies have addressed gastric MMPs and TIMPs and their role in gastric pathophysiology. MMP-1 (interstitial type I and type III-specific collagenase), the type IV-specific collagenases MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B), and TIMP-1 have been localized immunohistochemically in parietal cells, surface cells, and foveolar epithelial cells of normal human and rabbit gastric mucosa (14). In contrast, enhanced levels of mRNAs encoding MMP-1, MMP-3, MMP-7, and TIMP-1 were detected by in situ hybridization assays of human gastric mucosal samples representing peptic ulcers, Crohn's disease, and ulcerative colitis, and their involvement in tissue remodeling and epithelial regeneration was suggested (12). Overexpression of different MMPs has been reported in gastric cancer and appears to be correlated with increased metastatic potential (7).

Bacterial pathogens themselves synthesize and secrete a wide array of proteinases, of which the most common are metalloproteinases, particularly membrane-bound forms, which possess additional functional domains compared with the secreted forms (17). The functions of bacterial metalloproteinases are currently the focus of intense investigation and appear to include disruption of host defenses against invasive bacteria. Potential targets for these enzymes are host protease cascades, cytokine networks, extracellular matrix components, and host enzyme inhibitor inactivation (7). Analysis of the complete H. pylori genome revealed the presence of a nucleotide sequence encoding a putative zinc metalloprotease (16). At the same time, a 200-kDa zinc-dependent endoproteinase was found to be stably expressed on the surface of the bacterial outer membrane and also to be secreted into the culture medium, raising the possibility of metalloproteinase involvement in degradation of host proteins (21).

We recently investigated the effects of H. pylori infection on H+-K+-ATPase α-subunit promoter activity in human adenocarcinoma (AGS) cells (5). The responsiveness of AGS cells to the acid secretagogues histamine, epidermal growth factor, and phorbol ester, in terms of elevation of free intracellular Ca2+ and cAMP concentrations, suggested that AGS cells were an appropriate model in which to study the effect of H. pylori on gastric epithelial MMPs and TIMPs. In the present study, we tested the hypothesis that H. pylori infection of AGS cells modulates MMP and/or TIMP expression levels and activities. The results indicate that AGS cells secrete MMP-1 and MMP-3, as well as TIMP-1, -2, and -3, and that H. pylori secretes both MMP-3-like enzyme and TIMP-3-like protein. H. pylori infection of AGS cells also enhances activation of host MMP-1 and MMP-3.

MATERIALS AND METHODS

Materials. Ham's F-12, HEPES, and antibiotic-antimycotic solution (10,000 U/ml penicillin G, 25 mg/ml amphotericin B, and 10,000 mg/ml streptomycin) were acquired from Cellgro Mediatech (Herndon, VA). Fetal bovine serum was obtained from Atlanta Biologicals (Nocora, GA). MMP-1 (Antibody (Ab)-1), TIMP-1 (Ab-1), TIMP-2 (Ab-1), and MMP-3 (Ab-2) monoclonal antibodies were purchased from Oncogene (Cambridge, MA). TIMP-3 (AB802) polyclonal antibody and MMP-3 (MAB 1339) monoclonal antibody were purchased from Chemicon (Temecula, CA). Control MMPs and TIMPs prepared from human skin fibroblast-conditioned media were obtained from Sigma (St. Louis, MO). Secondary antibodies were obtained from Rockland Immunocchemicals (Gilbertsville, PA) and Jackson Immunoresearch Laboratories (West Grove, PA). Fluorogenic substrates for measurement of MMP enzyme activity were purchased from Bachem (Torrance, CA). All other reagents were of molecular biology grade or the highest grade of purity available.

Cells and bacteria. AGS cells [CRL 1739; American Type Culture Collection (ATCC)] were maintained in AGS medium (Ham's F-12, 10% fetal bovine serum, 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, and 100 μg/ml streptomycin) at 37°C in a 5% CO2-95% air incubator and used between passages 42 and 56. A strain of H. pylori positive for vacuolating cytotoxin (vacA), cytotoxin-associated protein (cagA), and urease (ATCC number 49603) was cultured on 5% horse blood agar plates (Remel, Lenexa, KS) incubated at 37°C in sealed microaerophilic pouches (BBL Campy Pouch; Becton Dickinson, Cockeysville, MD). Cultures were routinely screened for urease activity. For AGS cell infections, H. pylori were harvested between 48 and 72 h after inoculation of agar plates, resuspended in sterile PBS, and enumerated by absorbance at 600 nm [optical density (OD) of 2.4 × 109 colony-forming units/ml]. AGS cells (2.5 × 105) were seeded into T-75 flasks and infected at 90% confluence with H. pylori at a multiplicity of infection (MOI) of 50 in either serum-free Ham's F-12 or in fresh Ham's F-12 containing 10% serum. Infected AGS cells, uninfected AGS cells, and H. pylori alone (in the same amounts as used for infection) were cultured in T-75 flasks for 4, 6, 24, or 48 h as required. Conditioned media were centrifuged for 10 min at 4,000 g to remove cells and bacteria, and serum-free media were concentrated 10–200× by ammonium sulfate precipitation. Unconditioned serum-free medium was concentrated and used as a negative control. Media aliquots were stored at −70°C until used.

Zymography. Concentrated media were mixed with SDS-PAGE sample buffer (10% glycerol, 2% SDS, and 63 mM Tris, pH 7.0) without reducing agent and applied to 4.5% reducing gels or to nonreducing gels containing 4–16% acrylamide gels containing 0.1% casein (NuPAGE; Novex, Encinitas, CA). Electrophoresis was carried out for 90 min at 125 V at room temperature, and resolved proteins were renatured in situ by immersing the gels in 2.7% (wt/vol) Triton X-100 for 30 min at room temperature. The gels were then rinsed in zymogram developing buffer (Novex) for 30 min and incubated overnight at 37°C in the same buffer. Gelatinolytic or caseinolytic activity in the gels was visualized as negative staining with Coomassie brilliant blue. Metalloenzyme activity was stimulated by addition of 2 mM aminophenylmercuric acetate (APMA) to samples before electrophoresis. Metalloenzyme activity was inhibited similarly by using 20 mM EDTA and 2 mM 1,10-phenanthroline. Zymograms shown in this study are representative replicates selected from at least three experiments.

Enzyme activity assay. MMP activity of unconcentrated and 10–130× concentrated serum-free conditioned media from infected and uninfected AGS cells, as well as from H. pylori cultures, was measured using the fluorogenic MMP-1- and MMP-3-specific synthetic peptide substrates M-1905 and M2110 [Dnp-Pro-Leu-Gly-Cys(Me)-His-Ala-D-Arg-NH2 and
Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH₂, respectively). In all cases, specificity of enzyme activity was shown by enzyme inhibition with 2 mM 1,10-phenanthroline. For studying the interaction between AGS cells and bacterial MMPs and TIMPs, equal amounts of AGS-conditioned and H. pylori-conditioned concentrated media (with or without heat treatment) were combined and incubated at 37°C for 30 min. Heat treatment of the samples was carried out at 65°C for 20 min. Substrate fluorescence evoked by metalloproteinase activity was measured for 200 s in an Amino-SLM DW2 spectrofluorimeter at an emission wavelength of 399 nm with a slit width of 1 mm and sensitivity of 800 V.

Western blotting. Aliquots of concentrated media from H. pylori-infected or uninfected AGS cell cultures, or of medium from H. pylori alone, were mixed with SDS-PAGE sample buffer with or without reducing agent (dithiothreitol) and heated at 95°C for 5 min. Electrophoresis was carried out on 4–12% or on 10% acrylamide Bis-Tris gels (NuPAGE; Novex) for 35 min at 200 V. Resolved proteins were transferred to 0.2-μm nitrocellulose membranes (Osmonics, Westborough, MA) and blocked with 5% nonfat dry milk for 2 h. Protein replicas were washed three times in TTBS (20 mM Tris, 0.5M NaCl, pH 7.5, and 0.05% Tween 20) and incubated overnight at room temperature in the appropriate dilutions of MMP or TIMP antibodies. The replicas were then washed three times in TTBS and incubated for 1 h at room temperature in appropriate dilutions of secondary antibodies. Immunoreactive protein bands were visualized by using enhanced chemiluminescence and recorded on ECL Hyperfilm (ECL kit; Amersham Pharmacia, Piscataway, NJ).

Immunocytochemistry. Cells were seeded onto glass coverslips, cultured in 24-well plates to 90% confluence, and then incubated with H. pylori at an MOI of 50 for 3 h. H. pylori was also seeded alone and then concentrated onto glass coverslips by centrifugation (CytoSpin). Adherent cells on the coverslips were fixed with acetone for 6 min and washed several times with PBS (pH 7.4). Coverslips were blocked in 1% BSA in PBS, coverslips were incubated for 30 min at room temperature in 1:100 dilutions of FITC-labeled goat anti-rabbit antiserum diluted 1:50 in 1% BSA in PBS. After three washes in PBS, coverslips were incubated for 30 min in 1:100 dilutions in 1% BSA in PBS of FITC-labeled goat anti-rabbit IgG, and then washed three times in PBS. Negative controls included replacement of primary antibodies with nonimmune rabbit serum. Images of immunostained cells were recorded under epifluorescent illumination (490 nm) in a Zeiss Axiosvert 35 microscope equipped with a digital camera (CCD-100, Dage-MTI) and ImagePro 3.0 Plus software.

Protein measurement. Culture medium protein concentrations, measured with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), were as follows. The protein concentration of serum-free unconditioned medium was 0.38 ± 0.08 mg/ml, that of AGS-conditioned medium was 0.75 ± 0.17 mg/ml, that of infected AGS-conditioned medium was 1.96 ± 0.29 mg/ml, and that of H. pylori-conditioned medium was 0.82 ± 0.21 mg/ml. Data are expressed as means ± SE (n = 6). Five-microliter culture medium aliquots were used for enzyme activity assays. For zymographic and immunoblot assays, 18-μl culture medium aliquots were applied to each lane.

Data analysis. Densitometric analysis of zymogram gels was carried out using Scion Image scanning software. Data are expressed as percentage of control activity (the densitometric intensity of uninfected AGS cell-conditioned medium was set to 100%) and are shown next to the gels. Enzyme activity data were analyzed by linear regression and were expressed as initial reaction velocity (Δfluorescence unit/s).

Data comparisons were made by Student’s t-test and ANOVA, with P < 0.05 regarded as significant. All experiments were carried out at least three times.

RESULTS

As a first step toward the identification and characterization of putative gastric epithelial cell secretory MMPs, gelatinolytic and caseinolytic activities in AGS cell-conditioned culture media were assessed by zymography. Aliquots of 50–200× concentrated serum-free culture medium were electrophoresed on denaturing, nonreducing, gelatin-containing gels, which were subsequently stained with Coomassie blue; resolved gelatinolytic proteins were detected as unstained bands. The zymogram in Fig. 1 shows traces of gelatinolytic activity in unconditioned medium, and AGS cell-conditioned medium shows multiple bands of activity from >250 to <30 kDa, with gelatinolytic activity being most prominent at ~90 kDa. Pretreatment of AGS cell-conditioned medium for 30 min at 37°C with APMA increased activity at ~90 kDa. APMA treatment also increased activity at ~35 kDa at the expense of activity at ~160 and 60 kDa. Addition of either 20 mM EDTA or 2 mM 1,10-phenanthroline to AGS cell-conditioned medium inhibited activity at ~160, ~90, and ~60 kDa. EDTA treatment also inhibited activity at 120 kDa. These data indicate that AGS cells secrete a spectrum of gelatinases, a subset of which are Ca²⁺- and Zn²⁺-dependent metalloenzymes and are activated by APMA.

As shown in the zymogram in Fig. 2, coculture of AGS cells with H. pylori increased gelatinolytic activity at ~90 kDa and at lower molecular masses ranging
from 25 to 40 kDa compared with the uninfected control. No gelatinolytic activity was present in the concentrated serum-free medium or in medium conditioned by *H. pylori* alone. *H. pylori*-induced changes in three secreted gelatinolytic activities are expressed in Fig. 2 as percent densitometric intensity compared with uninfected AGS cell control (100%).

In complementary experiments, the presence of metalloproteinases of the stromelysin family (MMP-3, MMP-10, and MMP-11) in AGS cell-conditioned media was assessed by electrophoresis in casein-containing gels. As shown in the zymogram in Fig. 3, APMA-inducible caseinolytic activity at 110 and 22 kDa was detected in AGS cell-conditioned medium. *H. pylori* infection of AGS cells at an MOI of 50 resulted in increased APMA-inducible caseinolytic activity being detected in the cell-conditioned medium. As in Fig. 2, changes in caseinolytic activity following APMA treatment and/or *H. pylori* infection are expressed in the figure as percent densitometric intensity compared with uninfected AGS cell control (100%). Serum-free AGS cell medium conditioned by growth of *H. pylori* alone showed the presence of weak caseinolytic activity at ~50 kDa (Fig. 4). This activity may represent an activated form of the previously-reported 200-kDa *H. pylori* metalloproteinase (21).

To characterize further the metalloproteinase activities detected by zymography, we assessed the immunoreactivity of AGS cell-conditioned media by immunoblotting with a panel of MMP antibodies. Serum-free AGS cell culture medium was used to avoid detection of matrix metalloenzymes present in serum. Under serum-free conditions, the rate of AGS cell protein secretion is decreased, and therefore culture media were concentrated by ammonium sulfate precipitation. Concentrated (50–200 ×) AGS cell-conditioned media resolved on reducing 4–12% or 10% acrylamide SDS-PAGE gels showed immunoreactivity with both MMP-1 and MMP-3 antibodies at ~50 kDa (Fig. 5).

Fig. 2. Gelatin zymography of serum-free culture media conditioned by AGS cells alone, by AGS cells infected by *Helicobacter pylori* (AGS + *Hp*), and by *H. pylori* alone. Lane *M* refers to unconditioned serum-free medium. Calculated molecular masses of gelatinolytic bands are shown at left. Densitometric analysis of gelatinolytic bands is shown below the gel. Band intensities in lane AGS were set to 100%.

Fig. 3. Casein zymography of serum-free culture media conditioned by uninfected AGS cells (*lanes 1 and 2*) and by *H. pylori*-infected AGS cells (*lanes 3 and 4*) shows activation of caseinolytic activity by APMA (2 mM; *lanes 2 and 4*). Calculated molecular masses of caseinolytic bands are shown at left. Densitometric analysis of caseinolytic bands is shown below the gels. Band intensities in lane 1 were set to 100%.

Fig. 4. Casein zymography of *H. pylori*-conditioned serum-free culture medium shows weak caseinolytic activity at relative molecular weight (Mw) of ~60,000 (mol mass 60 kDa).
MMP-3 antibody (MAB 1339) also detected immunoreactivity at ~60 kDa (Fig. 5B), and another MMP-3 antibody (Ab-2) revealed the presence of 25-, 35-, and 45-kDa immunoreactive species corresponding to active forms of stromelysin-1 (Fig. 5C). *H. pylori* infection of the AGS cells increased MMP-1 immunoreactivity (Fig. 5A) and increased the intensity of MMP-3-immunoreactive bands representing both latent (~60 kDa) and active (~50, 45, and 25 kDa) forms of the enzyme (Fig. 5, B and C). No MMP-1 or MMP-3 immunoreactivity was detected in serum-free AGS cell medium conditioned by *H. pylori* alone (data not shown). These data indicate that AGS cells secrete the metallopro-teinases MMP-1 and MMP-3 and that *H. pylori* infection stimulates both MMP-1 and MMP-3 secretion and activates preformed MMP-3.

Further details of the functional properties of these AGS cell MMPs were acquired in synthetic peptidolytic activity assays. AGS cell or *H. pylori*-conditioned media (10× concentrated) were incubated with fluorogenic MMP-1- or MMP-3-specific substrates whose hydrolysis was measured by fluorometry as changes in emitted fluorescence as a function of time (fluorescence units/s). MMP-1-specific protease activity was undetectable in AGS cell-conditioned media and amounted to 0.0083 ± 0.0008 fluorescence units/s in *H. pylori*-conditioned media (Fig. 6A). In contrast, MMP-3-specific protease activities in AGS cell-conditioned media were 0.047 ± 0.0008 compared with 0.657 ± 0.001 fluorescence units/s in *H. pylori*-conditioned media. To determine whether *H. pylori* proteases activate AGS cell MMP-3, media conditioned by cocultured *H. pylori* and AGS cells, by AGS cells alone, or by *H. pylori* alone were combined before and after heat treatment, and the resulting MMP-3-specific protease activities were measured by fluorometry. Medium aliquots were concentrated 130 times to optimize detection of enzyme activity. As shown in Fig. 6B, conditioned media from cocultured *H. pylori* and AGS cells had 15-fold more MMP-3 specific protease activity than AGS cell-conditioned media and 4.5-fold more than in *H. pylori*-conditioned media. MMP-3-specific protease activity in conditioned media from AGS cells alone, *H. pylori* alone, and AGS-*H. pylori* cocultures was inactivated by heat treatment (Fig. 6B). As shown in Fig. 6C, MMP-3 activity of combined media was unaffected by heat treatment of AGS cell medium; however, heat treatment of the *H. pylori*-conditioned medium significantly reduced the MMP-3 activity of the combined media, and heat treatment of both AGS cell-conditioned and *H. pylori*-conditioned media eliminated all MMP-3 activity. These data indicate that AGS cells secrete MMP-3 activity and that *H. pylori* secretes both MMP-1- and MMP-3-like protease activity. Combined MMP-3-like activity is significantly potentiated by coculture of the bacteria with AGS cells. The persistence of low levels of MMP-3 activity in heat-treated *H. pylori*-conditioned media indicates that *H. pylori* MMP-3-like activity is more heat stable than its mammalian counterpart. The data show that host cell-bacterial interactions significantly affect MMP-3 activity and that bacterial factors are required for increased host MMP-3 production and activation, as suggested by immunoblot data (Fig. 5).

Reasoning that TIMPs are a possible source of MMP inhibition, we probed reducing SDS-PAGE replicas of conditioned media with antibodies directed against TIMP-1, TIMP-2, or TIMP-3. Positive controls for antibody specificity were provided by human skin fibroblast extracts (MMP-Control-1 and -2; Sigma). Figure 7A shows a time-dependent increase in TIMP-1 signal at ~29 kDa in both infected and uninfected AGS cell-conditioned media. *H. pylori* infection appeared not to affect the amount of secreted TIMP-1. No TIMP-1 im-

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munoreactivity was evident in *H. pylori*-conditioned media sampled after 24- or 48-h culture. With TIMP-2-specific antibody, ~21 kDa TIMP-2 immunoreactivity was evident at 24 and 48 h in *H. pylori*-infected and uninfected AGS cell-conditioned media (Fig. 7B); TIMP-2 signal strength was unaffected by *H. pylori* infection, and no TIMP-2 immunoreactivity was evident in *H. pylori*-conditioned media harvested after 24- or 48-h incubation. TIMP-3-specific antibody detected a prominent immunoreactive band at 70 kDa and two...
less reactive bands at 88 and 52 kDa in AGS cell-conditioned medium (Fig. 7C). H. pylori-infected AGS cell-conditioned medium also showed the 88- and 70-kDa bands, a prominent 24-kDa band, and two less reactive TIMP-3 bands at 45 and 35 kDa (Fig. 7C).

Significantly, both the 70- and 24-kDa TIMP-3-like immunoreactive bands were present in H. pylori-conditioned medium (Fig. 7C).

Further insights into TIMP-3 interaction with bacterial and host cell MMPs were acquired from nonreducing SDS-PAGE immunoblot analyses of conditioned media. As shown in Fig. 8, both the TIMP-3 and the MMP-3 blots detected immunoreactivity at ~98 kDa. In addition to the monomeric 24-kDa TIMP-3 detected on reducing blots (Fig. 7C), the nonreduced TIMP-3 immunoblot also detected 60- and 45-kDa bands in media conditioned by AGS cells, H. pylori, or cocultured AGS cells and H. pylori. The prominent lower band at ~52 kDa in the MMP-3 immunoblot (Fig. 8) represents the noncomplexed form of MMP-3. The MMP-3-specific antibody (MAB 1339) showed higher immunoreactivity with nonreduced MMP-3 than with the fully-reduced form shown in Fig. 5B, in which the same amount of protein was applied to the gel. These data indicate that heteromeric TIMP-3/MMP-3 complexes are formed in H. pylori-conditioned and in AGS cell-conditioned media and that H. pylori infection of AGS cells augments the formation of TIMP-3/MMP-3 complexes.

Direct visualization of TIMP-3 immunoreactivity in AGS cells and in H. pylori was accomplished by immunocytochemistry with TIMP-3 polyclonal antibody. In Fig. 9, A and B, a phase-contrast micrograph of immunostained AGS cells is compared with a fluorescent image of the same field showing a granular pancellular distribution of TIMP-3 immunoreactivity. The same antibody gave intense immunoreactivity with H. pylori alone (Fig. 9C), but immunostaining of H. pylori-infected AGS cells showed that the previously pancellular granular distribution of TIMP-3 immunoreactivity was now aggregated and polarized toward the side of the cells closest to the highest extracellular concentrations of H. pylori (Fig. 9D). These data suggest that H. pylori interaction with gastric epithelial cell surfaces...
activates cellular redistribution and exocytotic secretion of TIMP-3.

**DISCUSSION**

In this study, we tested the hypothesis that *H. pylori* infection of gastric epithelial cells modulates bacterial and host cell MMP and/or TIMP expression levels. The gelatinolitic and caseinolitic data from AGS cell culture or *H. pylori* coculture supernatants indicate that AGS cells express comparable levels of at least two different types of MMPs by immunoblot analysis: interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3). However, depending on the antibody used, the immunoblot data show more (antibody MAB 1339) or less (antibody Ab-2) MMP-3 than MMP-1, and activity data using MMP-1- and MMP-3-specific fluorogenic substrates indicates that stromelysin-1 contributes the majority of secreted protease activity in both AGS cells and *H. pylori*. Our observation of increased MMP-1 and MMP-3 activity in *H. pylori*-cocultured AGS cell supernatants is consistent with *H. pylori* proteinase activation of these host cell metalloproteinases. A potential candidate for this role is the ~60-kDa caseinolytic proteinase we detected in *H. pylori* culture medium. This enzyme has lower molecular mass than the previously described *H. pylori* metalloenzyme (21), which could indicate differences among the *H. pylori* strains or could represent an enzymatically active proteolytic fragment of the larger enzyme. The fact that the *H. pylori* ~60-kDa caseinolytic proteinase has similar substrate specificity to human stromelysin-1 (MMP-3) indicates that *H. pylori* expresses an MMP-3-like metalloproteinase.

Sequence comparison between human stromelysin-1 (MMP-3) gene and the *H. pylori* genome further supports expression of MMP-3-like enzymes in *H. pylori*.

Six open reading frames in the *H. pylori* genome have between 26 and 44% sequence similarity to a homologous 104-amino acid domain in the human MMP-3 gene sequence. Since the estimated molecular mass of these proteins is 25–44 kDa, the *H. pylori*-derived enzymatic activity detected at higher molecular masses on nondenaturating caseinolytic gels may represent complexes of bacterial MMPs with one another or with bacterial TIMPs.

Increased MMP activity is known to be followed by enhanced secretion of TIMPs (1). To date, four human TIMP isoforms have been identified (1). These TIMPs are two-domain proteins, with the NH₂ terminal domain forming inhibitory complexes with MMPs. The COOH terminal domains of vertebrate TIMPs mediate formation of noninhibitory complexes with MMPs, exemplified by the TIMP-2-proMMP-2 complex (6). The TIMP-3 COOH terminal domain anchors the protein to the extracellular matrix by virtue of specific molecular interactions with extracellular matrix constituents (11). TIMPs also have other biological activities, for example, cell growth-promoting and erythroid-potentiating properties that are independent from their MMP-inhibitory activity. Genomic and cDNA sequence comparisons have identified putative homologous TIMPs in *Drosophila* and *Caenorhabditis elegans* (1), although functional or structural properties of nonmammalian TIMPs have not been reported.

In the present study, immunoblot analysis of *H. pylori*-conditioned media, AGS cell-conditioned media, and *H. pylori*-AGS cell coculture supernatants showed that TIMP-3 isoforms are expressed independently by *H. pylori* and AGS cells. Furthermore, nonreducing immunoblots indicated that host and bacterial TIMP-3 isoforms participate in heteromeric complex formation with AGS cell MMP-3, a finding that is consistent with...
decreased MMP-3 activity in *H. pylori*-AGS cell culture supernatants as measured by fluorogenic substrate enzyme assays. Reducing and nonreducing immunoblots also revealed the presence of other SDS-stable TIMP-3-MMP complexes. Previous studies have reported inhibition of MMP activities by SDS-stable TIMP interactions with the enzymes (3, 22). Thus recombinant TIMP-2 was reported to inhibit rabbit fibroblast interstitial collagenase by formation of 1:1 molar ratio stoichiometric complexes with the 52-kDa procollagenase and the 46-kDa inactive intermediate of the enzyme. Both complexes and a third complex of TIMP-2 with the active 42-kDa collagenase were all stable in SDS (3). Human fibroblast collagenase and stromelysin-1 have also been reported to form SDS-stable inhibitory complexes with TIMP-1 (22).

Another characteristic of TIMPs is their spatially localized region of activity. The activity of TIMP-3, which appears to bind strongly to extracellular matrix components (11), is limited to areas close to its site of synthesis (1). Our immunocytochemical data are consistent with this localization, showing distribution of TIMP-3 immunoreactivity in infected cells close to the site of *H. pylori* attachment. We propose that *H. pylori* infection of AGS cells activates AGS cell TIMP-3, a process that may be mediated by the *H. pylori* MMP-3-like protease identified in this study, and that this locally-acting AGS cell TIMP-3 is then responsible for inhibition of both bacterial proteinases and activated host MMPs.

The TIMP-3 polyclonal antibody used in this study for immunoblot detection and immunocytochemical localization of TIMP-3 is directed against the COOH terminal domain of TIMP-3. Immunoactivity of an *H. pylori*-secreted protein with this antibody suggests that *H. pylori* TIMP-3-like protein shares sequence similarity with human TIMP-3 COOH terminal amino acids. Sequence comparisons of human TIMP-3 gene with the *H. pylori* genomic sequence revealed seven *H. pylori* open reading frames with 22–53% similarity to 71 COOH terminal amino acids of human TIMP-3. At this time, functional roles have not been ascribed to putative *H. pylori* TIMP-like protein. Clearly, as inhibitors of host cell metalloenzymes involved in facilitating neutrophil and macrophage infiltration of the gastric mucosa, bacterial TIMPs could have important roles in promoting bacterial colonization of host epithelial surfaces.

Detection of secreted MMPs and TIMP in culture media conditioned only by *H. pylori* suggests an additional novel functional role for the type IV secretion system of *H. pylori*. Macromolecular type IV secretory assemblies of gram-negative bacteria have been implicated in conjunctive transfer of DNA in *E. coli*, transfer of plasmid DNA from *Agrobacterium tumefaciens* to plant cell nuclei, *Bordetella pertussis* secretion of pertussis toxin, migration of *Rickettsia, Legionella*, and *Brucella* signaling factors to host cell cytoplasm and vacuolar spaces, and pedestal formation by *H. pylori* contact with epithelial cell plasma membranes (2). Recently, the type IV secretion system of *H. pylori* was shown to mediate transfer of the bacterial CagA protein into gastric epithelial cells, where its tyrosine phosphorylation modulated tyrosine phosphorylation of a spectrum of host cell proteins (10). Whether the type IV secretion system of *H. pylori* is also responsible for secretion of MMPs and TIMP remains to be determined. Delivery of these MMPs and TIMP into the extracellular matrix encompassing gastric epithelial cells would allow bacteria to modulate host cell MMP and TIMP mobilization, which could culminate in generation of β-defensins and other antibiotic peptides (18).

In summary, the significant results of this study are that gastric epithelial cells secrete MMP-1 and MMP-3 and that *H. pylori* infection increases secretion of both metalloproteinases. *H. pylori* also secretes MMP-1 and MMP-3-like metalloproteinases, the latter accounting for the majority of *H. pylori*-secreted proteinase activity. Gastric epithelial cells and *H. pylori* both express TIMP-3, and *H. pylori* infection of the cells induces cellular redistribution of TIMP-3 to the site of *H. pylori* attachment to the cells. We propose that the outcome of gastric mucosal infection by *H. pylori* depends in part on interactions between epithelial and bacterial MMPs and TIMPs identified in this study. To the extent that *H. pylori* MMPs and TIMP-3-like proteins promote bacterial colonization of gastric epithelium, these MMPs and their inhibitors may be considered a novel class of *H. pylori* virulence factors.

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