ORIGINAL ARTICLE

Involvement of mast cells in gastritis caused by *Helicobacter pylori*: a potential role in epithelial cell apoptosis

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Background: The role(s) of mast cells (MC) in gastric mucosal inflammation caused by *Helicobacter pylori* is (are) still debated.
Aim: To determine whether there is an association between MC density and epithelial cell apoptosis in antral gastric mucosa infected by *H pylori*.
Patients and methods: Biopsy specimens from 122 *H pylori*-positive subjects with chronic active gastritis, 84 patients with non-steroidal anti-inflammatory drug-induced gastritis and 48 volunteers were included. *H pylori* genotypes were determined by PCR amplification of bacterial cultures. Immunohistochemical analysis was performed on tissue microarrays with anti-CD117, anti-chymase, anti-tryptase, anti-myeloperoxidase, anti-Bcl-2, anti-Bcl-x, anti-Bax and anti-caspase 3 antibodies.
Results: Of the 122 patients infected with *H pylori*, 76 (62.3%) harboured *cagA* positive strains. *H pylori* isolates belonged to the *vacAs1/m1* genotype in 82 (67%) cases, to the *vacAs2/m2* genotype in 23 (18.8%) cases and to the *vacAs1/m2* genotype in 17 (13.9%) cases. 61 (50%) *H pylori* isolates were *babA2*+. In patients infected with *H pylori*, the density of MC, and in particular the number of MC-associated epithelial cells, was correlated with a high number of apoptotic epithelial cells. Moreover, the density of MC was correlated with the number of neutrophils infiltrating the antral gastric mucosa, and was strongly increased in patients infected with *cagA*, *vacAs1/m1* and *babA2* positive strains.
Conclusions: Taken together, these data show that the density of MC can be considered as a histopathological criterion of gastritis activity in patients infected with *H pylori*.

The specific location of mast cells (MC) within tissues in contact with the external environment, such as the digestive mucosa, and their ability to produce and secrete a wide spectrum of mediators and cytokines strongly suggest that they have a crucial role in innate immune responses.[1–4] However, it remains to be determined whether MC participate in innate immune responses that protect the human host against *Helicobacter pylori* infection.

In vitro approaches and in vivo studies in mice models have shown that different bacterial virulent factors produced by *H pylori* can bind and directly activate MC migration and the production of proinflammatory cytokines.[5] Indeed, the *H pylori*-neutrophil-activating protein (HP-NAP) produced by *H pylori* is a potent agonist of MC, capable of inducing degranulation of stored chemical mediators.[5] Moreover, oral administration of the vacuolating cytotoxin (vacA) of *H pylori* in mice causes MC accumulation in gastric mucosa.[6] However, the specific roles played by MC and the consequence of MC–gastric epithelial cell interactions during *H pylori* infection in humans remain to be elucidated.[7]

We undertook the present work to determine whether there is a correlation between the number of MC in gastric antral mucosa and the number of apoptotic epithelial cells in patients infected with *H pylori*. Results were evaluated according to the *H pylori* genotypes and the density of neutrophils seen in gastric mucosa, and were compared with data obtained for non-steroidal anti-inflammatory drug (NSAID)-induced gastritis.

MATERIALS AND METHODS

Patients, biopsy specimens and histological assessment

All patients included in this study were hospitalised in the Department of Gastroenterology (Archet II Hospital, Nice, France) for an upper digestive endoscopy, in order to evaluate gastrointestinal disease, mainly dyspepsia and/or gastric pain (318 patients), or before gastroplasties (74 asymptomatic patients). All patients were French Caucasians. Clinical information regarding associated gastrointestinal symptoms and conditions, use of NSAID, aspirin, antibiotics and proton pump inhibitors during the 8 weeks prior to the endoscopy were obtained from the medical database of the hospital. All patients signed an informed consent form, and the protocol was approved by the ethics committee of the University of Nice (Nice, France). For each patient, six large-cup antral biopsy specimens (three for diagnosis and three for building tissue microarrays (TMAs) only) were fixed in 10% buffered formalin, then processed, oriented on edge, embedded in paraffin, cut into sequential 4 μm sections, and stained by H&E and Giemsa for the evaluation of *H pylori* infection and inflammation. These sections were examined by two pathologists (VH and PH) who were blinded to the other experimental results. Two supplementary non-fixed antral biopsy specimens were sent to the Laboratory of Bacteriology for bacterial cultures. Urease test was performed in one antral biopsy specimen taken from each subject. Slides were coded and evaluated histologically for (1) activity of gastritis (neutrophil infiltration); (2) chronicity of gastritis (lymphocytic and plasma cell infiltration); (3) glandular atrophy; and (4) intestinal metaplasia. Each parameter was graded as none (0), mild (1), moderate (2) or severe (3), according to the Sydney classification.[8]

Abbreviations: HP-NAP, *H pylori*-neutrophil-activating protein; MC, mast cells; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate-buffered saline; TMA, tissue microarray; vacA, vacuolating cytotoxin

Tissue microarray construction and immunohistochemistry studies

Representative gastric antral biopsy specimens from each patient, selected from H&E-stained sections, were used for building TMAs. The TMAs were set up as described previously.[9 10] From each specimen, one tissue core (600 µm in diameter) from the upper part of the mucosa was obtained; pits and glands were always cut longitudinally. Two TMAs of gastric specimens were constructed, consisting of 624 and 600 µm-diameter tissue cores and 144 and 600 µm-diameter tissue cores from patients with symptoms and asymptomatic control patients, respectively. The TMA built with gastric specimens from patients with symptoms contained normal gastric antral mucosa (six tissue cores from biopsies performed on asymptomatic controls), which served both as a control and as a layout marker to set the spacing of 1 mm between core centres. A 4 µm H&E-stained section was reviewed to confirm the presence of morphologically representative areas of the original lesions.

Immunohistochemical staining was performed on serial 4 µm deparaffinised TMA sections. These sections were incubated with 0.1% trypsin (Sigma Chemical, St Louis, Missouri, USA) in phosphate-buffered saline (PBS, pH 7.5) for 10 min at 37°C. After washing with distilled water, sections were incubated with 0.03% hydrogen peroxide containing 0.2% sodium azide for 20 min (for blocking intrinsic peroxydase), washed with PBS and incubated with the following antibodies for 45 min: monoclonal mouse anti-MC tryptase (AA1; Dako A/S, Glostrup, Denmark), anti-MC chymase (MAB1254; Chemicon, Temecula, California, USA) and anti-Bcl-2 (124); polyclonal rabbit anti-CD117 (4502), anti-myeloperoxydase (MPO-7), anti-Bcl-x (A3535), anti-Bax (A3533) and anti-caspase 3 (A3537) (all from Dako). After rinsing with PBS, sections were incubated with peroxidase-labelled anti-mouse immunoglobulins or peroxidase-labelled anti-rabbit immunoglobulins (DAKO Envision System, DAKO Corp, Carpinteria, California, USA) for 45 min. Sections were then washed with PBS, coloured with 3-amino-9-ethylcarbazole in acetate buffer containing hydrogen peroxide, counterstained with haematoxylin and mounted with aqueous mounting medium. After staining, slides were evaluated by two pathologists (VH and SL). Results were scored by the method of quick score as described previously.[11] For each patient, the mean score of a minimum of two core biopsy specimens was calculated. Discrepancies were resolved by three pathologists (VH, SL, PH) using the multihead microscope.

H pylori culture

H pylori strains from patients were isolated, identified and stored. Two antral biopsy specimens were placed in selective transport medium and cultured on horse blood agar at 37°C under microaerophilic conditions, as described previously.[12] *H pylori* was identified by typical colony morphology, Gram stain and positive biochemical testing for urease, catalase and oxidase. Bacteria were harvested from the plates using sterile cotton swabs and stored at –70°C in brucella broth plus 30% (v/v) fetal calf serum and 20% (v/v) glycerol. All frozen isolates were controlled for contamination.

Detection of *H pylori* genotypes and sequencing

H pylori cagA, *vacA* (*vacAs1/s2* and *vacAm1/m2*) and *babA2* genotypes were determined by PCR. Genomic DNA was extracted from *H pylori* using the High Pure PCR Template preparation kit (Roche Diagnostics, Mannheim, Germany). The integrity of the DNA was assessed by 1.2% agarose gels stained with ethidium bromide. PCR reactions were performed in a total volume of 50 µl, which contained 50 pmol of primers, 100 ng of genomic DNA, 1.0 mM of each of the four dNTPs and 2 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Connecticut, USA). Primer sequences were described previously.[13–15] The amplified PCR products were resolved in 1.5% agarose gels, stained with ethidium bromide and visualised under a short-wavelength ultraviolet light source. The sequences of the PCR products were confirmed by automated sequencing (ABI Prism 310 Genetic Analyser; Perkin Elmer, Branchburg, New Jersey, USA) using the same primer pairs.

Statistics

MC density was compared between the groups of study subjects using the Mann–Whitney test. The correlation between MC density, and the intensity of inflammatory cell infiltration and/or the number of epithelial cells undergoing apoptosis and/or the different genotypes was evaluated by Spearman's rank correlation test. Values were expressed as mean (SEM).

RESULTS

Histological and bacteriological results

Among the 318 patients with gastric symptoms, 162 cases were positive for *H pylori*, detected by histology (162/162 cases), by urease test (157/162 cases) and by culture (142/162 cases). Twelve patients taking NSAID, who were positive for *H pylori* infection, detected by culture, and 8 patients positive for

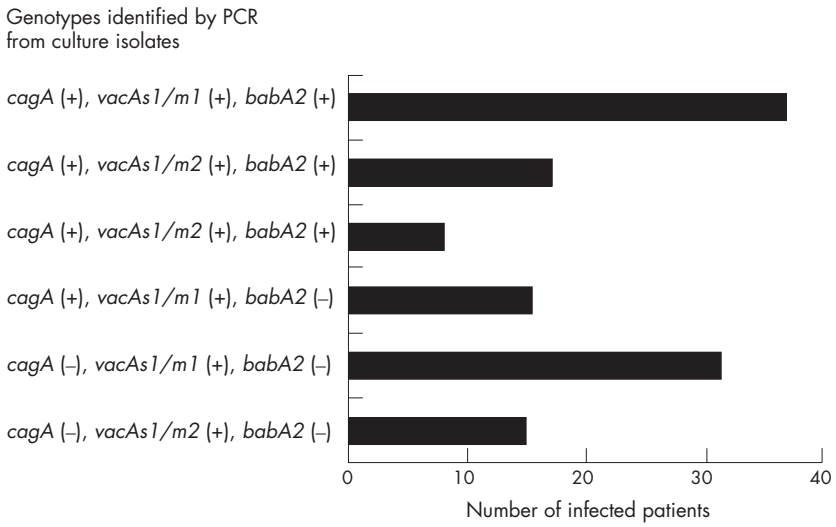

Figure 1 Different *Helicobacter pylori* genotypes characterised by PCR amplification from culture isolates in *H pylori*-infected gastric antral biopsy specimens.

H pylori infection, showing chronic active gastritis with metaplasia (8 cases) and low-grade dysplasia (4 cases), were not included in the present study. The study was performed on other selected patients positive for *H pylori* by culture (122 cases; group 1). Among these patients, 59 were men and 63 were women (mean (range) age 34.5 (19–45) years) not taking NSAID, antibiotics or proton pump inhibitors. The urease test was positive in all these cases. Selected biopsy specimens in group 1 showed antral-predominant non-atrophic gastritis as defined previously.[16] A total of 156 patients were negative histologically and by culture for *H pylori* infection. The urease test was negative in all these cases. Among these patients, 84 subjects (38 men, 46 women; mean (range) age 32.5 (23–43) years; group 2) used to take NSAID. Antral biopsy specimens performed in these 84 patients showed acute gastritis. No antibiotics or proton pump inhibitors were administered in these 84 patients for a period of 2 months before upper endoscopy. Among the 74 control volunteers, 48 patients (group 3) did not use NSAID, antibiotics or proton pomp inhibitors for a period of 2 months before endoscopy, and were negative histologically for *H pylori* infection, by the urease test and by culture. Biopsy specimens performed in this group of subjects did not show significant mucosal lesions.

The genotypes isolated by PCR from culture isolates in 122 patients infected with *H pylori* (group 1) are listed in fig 1. PCR amplification showed that of these 122 bacterial isolates, 76 (62.3%) harboured *cagA*(+) strains. *H pylori* isolates belonged to the *vacAs1/m1* genotype in 82 (67%) cases, to the *vacAs2/m2* genotype in 23 (18.8%) cases and to the *vacAs1/m2* genotype in 17 (13.9%) cases. In all, 61 (50%) cases were *babA*2 (+) strains. The simultaneous presence of *cagA*, *vacAs1/m1* and *babA2* genes (triple positive) was found in 36 (29.5%) cases of *H pylori* isolates, whereas 15 (12.2%) cases harboured the *cagA* (−), *vacAs2/m2* (+) and *babA2* (−) genotypes. The presence of *babA2* genotype did not correlate with the presence of *cagA* or the various *vacA* genotypes.

Gastric mucosal density of MC is 2–3-fold higher in patients with *H pylori*-chronic active gastritis and NSAID-induced gastritis in comparison to healthy volunteers

Evaluation of the density of MC was performed only in well representative spots (fig 2A.a1). In control subjects (group 3), the mean (SD) MC density was 118 (9) cells/mm^2. MC were observed in the lamina propria of the mucosa, predominantly around the small vessels (fig 2A.a1, inset). MC density was increased in all patients with gastritis (fig 2A.a2, 2A.a3). MC were noted in the upper portions of the mucosa. Densities of MC were significantly increased both in patients with NSAID-induced gastritis (group 2; 252 (12) cells/mm^2) and in patients with *H pylori*-chronic active gastritis (group 1; 267 (11) cells/mm^2), compared with controls ($p<0.05$). Increased numbers of MC positive for CD117, chymase and tryptase staining were similarly found in patients with NSAID-induced gastritis (group 2) and in those with *H pylori*-chronic active gastritis (group 1) in comparison to controls (group 3; fig 2B).

The number of MC in the epithelium increases in antral mucosa infected by *H pylori*

The number of MC in the epithelium of the antral mucosa was slightly increased in patients with NSAID-induced gastritis in comparison to healthy volunteers (fig 3). This number was also significantly increased in patients with *H pylori*-chronic active gastritis (group 1) and in patients with NSAID-induced gastritis (group 2), both in comparison to the antral mucosa of healthy volunteers (group 3; fig 3; $p<0.05$). These differences in the densities of MC seen in the epithelium were independent of the antibody (anti-chymase, anti-tryptase or anti-CD117) used to detect the MC (fig 3).

Gastric mucosal densities of MC and MC-associated epithelial cells are higher in antral biopsy specimens infected by *cagA* (+), *vacAs1/m1* (+) and *babA2* (+) *H pylori* strains than in those infected by *cagA* (−), *vacAs2/m2* (+) and *babA2* (−) *H pylori* strains

The number of MC was then evaluated both in the lamina propria and in the epithelium according to the genotype of *H pylori*. The global density of MC was 1–2-fold higher in mucosa infected by *cagA* (+), *vacAs1/m1*(+) and *babA2* (+) *H pylori* strains than in mucosa infected by *cagA* (−), *vacAs2/m2* (+) and *babA2* (−) *H pylori* strains (fig 4A). The density of MC associated with epithelial cells was significantly increased (1.7-fold) in mucosa infected by *cagA* (+), *vacAs1/m1*(+) and *babA2* (+) *H pylori* strains, as compared to mucosa infected by *cagA* (−), *vacAs2/m2* (+) and *babA2* (−) *H pylori* strains (fig 4B). No significant differences were found among the different antibodies used to detect the MC (anti-chymase, anti-tryptase and anti-CD117 antibodies; fig 4A,B). Finally, when considering each bacterial virulent factor independently, no differences in MC density were observed in mucosa infected by *cagA* (+) versus *cagA* (−) *H pylori* strains, in mucosa infected by *vacAs1/m1* (+) versus *vacAs2/m2* (+) *H pylori* strains, or in mucosa infected by *babA2* (+) versus *babA2* (−) *H pylori* strains (data not shown).

Significant correlation between the mucosal MC density (both in NSAID-induced gastritis and in *H pylori*-chronic active gastritis) and neutrophil infiltration

MC densities in both NSAID-induced gastritis (group 2) (fig 5A) and *H pylori*-chronic active gastritis (group 1) (fig 5B) exhibit a significant correlation with neutrophil infiltration. Although the number of neutrophils was higher in mucosa infected with *cagA* positive *H pylori strains* than in mucosa infected with *cagA* negative *H pylori* strains, correlations with the density of MC were similar (fig 5C,D).

The number of apoptotic epithelial cells and MC-associated epithelial cells is correlated

The number of apoptotic epithelial cells was then compared with the densities of MC observed in the epithelium and in the lamina propria in biopsy specimens from healthy volunteers (group 3), and from patients with NSAID-induced gastritis (group 2) and those with *H pylori*-chronic active gastritis (group 1). Epithelial cell apoptosis was evaluated by immunohistochemical staining of caspase 3 (fig 6a,c), Bax (fig 6b,d), Bcl-x and Bcl-2 (not shown) with specific antibodies. Interestingly, the number of apoptotic cells was correlated with the density of MC noted in the lamina propria of the different studied populations (fig 6A–C), and with the MC seen associated with the epithelium (fig 6D–F). In biopsy specimens from healthy volunteers (group 3), apoptotic cells were seen predominantly at the surface epithelium, more specifically between the crypts, whereas in biopsy specimens from patients with NSAID-induced gastritis (group 2) and *H pylori*-chronic active gastritis (group 1), apoptotic cells were mainly observed at the tip of the epithelium (fig7A–C). Similar results were obtained with anti-Bcl-2, anti-Bax, anti-Bcl-x and anti-caspase 3 antibodies. In mucosa infected by *cagA* (+), *vacAs1/m1* (+) and *babA2* (+) *H pylori* strains, the number of apoptotic epithelial cells was higher when the density of MC observed in epithelium was increased (not shown).

DISCUSSION

Using different specific antibodies raised against MC on TMAs built with a large series of biopsy specimens from homogeneous

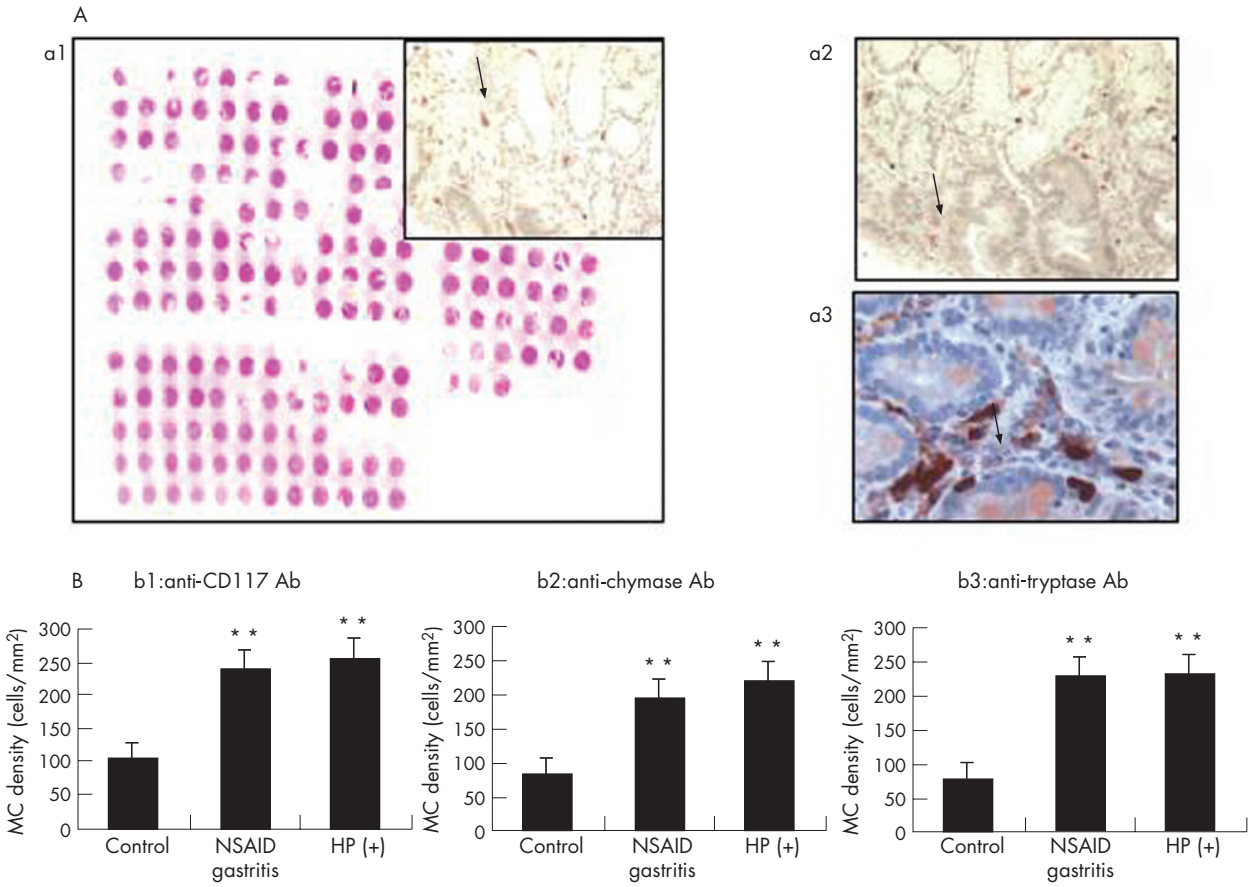

Figure 2 Gastric mucosal densities of mast cells (MC) in patients with *Helicobacter pylori*-chronic active gastritis, non-steroidal anti-inflammatory drug (NSAID)-induced gastritis and healthy volunteers. (A) Tissue microarray (TMA) in gastric antral mucosa. (a1) Representative TMA from gastric antral biopsy specimens stained by H&E. Only regular spots were evaluated. Inset: high magnification of one spot showing an immunostaining of the MC (arrow) of a control subject (anti-CD117 antibody, magnification ×400); (a2,a3): high magnification showing different immunostaining of MC (arrows) in NSAID-induced gastritis (a2) and in *H pylori*-chronic active gastritis (a3) (anti-chymase antibody, magnification ×400). (B) MC density in the antral mucosa in different groups; (b1): MC identified with anti-CD117 antibody; (b2): MC identified by anti-chymase antibody; (b3): MC identified by anti-tryptase antibody, **$p<0.05$.

populations, we demonstrated that *H pylori* infection, particularly infection by strains harbouring the *cagA* (+), *vacAs1/m1* (+) and *babA2* (+) genotypes, was associated with a significant increase in the MC density of the gastric antral mucosa. Interestingly, the density of MC, and more precisely the number of MC-associated epithelial cells, was correlated with an increase in apoptotic epithelial cells.

Chronic active *H pylori* infection produces a predominant infiltration of neutrophil cells, but little evidence of MC infiltration has thus far been reported in *H pylori*-infected gastric mucosa.[5 17 18] Here, we show for the first time that immunohistochemical staining of various MC provides a valuable means of quantifying MC infiltrates in gastric mucosa infected by *H pylori*. Although the TMA method has been developed largely for the analysis of tumour samples, our results demonstrate that this technology can also be used in inflammatory diseases.[9 10] Moreover, we have demonstrated that the TMA technology can be applied to small tissue

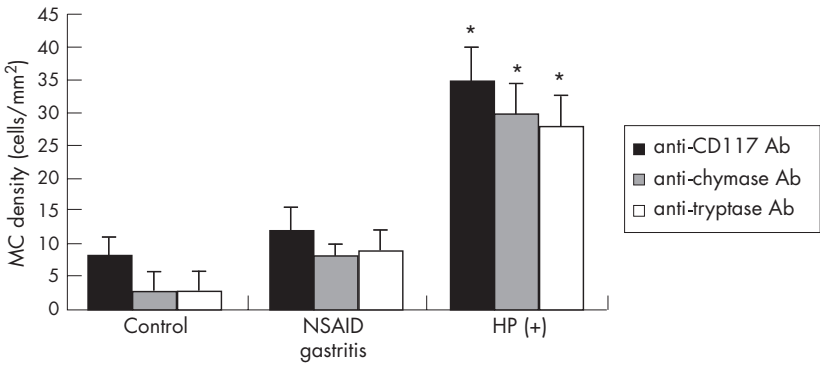

Figure 3 Epithelial mast cells (MC) density in patients with *Helicobacter pylori*-chronic active gastritis, non-steroidal anti-inflammatory drug (NSAID)-induced gastritis and control subjects. The MC density in the epithelium of subjects with *H pylori*-positive gastritis was significantly higher than in subjects with NSAID-induced gastritis.

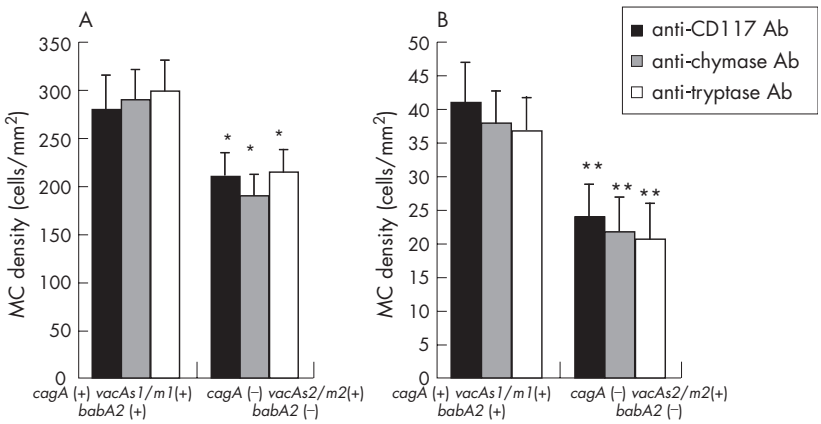

Figure 4 Mast cells (MC) density observed in gastric antral mucosa according to the genotype of *Helicobacter pylori*. (A) Lamina propria MC density in patients infected by *cagA* (+), *vacAs1/m1* (+), *babA2* (+) *H pylori* genotype versus that in patients infected by *cagA* (−), *vacAs2/m2* (+), *babA2* (−) *H pylori* genotype, *p<0.05. (B) Epithelial MC density in patients infected by *cagA* (+), *vacAs1/m1* (+), *babA2* (+) *H pylori* genotype versus that in patients infected by *cagA* (−), *vacAs2/m2* (+), *babA2* (−) *H pylori* genotype, **p<0.01.

specimens such as digestive biopsy specimens. Thus, the advantage of the high-density format of TMA technology can successfully be applied to lesions arising in gastritis, as shown in the present work.

Several studies have shown that activation of MC leads to the infiltration of neutrophils in tissues.[19–21] Indeed, our results confirm and extend this observation by showing that increased MC density in gastric antral mucosa is correlated with the score of neutrophil infiltration. Human MC produce several specific proteases, including chymase and tryptase.[4] Our study showed that, in *H pylori*-positive patients, the expression of both proteases is increased in the gastric mucosa when the neutrophil infiltrate is increased, indicating that both tryptase and chymase might have a role in the afflux of neutrophils in *H pylori*-associated gastritis. However, the migration of neutrophils to the infection site, after their encounter with activated endothelial cells, can be influenced by specific chemoattractants and cytokines, in particular leucotriene B4, platelet activating factor, IL8, GM-CSF and TNFα, released by activated MC.[4] Thus, *H pylori* infection can induce neutrophil infiltration in gastric mucosa via the basolateral release from epithelial cells of IL8, via the bacterial protein HP-NAP, or indirectly through activated MC.

The number of apoptotic epithelial cells detected with antibodies against proapoptotic proteins was correlated with the density of MC. Interestingly, the density of MC was higher in patients infected with *cagA*, *vacAs1/m1, babA2*, triple-positive *H pylori* strains than in patients infected with other *H pylori* strains, or in patients with NSAID-induced gastritis. The exact mechanisms involved in mediating the enhanced gastric epithelial cell apoptosis observed in vivo during infection with *H pylori* are not well determined to date.[22–25] Moreover, there are conflicting data both in vitro and in vivo regarding the mechanisms leading to the induction of apoptosis by *H pylori*.[26–32] The role of neutrophil cytotoxicity and/or transepithelial migration has been put forward in the apoptotic process of digestive epithelial cells.[33] By contrast, the implication of MC in the apoptosis of digestive epithelial cells has not

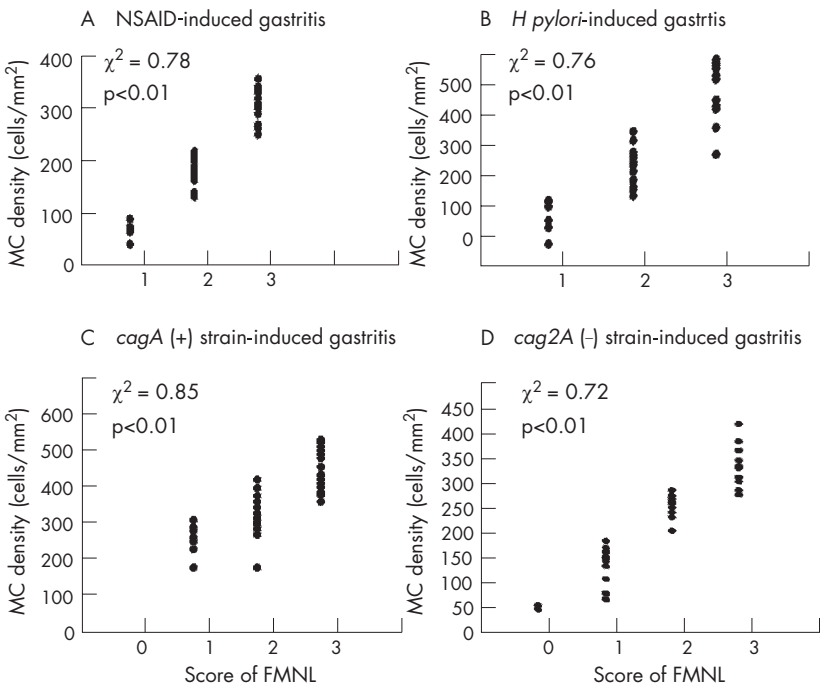

Figure 5 Correlation between mast cells (MC) density and neutrophil infiltration in gastric antral mucosa. Polymorphonuclear infiltrates: 0, none; 1, mild; 2, moderate; and 3, severe. MC density in the mucosa of non-steroidal anti-inflammatory drug (NSAID)-induced gastritis (A) and *Helicobacter pylori*-induced gastritis (B) has significant correlation with neutrophil infiltration. Moreover, cagA *H pylori* positive gastritis (C) and cagA *H pylori* negative induced gastritis (D) have similar significant correlations with neutrophil infiltration.

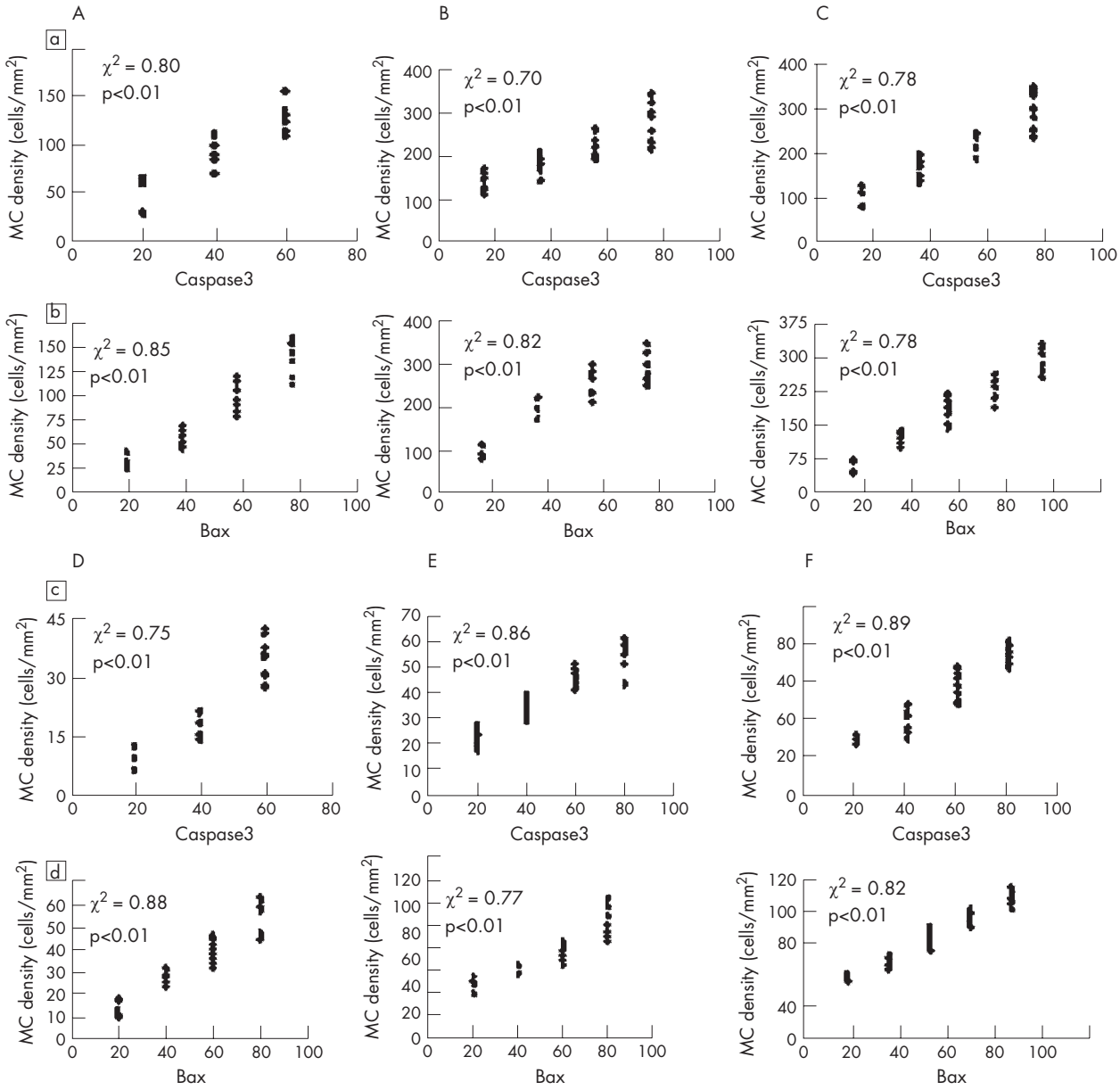

Figure 6 Correlation of epithelial cell apoptosis rate with the density of mast cells (MC) in gastric antral mucosa. The correlation of MC density in antral mucosa (A–C) and in the epithelium (D–F) was evaluated in healthy volunteers (A,D), and in patients with NSAID-induced gastritis (B,E) and *Helicobacter pylori*-chronic active gastritis (C,F). These correlations were made for caspase 3 (a and c) and Bax (b and d) protein expressions on epithelial cells.

been determined to date. Previous studies have shown that MC can mediate apoptosis of different cell types such as cardiomyocytes, smooth muscle cells, T cells, endothelial cells and keratinocytes.[34–39] In our study, high density of MC, particularly MC-associated epithelial cells, was highly correlated with both the decreased expression of Bcl-2 and Bcl-x anti-apoptotic proteins, and the increased expression of the proapoptotic Bax protein and caspase 3. Interestingly, HP-NAP and VacA can directly activate production of cytokines and migration in MC.[6 7] Thus, these bacterial products might have a role in inducing migration of MC into the epithelium. Although convincing evidence for this has not been found in the present work, it can be envisioned that gastric epithelial cell apoptosis may be induced by MC secretion of proapoptotic molecules, such as chymase and TNFα, during degranulation. Moreover, MC can increase epithelial cell apoptosis indirectly by potentiation of neutrophil afflux in antral gastric mucosa and then neutrophil transepithelial migration.

Our study strongly suggests a direct involvement of MC in epithelial cell apoptosis observed in gastric human mucosa infected by *H pylori* strains, particularly by *cagA* (+), *vacAs1/m1* (+) and *babA2* (+) *H pylori* strains.

In patients infected with *H pylori*, the number of MC detected in gastric antral biopsy specimens could be considered as a criterion of disease activity that should be taken into account, independently of the other known criteria.

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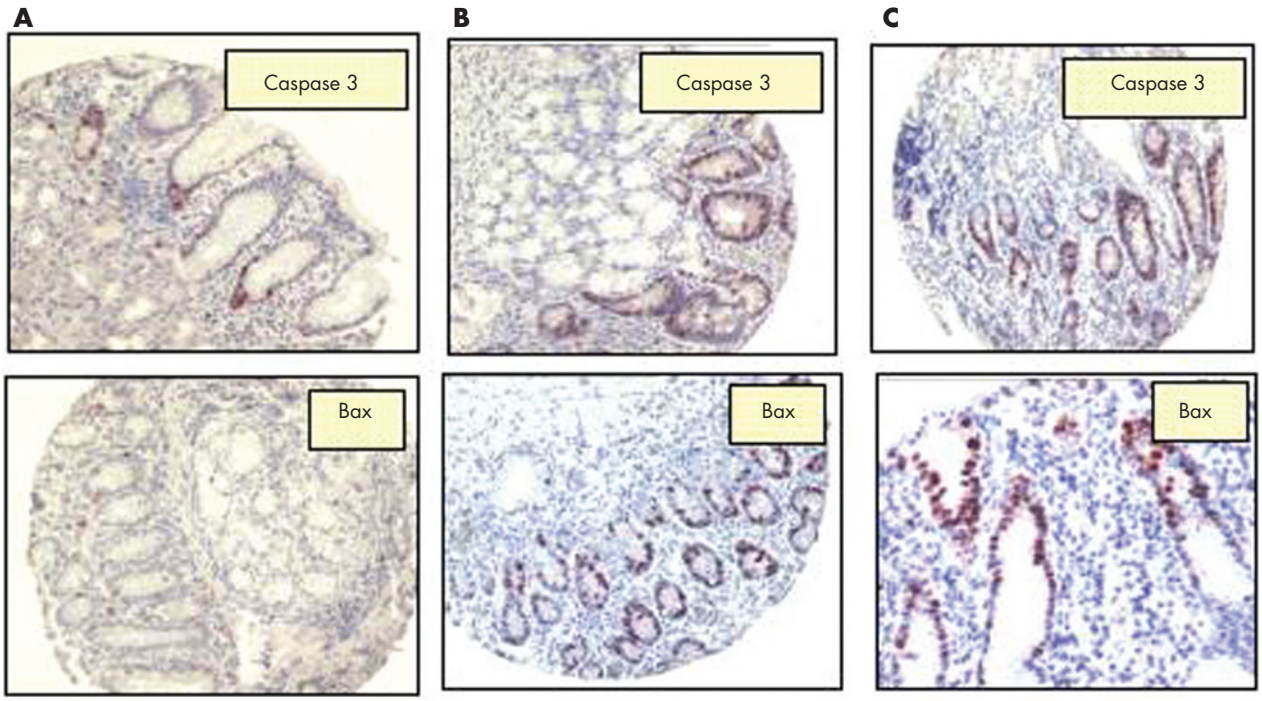

Figure 7 Immunostaining with anti-caspase 3 and anti-Bax antibodies in gastric antral biopsy specimens from healthy volunteers (A), and from patients with NSAID-induced gastritis (B) and *Helicobacter pylori*-chronic active gastritis (C).

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