Involvement of the CD95 (APO-1/Fas) Receptor and Ligand System in *Helicobacter pylori*–induced Gastric Epithelial Apoptosis

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Abstract

Helicobacter pylori **infection is associated with chronic gastritis, peptic ulceration, and gastric carcinoma. The potential role of CD95-mediated apoptosis was investigated in a panel of gastric biopsies obtained from patients with *H. pylori*–associated chronic gastritis ($n = 29$) and with noninfected normal mucosa ($n = 10$). Immunohistochemistry revealed increased CD95 receptor expression in epithelial and lamina propria cells in chronic gastritis. By in situ hybridization, CD95 ligand mRNA was absent or low in normal mucosa but expressed at high levels in lamina propria lymphocytes and, unexpectedly, in epithelial cells in chronic gastritis. Apoptotic cells were rare in normal mucosa but were observed regularly in chronic gastritis in close proximity to CD95 ligand mRNA expression throughout the epithelial and lamina propria cells.**

In a functional analysis gastric epithelial cell lines were incubated with supernatants of *H. pylori*. Treatment with the cytotoxic isolate *H. pylori* 60190 but not with the noncytotoxic isolate Tx30a upregulated CD95 in up to 50% of gastric epithelial cells and induced apoptosis in these cells. *H. pylori*–induced apoptosis was partially prevented by blocking CD95, demonstrating the functional role of the CD95 system. These findings suggest that *H. pylori*–associated chronic gastritis involves apoptosis of gastric epithelial cells by activation of the CD95 receptor and ligand system. (*J. Clin. Invest.* 1998. 102:1506–1514.) Key words: *Helicobacter pylori*–associated chronic gastritis • gastric epithelial cell line • lymphoma cell line • fluorescence-activated cell scanning • in situ hybridization

Introduction

Helicobacter pylori is an important etiologic factor in the development of chronic gastritis and ulcer disease (1). Infection with this bacterium induces infiltration of the mucosa with inflammatory cells (2, 3). In *H. pylori*–induced chronic gastritis, surface epithelial damage, erosions, and atrophy of the gastric mucosa as well as increased epithelial proliferation are striking histological features (3, 4). Epithelial proliferation does not seem to be counterbalanced by epithelial necrosis in *H. pylori*–induced gastritis (5), suggesting that apoptosis may account for the apparent cell loss in chronic gastritis.

Apoptosis, programmed cell death, is essential in development and homeostasis of multicellular organisms (for reviews see references 6–8). In addition it may also serve as a defense mechanism against bacterial and viral infections (9–11). Cytotoxic T lymphocytes and natural killer cells eliminate their targets by induction of apoptosis (12–16). Apoptosis can be mediated through activation of the CD95 receptor (CD95)[1] (APO-1/Fas) and ligand system. The CD95 belongs to the TNF receptor/nerve growth receptor superfamily and is a type I transmembrane protein (17, 18). CD95 is constitutively expressed in a wide range of tissues (19). CD95 ligand (CD95L) or agonistic antibodies induce apoptosis via CD95 (20, 21). CD95L belongs to the TNF family, is a type II transmembrane protein (22), and is expressed in various cells and tissues, including activated T lymphocytes, testis, lung, kidney, and liver of patients with alcoholic liver damage (22–24). The CD95 receptor and ligand system mediates T cell cytotoxicity (13–15).

In *H. pylori*–induced chronic gastritis, we hypothesized that increased epithelial proliferation may be accompanied by epithelial apoptosis to maintain cellular homeostasis. We found that apoptosis of gastric epithelium was increased in *H. pylori*–associated gastritis and involved upregulation of CD95 expression and increase in CD95L expression in lymphocytes and gastric epithelial cells. Subsequent cell culture studies confirmed that *H. pylori*–induced apoptosis is mediated by activation of the CD95 receptor and ligand system in gastric epithelial cells.

Methods

Patients

Gastric biopsy specimens were taken from the antrum in a series of consecutive patients who underwent upper gastrointestinal endoscopy for diagnostic reasons. 29 patients with *H. pylori*–positive chronic gastritis (age, 25–88 yr; median age 54 yr; 19 males) and 10 patients with non–ulcer dyspepsia who showed histologic normal mucosa and were not infected with *H. pylori* (age, 23–68 yr; median age 48 yr; 4 males) were included (Table I). All patients gave informed consent to biopsy sampling. The study was approved by the Ethics Committee of the University of Heidelberg.

J. Rudi and D. Kuck contributed equally to this study.

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1. *Abbreviations used in this paper: cagA*/CagA, cytotoxin-associated gene/protein; CD95, CD95 receptor; CD95L, CD95 ligand; DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; *vacA*/VacA, vacuolating cytotoxin gene/vacuolating cytotoxin.

Table I. Assessment of Gastritis, Apoptosis, and CD95-CD95L mRNA Expression in H. pylori–positive and –negative Antral Biopsies

	H. pylori–negative normal mucosa	*H. pylori*–associated gastritis	*P* value
	n = 10	*n = 29*	
Gastritis*			
Chronic infammation	0.1 (0.2)	1.7 (0.7)	< 0.0001
Activity	0	0.8 (0.9)	0.009
Apoptosis‡			
Number of positive cells (%)	1.9 (2.3)	8.0 (5.1)	0.002
CD95 expression‡			
Superficial epithelial cells (%)	0.8 (2.1)	30.0 (31.1)	0.003
Pyloric gland cells (%)	53.0 (24.3)	76.1 (14.2)	0.018
Lamina propria cells (%)	6.4 (5.2)	40.6 (27.5)	0.0002
CD95L mRNA expression§	0.3 (0.6)	1.4 (1.1)	0.012

The data are given as means (±SD). Differences were calculated by Wilcoxon two-sample test. *The degree of histological gastritis was scored according to the updated Sydney classification (3). ‡For assessment of apoptosis and CD95 expression, at least 300 cells were counted in each section and the number of positive cells per 100 cells was expressed as a percentage. In some biopsies from patients with normal *H. pylori*–negative mucosa, < 300 lymphocytes in each section were present. §Scoring of CD95L mRNA was performed as described in the text.

Gastric biopsies

The specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. Serial cryosections (4–5 μm thick) were fixed immediately for 5 min in cold acetone, air-dried overnight, and then stained with hematoxylin and eosin, and used for CD95 immunohistochemistry or stored at −80°C until use. For in situ hybridization cryosections were fixed with 4% paraformaldehyde. The diagnosis of active and chronic gastritis was based on the updated Sydney system (3) by histological determination of the density of infiltration with neutrophils (0, no infiltration; 1, mild; 2, moderate; and 3, marked infiltration) and mononuclear cells (0, no infiltration; 1, mild; 2, moderate; 3, marked infiltration). The presence of *H. pylori* in parallel biopsy samples was confirmed by the urease test and PCR of the 16S rRNA gene and the *vacA* gene of *H. pylori* as described previously (25, 26).

Detection of apoptosis in gastric biopsies

For identification of apoptotic cells, staining with 4′,6-diamidino-2-phenylindole (DAPI, 0.1 μg/ml; Boehringer Mannheim, Mannheim, Germany) was performed according to the manufacturer's protocol. Slides were mounted with Permafluor Mounting medium (Immunotech, Marseille, France) and viewed under a microscope (Axiophot, Zeiss, Göttingen, Germany).

DNA staining with the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed with the In Situ Cell Death Detection kit (Boehringer Mannheim). In brief, the tissue was permeabilized with 0.1% Triton X and 0.1% sodium citrate in PBS and then incubated with 50 μl TUNEL reaction mixture at 37°C for 1 h. At least 300 cells were counted in each section and the number of TUNEL-positive apoptotic cells per 100 cells was expressed as apoptotic index in percent.

Detection of CD95 in gastric biopsies by immunohistochemistry

Detection of CD95 was performed using a monoclonal IgG_1 mouse antibody directed against CD95 (19, 27). Acetone-fixed cryosections were incubated for 60 min at room temperature with the primary monoclonal antibody at a concentration of 10 μg/ml. Bound monoclonal antibody was visualized by the avidin-biotin-peroxidase method using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazol as chromogen. Counterstaining was performed with hemalaun (Merck, Darmstadt, Germany). CD95 expression was assessed by counting at least 300 epithelial cells or lymphocytes in each section. The number of CD95-positive cells per 100 cells was expressed in percent. However, in some biopsies from patients with normal *H. pylori*–negative mucosa < 300 lymphocytes in each section were present.

In situ hybridization of CD95L mRNA in gastric biopsies

Paraformaldehyde-fixed cryosections were dehydrated through an ascending series of ethanol and air dried. Digoxigenin-labeled antisense RNA corresponding to the sequence coding for amino acids 100–246 of human CD95L was generated by in vitro transcription with T7 polymerase (Boehringer Mannheim). The length was reduced to ~ 250 nucleotide fragments by limited alkaline hydrolysis (28). The hybridization mixture contained 100 pg/μl digoxigenin-labeled RNA, 50% deionized formamide, 10% dextran sulfate, 2× SSC, 1× Denhardt's mixture (0.02% wt/vol), 0.1% sodium dodecylsulfate, 500 μg/ml yeast tRNA, and 100 μg/ml herring sperm DNA. The pretreated, dried sections were incubated with this mixture and covered with parafilm. The slides were then incubated overnight at 37°C in a moist chamber containing 50% formamide. Subsequently, the slides were washed with 2× SSC (30 min), digested with 5 μg RNase A (20 min), and again washed with 0.5× SSC (20 min) at 37°C. Digoxigenin-labeled RNA was detected by a polyclonal antidigoxigenin sheep antibody conjugated with alkaline phosphatase (Boehringer Mannheim) and visualized for fluorescence microscopy using 2-hydroxy-3-naphthoic-acid-2′-phenylanilide phosphate (HNPP; Boehringer Mannheim) as chromogenic substrate. Subsequent staining with TUNEL or DAPI was performed as described in order to localize CD95L mRNA expression within the gastric mucosa. The slides were then viewed under a fluorescence microscope (Axiophot, Zeiss). CD95L mRNA expression was scored semiquantitatively (0, no expression; 1, mild; 2, moderate; 3, marked expression).

H. pylori strains, gastric epithelial cell lines, and human lymphoma cell lines

Two different *H. pylori* strains, *H. pylori* 60190 (ATCC 49503), a wild-type cytotoxic, *cagA*-positive strain with the *vacA* genotype s1a/m1, and Tx30a, a noncytotoxic strain, which has the *vacA* genotype s2/m2 and is *cagA* negative, were used (29). The strains were cultured in medium containing 10% brain heart infusion, 10% *Brucella* broth, 70% RPMI 1640, and 10% FCS for 3 d under microaerophilic conditions at 37°C. Supernatants were prepared by centrifugation (2,000 *g* at 4°C) and filtration through a 0.2-μm filter and then stored at −20°C.

Three gastric cancer cell lines, AGS (ATCC CRL 1739), Kato III (ATCC HTB 103), and Hs 746T (ATCC HTB 135) were cultured in Ham's F12 medium, RPMI 1640, or Dulbecco's modified Eagle medium (BioWhittaker, Walkersville, MD), supplemented with 10% FCS, 5 mmol/liter glutamine (Flow Laboratories, Meckenheim, Germany), 100 μg/ml streptomycin, and 100 μg/ml penicillin (both Flow Laboratories). The human lymphoma cell lines $Jurkat^S$, which is sensitive to CD95-mediated apoptosis, and $Jurkat^R$, which is insensitive to CD95-mediated apoptosis, were grown in Iscove's modified Dulbecco's medium supplemented with 10% FCS (30). Gastric cancer cells and lymphoma cells were subconfluently seeded in 25-cm^2 culture flasks and incubated with *H. pylori* supernatants at 37°C in 5% CO_2 in air.

Cytotoxicity assay

The cultured epithelial cell lines, AGS, Kato III, and Hs 746T were seeded into 96-well plates (10^5 cells/well) for 24 h. Then, serial dilutions of culture supernatants of *H. pylori* 60190 or Tx30a were added to the cells and incubated up to 24 h at 37°C. Experiments were per-

formed in quadruplicate and were repeated twice. After removal of the medium, the remaining adherent cells were stained with 0.75% crystal violet solution in 50% methanol. Cell viability was quantified by eluting the dye from the stained cells with 0.1 M sodium citrate/0.1 M citric acid and 50% ethanol. Absorbance was measured at 540 nm (31).

Detection of apoptosis in gastric cells and in lymphoma cells

FACS® analysis. Apoptosis in gastric epithelial cells and in lymphoma cells was assessed by FACS® analysis carried out in a FACScan® flow cytometer (Becton Dickinson, Heidelberg, Germany). Cells floating in the culture medium were collected by centrifugation at 100 *g*. Adherent cells were harvested by incubation with 1% trypsin for 2 min. The cells were washed with PBS, fixed in 70% ethanol, and stained with propidium iodide (50 μg/ml in PBS). DNA fluorescence was determined by FACScan® analysis according to the method of Nicoletti et al. (32) using LYSIS II software (Becton Dickinson). A minimum of 10,000 events was measured per sample.

DAPI and TUNEL staining. For DAPI staining of cellular DNA, cultured gastric epithelial cells were plated on chamber slides (Lab-Tek; Nunc, Naperville, IL). After removal of the culture medium, cells were briefly dipped twice into PBS containing 1 mM $MgCl_2$ at 37°C. The slides were then fixed in methanol for 10 min followed by acetone for 5 min at −20°C and air dried. DNA staining with DAPI and with TUNEL reagent was performed according to the manufacturer's protocols (Boehringer Mannheim) as described above. The slides were viewed under a fluorescence microscope.

CD95 expression on gastric cells

CD95 expression on the cell surface of cultured gastric epithelial cells was assessed by FACS® analysis. A mouse anti–APO-1 IgG_1 antibody (100 ng/ml) was used as primary antibody (27). Phycoerythrin labeled goat anti–mouse antibody (Jackson ImmunoResearch, West Grove, PA) was used as secondary reagent for indirect immunofluorescence according to the manufacturer's instructions. Gastric epithelial cells were washed with PBS and incubated with anti–APO-1 antibody. After a 15-min incubation, cells were washed twice, incubated for 15 min with the secondary antibody, washed twice, resuspended in PBS containing 1% formaldehyde at 4°C, and assayed by FACS® analysis. For data acquisition a gate was set on intact cells by forward/side scatter analysis and a minimum of 10,000 viable cells was analyzed.

Treatment with IgG_3 anti–APO-1 antibody and $F(ab')_2$ anti–APO-1 fragments

To investigate the susceptibility of gastric epithelial cells to undergo apoptosis upon CD95 stimulation, cells were treated for 12–48 h after plating with the monoclonal antibody IgG_3 anti–APO-1 at a concentration of 1 μg/ml (20, 27). CD95 blocking experiments were performed by incubation with $F(ab')_2$ anti–APO-1 fragments as described previously (33). Experiments were performed at least three times.

RT-PCR analysis of CD95 mRNA and CD95L mRNA

RNA was prepared from $\sim 10^7$ cultured AGS, Kato III, or Hs 746T cells treated with *H. pylori* supernatants (100 μl/ml) for 24 h, using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. RT-PCR was performed using the GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT) according to the manufacturer's protocol. cDNA synthesis was performed with oligo $d(T)_{16}$ in the presence of 75 U MuLV RT (Perkin Elmer), 67 μmol $MgCl_2$, and 63 μM of each dNTP in 20 μl for 45 min at 42°C. The efficiency of the reverse transcriptase and the integrity of RNA used in the RT-PCR were controlled by detection of human β-actin mRNA. For amplification of CD95 DNA, the primers 5′ TGA AGT TGA TGC CAA TTA CG 3′ and 5′ CAA GTG CAG ATG TAA AC 3′ were used. The primers used for amplification of the CD95L have been described previously (30). 35 PCR cycles were performed at 94°C for 30 s, at 56°C for 30 s, and at 72°C for 2 min in a volume of 100 μl. PCR-amplified products (10 μl each) were analyzed on 1.5% agarose gels after ethidium bromide staining.

Results

Detection of apoptosis in gastric biopsies. A high number of TUNEL-positive apoptotic cells was found in antral samples in 24 (82.8%) of 29 patients with *H. pylori*–associated gastritis (Table I). The apoptotic index in biopsies from all *H. pylori*–infected patients was 8.0±5.1% ($P = 0.002$). Apoptotic cells were present in the superficial epithelial cells, in the pyloric gland cells, and in the lamina propria. As shown in Fig. 1, apoptotic cells were typically localized in the neighborhood of CD95L mRNA-positive cells. In contrast, samples from *H. pylori*–negative patients showed no apoptotic TUNEL-positive cells in 7 of 10 patients. Only a small number of apoptotic epithelial cells was found in the pyloric gland cells and lamina propria cells in biopsies of the other three patients. The apoptotic index in biopsies from all patients without *H. pylori* infection was 1.9±2.3%.

CD95 expression in gastric biopsies. The above patients were studied for expression of CD95 in antral biopsies (Fig. 2). In all biopsies with *H. pylori*–associated gastritis, CD95 expression on surface epithelium was significantly increased (30.0±31.1%, $P = 0.003$; Table I). Lamina propria cells displayed CD95 expression in 40.6±27.5% of the cells ($P = 0.0002$). In contrast, in *H. pylori*–negative biopsies CD95 expression was detected only on a few surface epithelial cells (0.8±2.1%). Lamina propria cells showed low CD95 expression (6.4±5.2%). In pyloric gland cells strong expression of CD95 was found in both *H. pylori*–positive and *H. pylori*–negative biopsies, but it was more pronounced in chronic gastritis (76.1±14.2 vs. 53.0±24.3%; $P = 0.018$). These findings suggest that CD95 expression is upregulated in *H. pylori*–induced chronic gastritis.

CD95L mRNA expression in gastric biopsies. To assess whether CD95L is expressed in *H. pylori*–associated chronic gastritis, CD95L mRNA was investigated by in situ hybridization. In 21 (72.4%) of 29 *H. pylori*–infected patients, antral biopsies showed moderate or intense expression of CD95L mRNA (Fig. 1), located in the lamina propria (Fig. 3), thus, in the area of infiltration with mononuclear cells. In addition, CD95L mRNA was also localized in superficial epithelial cells and pyloric gland cells, demonstrating that gastric epithelial cells are capable of expressing CD95L mRNA in *H. pylori*–associated chronic gastritis. Uniformly, CD95L mRNA expression by epithelial cells was in the same range as the expression by lamina propria cells. The mean score of CD95L mRNA expression in biopsies from all *H. pylori*–infected patients was 1.4±1.1 ($P = 0.012$; Table I). CD95L mRNA expression was absent or low in biopsies from patients without *H. pylori*–associated gastritis (score, 0.3±0.6). These results indicate that CD95L mRNA expression is not restricted to lymphocytes but can also be expressed by gastric epithelial cells.

Induction of apoptosis and cytolysis in gastric epithelial cell lines. In addition to the above in vivo experiments, induction of apoptosis was evaluated in vitro in the three different gastric epithelial cell lines AGS, Kato III, and Hs 746T by treating with supernatants of the *H. pylori* strains 60190 (cytotoxic) and Tx30a (noncytotoxic). FACS® analysis after propidium iodide staining (Fig. 4, *A* and *B*) and staining with TUNEL or DAPI

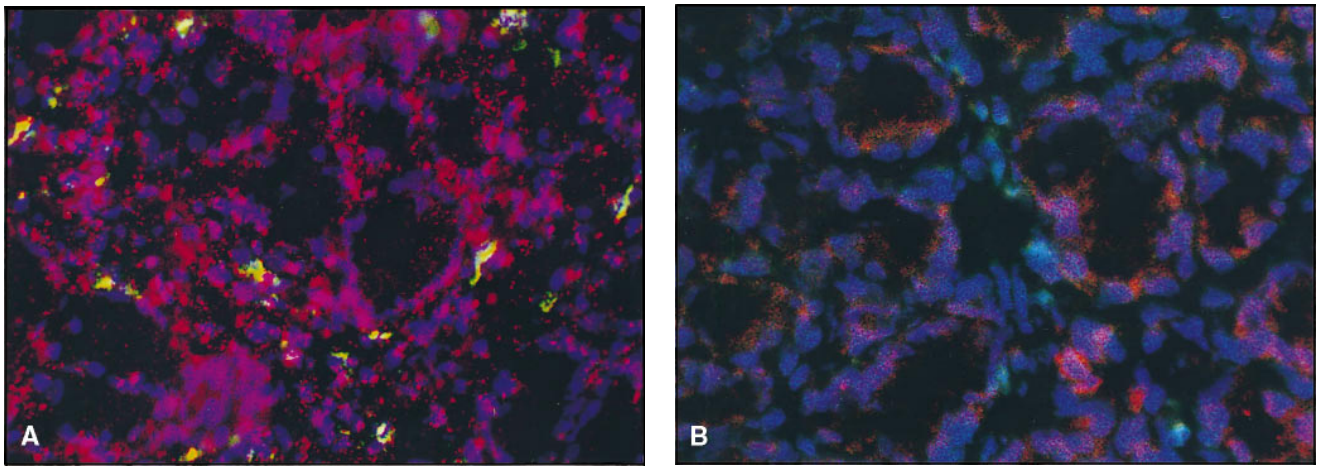

Figure 1. (*A*) Detection of apoptotic cells by TUNEL staining (green fluorescence) and in situ hybridization for CD95L mRNA (red staining) in *H. pylori*–associated chronic antral gastritis. Nuclei are stained blue by DAPI. Note that apoptotic cells are localized next to the CD95L signal. (*B*) Normal mucosa with few apoptotic cells and low levels of CD95L mRNA expression. ×300.

reagents (not shown) demonstrated apoptosis as the predominant form of cell death in a dose- and time-dependent fashion after incubation of all three cell lines with *H. pylori* 60190 supernatant. Apoptosis, as detected by the appearance of a typical sub-G1 fraction of fragmented nuclei in the FACS® analysis, started after 2 h of incubation with *H. pylori* 60190 supernatant amounting to up to 30% in AGS cells, 49% in Kato III cells (Fig. 4 *B*), and 34% in Hs 746T cells (not shown) at 4–6 h, and to 66% in AGS cells, 65% in Kato III cells, and 48% in Hs 746T cells at 12 h (not shown). Comparable results were obtained by TUNEL and DAPI staining (not shown). When *H. pylori* 60190 supernatant was fractionated by ultrafiltration, the fraction containing bacterial products with a molecular mass > 50 kD induced apoptosis in gastric epithelial cells. In contrast, no induction of apoptosis was seen after incubation of gastric epithelial cells using the fraction with bacterial products < 50 kD (data not shown). Anti–APO-1 treatment resulted exclusively in apoptotic cell death as indicated by the sub-G1 peak in FACS® analysis (Fig. 4 *A*). FACS® analysis after treatment with *H. pylori* 60190 supernatant revealed a minor fraction of very small nuclear fragments close to the *y* axis in addition to the typical sub-G1 fraction (Fig. 4 *A*), indicating that *H. pylori* induced cytolysis as well. Accordingly, apoptotic cell death and cytolysis accounted for complete cell death in all three cell lines after treatment with *H. pylori* 60190 for 12 h as assessed by the crystal violet assay (Fig. 5). In contrast, incubation of gastric epithelial cell lines with *H. pylori* strain Tx30a supernatant resulted in no or only a slight increase in apoptotic or necrotic cells compared with cells incubated with medium alone (Figs. 4 and 5).

These data show that *H. pylori* induces apoptotic and cytolytic cell death in gastric epithelial cell lines in vitro.

Upregulation of CD95 in gastric epithelial cell lines by H. pylori. Untreated gastric epithelial cells showed a low basal expression of CD95 in 7% or less of the cells (AGS cells, 7%; Kato III cells, 5%; and Hs 746T cells, 7%) as detected by FACS® analysis after phycoerythrin staining. Incubation with *H. pylori* 60190 supernatant resulted in a time-dependent, substantial increase in CD95-positive epithelial cells. CD95 expression was detectable in up to 50% of AGS cells, in 28% of Hs 746T cells (Fig. 6), and in 25% of Kato III cells (not shown) after a 48-h treatment. In contrast, no or only slight upregula-

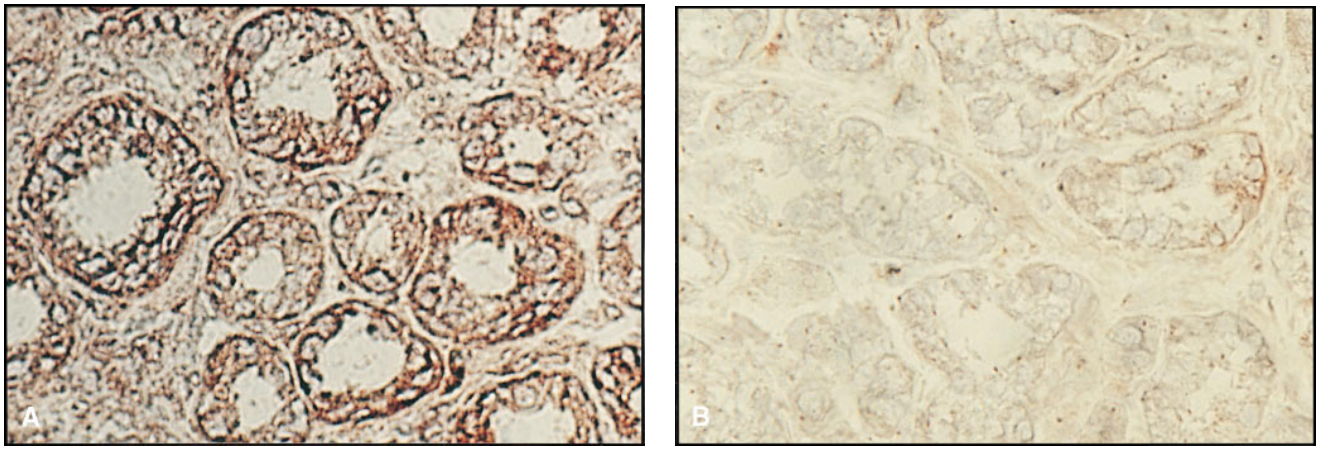

Figure 2. CD95 expression in antral mucosa by immunocytochemistry. Expression of CD95 at the surface of pyloric gland cells is significantly increased in *H. pylori*–associated chronic antral gastritis (*A*) compared with uninfected normal mucosa (*B*). ×400.

tion of CD95 in all three cancer cell lines was observed after treatment with supernatant of *H. pylori* strain Tx30a. In addition, incubation of gastric epithelial cells with TNF-α (0–50 ng/ml) or IFN-γ (0 to 250 U/ml) or both had only slight or no effects on CD95 and CD95L expression in these cells (data not shown).

After incubation with *H. pylori* 60190 supernatant the percentage of cells expressing CD95 was lower than the percentage of cells undergoing apoptosis. This could be explained by the different doses of *H. pylori* supernatant used in the studies. Doses used for CD95 upregulation (0–125 μl/ml) were lower than those used for induction of apoptosis (≥ 100 μl/ml) in order to avoid apoptotic cell death within the incubation period.

CD95 mRNA was detectable by semiquantitative RT-PCR after treatment with *H. pylori* 60190 supernatant (100 μl/ml) for 24 h (not shown). No CD95 mRNA expression was found in untreated gastric epithelial cell lines or in epithelial cells treated by supernatant of strain Tx30a.

Thus, *H. pylori* 60190 increases CD95 expression at the mRNA and protein level.

Induction of CD95L mRNA expression in gastric epithelial cell lines by H. pylori. The unexpected finding of CD95L mRNA expression not only by lymphocytes but also by gastric epithelial cells in chronic gastritis prompted us to study the induction of CD95L mRNA in gastric epithelial cell lines in vitro after treatment with *H. pylori*. CD95L mRNA was constitutively expressed in untreated gastric epithelial cells. CD95L mRNA levels increased after incubation with *H. pylori* 60190 supernatant (100 μl/ml) reaching a maximum after 24 h (Fig. 7). In contrast, the amount of CD95L mRNA remained unchanged after incubation of gastric epithelial cells with supernatant of *H. pylori* strain Tx30a (Fig. 7) or with TNF-α and/or IFN-γ (data not shown). These findings are consistent with the results obtained by in situ hybridization of CD95L mRNA showing increased expression of CD95L mRNA by gastric epithelial cells in biopsies of patients affected by chronic gastritis.

H. pylori–induced apoptosis is mediated by the CD95 receptor and ligand system. To further demonstrate the functional relevance of the CD95 receptor and ligand system in *H. pylori*–induced apoptosis, gastric epithelial cells were incubated with the agonistic anti–APO-1 antibody or with antagonistic $F(ab')_2$ anti–APO-1 fragments. In untreated gastric epithelial cells, incubation with anti–APO-1 antibody resulted in apoptotic cell death in up to 15% of the cells. However, when gastric epithelial cells were pretreated with sublethal doses of *H. pylori* 60190 supernatant (100 μl/ml) for 48 h, additional stimulation with anti–APO-1 antibody (1 μg/ml) for 24 h induced apoptosis in up to 46 and 72% of the gastric epithelial cells, respectively (data not shown). In contrast, induction of apoptosis in untreated cells by *H. pylori* 60190 supernatant (300 μl/ml) was substantially inhibited from 62±6 to 25±6% in AGS cells and from 53±6 to 27±4% in Kato III cells after 24 h of culture in the presence of $F(ab')_2$ anti–APO-1 fragments (1 μg/ml).

Incubation of apoptosis-sensitive Jurkat^S cells for 12 h with culture supernatant from AGS cells pretreated with *H. pylori* 60190 supernatant (100 μl/ml) for 24 h induced apoptosis in 24% of the lymphocytes. In contrast, cultivation of CD95L insensitive Jurkat^R cells resulted in a low level of apoptotic cell death (< 7%), which was comparable to the level of apoptosis seen in Jurkat^S cells incubated with culture supernatants from untreated or with Tx30a supernatant-treated AGS cells or with supernatants from *H. pylori* 60190 or Tx30a (data not shown). This suggests that *H. pylori* 60190 induces CD95L expression in AGS cells leading to apoptosis in Jurkat^S cells. Taken together, the results show that *H. pylori*–induced apoptosis in gastric epithelial cells is mediated by activation of the CD95 receptor and ligand system.

Discussion

In this study we demonstrate that *H. pylori*–associated chronic gastritis involves apoptotic cell death. A markedly elevated number of apoptotic cells was identified in surface epithelium, antral pyloric glands, and lamina propria in 83% of biopsies from patients with *H. pylori*–associated gastritis. Increased epithelial apoptosis has been reported in gastric biopsies from patients with duodenal ulcer and nonatrophic gastritis (34–36). Successful treatment of *H. pylori* resulted in a decrease of apoptosis to levels found in normal mucosa (34). This implies that *H. pylori* induces gastric epithelial apoptosis.

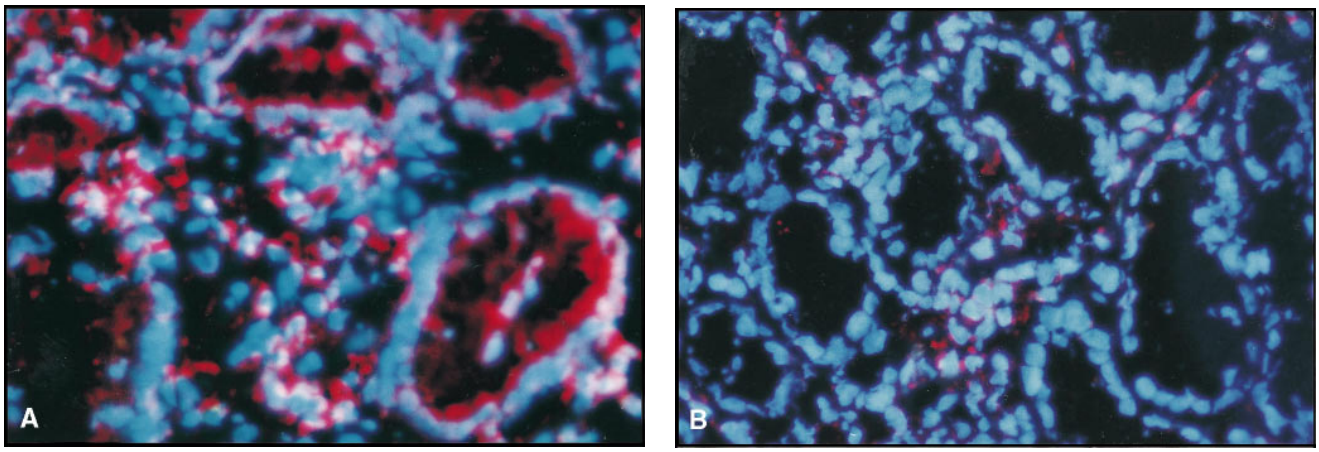

Figure 3. (*A*) DAPI staining and in situ hybridization for CD95L mRNA in *H. pylori*–associated chronic antral gastritis. Nuclei are stained blue and CD95L mRNA is indicated by red staining. CD95L mRNA is expressed extensively by epithelial gland cells and lamina propria lymphocytes. Lymphocytes are characterized by large nuclei and small cytoplasm. (*B*) Normal mucosa with low levels of CD95L mRNA expression. ×400.

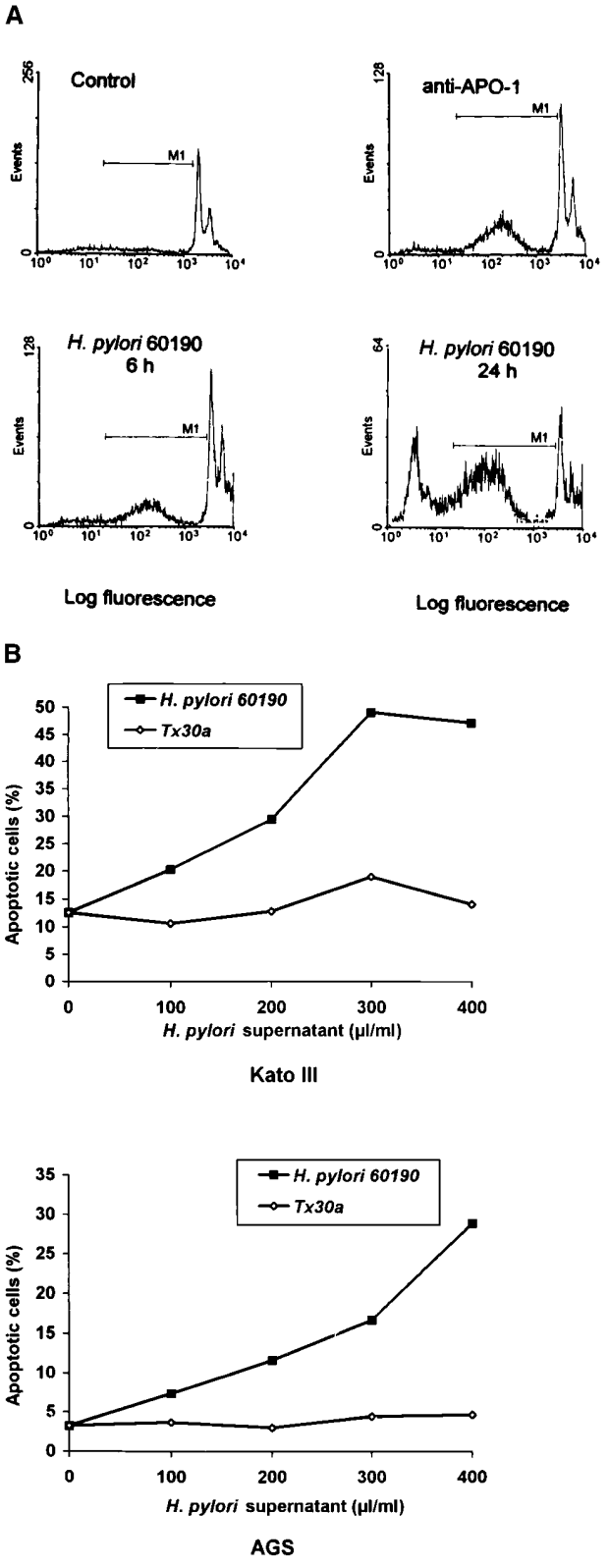

Figure 4. Dose-dependent induction of apoptosis in the gastric epithelial cell lines Kato III and AGS by supernatants of *H. pylori* strains 60190 and Tx30a. (*A*) Flow cytometry of untreated AGS epithelial cells (*top left*) and AGS epithelial cells treated with agonistic anti–APO-1 antibody (1 μg/ml) for 6 h (*top right*) or *H. pylori* 60190 supernatant (300 μl/ml) for 6 h (*bottom left*) and 24 h (*bottom right*). Apoptotic cells are characterized by a shift to the left representing the sub-G1 state of the cells (marker *M1*). Note the increases in very small nuclear fragments close to the *y* axis and at the left of marker M1 which represent additional nonapoptotic cell death after treatment with *H. pylori* 60190 for 24 h. (*B*) Increase in the number of apoptotic Kato III and AGS cells after treatment with different doses of *H. pylori* supernatants for 6 h as determined by FACS® analysis.

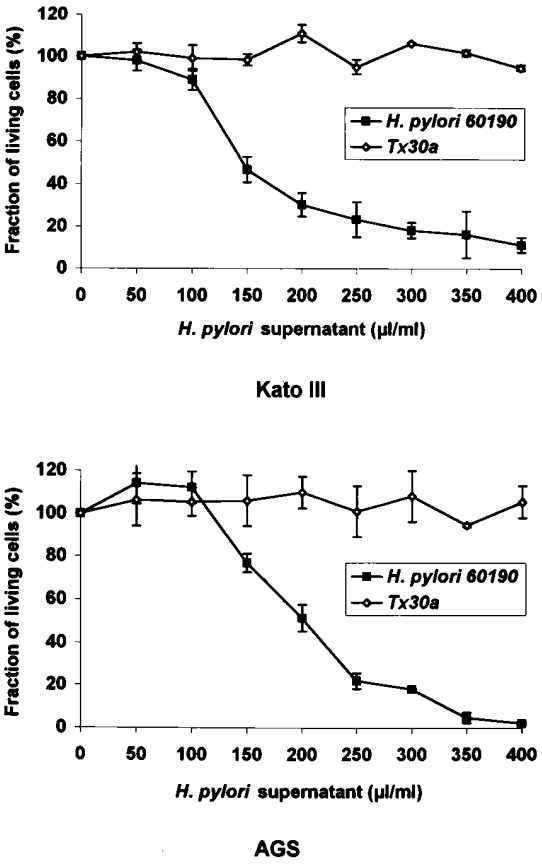

Figure 5. Cytotoxicity assay with crystal violet staining of viable cells. Kato III and AGS epithelial cells were incubated with different doses of supernatants obtained from strain *H. pylori* 60190 and strain Tx30a for 12 h. Data are expressed as fraction of living cells (mean±SD).

The mechanisms of apoptosis induction by *H. pylori* have not been evaluated. Our data suggest that apoptosis in *H. pylori*–associated gastritis involves CD95 and CD95L activation. In *H. pylori*–infected mucosa CD95 expression was upregulated in surface epithelium, lamina propria lymphocytes, and pyloric gland cells. In addition, increased levels of CD95L mRNA found by in situ hybridization in lamina propria cells suggest that lymphocytes in the lamina propria might express CD95L and induce apoptosis via CD95L in CD95-positive neighboring cells. Activated T cells express CD95L, and the CD95-CD95L system is one of the major pathways of T cell–mediated cytotoxicity (13–15, 33, 37). CD95L-expressing lymphocytes play a key role in diseases with chronic inflammation such as chronic viral hepatitis B and C (24, 38). However, CD95L is not confined to the immune system, as thyrocytes from patients with Hashimoto's thyroiditis, hepatocytes from patients with alcoholic liver damage, or hepatoma cells express CD95L (24, 39, 40). Interestingly, CD95L mRNA was also expressed on surface epithelium and pyloric gland cells of *H. py-*

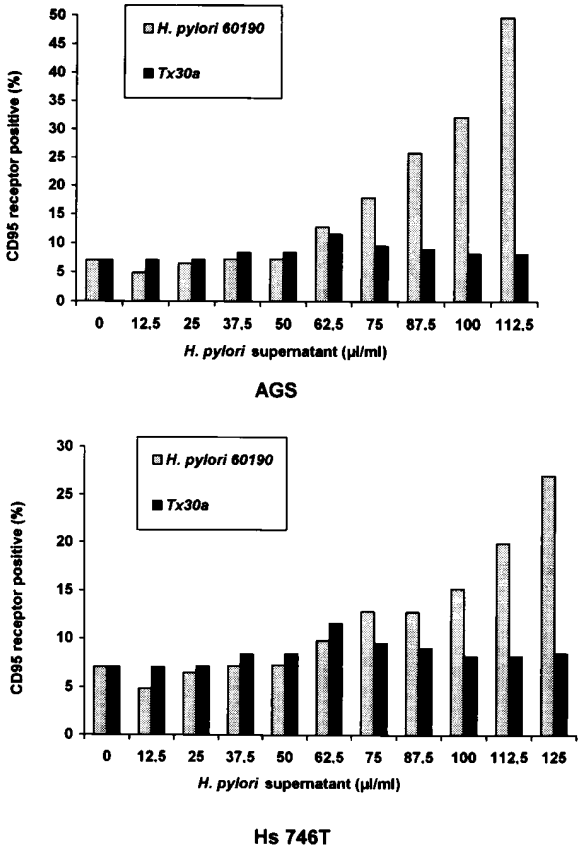

Figure 6. Upregulation of CD95 in gastric epithelial cells after treatment with *H. pylori* supernatant. CD95 expression as assessed by FACS® analysis significantly increased after incubation of AGS and Hs 746T cells with supernatant of *H. pylori* 60190 for 48 h.

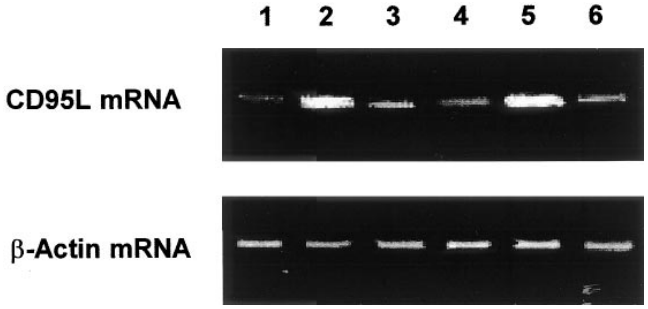

Figure 7. Semiquantitative RT-PCR analysis of CD95L mRNA expression after incubation with *H. pylori* supernatants (100 µl/ml) for 24 h. Incubation with *H. pylori* 60190 increased CD95L mRNA expression in AGS (lanes *1–3*) and Kato III (lanes *4–6*) cells. Expression of β-actin mRNA was used as control. Lanes: *1* and *4*, control; *2* and *5*, *H. pylori* 60190; *3* and *6*, *H. pylori* strain Tx30a.

lori–infected mucosa at levels comparable to those found on lamina propria lymphocytes. The presence of CD95L mRNA and upregulation of CD95 in the same cell points to a new mechanism of damage in *H. pylori*–induced chronic gastritis. By TUNEL staining, apoptotic cells were regularly detected in close proximity to CD95L mRNA–expressing cells. CD95L might be expressed as a membrane-bound form on epithelial cells and mediate apoptosis by "fratricide" interacting with CD95 on neighboring epithelial cells (41) or through suicide of the CD95L-CD95–expressing cell itself. Thus, gastric epithelial apoptosis in *H. pylori*–associated gastritis may be induced not only by CD95L-expressing lymphocytes but also by CD95L-expressing epithelial cells.

The causative relationship between *H. pylori*, induction of apoptosis, and activation of the CD95 receptor and ligand system was further established in in vitro studies. Treatment with cytotoxic *H. pylori* supernatant induced mRNA production of CD95L and upregulated CD95 expression in gastric epithelial cells. Agonistic anti–APO-1 antibody induced apoptotic cell death in gastric epithelial cells pretreated with low concentrations of *H. pylori* supernatant, demonstrating functional CD95 expression. In contrast, incubation of gastric epithelial cells with cytotoxic *H. pylori* supernatant in the presence of $F(ab')_2$ anti–APO-1 fragments partially protected from induction of apoptosis. Cultivation of CD95L-sensitive Jurkat^S cells with supernatants of AGS cells pretreated with *H. pylori* 60190 supernatant resulted in apoptotic cell death of the lymphoma cells, suggesting CD95L expression of AGS cells by *H. pylori*. The activation of the CD95-CD95L system by *H. pylori* raises the question of whether this effect is specific for gastric epithelial cells. Parallel experiments with HeLa and HepG2 cells (data not shown) revealed that treatment with *H. pylori* 60190 supernatant resulted in a similar increase in sensitivity towards apoptosis and to induction of apoptosis arguing for a nonspecific effect of *H. pylori* on different cells.

These data strongly support the important pathogenic and functional role of the CD95 receptor and ligand system in *H. pylori*–induced apoptosis. *H. pylori* induced simultaneous expression of functional CD95L and CD95, possibly as the result of cytokine induction released from activated epithelial cells and/or infiltrating lymphocytes (42–44). However, TNF-α and/or IFN-γ had no or only slight effects on CD95-CD95L upregulation in gastric cancer cell lines in vitro (45). These findings may be cell line specific and do not necessarily exclude a possible upregulation of the CD95 system in gastric epithelial cells by infiltrating lymphocytes in vivo.

Upregulation of CD95 receptor expression and induction of apoptosis was achieved with the cytotoxic *H. pylori* strain 60190. Cytotoxic *H. pylori* strains produce high concentrations of VacA and induce vacuolation in gastric epithelial cells in vitro (46–48). These strains are more virulent and are associated with more severe disease such as peptic ulceration (29, 49). In addition, CagA producing *H. pylori* strains are associated with increased cytokine production in the gastric mucosa and in gastric epithelial cell lines (50–54), which may be responsible for activation of the CD95-CD95L system in these cells. In a recent study in rats lipopolysaccharide isolated from a cytotoxic *H. pylori* strain was suggested to be responsible for induction of apoptosis (55). However, whether cytokines or bacterial products from virulent *H. pylori* strains initiate CD95 activation in vivo is still elusive and needs further investigation.

Incubation of gastric epithelial cells with *H. pylori* supernatant in the presence of $F(ab')_2$ anti–APO-1 fragments could not completely protect from apoptosis. In addition, the number of dead cells by the cytotoxicity assay was higher than the fraction of apoptotic cells measured by FACS® analysis, indicating that cytolytic cell death might also occur in addition to apoptosis. Cytolytic cell death is caused by the bacterial VacA

protein, which induces vacuolar degeneration in gastric epithelial cells (46–49) probably via a V-ATPase proton pump (56). Vacuolar degeneration has not been identified as a typical morphological feature in cells undergoing apoptosis (57). Thus, vacuolar degeneration and CD95-mediated apoptosis might represent two different mechanisms contributing to the toxic activity of *H. pylori*.

In conclusion, this study provides evidence that apoptosis is involved in *H. pylori*–induced epithelial cell damage. Apoptotic cell death in *H. pylori*–associated chronic gastritis may not only involve killing of epithelial cells by CD95L-expressing lymphocytes but also may occur by fratricide and/or suicide mediated by CD95L-CD95 interaction among gastric epithelial cells. The involvement of the CD95 pathway in *H. pylori*–induced gastric epithelial apoptosis offers a new understanding of the pathogenesis of chronic *H. pylori*–induced gastritis.

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