Helicobacter pylori induces apoptosis via NF-κB activation in human gastric AGS cells

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Helicobacter pylori induces apoptosis via NF-κB activation in human gastric AGS cells

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감사의 글

한 해를 마무리하는 시기에 지난 3년간의 시간을 되돌아보니 약리학교실에서의 시간들이 모두 소중하고 기분 좋은 추억으로 남습니다.

무엇보다도 처음부터 지금까지 이끌어 주시고 배려해 주신 김경환 교수님과 늘 학문에 대한 진지함을 보여주시고 세심하게 지도해 주신 김혜영 교수님께 감사를 드립니다. 가장 가까운 곳에서 늘 가르쳐 주시고 도움을 주신 임주원 선생님께 감사를 드립니다. 연구 결과에 대한 비판적인 사고를 키울 수 있도록 끊임없는 조언과 지도를 해주신 안영수 교수님, 김동구 교수님, 이민구 교수님, 장정원 교수님, 서정택 교수님, 정광철 교수님께 감사를 드립니다. 논문의 심사 및 교정에 많은 도움을 주신 김원호 교수님, 송시영 교수님, 정명희 교수님께 감사를 드립니다. 졸업 후에도 많은 관심과 배려로 이끌어 주신 유지수 교수님, 황애란 교수님, 이정렬 교수님, 이경은 교수님께 감사를 드립니다. 지훈, 지혜, 지연, 혜연, 이장원 선생님, 서정연 선생님께도 감사의 마음을 전하며, 많은 시간을 함께 한 약리학 교실의 교실원 모두와 졸업의 기쁨을 함께 하고 싶습니다.

공부하는 며느리를 항상 딸처럼 대해주시고 격려해주신 시부모님께 감사를 드립니다. 경태를 선뜻 맡아주시고 돌봐주신 어머니, 정훈 오빠, 동생 양호에게 감사를 드립니다.

마지막으로 지금까지 한결같은 믿음과 사랑으로 함께 하면서 격려해주고 지지해준 남편에게 감사의 마음을 전하며, 곁에 있어 주지 못했는데도 건강하게 잘 자라준 아들 경태에게 고마움을 느낍니다.

2002년 12월

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Helicobacter pylori induces apoptosis via NF - κB activation in human gastric AGS cells

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(Directed by Professor Kyung Hwan KIM)

Helicobacter pylori (H. pylori) causes gastric epithelial cell damage. But thorough investigations into *H. pylori*-induced apoptosis and subsequent molecular mechanisms are rare. The present study examined the role of NF-κB in *H. pylori*-induced apoptosis in human gastric epithelial cells. To investigate the relationship between NF-κB and apoptosis, human gastric epithelial cells (AGS) were treated with antisense ODN for NF-κB p50 (AS ODN) subunit or were transfected with a mutated IκBα gene (MAD-3 mutant). AGS cells were stimulated with *H. pylori* (NCTC11637) in antibiotic-free culture medium for 24 hr (bacterium:cell, 300:1). Apoptosis was determined by cell counting, quantification of DNA fragmentation, and Hoechst staining. As apoptotic indices, expression of p53, bax and bcl-2 were examined using Western blotting and

RT-PCR analysis. As a result, *H. pylori* induced apoptosis, accompanied with the increase of p53 and bax and decrease of bcl-2. *H. pylori*-induced apoptosis was inhibited in the cells treated with AS ODN or transfected with a mutated IκBα gene, while apoptosis was shown in the cells treated with sense ODN of NF-κB p50 subunit (S ODN) or transfected with a control vector (pcDNA-3), by *H. pylori*. *H. pylori*-induced alterations of p53, bax and bcl-2 were inhibited in the cells treated with AS ODN or transfected with a mutated IκBα gene. Moreover, overexpression of bcl-2 attenuated *H. pylori*-induced apoptosis. These results suggest that *H. pylori* induces apoptosis in gastric epithelial cells through activation of NF-κB, mediating regulation of p53, bax and bcl-2.

Key words: Helicobacter pylori, Apoptosis, NF-κB, bax, and bcl-2.

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I. INTRODUCTION

Helicobacter pylori (H. pylori) has been shown to be an important pathogen of a wide variety of diseases including duodenal ulceration, gastritis, gastric adenocarcinoma, and gastric B-cell lymphoma. However, the pathogenic mechanisms are not well defined. One of the potential toxic factors involving *H. pylori*-induced gastric injury is oxygen radicals which are released from activated neutrophils, because *H. pylori* exhibits chemotactic activity for neutrophils.[1] However, *H. pylori* itself induced the production of oxygen radicals in gastric epithelial cells, and enhances membrane damage.[2,3] *H. pylori* infection has been reported to be associated with increased epithelial cell

apoptosis,[4,5] but the exact mechanisms responsible for this phenomenon are unknown.

The bcl-2 family is one of the best-studied groups of proteins involved in the regulation of apoptosis. The first member of this family, bcl-2, was discovered in human B-cell lymphoma at a chromosomal translocation T(14:18), where it was thought to lead to malignant transformation because of its ability to prevent apoptotic cell death.[6] Meanwhile, several other bcl-2 related genes were discovered. Some members of the bcl-2 family, such as bax, bad, or bik, promote apoptosis, whereas the other members, such as bcl-2 and bcl-x_L, inhibit this process. The susceptibility of a cell to apoptosis depends on the balance between pro-apoptotic and anti-apoptotic factors.[7] It has been reported that *H. pylori*-induced programmed cell death in the gastric mucosa was induced by up-regulation of pro-apoptotic bax and attenuation of anti-apoptotic bcl-2 at transcriptional and protein levels.[8] The expression of bax, bcl-2 and bcl-x_L all decreased moderately whereas the pro-apoptotic protein bak was markedly increased in the *H. pylori* stimulated AGS cells in the other study.[9] However, both expression and function of bcl-2 and bax in gastric epithelial cells remain poorly understood.

NF-κB is an inducible transcription factor that mediates signal transduction between cytoplasm and nucleus in many cell types.[10] NF-κB is a member of the Rel family including p50 (NF-κB1), p52 (NF-κB2), Rel A (p65), c-Rel, rel B, and Drosophila morphogen dorsal gene product.[11] In resting cells, NF-κB is localized in the cytoplasm as a hetero- or homodimer, which are noncovalently associated with cytoplasmic inhibitory proteins, including IκB. Upon stimulation by a variety of pathogenic inducers such as viruses, mitogens, bacteria, agents providing oxygen radicals and inflammatory cytokines, the NF-κB complex migrates into the nucleus and binds DNA recognition

sites in the regulatory regions of the target genes.[12] Our previous studies[13,14] demonstrated that the constitutive level of NF-κB was observed in the nucleus of gastric epithelial AGS cells. NF-κB dimers (a p50/p65 heterodimer and a p50 homodimer) were detected and the major NF-κB band was a p50 homodimer even in resting cells. Upon stimulation by *H. pylori* infection, a p50/p65 heterodimer was highly elevated and a p50 homodimer was relatively slightly increased in the nucleus of AGS cells. *H. pylori*-induced cytotoxicity and apoptosis were inhibited by antioxidant enzyme catalase, an inhibitor of NF-κB activation PDTC, iNOS inhibitors and antisense oligonucelotides (AS ODN) for p50 to inhibit NF-κB expression.[15]

Antisense oligonucleotides (AS ODN) are small, synthetic molecules, 15-25 base pairs in length, and are usually single-stranded DNA complementary to the mRNA transcribed from the target gene. Formation of a duplex structure occurs through base-pairing between the antisense DNA and its target mRNA, inhibiting gene expression.[16] Postulated mechanism of AS ODN include steric hindrance of gene transcription or gene translocation, blockage of mRNA processing or splicing, and degradation of mRNA through the action of RNAse H activity.[17] An additional benefit is that ODN can be chemically manipulated by replacement of a nonbridging oxygen with sulfur in a phosphate linkage to form phosphorothioate-modified ODN, making ODN more resistant to degradation by nucleases.[18] Thus, AS ODN can be made to bind to a single gene or its transcription product and act within the constraints of that gene's expression, minimizing side effects. *H. pylori* induced the activation of two species of NF-κB dimmers (a p50/p65 heterodimer and a p50 homodimer) in gastric epithelial cells.[13,14] As a choice for targeting by AS ODN, p50 offers the best potential for effects

upon NF-κB expression and the genes that NF-κB regulates.

NF-κB activation is tightly regulated by its endogenous inhibitor, IκB, which complexes NF-κB in the cytoplasm. To date, the most extensively studied IκB protein is IκBα (36 kDa) encoded by the human MAD-3 gene or its homologues in different species.[19] The mechanisms that lead to the degradation of IκBα proteins are poorly understood, but involve changes in the phosphorylation state of IκBα.[12,20] Two serines in the N-terminal domain of IκBα, serine residues 32 and 36, were shown to be critical for IκBα stability.[18] Substitution of these two serine residues by alanine residues rendered IκBα undegradable by cellular activators.[18,21] Among many proteins exhibiting IκB function, IκBα is the only inhibitor that dissociates from NF-κB complex in response to cell stimulation, with kinetics matching NF-κB translocation to the nucleus.[22] It was therefore suggested that the activation of NF-κB is mainly regulated by NF-κB/ IκBα dissociation.[23,24] Such a mutant IκBα has been shown to act as an NF-κB super-repressor.[25]

The present study was conducted to evaluate the role of NF-κB in *H. pylori*–induced apoptosis in gastric epithelial AGS cells by using phosphorothioate-modified AS ODN for p50 or a mutant IκBα gene to inhibit NF-κB activation. In order to investigate the role of bcl-2 in gastric epithelial cells, human bcl-2 gene was transfected into AGS cells. Cell viability (viable cell number) and apoptosis indices (quantification of DNA fragmentation, Hoechst staining, and changes in expression of p53, bax and bcl-2) were determined.

II. MATERIALS AND METHODS

1. Materials

All chemicals were of analytical reagent grade and purchased from Sigma (Sigma, St. Louis, MO, U.S.A.) unless stated otherwise.

2. Bacterial strain and culture condition

H. pylori, strain NCTC 11637, was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The bacteria were inoculated on chocolate agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD, U.S.A.) at 37℃ under microaerophilic conditions using an anaerobic chamber (BBL Campy Pouch® System, Becton Dickinson Microbiology Systems).

3. Gastric epithelial cell culture

A human gastric epithelial cell line AGS (ATCC CRL 1739) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were grown in complete medium, consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (GIBCO-BRL, Grand Island, NY, U.S.A.). The cells were seeded onto cell culture plate and cultured to reach 80% confluency. Prior to stimulation, each well was washed twice with 1 ml of fresh cell culture medium containing no antibiotics.

4. Extraction of nuclei

The cells were rinsed with ice-cold PBS, harvested by scraping into phosphate buffered saline (PBS), and pelleted by centrifugation at 1,500 *g* for 5 minutes. The cells were lysed in buffer containing 10 mM Hepes, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.5 mM $MgCl_2$, 0.2% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (PMSF). The nuclear pellet was resuspended on ice in nuclear extraction buffer containing 20 mM Hepes, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM DTT, and 0.5 mM PMSF, and the nuclear protein concentration was determined by the method of Bradford.[26]

5. Electrophoretic mobility shift assay

NF-κB gel shift oligonucleotide, 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Promega, Madison, WI, U.S.A.) was labelled with [^{32}P] dATP (Amersham Biosciences, Piscataway, NJ, U.S.A) using T4 polynucleotide kinase (GIBCO-BRL). End-labelled probe was purified from unincorporated [^{32}P] dATP using a Bio-Rad purification column (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and recovered in tris-EDTA buffer (TE). Nuclear extracts (1 μg) were preincubated in buffer containing 12% glycerol; 12 mM Hepes, pH 7.9; 4 mM Tris-HCl, pH 7.9; 1 mM EDTA; 1 mM DTT; 25 mM KCl; 5 mM $MgCl_2$; 0.04 μg/ml poly[d(I-C)] (Boehringer Mannheim, Indianapolis, IN, U.S.A.); 0.4 mM PMSF; and TE. The labelled probe was added and samples were incubated on ice for 10 min. Samples were subjected to electrophoretic separation at room temperature on a nondenaturing 5% acrylamide gel at 30 mA using 0.5× Tris

borate EDTA buffer. The gels were dried at 80℃ for 1 h and exposed to the radiography film for 6-18 h at –70℃ with intensifying screens.[27]

6. Determination of cell death

Cells were plated at 1×10^5 cells/well in a 24-well culture plate and incubated with *H. pylori* for 24 h. After the end of incubation, cell death was evaluated by cell counting or release of lactate dehydrogenase (LDH) in the medium. Cell number and their viability were determined in a hemocytometer by trypan blue exclusion (0.2% trypan blue) using a phase-contrast microscopy (Labophot, Nikon, Japan). LDH release was determined photometrically with a commercial test (Sigma) and was expressed as the percent of the total LDH of cells.

7. Cell viability assay

The differential uptake of fluorescent DNA binding dyes, acridine orange (AO) and ethidium bromide (EtBr) was used to determine viable and nonviable cells.[28] These two dyes can be used to determine which cells in the population have undergone apoptosis, and whether the cell is in the early or late stages of apoptosis based on membrane integrity. AO intercalates into the DNA, giving it a green appearance. This dye also binds to RNA but because it cannot intercalate, the RNA stains red-orange. Thus viable cells will have bright green nuclei and slightly red cytoplasm. EtBr is only taken up by nonviable cells. This dye also intercalates into DNA, making it appear orange. Both normal and apoptotic nuclei in live cells will fluoresce bright green. In contrast, apoptotic nuclei in dead cells will fluoresce bright orange. Thus, we could differentiate

between early and late apoptotic cells using these two dyes. 100 ug/ml AO was mixed with 100 ug/ml EtBr in PBS. 1×10^5 cells cultured in 8 well plates were incubated with dye mix for 5 min and then washed with PBS twice. The cells were observed under the inverted fluorescence microscope (Axiovert 135, Zeiss, Germany) with the filter of excitation 480nm and emission 530nm.

8. Assessment of apoptosis

To assess DNA fragmentation, nucleosomes were quantified in the cytoplasm by means of a sandwich enzyme-linked immunosorbent assay (ELISA) (Cell Death Detection ELISA ^{plus} kit; Boehringer Mannheim). In brief, adhesion cells incubated for 24 h with *H. pylori* were detached, and 1 x 10^5 cells were lysed, after which cytosolic oligonucleosome was quantified by mean of biotin-coupled mouse monoclonal anti-histone antibody as the capturing antibody, peroxidase-conjugated mouse monoclonal anti-DNA antibody as the detecting antibody, and ABTC (2, 2'-azio-di[3-ethylbenhioazolin-sulfonate]) as the developing reagent. Absorbance of treated and control cells were measured at 405 nm. The relative increase in nucleosomes in the cytoplasm was expressed as an enrichment factor, which was calculated as the ratio of specific absorbance of *H. pylori*-infected cells compared with uninfected cells, as described by the manufacturer of the assay.

To assess apoptotic cell death, Hoechst 33258, a DNA-specific dye, staining was performed. AGS cells (4×10^5 cells/well) were cultured in the presence of *H. pylori* (at a bacterium/cell ratio, 300:1) for 24 h. The cells were washed with PBS (pH 7.4), fixed with 4% paraformaldehyde for 30 min, and then stained with 1 mM Hoechst 33258 for

10 min at 37 °C. Cells were washed again with PBS prior to viewing stained nuclei under a microscope. Percentage of apoptotic cells, assessed by staining with Hoechst 33258, was calculated based on total numbers of the cells at the end of the experiment.

9. ODNs preparation

Single-stranded ODNs were produced commercially (GIBCO-BRL). ODNs were phosphorothioate-modified to reduce intracellular nuclease digestion. Antisense (AS) and sense (S) ODNs targeted the ATG start codon of the p50 mRNA. The sequence of the p50 AS ODN was 5' GGA TCA TCT TCT GCC ATT CTG 3'. The sequence of p50 S ODN was 5' CAG AAT GGC AGA AGA TGA TCC 3'.

10. Treatment with ODNs using cationic liposome

AGS cells were transfected with ODNs using a cationic liposome, a commercially available transfection-reagent DOTAP (N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, Ntrimethyl ammonium methylsulfate) (Boehringer-Mannheim) to improve stability and intracellular delivery of ODNs.[29] When DOTAP was employed, the appropriate amount of ODNs were incubated with DOTAP (15 μl/ml) to achieve the respective final concentration of the ODNs to 0.5 μM at 37℃ for 15 min. Then the mixture was added directly to the cells, plated at 5 x 10^5 cells/ml in 6 well plates, and incubated for 55 h. Cells were trypsinized and plated again at the density of 5 x 10^5 cells/ml in 6 well plates. 0.5 μM (final concentration) of ODN were added to the cells and incubated for another 17 h. After medium was changed with antibiotic-free medium, ODN-transfected cells were cultured in the presence of *H. pylori.* The time points were based on the present

time-response of AGS cells for p53, bax and bcl-2 expression.

11. Transfection with a mutated IκBα gene

As a mutated IκBα gene, MAD-3 double point mutant (substitution of two serine residues at position 32 and 36 by alanine residues) construct was prepared as described previously.[21] The control vector pcDNA (Invitrogen, Carlsbad, CA, U.S.A.) was transfected to the cells instead of mutated IκBα gene. These cells were considered as a relative control and named pcN-3. Subconfluent AGS cells, plated in 10 cm culture, were transfected with each 10 μg of expression construct using DOTAP for 16 h. After transfection, the cells were trypsinized and plated at 1 x 10^3 cells per 10 cm tissue culture plate. The cells were cultured in medium containing 400 μg/ml G-418 (GIBCO-BRL) for 15-17 days, 3-4 resistant clones were isolated from each plate and examined for mutated IκBα expression by Western blotting. The positive clones for mutated IκBα were maintained in culture medium containing 400 μg/ml G418 for more than two months and named IW-6 and IW-10. To confirm the establishment of stably transfected cell lines with mutated IκBα genes, Western blotting for wild type IκBα, phosphorylated IκBα and mutated IκBα and electrophoretic mobility shift assay for NF-κB were performed in the cells transfected with a control vector or a vmutated IκBα gene and control cells which received no transfection in the previous study.[30]

12. Transfection with a human bcl-2 gene

A full-length human bcl-2 expression vector (CMVbcl-2nl) and control plasmid (CMVneo) were constructed by replacing the splenic focus forming virus (SFFV)

promoter in SFFVbcl-2nl and SFFVneo with a 760 bp Sau3A1 fragment containing the human cytomegalovirus (CMV) major immediate early enhancer/promoter.[31] AGS cells were transfected with either a CMVbcl-2nl or CMVneo expression vector using DOTAP as manufacturer's guide.

13. Western blottings

Western blotting was performed for the analysis of poly (ADP-ribose) polymerase (PARP), IκBα, bcl-2, bax and p53 proteins. After stimulation with *H. pylori*, the proteins were extracted from the collected cells. Cells were boiled with Tris-HCl buffer containing (125 mM Tris, pH6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol). Protein concentration of 14,000 *g* soluble supernatants from each sample was measured by the method of Bradford.[26] 100 μg of protein for PARP or bcl-2 or p53 determination, and 10 μg of protein for IκBα or bax determination was loaded per lane, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences) by electroblotting. The transfer of protein and equality of loading in all lanes was verified using reversible staining with Ponceau S. Membranes were blocked using 5% nonfat dry milk in TTBS (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% Tween 20) for 1 h. Blots were incubated with a rabbit polyclonal antibody to PARP (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), a rabbit polyclonal antibody to IκBα (1:500; Santa Cruz Biotechnology), a mouse monoclonal antibody to bcl-2 (1:500; Transduction laboratories, San Diego, CA, U.S.A.) or a mouse monoclonal antibody to bax (1:1000; Transduction laboratories) or a mouse monoclonal antibody to p53 (1:500, Pharmingen, San Diego, CA, U.S.A.) at 4℃

overnight. Blots were washed with TTBS three times for 10 min and followed by goat anti-rabbit secondary antibodies (1:5000; Zymed Laboratories, San Francisco, CA, U.S.A.) or goat anti-mouse secondary antibodies (1:1000; Santa Cruz Biotechnology) conjugated to horseradish peroxidase for 1 h at room temperature. After washing the membrane three times with TTBS for 10 min, reactive proteins were visualized with the enhanced chemiluminescence detection kit (Amersham Biosciences).

14. Reverse transcription-polymerase chain reaction analysis for bcl-2, bax and p53

Gene expression of bcl-2, bax and p53 mRNA was assessed using reverse transcription-polymerase chain reaction (RT-PCR) standardized by co-amplifying each gene with the housekeeping gene, β-actin, which served as an internal control. Total RNA was isolated from the cells, treated with ODNs by guanidine thiocyanate extraction method.[32] Total RNA was reverse transcribed into cDNA and used for PCR with human specific primers for bcl-2, bax and p53 and β-actin. Sequences of bcl-2 primers were 5'-GTCATGTGTGTGGAGAGCGTCAA-3' (forward primer) and 5'-AGAGGAGGAGGTAGGGACACGC-3' (reverse primer), giving a 273 bp PCR product. For bax, forward primer was 5'-ACCAAGAAGCTGAGCGAGTGTC-3' and reverse primer was 5'-CAACCACCCTGGTCTTGGATC-3', giving a 308 bp PCR product.[33] For p53, forward primer was 5'-CAGTCAGATCCTAGCGTCGAGC-3' and reverse primer was 5'-GCACCACCACACTATGTCGAAA, giving a 542 bp PCR product.[34] For β-actin, forward primer was 5'-ACCAACTGGGACGACATGGAG-3' and reverse primer was 5'-GTGAGGATCTTCATGAGGTAGTC-3', giving a 353 bp

PCR product.[35] Briefly, the PCR was amplified by 25-33 repeat denaturation cycles at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. During the first cycle, the 95°C step extended to 2 minutes, and on the final cycle the 72°C step extended to 5 minutes. PCR products were separated on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and visualized by UV transilumination.

15. Statistical analysis

Each experiment was performed at least three times. Results are expressed as means ± standard error of four separate experiments. Analysis of variance (ANOVA) followed by Newman-Keul's test was used for statistical analysis.[36] $P<0.05$ was considered statistically significant.

III. RESULTS

1. *H. pylori*-induced NF-κB activation in AGS cells

Treatment of the cells with *H. pylori* (bacterium: cell, 300:1) resulted in NF-κB activation (Fig.1). An increased amount of activated NF-κB was detected at 1 h and the levels of activated NF-κB decreased thereafter.

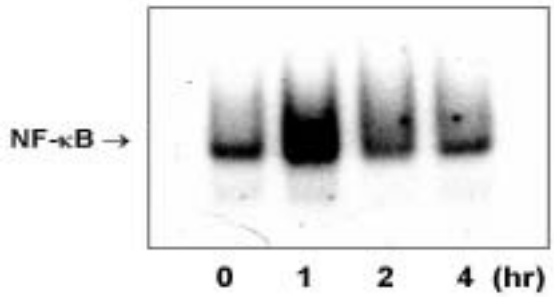

Fig. 1. *H. pylori*–induced NF-κB activation. Electrophoretic mobility shift assay was performed using nuclear extracts prepared from the cells cultured in the presence of *H. pylori* for the indicated time point. Activated NF-κB bands are indicated by the arrow.

2. *H. pylori*-induced cell death

To determine whether *H. pylori* infection induces cell death, the cells were cultured in the presence of *H. pylori* and the viable cells were counted. After treatment of *H. pylori* (bacterium: cell, 300:1), the viable cells were counted at 0, 12, 24, 36 h (Fig.2A). Reduction in viable cell number was shown after 12 h infection. When the cells were cultured in the presence of *H. pylori* for 24 h with the indicated ratio of bacterium to cell (Fig.2B), cell death increased with the number of bacterium treated to the cells. The

number of viable cells, cultured in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 24 h, decreased 49% of that of the cells cultured in the absence of *H. pylori*. Therefore, a bacterium/cell ratio of 300:1 and a 24h culture period were used in this experiment.

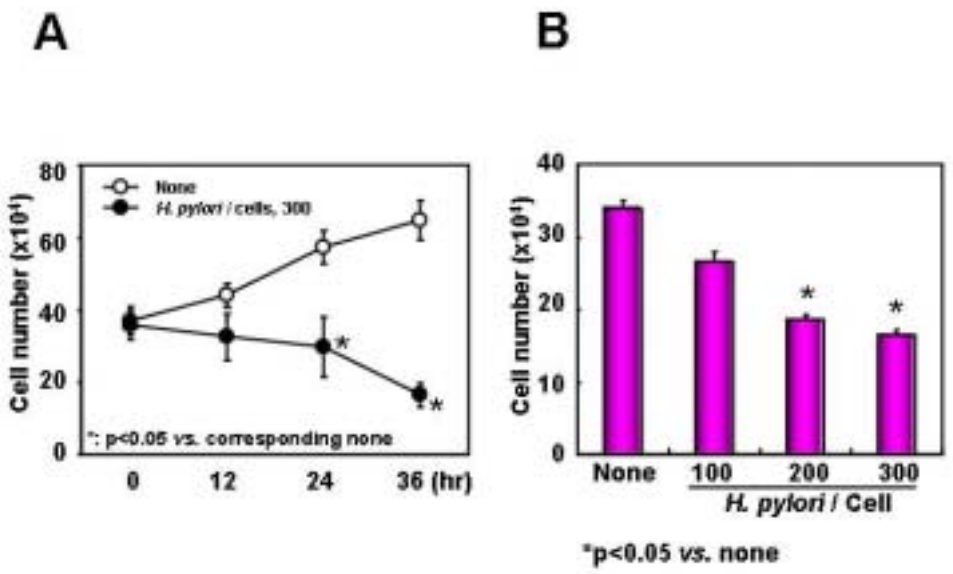

Fig. 2. *H. pylori*–induced cell death in AGS cells. (A) AGS cells (4×10^5 cells/well) were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 36 h. At each indicated time point, cell number was determined by trypan blue exclusion test. (B) AGS cells (2×10^5 cells/well) were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 100:1, 200:1, and 300:1 for 24 h. Cell number was determined by trypan blue exclusion test. Data represent means ± SE. *$p < 0.05$ compared to the corresponding none. None = the cells cultured in the absence of *H. pylori*.

3. *H. pylori*-induced PARP cleavage

To determine whether apoptosis induced by *H. pylori*, the specific cleavage of certain substrates during apoptosis, such as PARP was examined. An anti-PARP antibody capable of detecting both the uncleaved 116 kDa proform and the active cleaved 85 kDa fragments of PARP was used in Western blotting. The 85 kDa fragment cleaved from the 116 kDa PARP was detected in extracts from AGS cells treated with *H. pylori* with a bacterium/cell ratio of 300:1 for 24 h (Fig.3).

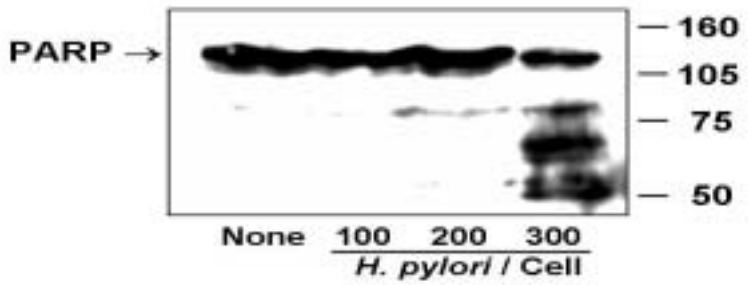

Fig. 3. *H. pylori*–induced PARP cleavage in AGS cells. AGS cells (1×10^6 cells/well) were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 100:1, 200:1, and 300:1 for 24 h. Cell lysates (100 μg of protein) were subjected to 8% SDS-PAGE and transferred onto membranes. PARP cleavage was visualized with a PARP-specific antibody.

4. *H. pylori*-induced down-regulation of bcl-2 and up-regulation of bax and p53

To study the molecular mechanism of *H. pylori*-induced apoptosis, Western blotting for two apoptotic related proteins, bcl-2 and bax, was performed. With *H. pylori* infection, bcl-2 protein level was significantly decreased, whereas bax was significantly increased after 8 h (Fig.4). These data suggest that the mechanisms of apoptosis induced

by *H. pylori* involve bcl-2 pathway. Thus the ratio of bcl-2 to bax that switch the intracellular signal to cell death may be significantly increased in gastric epithelial cells by *H. pylori* infection. Recent studies demonstrated that NF-κB regulates p53 gene expression.[37,38] p53 is an important factor in apoptosis in various cells.[39-42] Also the induction of p53 was observed in the treatment of *H. pylori* in AGS cells.

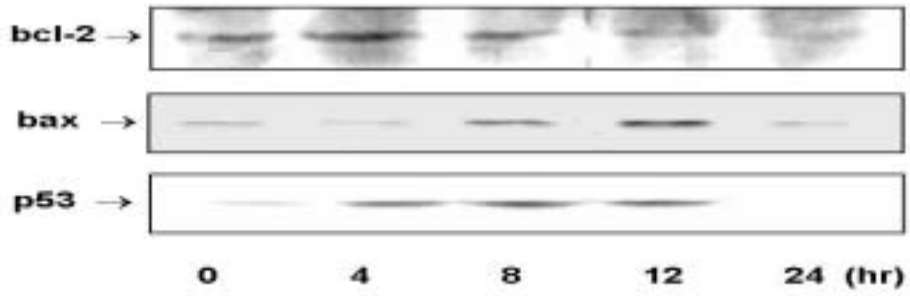

Fig. 4. Protein levels of bcl-2, bax, and p53 in AGS cells. AGS cells (1×10^6 cells/well) were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 24 h. Cells were collected at an indicated time. The protein level of bcl-2, bax, and p53 were determined by Western blotting using specific antibodies.

5. *H. pylori*–induced apoptosis in AGS cells treated with or without ODNs

Either AS ODN or S ODN (0.5uM) was treated directly to the cells and cultured in the presence of *H. pylori* for 24 h. Viable cell number was determined in the cells, treated with or without 0.5 uM of AS ODN or S ODN in the presence of *H. pylori* for 24 h. As shown Fig.5A, treatment of AS ODN showed inhibition of *H. pylori*-induced cell death while S ODN had no inhibitory effect on the reduction of viable cells caused by *H.*

pylori. Treatment of ODNs itself did not induce death of the cells in the absence of *H. pylori,* determined by viable cell number. *H. pylori*-induced DNA fragmentation was determined by the amount of oligonucleosome-bound DNA in the cell lysate. Apoptosis–associated increase in nucleosome-associated low-molecular-weight DNA was inhibited by AS ODN, but not by S ODN (Fig.5B). Staining of DNA-specific dye Hoechst 33258 demonstrated that *H. pylori* treatment caused morphological change of the nuclei, which contained highly condensed chromatin (Fig.6A). This apoptotic phenomenon was inhibited by AS ODN. Treatment of S ODN showed similar apoptotic nuclear morphology as that shown in the cells receiving no treatment, but cultured in the presence of *H. pylori* (*H. pylori* alone). Percentage of apoptotic cells determined by staining with Hoechst 33258, was calculated based on total numbers of the cells (Fig.6B). *H. pylori* induced 5-fold increase in the number of apoptotic cells, which was inhibited by AS ODN.

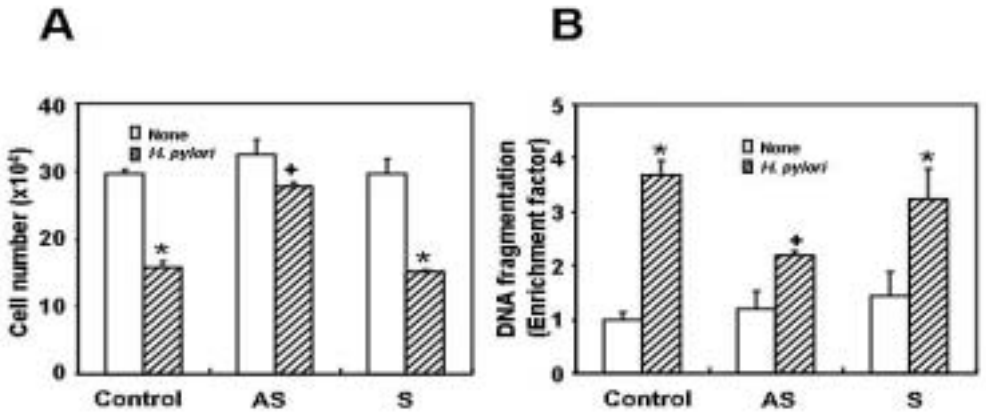

Fig. 5. *H. pylori*–induced cell death and apoptosis in AGS cells treated with ODNs. AGS cells (2×10^5 cells/well) treated with 0.5 μM of antisense (AS) ODN or sense (S) ODN were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 24 h. (A) Cell number was determined by trypan blue exclusion test. (B) DNA fragmentation was determined by the amount of oligonucleosome-bound DNA in the cell lysate. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. Enrichment factor of none control was considered as 1. Data represent means ± SE. *$p < 0.05$ compared to the cells received no treatment in the absence of *H. pylori* (none control). $^{+}p < 0.05$ compared to the cells received no treatment, but cultured in the presence of *H. pylori* (*H. pylori* control). None = the cells cultured in the absence of *H. pylori*.

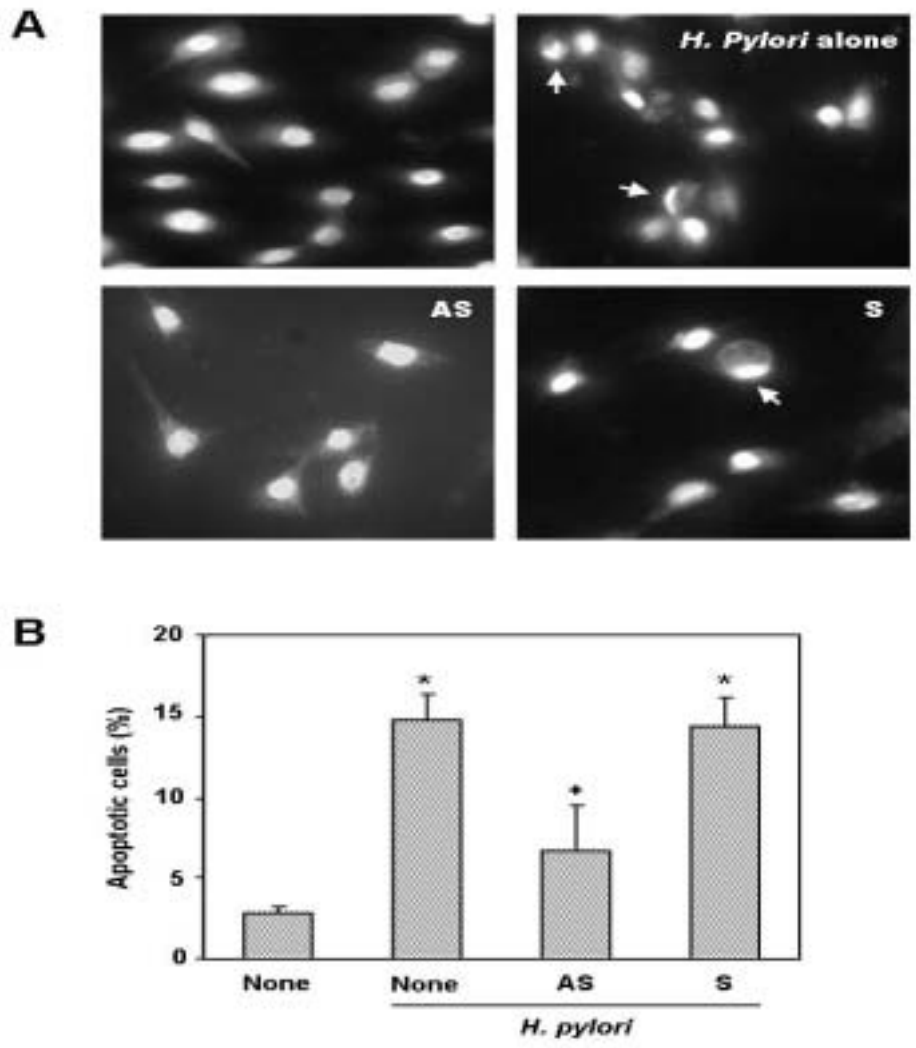

Fig. 6. Hoechst staining of AGS cells treated with ODNs. (A) AGS cells (4×10^5 cells/well) were treated either with AS ODN or S ODN, and cultured in the presence of *H. pylori* (at a bacterium/cell ratio, 300:1) for 24 h. Monolayers of cells onto coverslips were fixed with 4% paraformaldehyde and were stained with DNA-specific dye Hoechst 33258. Nuclear morphology was observed under a fluorescence microscope at 400×. Arrows indicate the condensed nuclei in the cells. (B) % of apoptotic cells were assessed based on total numbers of the cells. Data represent means ± SE. *$p < 0.05$ compared to the cells received no treatment in the absence of *H. pylori*. $^{+}p < 0.05$ compared to the cells received no treatment, but cultured in the presence of *H. pylori* .

6. *H. pylori*-induced alterations of expression of bcl-2, bax and p53 in AGS cells treated with or without ODNs

At the time point that each protein was significantly increased or decreased in the previous Western blotting, AGS cells were collected and used for Western blotting for bcl-2, bax and p53 to determine the relationship between NF-κB and those protein. Treatment of AS ODN significantly inhibited decrease of bcl-2 and increase of bax and p53 (Fig.7A). The inhibition on *H. pylori*-induced alteration for bcl-2, bax and p53 proteins in the cells treated with AS ODN was also confirmed by RT-PCR analysis for mRNA expressions of these genes in the cells treated with AS ODN (Fig.7B).

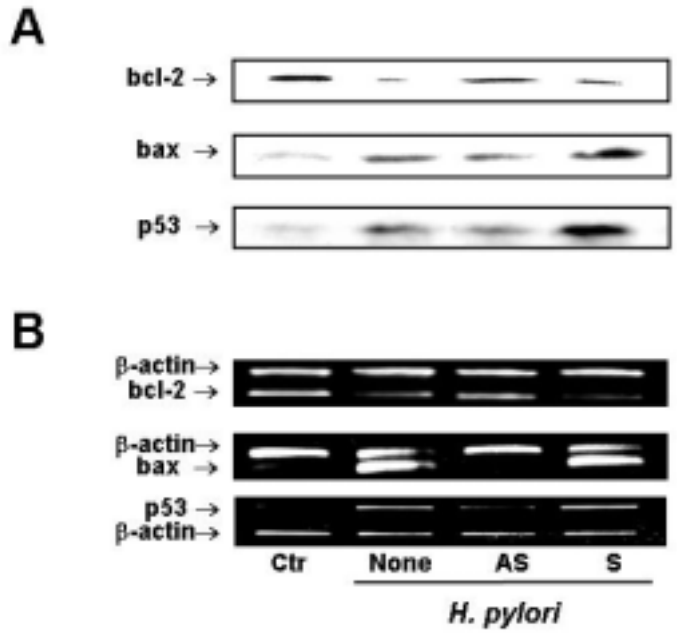

Fig. 7. Western blotting and reverse transcription polymerase chain reaction for bcl-2, bax, and p53 on AGS cells treated with ODNs. (A) For Western blotting, AGS cells (1 x 10^6 cells/well) were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for

24 h. Cells were collected at the time point that each protein was significantly increased or decreased in the previous Western blotting (for bcl-2 at 24 h, for bax at 8 h, and for p53 at 6 h). The protein level of bcl-2, bax, and p53 were determined by Western blotting using specific antibodies. (B) For RT-PCR analysis, cells were collected at the expected time point that each mRNA would significantly increase or decrease concerning on previous western blot analysis (for bcl-2 at 12 h, for bax at 6 h, and for p53 at 4 h). Total RNA was isolated from the cells by the guanidine thiocyanate extraction method. mRNA expression of each gene was assessed using RT-PCR standardized by coamplifying this gene with the housekeeping gene, β-actin.

7. *H. pylori*-induced IκBα degradation in AGS cells transfected with the mutated IκBα gene

To inhibit NF-κB activation, the cells were transfected with a control pcDNA3 vector (pcN-3) or a mutated IκBα gene (IW-6 and IW-10). AGS cells were stably transfected with the mutated IκBα gene, which was unsusceptible to phosphorylation at position 32 and 36, and found to resist degradation in transient transfection.[20] The stable transfected clones of the control vector pcDNA-3 (pcN-3) and those of the mutated IκBα gene (IW-6 and IW-10) were selected. To investigate whether the introduction of exogeneous mutated IκBα gene lead to an inhibition of degradation of IκBα, Western blotting with whole cell lysate from the wild type and transfected cells in the presence of *H. pylori* was performed. As shown in Figure 8, IκBα was rapidly degraded within 30 min with *H. pylori* stimulation in the wild type cells and the cells transfected with the control vector (pcN-3). However, IκBα of the cells transfected with the mutated IκBα (IW-6 and IW-10) remained resistant to *H. pylori* stimulation. These results show that mutated IκBα expression is directly related to inhibition on IκBα degradation.

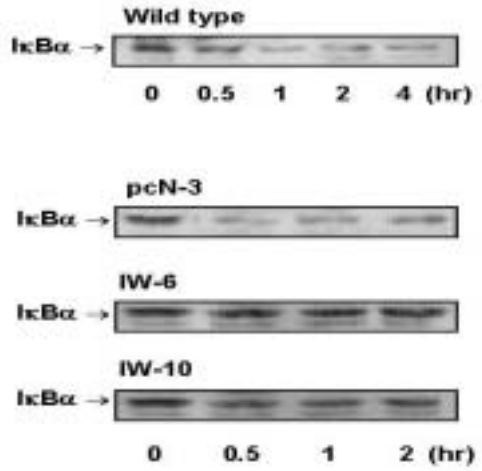

Fig. 8. Western blotting for IκBα on AGS cells transfected with the mutated IκBα gene. For IκBα analysis, cells transfected with a pcDNA3 vector (pcN-3) or the mutated IκBα gene (IW-6 and IW-10) were cultured in the presence of *H. pylori*. Cell lysates (10 μg of protein) were subjected to 10% SDS-PAGE and transferred onto membranes. Degradations of IκBα were visualized with a IκBα -specific antibody. Wild type cells received no transfection.

8. *H. pylori*-induced cell death and apoptosis in AGS cells transfected with the mutated IκBα gene

To evaluate the relationship between NF-κB inhibition and cell death, the cells transfected with the mutated IκBα gene were cultured for 24 h in the presence or in the absence of *H. pylori*, and the cell number was counted usingr trypan blue exclusion (Fig.9A). *H. pylori*-induced cell death was inhibited in the cells transfected with the mutated IκBα gene. These results showed that inhibition of NF-κB activation caused significant rescue of the cells compared with wild type and the cells transfected with the control vector. Apoptosis–associated increase in nucleosome-associated low-

molecular-weight DNA was inhibited in the cells transfected with the mutated IκBα gene, comparing with the cells transfected with the control vector (Fig.9B). Staining of DNA-specific dye Hoechst 33258 showed that apoptosis-associated condensation of nuclei was inhibited in the cells transfected with the mutated IκBα gene, comparing with the cells transfected with the control vector (Fig.10).

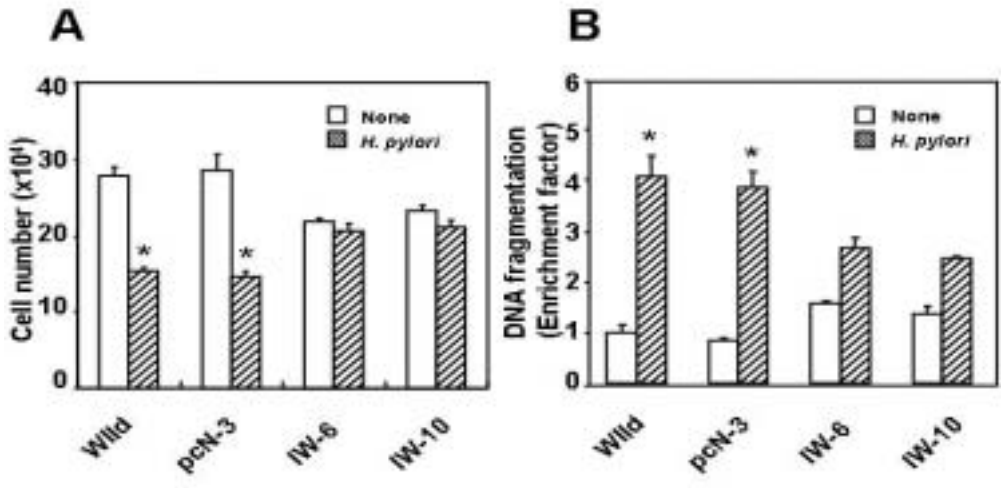

Fig. 9. Cell death and apoptosis in AGS cells transfected with the mutated IκBα. Cells (2×10^5 cells/well) were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 24 h. (A) Cell number was determined by trypan blue exclusion test. (B) Cell lysates were isolated from 1×10^5 cells. DNA fragmentation was determined by the amount of oligonucleosome-bound DNA in the cell lysate. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. Enrichment factor of none control was considered as 1. Data represent means ± SE. $*p < 0.05$ compared to the cells received no treatment in the absence of *H. pylori* (none wild).

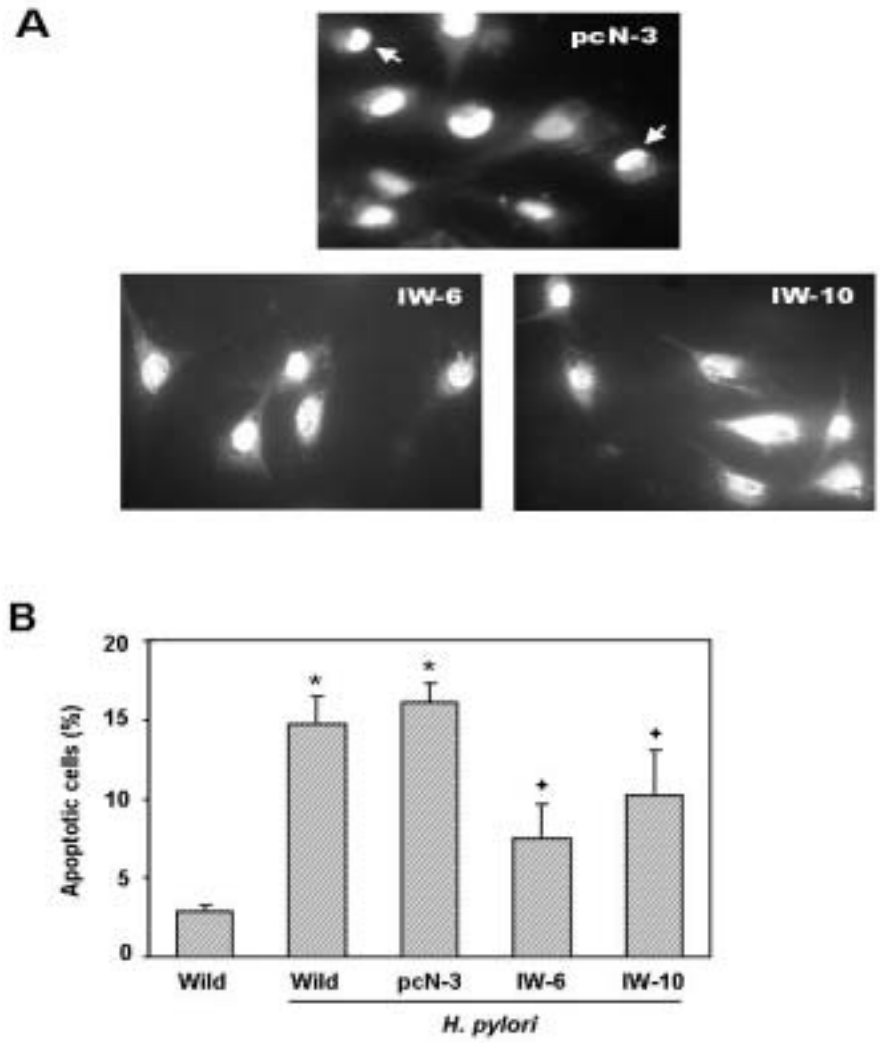

Fig. 10. Hoechst staining of AGS cells transfected with the mutated IκBα. (A) Cells (4×10^5 cells/well) were cultured in the presence of *H. pylori* (at a bacterium/cell ratio, 300:1) for 24 h. Monolayers of cells onto coverslips were fixed with 4% paraformaldehyde and were stained with DNA-specific dye Hoechst 33258. Nuclear morphology was observed under a fluorescence microscope at 400×. Arrows indicate the condensed nuclei in the cells. (B) % of apoptotic cells were assessed based on total numbers of the cells. Data represent means ± SE. *$p < 0.05$ compared to the cells received no treatment in the absence of *H. pylori*. $^{+}p < 0.05$ compared to the cells received no transfection, but cultured in the presence of *H. pylori*.

9. *H. pylori*-induced bax and bcl-2 expression in AGS cells transfected with the mutated IκBα gene

Wild type and the transfected cells were cultured in the presence or absence of *H. pylori* for 12 h and after that protein levels of bax and bcl-2 were analyzed by Western blotting. Induction of bax was lower in the cells transfected with the mutated IκBα gene (IW-6 and IW-10) than that in wild type or control cells (pcN-3). Western blotting for bcl-2 protein in the cells transfected with the mutated IκBα gene showed similar inhibition on reduction of bcl-2 with *H. pylori* stimulation. From these it is postulated that bax and bcl-2 are regulated by NF-κB in AGS cells. Thus, inhibition of NF-κB by suppressing the phosphorylation of IκBα (IW-6 and IW-10) induces low bax gene expression and conserving bcl-2 expression, which inhibits cell death by *H. pylori* in AGS cells.

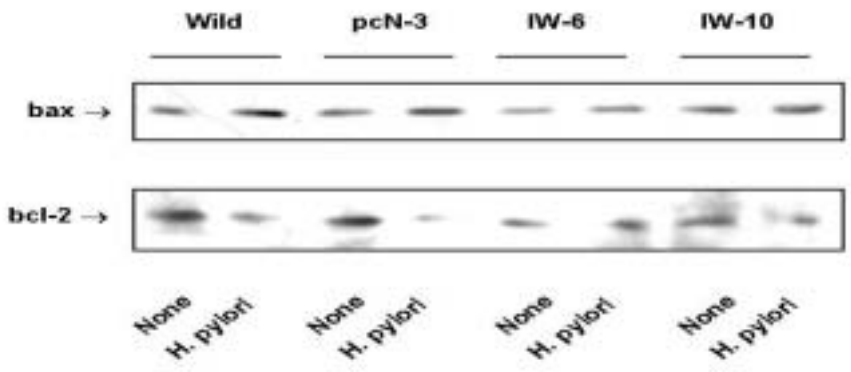

Fig. 11. Western blotting for bcl-2 and bax on AGS cells transfected with the mutated IκBα. Cells (1×10^6 cells/well) were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 12 h. Cells were collected and the protein level of bcl-2 and bax were

determined by Western blotting using specific antibodies.

10. *H. pylori*-induced cell death in AGS cells transfected with the human bcl-2 gene

To confirm whether bcl-2 can act as an antiapoptotic factor in *H. pylori*-induced gatric epithelial cell death, we transfected a human bcl-2 expression vector into AGS cells. Successful transfection of bcl-2 gene into AGS cells was confirmed by Western blotting. As shown in Fig.12, reduced expression of bcl-2 in the cells transfected with bcl-2 by *H. pylori* was more higher than constitutive expression of bcl-2 in non-treated wild type AGS cells. Expectedly, transfection of human bcl-2 gene resulted in significant inhibition of cell death induced by *H. pylori* (Fig.13). Apoptotic cells were also significantly decreased by transfection of bcl-2 gene as assessed by differential uptake of AO/EtBr (Fig.14), by DNA fragmentation (Fig.15), and by Hoechst 33258 staining (Fig.16). To investigate whether the activation of NF-κB can be inhibited by transfection of bcl-2 gene, electrophoretic mobility shift assay (EMSA) and Western blotting for IκBα were performed. As a result, there were no significant differences in the activation of NF-κB and degradation of IκBα between the cells transfected with the bcl-2 gene or the control vector (Fig.17).

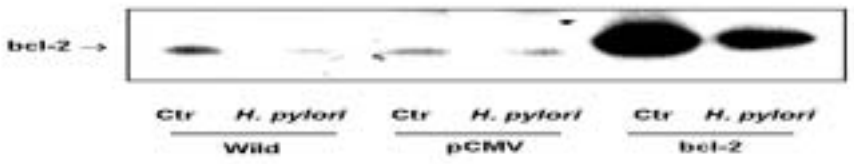

Fig. 12. Western blotting for bcl-2 expression on cells transfected with the human bcl-2

gene. Cells transfected with a control vector, CMVneo, or a full-length human bcl-2 expression vector, CMVbcl-2nl, were cultured in the absence of of *H. pylori* or in the presence of *H. pylori* for 24 h. Cell lysates (100 μg of protein) were subjected to 12% SDS-PAGE and transferred onto membranes. Expressions of bcl-2 were visualized with a bcl-2-specific antibody. Wild type cells received no transfection. pCMV cells were transfected with a control vector, CMVneo. bcl-2 cells were transfected with a full-length human bcl-2 expression vector, CMVbcl-2nl.

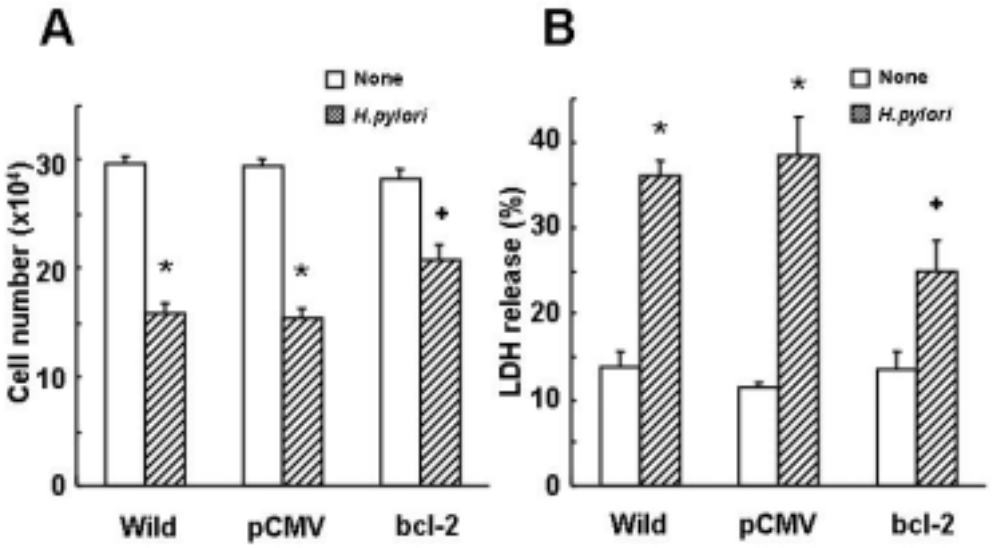

Fig. 13. *H. pylori*–induced cell death in AGS cells transfected with the human bcl-2 gene. Cells (2×10^5 cells/well) were cultured in the abesence of *H. pylori* or in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 24 h. Cell number was determined by trypan blue exclusion test. LDH release was determined photometrically with a commercial test and was expressed as released percent of the total LDH of the cells. Data represent means ± SE. $*p < 0.05$ compared to the cells received no treatment in the absence of *H. pylori*. $^{+}p < 0.05$ compared to the cells transfected with a control vector and cultured in the presence of *H. pylori* (pCMV *H. pylori*).

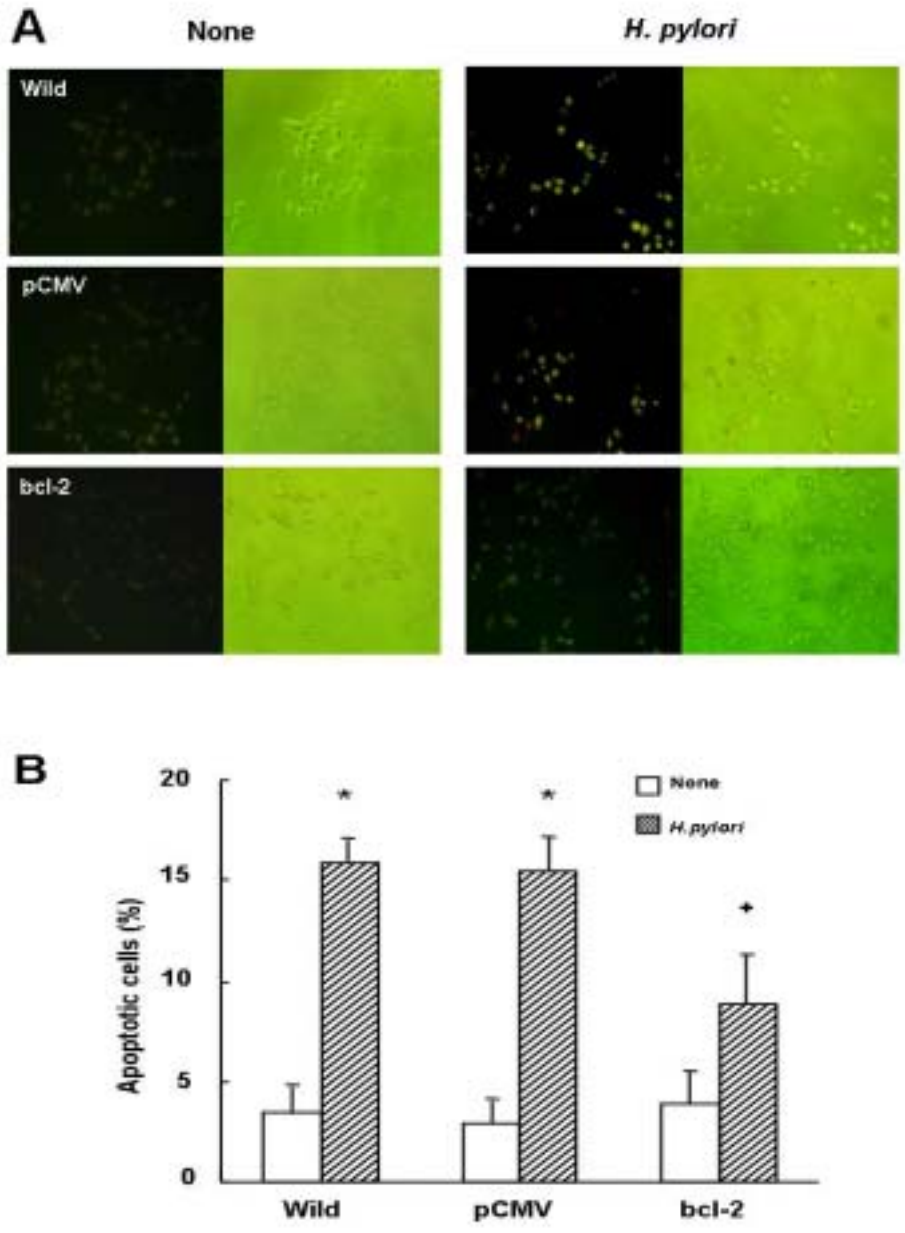

Fig. 14. Acridine orange/ethidium bromide staining of AGS cells transfected with the human bcl-2 gene. Cells (1×10^5 cells/well) were cultured in the abesence or the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 24 h. Monolayers of cells were stained with acridine orange and ethidium bromide. Morphology was observed under a fluorescence microscope or a phase contrast microscope at 200×. Wild type cells received no transfection.

pCMV cells were transfected with a control vector, CMVneo. bcl-2 cells were transfected with a full-length human bcl-2 expression vector, CMVbcl-2nl. *$p < 0.05$ compared to the cells in the absence of *H. pylori* (corresponding none). $^{+}p < 0.05$ compared to the cells transfected with a control vector and cultured in the presence of *H. pylori* (pCMV *H. pylori*).

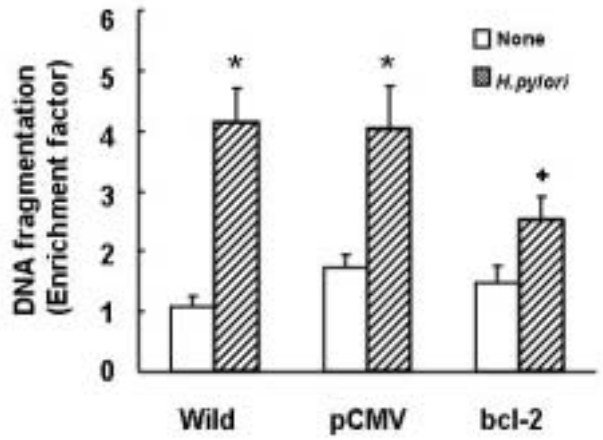

Fig. 15. *H. pylori*–induced apoptosis in AGS cells transfected with the human bcl-2 gene. Cells (2×10^5 cells/well) were cultured in the presence of *H. pylori* at a bacterium/cell ratio of 300:1 for 24 h. Cell lysates were isolated from 1×10^5 cells. DNA fragmentation was determined by the amount of oligonucleosome-bound DNA in the cell lysate. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. Enrichment factor of none control was considered as 1. Data represent means ± SE. *$p < 0.05$ compared to the cells in the absence of *H. pylori* (corresponding none). $^{+}p < 0.05$ compared to the cells transfected with a control vector and cultured in the presence of *H. pylori* (pCMV *H. pylori*).

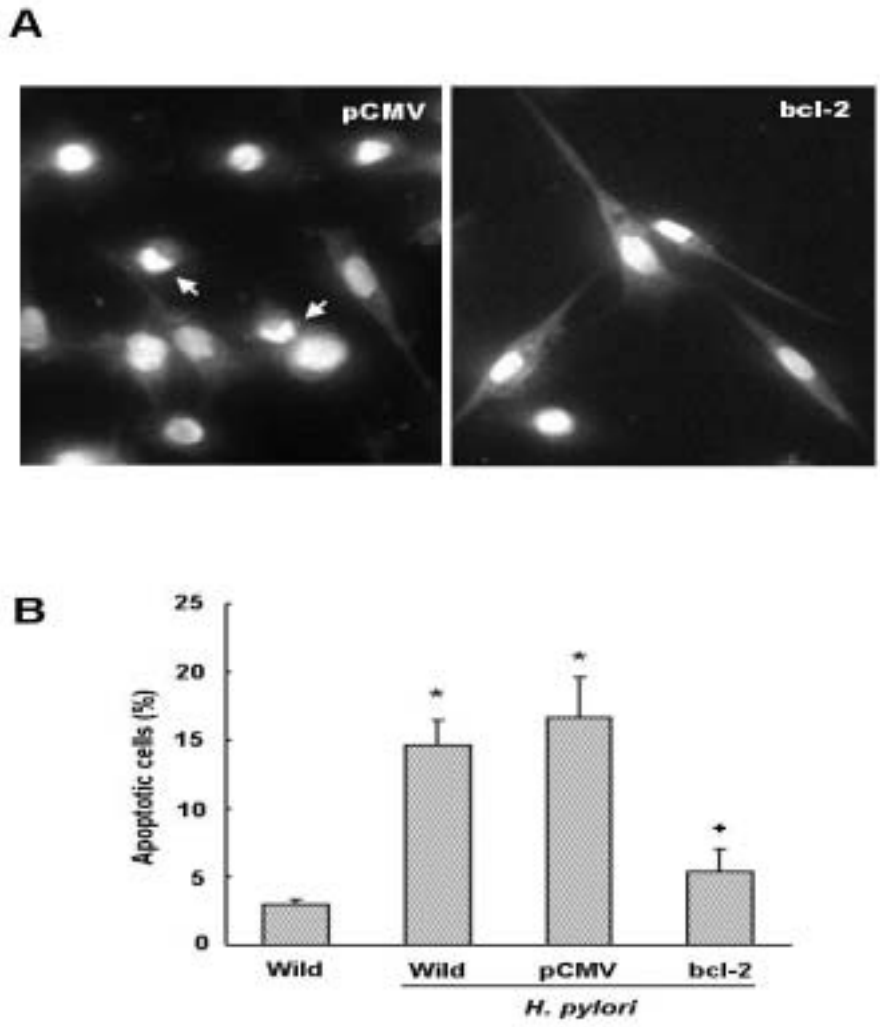

Fig. 16. Hoechst staining of AGS cells transfected with the human bcl-2 gene. (A) Cells (4×10^5 cells/well) were cultured in the presence of *H. pylori* (at a bacterium/cell ratio, 300:1) for 24 h. Monolayers of cells onto coverslips were fixed with 4% paraformaldehyde and were stained with DNA-specific dye Hoechst 33258. Nuclear morphology was observed under a fluorescence microscope at 400×. Arrows indicate the condensed nuclei in the cells. (B) % of apoptotic cells were assessed based on total numbers of the cells. Data represent means ± SE. *$p < 0.05$ compared to the cells received no treatment in the absence of *H. pylori*. $^{+}p < 0.05$ compared to the cells received no transfection, but cultured in the presence of *H. pylori* .

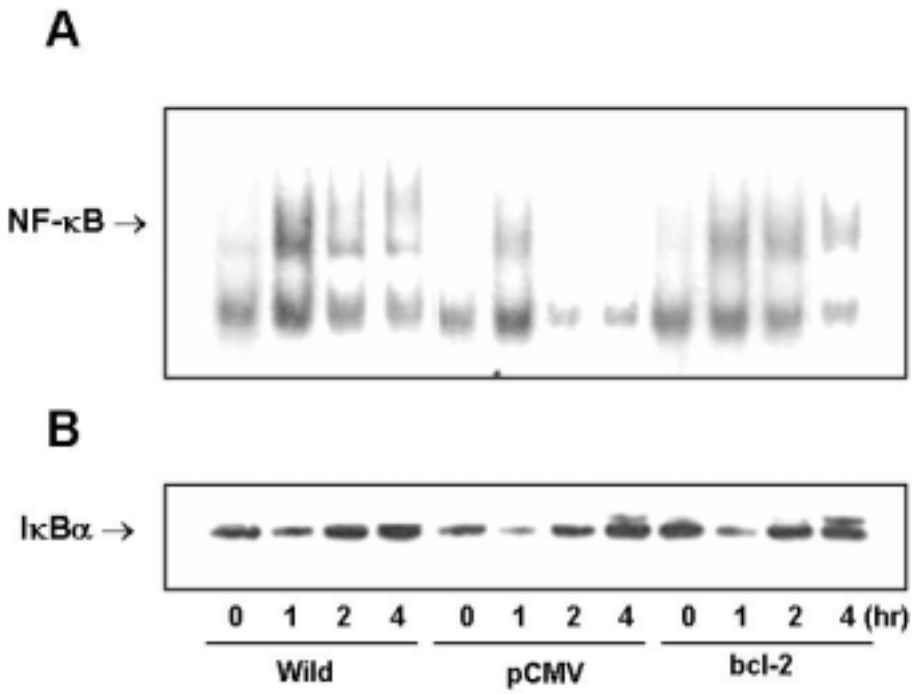

Fig. 17. *H. pylori*-induced NF-κB activation and IκBα degradation in the cells transfected with the human bcl-2 gene. (A) Electrophoretic mobility shift assay was performed using nuclear extracts prepared from the cells cultured in the presence of *H. pylori* for the indicated time point. Activated NF-κB bands are indicated by arrows. (B) For IκBα analysis, cells transfected with a pcDNA3 vector (pCMV) or the human bcl-2 gene (bcl-2) were cultured in the presence of *H. pylori*.

IV. DISCUSSION

The main finding of the present study is that NF-κB has an important role in *H. pylori*-induced apoptosis by regulating gene expression of bcl-2, bax and p53. Especially, up-regulation of bax and down-regulation of bcl-2 were attenuated by inactivation of NF-κB, using AS ODN or IκBα mutant vector, suggesting that alteration of bcl-2 and bax by *H. pylori* may depend on NF-κB activation in gastric epithelial cells. Thus, NF-κB activation is a critical step in *H. pylori*-induced apoptosis.

NF-κB belongs to a Rel family of transcription factors regulating the activation of a wide variety of genes that respond to immune or inflammatory signals.[12] *H. pylori* is known to increase LPO production, an indication for oxidative damage, in gastric epithelial AGS cells, which was accompanied by NF-κB activation.[13] *H. pylori* activates the transcription factor, NF-κB, and induces nuclear translocation of p50/p65 NF-κB heterodimers and p50 NF-κB homodimers in gastric epithelilal cells (AGS, KATO III).[14] Inhibition of NF-κB activation using antioxidants, catalase, PDTC and AS ODN to p50 of NF-κB suppresses *H. pylori*-induced apoptosis in AGS cells, suggesting main cause of gastric epithelial cell damage by *H. pylori* is oxidative stress and activation of NF-κB.[15]

The mechanism by which NF-κB activation induces apoptosis is not completely clear, but it is assumed that this mechanism involves the regulation of one or more genes known to play a role in apoptosis. Among the potential downstream regulators, the expressions of bax and bcl-2, pro- and anti-apoptotic members of the bcl-2 family that have been reported to be up- and down-regulated, respectively, in various models of

apoptosis were determined.[43-46] The present studies revealed a significant decrease in bcl-2, an anti-apoptotic factor, and an increase in bax, a pro-apoptotic factor, by *H. pylori* infection. These findings are in agreement with earlier findings that *H. pylori* induces apoptosis in the gastric epithelium due to an up-regulation of bax and down-regulation of bcl-2 in both protein and mRNA level.[8] Therefore, it was hypothesized that increase in NF-κB binding activity might be involved in *H. pylori*-induced cell death through bcl-2 pathway. To resolve this important question, AS ODN for p50 or mutated IκBα gene was used. Expectedly, transfection of either AS ODN or mutated IκBα gene into AGS gastric epithelial cells attenuated apoptosis. More importantly, a significant decrease in bcl-2 protein and an increase in bax protein were also attenuated by the transfection of either AS ODN or a mutated IκBα gene, but not by S ODN or a control vector. These findings suggested that *H. pylori*-induced activation of NF-κB caused gastric epithelial cell death through bcl-2-dependent apoptosis. These results are supported by the observation that overexpression of bcl-2 attenuates apoptosis in AGS cells. Although Matsushita et al also previously suggested that NF-κB activation suppressed bcl-2 in hypoxia-induced endothelial apoptosis, they failed to discover how NF-κB regulated bcl-2 expression. Recently, several studies have reported the effect of NF-κB on expression of bcl-2. For instance, p50 homodimers activate bcl-2 expression by directly binding to κB sequences in murine B cell lymphoma cell lines.[47] In another report, NF-κB activates bcl-2 expression in t(14;18) lymphoma cells through activating the bcl-2 promoter by interactions with the ATF/CREB and Sp1 factors and their binding sites.[48] The role of NF-κB in apoptosis is still controversial. NF-κB may exert either anti- or pro-apoptotic effects, depending on cell types and apoptotic stimuli. But

this type of regulation on bcl-2 expression by NF-κB only attributes the anti-apoptotic effect of NF-κB. Little is known about the pro-apoptotic effect of NF-κB when its activation triggers the decrease of bcl-2. However, Sohur et al showed that the bcl-2 gene is one downstream target of the transcriptional regulatory activity of NF-κB transcription complex during early B lineage apoptosis. In that model, withdrawal of survival signals in the bone marrow microenvironment activates NF-κB, which in turn represses the bcl-2 promoter. Thus, a subtle decrease in the level of bcl-2 through both transcriptional repression and active degradation of bcl-2 protein results in a rapid apoptosis.

Other investigators demonstrated that bcl-2 decreased NF-κB activity,[49-51] although there also have been studies showing that bcl-2 overexpression activates NF-κB by promoting degradation of inhibitory κB.[52,53] When bcl-2 gene was overexpressed in AGS cells, expression of IκBα in resting state and activation of NF-κB by *H. pylori* was not changed in the cells transfected with bcl-2, compared with those in wild type cells or cells transfected with a control vector in this experiment.

The prior appearance of increased NF-κB activity could be related to the p53 up-regulation in this experiment. One of the functions of p53 is to repress expression of bcl-2 gene, which exhibits an anti-apoptotic action through a *cis*-acting p53 negative-response element located in the 5'-untranslated region.[54] Aoki et al suggested that the down-regulation of bcl-2, translocation of bax, and up-regulation of p53 mediate oxidative stress-induced apoptosis in human aortic endothelial cells through NF-κB activation.[55] Although the regulation of NF-κB on p53 promoter activation is not fully understood, several reports have demonstrated the direct or indirect interaction of

NF-κB to p53.[22,38,56] Since AGS cells contains only wild type p53 with no mutations,[9] it can be supposed that oxidative stress caused by *H. pylori* infection induces p53 expression through the activation of NF-κB. This overexpressed p53 may down-regulate bcl-2.

On the other hand, a pro-apoptotic member of bcl-2 family, bax, has been reported as a target of NF-κB as well as a downstream mediator of p53.[57-59] The bax promoter contains an imperfect NF-κB consensus sequence.[60] Romeo et al proved a role for NF-κB in bax up-regulation by overexpressing the p65 subunit in bovine retinal pericytes,[61] indicating that bax should be added to the known proapoptotic effectors of NF-κB activation in selected contexts.[62] In this study, activation of NF-κB leads to up-regulation of bax as well as induction of p53. Thus, transcription factor NF-κB can be detrimental in *H. pylori*-infected gastric cell damage.

Present study demonstrates the role of NF-κB in *H. pylori*-induced apoptosis in gastric epithelial cells. Two important pro- and anti-apoptotic indices, bax and bcl-2, were regulated by NF-κB in response to *H. pylori* infection might be a critical to understanding the pathophysiology of *H. pylori*-related gastric injury. The controlling of NF-κB activation in the patients of gastritis or gastric ulcer will provide a better therapeutic approach towards curing *H. pylori*-related gastric apoptosis and disorders.

V. CONCLUSION

This study was conducted to evaluate the role of NF-κB in *H. pylori*–induced expression of bcl-2, bax and apoptosis in gastric epithelial AGS cells by inhibiting NF-κB expression using AS ODN for p50 or a mutant IκBα gene or by over-expressing bcl-2 gene. Cell death (viable cell number), apoptosis indices (quantification of DNA fragmentation, morphological assessment by Hoechst staining or AO/EtBr staining), and expression of bcl-2, bax and p53 (Western blotting, RT-PCR) were determined.

1. *H. pylori* induced apoptosis in human gastric epithelial AGS cells.
2. *H. pylori*-induced apoptosis in AGS cells was mediated by NF-κB.
3. *H. pylori* down-regulated an anti-apoptotic protein, bcl-2, and up-regulated pro-apoptotic proteins, bax and p53.
4. Alterations on expressions of bcl-2, bax and p53 caused by *H. pylori* were attenuated by inhibiting NF-κB activation.
5. Bcl-2 overexpression reduced *H. pylori*-induced cell death in AGS cells.

The main finding of this study is that NF-κB has an important role on the expression of bcl-2, bax and p53 in *H. pylori*-induced apoptosis. These suggest that alteration of bcl-2, bax and p53 by *H. pylori* may depend on NF-κB activation and is a critical step in the *H. pylori*-induced apoptosis.

REFERENCES

1. Grisham DY. Pathogenic mechanisms loading to *Helicobacter pylori*-induced inflammation. Eur J Gastroenterol Hepatol 1992;4:S9-16.

2. Bagchi D, Bhattacharya G, Stohs SJ. Production of reactive oxygen species by gastric cells in association with *Helicobacter pylori*. Free Radic Res 1996;24: 439-50.

3. Zhang Y, Broser M, Rom WN. Activation of the interleukin 6 gene by Mycobacterium tuberculosis or lipopolysaccharide is mediated by nuclear factors NF-IL6 and NF-kappa B. Proc Natl Acad Sci U S A 1994;91:2225-9.

4. Moss SF, Calam J, Agarwal B, Wang S, Holt PR. Induction of gastric epithelial apoptosis by *Helicobacter pylori*. Gut 1996;38:498-501.

5. Wagner S, Beil W, Westermann J, Logan RP, Bock CT, Trautwein C, Bleck JS, Manns MP. Regulation of gastric epithelial cell growth by *Helicobacter pylori*: offdence for a major role of apoptosis. Gastroenterology 1997;113:1836-47.

6. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. Science 1985;228:1440-3.

7. Rudin CM, Thompson CB. Apoptosis and disease: regulation and clinical relevance of programmed cell death. Annu Rev Med 1997;48:267-81.

8. Konturek PC, Pierzchalski P, Konturek SJ, Meixner H, Faller G, Kirchner T, Hahn EG. *Helicobacter pylori* induces apoptosis in gastric mucosa through an upregulation of Bax expression in humans. Scand J Gastroenterol 1999;34:375-83.

9. Chen G, Sordillo EM, Ramey WG, Reidy J, Holt PR, Krajewski S, Reed JC, Blaser

MJ, Moss SF. Apoptosis in gastric epithelial cells is induced by *Helicobacter pylori* and accompanied by increased expression of BAK. Biochem Biophys Res Commun. 1997; 239: 626-32.

10. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994; 12: 141-79.
11. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-κB. Annu Rev Cell Biol 1994;10:405-55.
12. Thanos D, Maniatis T. NF-kappa B: a lesion in family values. Cell 1995;80:529-32.
13. Kim H, Seo JY, Kim KH. Effects of mannitol and dimethylthiourea on *Helicobacter pylori*-induced IL-8 production in gastric epithelial cells. Pharmacology 1999;59:201-11.
14. Kim H, Seo JY, Kim KH. Inhibition of lipid peroxidation, NF-kappaB activation and IL-8 production by rebamipide in *Helicobacter pylori*-stimulated gastric epithelial cells. Dig Dis Sci 2000;45:621-8.
15. Lim JW, Kim H, Kim KH. NF-kappaB, inducible nitric oxide synthase and apoptosis by *Helicobacter pylori* infection. Free Radic Biol Med 2001;31:355-66.
16. Milligan JF, Matteucci MD, Martin JC. Current concepts in antisense drug design. J Med Chem 1993;36:1923-37.
17. Crooke ST. Therapeutic applications of oligonucleotides. Annu Rev Pharmacol Toxicol 1992;32:329-76.
18. Brown K, Gersberger S, Carlson L, Franzoso G, Siebenlist U. Control of IκBα proteolysis by site-specific, signal-induced phosphorylaiton. Science 1995; 267: 1485-8.

19. Haskill S, Beg AA, Tompkins SM, Morris JS, Yurochko AD, Sampson-Johannes A, Mondal K, Ralph P, Baldwin AS Jr. Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. Cell 1991;65:1281-9.

20. Le Bail O, Schmidt-Ullrich R, Israel A. Promoter analysis of the gene encoding the IκB/MAD-3 inhibitor of NF-κB: positive regulation by members of the Rel/NF-κB family. EMBO J 1993; 12: 5043-8.

21. Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA. Phosphorylation of human Icon serines 32 and 36 controls IκBα proteolysis and NF-κB activation in response to diverse stimuli. EMBO J 1995;14:2876-83.

22. Sun SC, Ganchi PA, Beraud C, Ballard DW, Greene WC. Autoregulation of the NF-κB transactivator Rel A(p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. Proc Natl Acad Sci USA 1994;91:1346-50.

23. Beg AA, Baldwin AS Jr. The I kappa B proteins: multifunctional regulators of Rel/ NF-kappa B transcription factors. Genes Dev 1993; 7: 1564-70.

24. Liou HC, and Baltimore D. Regulation of the NF-κB/rel transcripton factor and IκB inhibitor system. Curr Opin Cell Biol 1993;5:477-87.

25. Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science 1996;274: 784-7.

26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

27. Li JJ, Westergaard C, Ghosh P, Colburn NH. Inhibitiors of both nuclear factor-κB

and activator protein-1 activation block the neoplastic transformation response. Cancer Res 1997;57:3569-76.

28. McGahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, Nishioka WK, Green DR. The end of the cell line: methods for study of apoptosis *in vitro*. In: Schwartz LM, Osborne BA, Wilson L, Matsudaira P, editors. Methods in cell biology: Cell death. 2nd ed. London: Academic press; 1995. p.153-85.

29. Lappalainen K, Urtti A, Soderling E, Jaaskelainen I, Syrjanen K, Syrjanen S. Cationic liposomes improve stability and intracellular delivery of antisense oligonucleotides into CaSki cells. Biochem Biophys Acta 1994;1196:201-8.

30. Lim JW, Kim H, Kim KH. Nuclear factor-kappaB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells. Lab Invest 2001;81:349-360.

31. Hockenbery D, Nunez G, Milliman C, Schreber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990;348:334-6.

32. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidine isothiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-9.

33. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74:609-19.

34. Chang NS, Pratt N, Heath J, Schultz L, Sleve D, Carey GB, Zevotek N. Hyaluronidase induction of a WW domain-containing oxidoreductase that enhances tumor necrosis factor cytotoxicity. J Biol Chem 2001; 276: 3361-70.

35. Nakajima-Iijima S, Hamada H, Reddy P, Kakunaga T. Molecular structure of the

human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. Proc Natl Acad Sci USA 1985;82:6133-7.

36. Zar BH. Biostatistical Analysis. 4th ed. Englewood Cliffs(NJ): Prentice-Hall; 1998.

37. Royds JA, Dower SK, Qwarnstorm EE, Lewis CE. Response of tumour cells to hypoxia: role of p53 and NF-kB. J Clin Pathol 1998;51:55-61.

38. Wu H, Lozano G. NF-kB activation of p53: a potential mechanism for suppressing cell growth in response to stress. J Biol Chem 1994;269:20067-74.

39. Vogelstein B, Kinzler KW. P53 function and dysfunction. Cell 1992;70:523-6.

40. Lane DP. p53: guardian of the genome. Nature 1992;258:15-6.

41. Symonds H, Krall L, Remington L, Saenz-Robles M, Lowe S, Jacks T, Dyke TV. p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell 1994;78:703-11.

42. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzeler KW, Vogelstein B. WAF 1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-25.

43. Miller TM, Moulder KL, Knudson CM, Creedon DJ, Deshmukh M, Korsmeyer SJ, Johnson EM Jr. Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. J Cell Biol 1997;139:205-17.

44. Niwa M, Hara A, Iwai T, Sassa T, Mori H, Uematsu T. Expression of Bax and Bcl-2 protein in the gerbil hippocampus following transient forebrain ischemia and its modification by phencyclidine. Neurol Res. 1997;19:629-33.

45. Reed JC. Bcl-2 family proteins. Oncogene. 1998;17:3225-36.

46. Ravishankar S, Ashraf QM, Fritz K, Mishra OP, Delivoria-Papadopoulos M.

Expression of Bax and Bcl-2 proteins during hypoxia in cerebral cortical neuronal nuclei of newborn piglets: effect of administration of magnesium sulfate. Brain Res. 2001;901:23-9.

47. Kurland JF, Kodym R, Story MD, Spurgers KB, McDonnell TJ, Meyn RE. NF-kappaB1 (p50) homodimers contribute to transcription of the bcl-2 oncogene. J Biol Chem. 2001;276:45380-6.

48. Heckman CA, Mehew JW, Boxer LM. NF-kappaB activates Bcl-2 expression in t(14;18) lymphoma cells. Oncogene. 2002;21:3898-908.

49. Grimm S, Bauer MK, Baeuerle PA, Schulze-Osthoff K. Bcl-2 down-regulates the activity of transcription factor NF-kappaB induced upon apoptosis. J Cell Biol. 1996;134:13-23.

50. Badrichani AZ, Stroka DM, Bilbao G, Curiel DT, Bach FH, Ferran C. Bcl-2 and Bcl-XL serve an anti-inflammatory function in endothelial cells through inhibition of NF-kappaB. J Clin Invest. 1999;103:543-53.

51. Hour TC, Chen L, Lin JK. Suppression of transcription factor NF-kappaB activity by Bcl-2 protein in NIH3T3 cells: implication of a novel NF-kappaB p50-Bcl-2 complex for the anti-apoptotic function of Bcl-2. Eur J Cell Biol. 2000;79:121-9.

52. de Moissac D, Zheng H, Kirshenbaum LA. Linkage of the BH4 domain of Bcl-2 and the nuclear factor kappaB signaling pathway for suppression of apoptosis. J Biol Chem. 1999 ;274:29505-9.

53. Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T, Tohyama M. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. J Biol Chem. 1999;274:8531-8.

54. Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res. 1994;54:3131-5.

55. Aoki M, Nata T, Morishita R, Matsushita H, Nakagami H, Yamamoto K, Yamazaki K, Nakabayashi M, Ogihara T, Kaneda Y. Endothelial apoptosis induced by oxidative stress through activation of NF-kappaB: antiapoptotic effect of antioxidant agents on endothelial cells. Hypertension 2001; 38: 48-55.

56. Koong AC, Chen EY, Mivechi NF, Denko NC, Stambrook P, Giaccia AJ. Hypoxic activation of nuclear factor-kappa B is mediated by a Ras and Raf signaling pathway and does not involve MAP kinase (ERK1 or ERK2). Cancer Res. 1994;54:5273-9.

57. Shou Y, Li N, Li L, Borowitz JL, Isom GE. NF-kappaB-mediated up-regulation of Bcl-X(S) and Bax contributes to cytochrome c release in cyanide-induced apoptosis. J Neurochem. 2002;81:842-52.

58. Bentires-Alj M, Dejardin E, Viatour P, Van Lint C, Froesch B, Reed JC, Merville MP, Bours V. Inhibition of the NF-kappa B transcription factor increases Bax expression in cancer cell lines. Oncogene 2001;20:2805-13.

59. Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 1994;9:1799-1805.

60. Dixon EP, Stephenson DT, Clemens JA, Little SP. Bcl-X_{short} is elevated following severe global ischemia in rat brains. Brain Res 1997;776:222–9.

61. Romeo G, Liu WH, Asnaghi V, Kern TS, Lorenzi M. Activation of nuclear factor-kappaB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. Diabetes. 2002;5:2241-8.

62. Barkett M, Gilmore TD. Control of apoptosis by Rel/NF-κB transcription factors. Oncogene 1999;18:6910–24.

국문요약

Helicobacter pylori 감염 AGS 세포에서의 NF-κB 활성화와 세포고사

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*Helicobacter pylori (H. pylori)*는 비침습성, 그람 음성, 미호기성(microaerophilic) 세균으로, 위염, 소화성 궤양, 위 림프종, 위암의 중요한 원인균으로 여겨지고 있다. 그러나 *H. pylori*의 질병유발기전에서 위 상피 세포와 상호작용을 통한 위 상피 세포고사와 그에 따른 분자생물학적인 기전에 대해서는 아직 명확하게 밝혀져 있지 않다. 본 연구에서는 *H. pylori* 에 의한 위 상피 세포고사에서 중요한 역할을 하는 것으로 추정되는 NF-κB의 역할을 밝히고자 하였다. 실험 재료로는 인간 위상피 세포주인 AGS를 사용하였으며, NF-κB와 세포고사의 관계를 규명하기 위해 AGS 세포에 NF-κB 아형인 p50에 대한 안티센스(antisense)를 처리하거나, 돌연변이 IκBα 유전자를 도입하였다. AGS 세포를 항생제가 없는 배양액에서 *H. pylori* (NCTC11637, 세균:세포, 300:1)와 24시간 동안 배양한 후, 세포 수 측정, DNA 분절 정량화, Hoechst 염색법으로 세포고사를 측정하였다. 또 다른 지표로 p53, bax, bcl-2의 발현을 역전사중합연쇄법 (RT-PCR)과 웨스턴 블럿법 (Western blotting)으로 측정하였다. 그 결과 *H. pylori*는 p53과 bax의 증가,

bcl-2의 감소를 동반하여 세포고사를 유도함을 관찰할 수 있었다. *H. pylori*로 유도된 세포고사는 안티센스를 처리하거나, 또는 돌연변이 IκBα 유전자를 도입한 경우 억제된 반면, 센스를 처리하거나, 또는 대조 유전자를 도입한 경우 세포고사가 유도되었다. *H. pylori*에 의한 p53, bax, bcl-2의 변동도 안티센스를 처리하거나, 또는 돌연변이 Iκ B-α 유전자 도입으로 억제되었다. 더 나아가 bcl-2 유전자를 과발현 시킴으로 *H. pylori*로 유도된 세포고사가 감소됨을 확인할 수 있었다. 이상의 결과들을 종합하여 보면 *H. pylori*는 위 상피 세포에서 NF-κ B를 활성화시키고, p53, bax, bcl-2의 조절을 매개하여 세포고사를 유도한다고 생각한다.

핵심되는 말 : *H. pylori*, 세포고사, NF-κ B, bcl-2, bax