Analysis of apoptotic and antiapoptotic signalling pathways induced by *Helicobacter pylori*

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**Background and aims:** Although it is reported that *Helicobacter pylori* induces apoptosis on gastric epithelial cells, the mechanism remains unknown. Antiapoptotic effects generated by *H pylori* have not yet been evaluated.

**Methods:** (1) *H pylori* strains (type 1 wild, TN2-cagE, TN2-vacA) were cocultured with MKN45, TMK1, and Hela cells, and cell viability and apoptosis were assessed by trypan blue exclusion and DNA laddering, respectively. (2) Activation of caspases-3, 7, and 8, cytochrome c release from the mitochondria, and Fas, Fas associated death domain protein (FADD), Bax, Bak, and Bcl-X expression were evaluated by immunoblot analysis. (3) To investigate whether nuclear factor kappa B (NFκB) activation induced by cag pathogenicity island (PAI) positive *H pylori* affects antiapoptosis, MKN45 cells stably expressing super-repressor ΔικΒα were cocultured with *H pylori*, and cell viability and caspase activation were evaluated. NFκB regulated gene expression was also evaluated by ribonucleotide protection assay.

**Results:** (1) Wild-type and ΔvacA mutant *H pylori* induced apoptosis more potently than the ΔcagE mutant. Inhibition of cell contact between *H pylori* and cancer cells and heat killing *H pylori* diminished cell death. (2) Caspases-3, 7, and 8 were activated time dependently by *H pylori* as well as by the agonist anti-Fas. Cytochrome c release from mitochondria was observed and was not inhibited by caspase-8 inhibitor. Although protein expression of Fas, FADD, Bax, Bak, and Bcl-X in the whole cell lysates was not changed by *H pylori*, Bax was decreased from mitochondria free cytosol suggesting that Bax was translocated into mitochondria. (3) Cell death and the activities of caspases-3 and 8 were promoted in MKN45 cells stably expressing super-repressor ΔικΒα that inhibits NFκB activation. Antiapoptotic proteins c-IAP1 and c-IAP2 were upregulated by the wild-type strains.

**Conclusion:** cag PAI positive *H pylori* is capable of inducing apoptotic effects mainly through the mitochondrial pathway. Antiapoptotic effects mediated by NFκB activation were also observed.

*Helicobacter pylori* is a gram negative bacterium that infects the human stomach and plays an important role in the pathogenesis of chronic gastritis and peptic ulcer diseases. In addition, epidemiological studies have consistently identified an association between *H pylori* infection and the development of gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma. However, the mechanisms underlying the carcinogenic potential of *H pylori* are not completely understood.

Homeostasis of the gastrointestinal mucosa is maintained through a balance between the proliferation and apoptosis of mucosal cells. Apoptosis is also implicated in carcinogenesis, autoimmune diseases, and various infectious diseases. Although infection with *H pylori* is associated with significant epithelial cell damage, including an increased level of apoptosis, the mechanism underlying *H pylori* induced apoptosis in gastric epithelial cells remains unclear.

Two major pathways leading to apoptosis have been described. One pathway involves apoptosis mediated by death receptors, such as CD95 (Fas) and tumour necrosis factor receptors. When the Fas ligand binds to the Fas receptor, formation of the death inducing signal complex comprising the adapter molecule Fas associated death domain protein (FADD) and caspase-8 results in the active caspase-8 and process effector caspases (caspases-3, 6, and 7), thereby inducing apoptosis. In the other pathway, various proapoptotic signals converge at the mitochondria level, provoking translocation of cytochrome c from the mitochondria to the cytoplasm. Once cytochrome c is released into cytoplasm, it binds to Apaf-1 and induces recruitment of procaspase-9. Activated caspase-9 then cleaves and activates procaspase-3. Bcl-2 family members are associated with mitochondria related apoptosis. While cell survival-promoting molecules Bcl-2 and Bcl-X, localised at the outer mitochondrial membrane, prevent translocation of cytochrome c from the mitochondria, induced expression or enforced dimerisation of Bax results in mitochondrial dysfunction leading to cytochrome c release.

Several studies reported that the Fas/Fas ligand system was involved in *H pylori* induced apoptosis. In these reports, *H pylori* strains or supernatant upregulated Fas/Fas ligand expression and induced apoptosis indirectly. However, it is not known if these systems are major pathways of *H pylori* mediated apoptosis. Moreover, the other main apoptotic pathway, the mitochondrial pathway, was not investigated. In contrast, there are a few reports of an association between the Bcl-2 family, which is involved in the mitochondrial pathway, and *H pylori* induced apoptosis, where upregulation of Bak or Bax was associated with *H pylori* induced apoptosis in vitro or in vivo. However, these studies did not investigate most of the other proteins associated with the apoptotic pathway.

**Abbreviations:** PAI, pathogenicity island; NFκB, nuclear factor kappa B; FADD, Fas associated death domain protein; IFN, interferon; PBS, phosphate buffered saline; BSA, bovine serum albumin; VacA, vacuolating cytotoxin; FBS, fetal bovine serum; MyD88, myeloid differentiation factor 88.
Several factors have been proposed as possible virulence determinants of \textit{H. pylori}. In particular, \textit{cag} pathogenicity island (\textit{cag} PAI), a 40 kb region of possibly extraneous origin, is responsible for transcriptional factor nuclear factor kappa B (NFkB) activation.\textsuperscript{20–22} Isogenic mutant studies demonstrated that some proteins encoded by \textit{cag} PAI genes are responsible for NFkB activation.\textsuperscript{23} NFkB is a regulator of genes involved in inflammation, cell proliferation, and apoptosis.\textsuperscript{24,25} Recent studies suggest that NFkB may play a critical role in protecting cells against apoptosis.\textsuperscript{26–27} The antiapoptotic role played by NFkB involves the ability of this transcriptional factor to induce expression of genes that promote cell survival such as the genes coding for TRAF1, TRAF2, and the cellular inhibitors of apoptosis 1 and 2 (c-IAP1, c-IAP2).\textsuperscript{28} Curiously, NFkB has been found to be associated with proapoptotic as well as antiapoptotic mechanisms. For instance, NFkB activation appears to induce apoptosis in cells exposed to hydrogen peroxide.\textsuperscript{29} The magnitude of the stimulus and the cell type involved may determine whether NFkB leads to cell survival or cell death.

Although \textit{H. pylori} infection induces apoptosis in gastric epithelial cells, the mechanism of intracellular signal conduction that leads to apoptosis is scarcely known. In addition, it is not known whether \textit{H. pylori} mediated NFkB activation plays an apoptotic or antiapoptotic role. The aims of this study were to clarify the molecular mechanism of the proapoptotic pathway induced by \textit{H. pylori}, and to investigate the relationship between \textit{H. pylori} induced NFkB activation and apoptosis.

**MATERIALS AND METHODS**

**Bacterial strains**

TN2, a strain positive for \textit{cagA}, \textit{cag PAI}, and \textit{VacA} (vacuolating cytotoxin), were generously provided by Dr Nakao (Takeda Chemical Industries Ltd, Osaka, Japan). Infection with this strain induces gastric cancer in Mongolian gerbils.\textsuperscript{30} Isogenic \textit{cagE} negative and \textit{VacA} negative mutants, TN2-\textit{ΔcagE}, and TN2-\textit{ΔVacA} were prepared by insertion of a kanamycin resistant gene into the \textit{cagE} and \textit{vacA} locus of TN2, as previously described.\textsuperscript{31–33} \textit{H. pylori} strains were cultured on Columbia agar with 5% (vol/vol) horse blood and Dent antibiotic supplement (Oxoid, Basingstoke, UK) at 37°C for three days under microaerobic conditions (Campy-Pak Systems; BBL, Cockeysville, Maryland, USA). The isolates were kept at −80°C in Brucella broth with 5% (vol/vol) fetal bovine serum (FBS) containing 16% (vol/vol) glycerol. In coculture experiments, \textit{H. pylori} was cultured in Brucella broth containing 7.5% FBS for 24 hours, centrifuged, and resuspended in cell culture medium (RPMI 1640) containing 10% FBS, and then applied immediately to assays. The range of bacteria/cancer cell ratio was 50:1 to 75:1.

**Immunoblot analysis**

Cells were seeded in 50 mM Tris HCl (pH 7.4) buffer containing 1 mM EGTA, 2 mM DTT, 25 mM sodium β-glycophorhate, 0.1 mM PMSF, and 10 µg/ml aprotinin. An equal amount of protein extracts was fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoretically transferred to a PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was probed with the antibodies described above. An ECL detection assay (Amersham Pharmacia Biotech) was performed according to the manufacturer’s instructions.

**Immunoprecipitation**

MNK45 cells (5×10⁶) were incubated with \textit{H. pylori} or the agonist anti-Fas (CH-11) (approximately 1×10⁶ cells) were incubated in PBS and lysed in 40 µl of lysis buffer containing 200 mM NaHPO₄, and 4 mM citric acid. Samples were centrifuged and the supernatants were incubated with 3 µl of 0.25% NP-40 and 3 µl of Dnase-free RNase (10 mg/ml), and followed with a 10 mg/ml proteinase K digestion. Aliquots (10 µl) from a 50 µl DNA solution were electrophoresed on a 2% agarose gel.

**Cell viability assay**

Cells were collected and washed in phosphate buffered saline (PBS). Cell viability was assayed by trypan blue dye exclusion assay by counting 300 cells. Viable cells were quantitated under bright field microscopy.

**Analysis of DNA fragmentation**

Cancer cells cocultured with \textit{H. pylori} or treated with anti-Fas (CH-11) (approximately 1×10⁶ cells) were washed in PBS and lysed in 40 µl lysis buffer containing 200 mM NaHPO₄ and 4 mM citric acid. Samples were centrifuged and the supernatants were incubated with 3 µl of 0.25% NP-40 and 3 µl of Dnase-free RNase (10 mg/ml), and followed with a 10 mg/ml proteinase K digestion. Aliquots (10 µl) from a 50 µl DNA solution were electrophoresed on a 2% agarose gel.

**Generation of stable transfectants**

MNK45 (5×10⁶) was seeded onto 10 cm plates and transfected 24 hours later with 3 µg of FLAG-IκBα (SS32/36AA) subcloned in pcDNA3 or control vector pCDNA3 using Effectene transfection reagent (Quiagen, Hilden, Germany). Cells stably expressing FLAG-IκBα (SS32/36AA) were selected in medium containing 1 mg/ml G418 (Gibco BRL, Life Technologies, Inc., Rockville, Maryland, USA) for two weeks. MNK45 cells transfected with the pCDNA3 vector and selected by G418 were used as controls.

**RNA isolation and nuclease protection assay**

Total RNA was extracted using an acid guanidinium thiocyanate-phenol-chloroform method according to the manufacturer’s instructions (Isogen; Nippongene, Tokyo, Japan). The super-repressor mutant of IκBα, IκBα (SS32/36AA) subcloned in pcDNA3, was generously donated by Dr Suzuki (Yamanouchi Pharmaceutical Co, Ltd, Ibaraki, Japan). The super-repressor mutant of IκBα, IκBα (SS32/36AA) subcloned in pcDNA3, was generously donated by Dr Suzuki (Yamanouchi Pharmaceutical Co, Ltd, Ibaraki, Japan).
Absorbance was measured at 405 nm.

12 hours and lysed, and supernatants were used for ELISA.

was considered significant.

were examined by ANOVA with ad hoc test. A p value <0.05 was performed using a Fujix bioimaging analyser FLA 3000.

borate-EDTA at 4°C. The gel was dried and autoradiography with mide gel. Gel electrophoresis was executed in 0.5 mplexes were loaded onto a chilled 4% non-denaturing acrylamide gel. Gel electrophoresis was executed in 0.5 µg of poly(dI-dC). Supershift analysis was performed µ g after 24 and 36 hours of coculture (fig 2A). The DNA fragmentation was significantly different between the control group at 36 hours and the H pylori coculture or anti-Fas treatment without interferon γ (IFN-γ) group. * Percentage viability was significantly (p<0.05) different between the H pylori coculture or anti-Fas without IFN-γ group and the IFN-γ group.

DNA fragmentation ELISA

DNA fragmentation was quantified using a commercially available ELISA (Boehringer Mannheim Biochemicals, Mannheim, Germany) that detects nucleosomal fragments in the cytoplasmic fractions of cells undergoing apoptosis but not necrosis. For these experiments, 5×10⁶ cells were incubated in triplicate with H pylori, anti-Fas (CH-11), or medium alone for 12 hours and lysed, and supernatants were used for ELISA. Absorbance was measured at 405 nm.

Electrophoretic mobility shift assay

Detection of NFkB was performed with a 32P-dATP labelled oligo probe containing the NFkB recognition site purchased from Promega (Madison, Wisconsin, USA). The DNA binding reactions were performed at room temperature for 30 minutes in a 10 µl mixture consisting of 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 0.5 µg of poly(dI-dC). Supershift analysis was performed using antibodies against p65 and p50. DNA-protein complexes were loaded onto a chilled 4% non-denaturing acrylamide gel. Gel electrophoresis was executed in 0.5xTris borate-EDTA at 4°C. The gel was dried and autoradiography was performed using a Fujix bioimaging analyser FLA 3000 (Fuji Photo Film).

Statistics

Data are presented as means (SEM). Differences in means were examined by ANOVA with ad hoc test. A p value <0.05 was considered significant.

RESULTS

H pylori induced apoptosis in cancer cells

Apoptosis induction was evaluated in vitro in three cancer cell lines, MKN45, TMK1, and HeLa. Cells were treated with or without IFN-γ (10 ng/ml) for 24 hours and then incubated with H pylori (TN2) (bacteria:cancer cell ratio 50:1–75:1) or the agonist anti-Fas (CH-11). DNA fragmentation was induced in all cell lines 16 hours after coculture with H pylori as well as after anti-Fas treatment with IFN-γ (data not shown). Weak DNA fragmentation was observed both after coculture with H pylori and after treatment with anti-Fas without IFN-γ (data not shown). We tested other bacterial ratios, 5:1 and 500:1. At 5:1, neither apparent cell death nor apoptosis was observed. At 500:1, cells lost viability immediately after addition of bacteria, with or without IFN-γ priming, making it impossible to analyse apoptosis. A bacteria:cancer cell ratio of 50:1–75 was used in the following studies. Exposure of MKN45, TMK-1, and HeLa (with or without IFN-γ) cells to H pylori or anti-Fas caused a time dependent decrease in the number of viable cells, as determined by the trypan blue dye exclusion assay (fig 1). These results indicate that H pylori directly induces cell apoptosis. Pretreatment with IFN-γ strengthened the apoptotic effects by H pylori and the agonist anti-Fas in these cell lines. Pretreatment with IFN-γ was performed in the following studies.

Effects of H pylori virulence factors

To evaluate the effects of H pylori virulence factors, the cagE mutant TN2 ΔcagE and the vacA mutant TN2 ΔvacA were used. In the cell viability assays, wild-type and TN2 ΔvacA significantly decreased the viability of MKN45 and TMK-1 cells after 24 and 36 hours of coculture (fig 2A). The DNA fragmentation assay revealed that wild-type and TN2 ΔvacA induced apoptosis after 16 hours of infection whereas TN2 ΔcagE did not induce apoptosis at this time (fig 2B). However, cagE also induced apoptosis after 36 hours of infection (data not shown). These results indicate that the cagE mutant induces apoptosis less effectively than the wild-type or vacA mutant.

Effects of heat killed H pylori and inhibition of direct contact with host cells

To assess the effect of direct contact, cancer cells and bacteria were separated by a membrane filter (Nunc Tissue Culture Inserts No 162138; Nunc, Roskilde, Denmark). We also used heat killed bacteria heated at 80°C for 30 minutes. These procedures suppressed the H pylori mediated decrease in cell viability (fig 3). No DNA fragmentation was observed with heat killed bacteria or with viable bacteria separated by a permeable membrane, indicating that direct contact with viable bacteria is necessary for induction of apoptosis by H pylori.
Apoptotic and antiapoptotic signalling pathways induced by *H. pylori*

**Figure 1** Attenuation of the apoptotic effect by *cagE* negative mutant. (A) Cells were treated with interferon-γ (IFN-γ 10 ng/ml) for 24 hours and incubated with *H. pylori* (TN2), TN2-ΔcagE, and TN2-ΔvacA. Cell viability was assessed by trypan blue dye exclusion assay at the indicated times by counting 300 cells. Viable cells were quantitated under bright field microscopy. Results are expressed as percentages of viable cells. Values are mean (SD) of three independent experiments. *Percentage viability was significantly (p<0.05) different between the independent experiments.* *Percentage viability was significantly (p<0.05) different between the independent experiments.* Percentage viability was significantly (p<0.05) different between the independent experiments. *Percentage viability was significantly (p<0.05) different between the independent experiments.*

**Figure 2** Apoptotic and antiapoptotic signalling pathways induced by *H. pylori* coculture group and the *cagE* coculture group. (B) DNA fragmentation was evaluated after 16 hours of infection.

**Figure 4** *Helicobacter pylori* induces apoptosis via a caspase dependent pathway. Immunoblot analysis was performed using anti-caspases-8, 3, 7, and cleaved caspase-3 antibodies in MKN45 cells. Incubation with *H. pylori* and anti-Fas resulted in a time dependent degradation of the primary forms of caspases-8, 3, and 7. Processing into two fragments (43 kDa and 41 kDa) of the caspase-8 intermediate form and the 20 kDa active caspase-3 was also observed.

**Figure 3** Viable bacteria and direct contact with cells were necessary for inducing apoptosis. MKN45 cells were treated with interferon-γ (IFN-γ 10 ng/ml) for 24 hours. Cells and bacteria were separated by membrane filter (Nunc Tissue Culture Inserts No. 162138; Nunc, Roskilde, Denmark). We also used heat killed bacteria at 80°C for 30 minutes. Cell viability was assessed by trypan blue dye exclusion assay at 24 hours by counting 300 cells. Viable cells were quantitated under bright field microscopy. Results are expressed as percentages of viable cells. Values are mean (SD) of three independent experiments. *Percentage viability was significantly (p<0.05) different between the *H. pylori* (HP) wild-type and vacA coculture group and the *cagE* coculture group. (B) DNA fragmentation was evaluated after 16 hours of infection.

**Figure 2** Attenuation of the apoptotic effect by *cagE* negative mutant. (A) Cells were treated with interferon-γ (IFN-γ 10 ng/ml) for 24 hours and incubated with *H. pylori* (TN2), TN2-ΔcagE, and TN2-ΔvacA. Cell viability was assessed by trypan blue dye exclusion assay at the indicated times by counting 300 cells. Viable cells were quantitated under bright field microscopy. Results are expressed as percentages of viable cells. Values are mean (SD) of three independent experiments. *Percentage viability was significantly (p<0.05) different between the *H. pylori* (HP) wild-type and vacA coculture group and the *cagE* coculture group. (B) DNA fragmentation was evaluated after 16 hours of infection.

**Activation of caspases-8, 3, and 7**

To assess whether *H. pylori* induces apoptosis via caspase activation, immunoblot analysis was performed using anti-caspases-8, 3, 7, and cleaved caspase-3 antibodies in MKN45 cells. In untreated MKN45 cells, caspase-8 was present primarily as a molecule of approximately 55 kDa. Incubation with *H. pylori* or the agonist anti-Fas resulted in a time dependent degradation of the primary form and processing into two fragment of 43 and 41 kDa intermediate forms. Caspase-8 processing increased eight hours after *H. pylori* treatment. In untreated MKN45 cells, caspase-3 was present primarily as a molecule of approximately 32 kDa. Incubation with *H. pylori* or anti-Fas resulted in a time dependent degradation of the primary form that was processed to a 20 kDa active form. The active form of caspase-3 was observed 12 hours after *H. pylori* treatment. In untreated MKN45 cells, caspase-7 was present primarily as a molecule of approximately 35 kDa. Incubation with *H. pylori* and anti-Fas resulted in a time dependent degradation of the primary form. An increase in the processing of caspase-7 was observed 12 hours after *H. pylori* treatment. These results indicate that *H. pylori* and anti-Fas activated caspases-8, 3, and 7 in MKN45 cells (fig 4). Similar results were obtained using TMK-1 cells (data not shown).

**Caspase-8 and Fas do not play a major role in *H. pylori* induced apoptosis**

We evaluated the effect of the caspase-8 inhibitor Ac-IETD-CHO on the *H. pylori* mediated apoptosis in MKN45 cells. While the inhibitor (100 µM) abolished anti-Fas induced cell death (from 50% to 15%), *H. pylori* mediated cell death was not affected (fig 5A), and DNA fragmentation induced by *H. pylori* was not inhibited (fig 5B). These results indicate that caspase-8 activation by *H. pylori* does not function as a major pathway in *H. pylori* induced apoptosis. Similar results were obtained using TMK-1 cells (data not shown).

We evaluated whether FADD was recruited to Fas and found that under apoptosis induced conditions, anti-Fas but not *H. pylori* recruited FADD to Fas (fig 6A). In evaluating the effect of neutralising anti-Fas antibody (ZB-4), we found that ZB-4 decreased cell cytotoxicity induced by the agonist anti-Fas antibody (CH-11) (from 42% to 21%) but not *H. pylori* mediated cell cytotoxicity (from 35% to 38%) (fig 6B). These results suggest that Fas does not play a major role in *H. pylori* induced apoptosis.

**H. pylori** induces cytochrome c release from the mitochondria

To examine whether or not *H. pylori* induced apoptotic signalling involves a mitochondrial pathway, MKN45 cells were treated with *H. pylori* and mitochondria free cytosolic extracts were prepared and analysed by immunoblotting. Cytochrome c accumulated in cytosolic extracts after 12 hours of coculturing with *H. pylori*. In examining the effects of the agonist anti-Fas antibody, as previously reported in the other cell lines, cytochrome c also accumulated after exposure to anti-Fas in MKN45 cells (fig 7A). Similar results were obtained using...
Mitochondria is not caspase-8 dependent (fig 7B).

Indicates that the caspase-8 inhibitor abolished anti-Fas mediated cytochrome c release and caspase activity caused by H. pylori (fig 6).

Similar to the caspase-8 inhibitor, the pan-caspase inhibitor Z-VAD-FMK, which is a broad-spectrum caspase inhibitor, also abolished anti-Fas mediated cytochrome c release from the mitochondria (fig 5A).

Exposure to H. pylori induced apoptosis. (A) MKN45 cells were treated with interferon γ (IFN-γ 10 ng/ml) for 24 hours. Cells were then incubated with H. pylori (HP) or anti-Fas (CH-11) for 24 hours. Cell viability was assessed by trypan blue dye exclusion assay by counting 300 cells. Viable cells were quantitated under bright field microscopy. Results are expressed as percentages of dead cells. Values are mean (SD) of three independent experiments. *Percentage cytotoxicity was significantly (p < 0.05) different between the anti-Fas treatment group and the anti-Fas treatment with caspase-8 inhibitor treatment group. NS, no significant difference was found. (B) DNA fragmentation was also evaluated after 24 hours of infection.

Figure 6 Fas (CD95) was not associated with Helicobacter pylori induced apoptosis. (A) MKN45 cells were treated with interferon γ (IFN-γ 10 ng/ml) for 24 hours. Cells were then incubated with H. pylori (HP) or anti-Fas (CH-11) for 24 hours. Total cell lysates were extracted and immunoprecipitated with anti-Fas antibody and immunoblotted with anti-Fas associated death domain protein (FADD) and anti-Fas antibody. (B) MKN45 cells were treated with IFN-γ (10 ng/ml) for 24 hours, with or without neutralising anti-Fas antibody (ZB-4) for one hour, and incubated with H. pylori or anti-Fas (CH-11). Cell viability was assessed by trypan blue dye exclusion assay at the indicated times by counting 300 cells. Viable cells were quantitated under bright field microscopy. Results are expressed as percentages of viable cells. Values are mean (SD) of three independent experiments. *Percentage cytotoxicity was significantly (p < 0.05) different between the anti-Fas treatment group and the anti-Fas treatment with ZB-4 treatment group. NS, no significant difference was found.

TMK-1 cells. To examine whether or not cytochrome c accumulation is caspase-8 dependent, cells were pretreated with caspase-8 inhibitor (Ac-IETD-CHO) for one hour before exposure to H. pylori or anti-Fas. Although anti-Fas mediated cytochrome c release from the mitochondria was inhibited by the caspase-8 inhibitor, H. pylori mediated release was not inhibited. We also used the pan-caspase inhibitor Z-VAD-FMK. Similar to the caspase-8 inhibitor, the pan-caspase inhibitor abolished anti-Fas mediated cytochrome c release from the mitochondria but not H. pylori mediated release. This indicates that H. pylori induced cytochrome c release from the mitochondria is not caspase-8 dependent (fig 7B).

Expression of apoptosis related proteins

We determined whether H. pylori mediated apoptosis correlated with expression of apoptosis related proteins. Coculture with H. pylori cells did not change levels of Fas, FADD, Bax, Bak, or Bcl-X in total cell lysates extracted from MKN45 at the indicated times (fig 8A). Similar results were obtained using TMK-1 cells. We also evaluated mRNA expression of Bax, Bak, and Bcl-X using a ribonuclease protection assay and found no apparent changes. It is known that Bax may be translocated from the cytosol to the mitochondrial membrane without changing total cell amount. Thus we examined the immunoblot analysis of Bax using only the cytosolic fraction and found that cytosolic levels of Bax were decreased in H. pylori mediated apoptosis as well as in Fas mediated apoptosis, indicating that Bax is translocated into mitochondria in H. pylori mediated apoptosis (fig 8C). We also evaluated the effect of the caspase-8 inhibitor. The inhibitor abolished the decrease in cytosolic Bax induced by anti-Fas but did not affect the decrease induced by H. pylori (fig 8C).

H. pylori mediated NFκB activation suppresses apoptotic effects

We previously described cag PAI positive H. pylori activation of NFκB in gastric cancer cells. There was a significant decrease in induction of NFκB transiently transfected with mutant IκBα (S32A/S36A) (super-repressor), indicating that phosphorylation of IκBα at Ser32 and Ser36 is critical for H. pylori mediated activation of NFκB. To determine whether NFκB activation correlated with H. pylori induced apoptosis, we generated stable transformants of MKN45 cells expressing mutant IκBα (MKN45 IκBα SR) (fig 9A). To examine the effect of stable expression of IκBα super-repressor, we performed a electrophoretic mobility shift assay. H. pylori and tumour necrosis factor α activated NFκB in MKN45-IκBα SR cells significantly less than in wild-type MKN45 cells. This result indicated that stable expression of IκBα super-repressor construct is functioning as an inhibitor (fig 9B). Cell cytotoxicity caused by H. pylori or the agonist anti-Fas was evaluated in the IκBα (S32A/S36A) transformants and compared with...
Apoptosis was increased in MKN45 cells compared with MKN45 cells, indicating that specific NFκB activated more rapidly (fig 9E). These results indicate that the mitochondria is a major pathway in effects. To evaluate expression of antiapoptosis related genes, we used fragmentation ELISA at 12 hours after infection and in vitro. The importance of this pathway in vivo remains unknown.

Various proapoptotic signals, such as those induced by radiation, anticancer drugs, and stress, converge at cytochrome c release from the mitochondria. Although the current data suggest that cytochrome c release also plays a pivotal role in H pylori mediated apoptosis, the exact mechanism has yet to be investigated. The status of Bcl-2 family proteins determines whether a cell will live or die through regulation of cytochrome c release from the mitochondria. Increased Bak expression, a proapoptotic member of the Bcl-2 family, in H pylori infection was recently reported. However, we did not find changes in expression of Bak, Bax, or Bcl-X in either mRNA or protein levels when cocultured with H pylori. These findings do not exclude the possibility that some Bcl-2 family proteins are involved in H pylori mediated apoptosis. For example, Bax may translocate from the cytosol to the mitochondria for integration into the membrane following a proapoptotic stimulus. This action then results in cytochrome c release while the total amount of protein remains constant. Thus we checked by immunoblot analysis the level of Bax in the cytosolic fraction and found that cytosolic Bax was decreased in H pylori mediated apoptosis as well as in Fas mediated apoptosis, suggesting that Bax was translocated into mitochondria in H pylori mediated apoptosis.

p53 is an important molecule that affects apoptosis. Gene expression caused by p53 may be an important part of p53 mediated apoptosis. Reportedly, H pylori infection in patients resulted in nuclear staining for p53 in the glandular cells of the mucosa, and bacterial eradication caused a decrease in p53 accumulation in epithelial cells. However, we did not find upregulation of Bax or MDM2, which are regulated by p53 in cells with normal p53, including MKN45 cells, suggesting that H pylori cannot activate p53 directly in vitro (unpublished observation).

We have previously shown that H pylori induces NFκB activation. In this study, we confirmed that H pylori mediated NFκB activation exerts antiapoptotic effects in MKN45 cells where upregulation of c-IAP1 and 2 may be involved. Thus H pylori has both apoptotic and antiapoptotic effects. Similarly, the cytokine tumour necrosis factor α can produce bidirectional effects on apoptosis. The signal triggered by tumour necrosis factor α binding to its receptor bifurcates at TRADD: one signal induces NFκB activation via RIP promoting cell survival and the other induces apoptosis via FADD. The adapter molecule myeloid differentiation factor 88 (MyD88) mediates both apoptosis and NFκB activation through Toll-like receptors. Inhibition of the NFκB pathway downstream of MyD88 potentiates apoptosis, indicating that these two pathways bifurcate at the level of MyD88. Moreover, MyD88 binds FADD and is sufficient to induce apoptosis. Recently, we revealed that NFκB activation caused by H pylori was associated with TRAF6, a molecule downstream of MyD88. In addition, as H pylori is a gram
were prepared from MKN45 and MKN45 I ribonuclease protection assay was performed according to the figure 9 (A) Detection of stable MKN45 transfectants expressing antibody to FLAG and polyclonal antibody to 292 Maeda, Yoshida, Mitsuno, et al. www.molpath.com

This complex was found to be specific, as judged using supershifting with anti-Fas or cocultured with MKN-45 antibody. (F) MKN45 and THP-1 cells were incubated with medium alone for 12 hours. Immunoblot analysis was performed using anti-Fas or cocultured with MKN45 IxIbα SR cells were treated with interferon γ (IFN-γ 10 ng/ml) for 24 hours. Cells were incubated with H pylori (HP) or anti-Fas (CH-11) for 24 hours. Cell viability was assessed by trypan blue dye exclusion assay by counting 300 cells. Viable cells were quantitated under bright field microscopy. Results are expressed as percentages of dead cells. Values are mean (SD) of three independent experiments. *Percentage cytotoxicity was significantly (p<0.05) different between the MKN45 treated cells with anti-Fas or cocultured with H pylori and MKN45 IxIbα SR treated cells with anti-Fas or cocultured with H pylori. (D) DNA fragmentation was quantified using a commercially available ELISA (Boehringer Mannheim Biocemicals, Mannheim, Germany): 5x10⁵ cells were incubated in triplicate with H pylori (HP), anti-Fas (CH-11), or medium alone for 12 hours and lysed, and the supernatants were used for ELISA. Absorbance was measured at 405 nm. *Absorbance was significantly (p<0.05) different between the control and treated with anti-Fas or cocultured with H pylori in the MKN45 IxIbα SR groups; NS, no significant difference was found. (E) MKN45 and MKN45 IxIbαSR cells were treated with IFN-γ (10 ng/ml) for 24 hours. Cells were incubated with H pylori (HP) or anti-Fas (CH-11) for 12 hours. Immunoblot analysis was performed using anti-caspase-8, caspase-3, cleaved caspase-3, and -actin antibody. (F) MKN45 and THP-1 cells were incubated with H pylori and total RNA was extracted at the indicated times. The ribonuclease protection assay was performed according to the supplier’s instructions.

negative bacterium, the relation to Toll-like receptors should be investigated.

Knockout of cagE, one of the cag PAI genes, decreased both the apoptotic effects and potential to activate NFκB. Thus it may be speculated that both apoptosis and NFκB activation are triggered by the same signal that is dependent on cag PAI. Several cag PAI proteins, including CagE, are thought to constitute a type IV secretion system. CagE itself is a homologue of a transporter component in Agrobacterium tumefaciens and Bordetella pertussis that engage in the transcellular transport of toxins or T-DNA. Recent reports suggest that the system transports CagA protein, another product of the cag PAI, into the cytoplasm of host cells, where CagA undergoes tyrosine phosphorylation. As the system may also transport other molecules, it is possible that a certain protein produced by H pylori is transported into host cells where it triggers both apoptotic and NFκB activating signalling pathways. Recently, we have revealed that knockout of the cagE gene deprived wild-type H pylori on the pathogenicity for gastric ulcer, gastritis, and intestinal metaplasia in an in vivo model. This observation may indicate that the pro- and antiapoptotic effect induced by wild-type H pylori is an important factor in the pathogenesis of H pylori mediated gastric diseases.

In conclusion, we demonstrated that H pylori directly induced apoptosis mainly through cytochrome c release from the mitochondria. H pylori also exerted antiapoptotic effects through NFκB activation. Both apoptotic and antiapoptotic effects were dependent on cag PAI.

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