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Dual effects of *Helicobacter pylori* vacuolating cytotoxin on human eosinophil apoptosis in early and late periods of stimulation

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Although *Helicobacter pylori* infections of the gastric mucosa are characterized by the infiltration of inflammatory cells such as eosinophils, the responses of eosinophils to *H. pylori* vacuolating cytotoxin (VacA) have not been fully elucidated. This study investigates the role of VacA in the apoptosis of human eosinophils. We treated human eosinophils with purified *H. pylori* VacA and observed that induction of apoptosis is a relatively late event. Expression of cellular inhibitor of apoptosis protein (c-IAP)-2 was upregulated during the early period of VacA stimulation, and transfection with *c-IAP2* siRNA augmented apoptotic cell death. VacA caused the translocation of cytoplasmic Bax to the mitochondria and increased cytochrome c release from mitochondria in eosinophils. Transfection of an EoL-1 eosinophil cell line with *Bax* siRNA decreased the release of cytochrome c and DNA fragmentation. Furthermore, apoptosis facilitated by Bax and cytochrome c was primarily regulated by p38 MAPK in VacA-treated eosinophils. These results suggest that the exposure of human eosinophils to *H. pylori* VacA induces the early upregulation of c-IAP2 and a relatively late apoptotic response, with the apoptosis progressing through a sequential pathway that includes p38 MAPK activation, Bax translocation, and cytochrome c release.

Key words: Apoptosis · Eosinophils · *H. pylori* vacuolating cytotoxin

Introduction

Active chronic gastritis due to *Helicobacter pylori* infection is characterized by the infiltration of inflammatory cells such as neutrophils, lymphocytes, and eosinophils into the gastric mucosa [1–3]. Levels of infiltrating eosinophils decrease slowly and remain elevated even 1 year after *H. pylori* is eradicated [2]. One follow-up study showed that neutrophil infiltration disappears within 2 months after *H. pylori* eradication, while mononuclear cell infiltration persists into the second year [4]. Although these studies suggest that eosinophils play a role in the prolongation of gastric inflammation after infection with *H. pylori*, the specific pathogenic roles have not been fully investigated.

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Eosinophils are bone marrow-derived granulocytes that have specific granules containing large amounts of toxic proteins such as major basic protein, eosinophil-derived neurotoxin, eosinophil cationic protein, and eosinophil peroxidases. Eosinophil degranulation in mucosal tissues plays pathogenic roles in several diseases [5–7]. Considering that *H. pylori*-induced chronic gastritis results in increased infiltration of eosinophils and that infiltrated eosinophils may mediate pathogenic effects in *H. pylori*-infected chronic gastric patients [8], it is possible that the infiltrated eosinophils cause tissue damage. However, the status of eosinophils induced by *H. pylori* infection remains unclear.

H. pylori vacuolating cytotoxin (VacA) is a major virulence factor in the pathogenicity of *H. pylori* [9, 10]. Higher VacA activity during *H. pylori* infections is significantly associated with severe chronic gastritis [11–13]. Since human eosinophils induce proinflammatory cytokines such as IL-8 in response to the *H. pylori* VacA [14], VacA may affect eosinophils by inflammatory signals.

Programmed cell death or apoptosis can be induced in granulocytes by intrinsic or mitochondrial pathways. Stress conditions influence the expression and location of Bcl-2 family proteins such as the pro-apoptotic Bax protein [15]. In certain situations, anti-apoptotic members of the Bcl-2 family such as inhibitor of apoptosis protein (IAP) may be involved in delaying apoptosis [16]. Although normal eosinophils are sensitive to apoptosis, *Trichinella spiralis*-infected eosinophils show resistance to apoptotic cell death [17]. Therefore, apoptosis may regulate pathogenesis of several infectious diseases [18–21]. Considering that *H. pylori* VacA induces apoptosis in gastric epithelial cells *via* a mitochondria-dependent pathway [22], VacA may influence the apoptotic processes of eosinophils.

In this study, we investigated the role of VacA in human eosinophil apoptosis, and observed early induction of the anti-apoptotic cellular inhibitor of apoptosis protein (c-IAP)-2, along with relatively late induction of apoptosis through a signaling pathway including activation of p38 MAPK, Bax translocation, cytochrome c release from mitochondria, and DNA fragmentation in VacA-stimulated human eosinophils.

Results

H. pylori VacA induces apoptosis of human eosinophils

Freshly isolated human eosinophils were treated with purified VacA and apoptosis was evaluated. As shown in Fig. 1A, nuclear fragmentation was observed in VacA-stimulated eosinophils. Under transmission electron microscopy (TEM), VacA treatment resulted in characteristic features of apoptosis such as condensation of chromatin and apoptotic body formation (Fig. 1B). VacA-treated mitochondria were stained using a fluorescent dye, JC-1 (5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethyl benzimidazl-carbocyanine iodide). In normal cells, JC-1 dye was concentrated

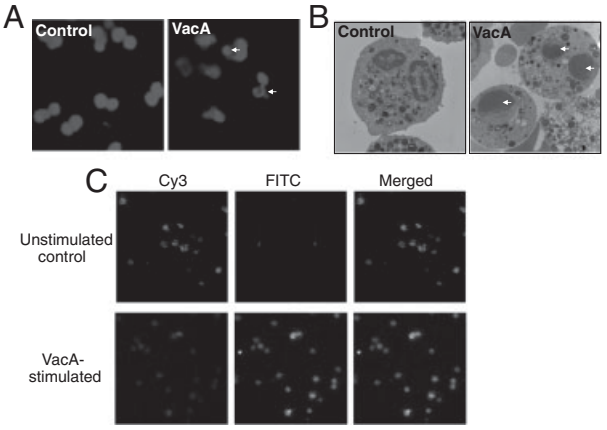

Figure 1. Apoptosis in VacA-stimulated human eosinophils. Freshly isolated human eosinophils were stimulated with VacA (10 μg/mL) for 24 h. (A) Cells were fixed with 2% paraformaldehyde and stained with Hoechst dye 33258 (× 400). Apoptotic bodies are shown in VacA-treated cells (arrowhead). (B) Cells were analyzed by TEM (× 10 000). VacA-treated cells demonstrated the characteristic features of apoptosis such as condensation of chromatin (arrowhead). (C) Cells were stained with JC-1 dye and visualized under a fluorescent microscope (× 400). Normal cells show granular mitochondrial staining and apoptotic cells show diffuse cytoplasmic staining.

in the mitochondrial matrix, where it formed JC-aggregates (Fig. 1C). In VacA-induced apoptotic cells, the mitochondrial membrane dissipated and the dye dispersed throughout the entire cell, leading to a shift from JC-aggregates to JC-1 monomers.

Biochemical assessments of apoptosis following VacA stimulation were performed by several methods, including staining eosinophils with annexin V to detect the externalization of phosphatidylserine to the outer leaflet of the cell membrane or staining with tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) to detect reduced mitochondrial transmembrane potential. We observed increasing numbers of apoptotic cells in VacA treatment (Fig. 2A and B). Peripheral blood eosinophils (PBE) are programmed to die in ∼3 days, unless they encounter prosurvival cytokines such as IL-5 and GM-CSF [23, 24]. Tissue eosinophils, in contrast, are exposed to prosurvival cytokines *in vivo*, which prolongs their viability. Since observations regarding the effects of VacA on prosurvival cytokine-treated eosinophils is much more relevant to *in vivo* situation, isolated eosinophils were treated with GM-CSF and VacA, and apoptosis was then measured. As shown in Fig. 2A and B, prosurvival cytokine GM-CSF decreased the numbers of apoptotic cells compared with untreated controls. In this experimental condition, the addition of VacA to GM-CSF-treated PBE significantly increased apoptotic cell populations. Although the absolute numbers of apoptotic cells observed differed according to each method, the ratios of apoptotic cells in VacA-stimulated and control cultures were similar (Fig. 2C–E). These results indicate that VacA enhances apoptosis in both PBE and prosurvival cytokine-treated eosinophils.

We next asked whether VacA could induce cytochrome c release from mitochondria. As shown in Fig. 3A and B, treatment

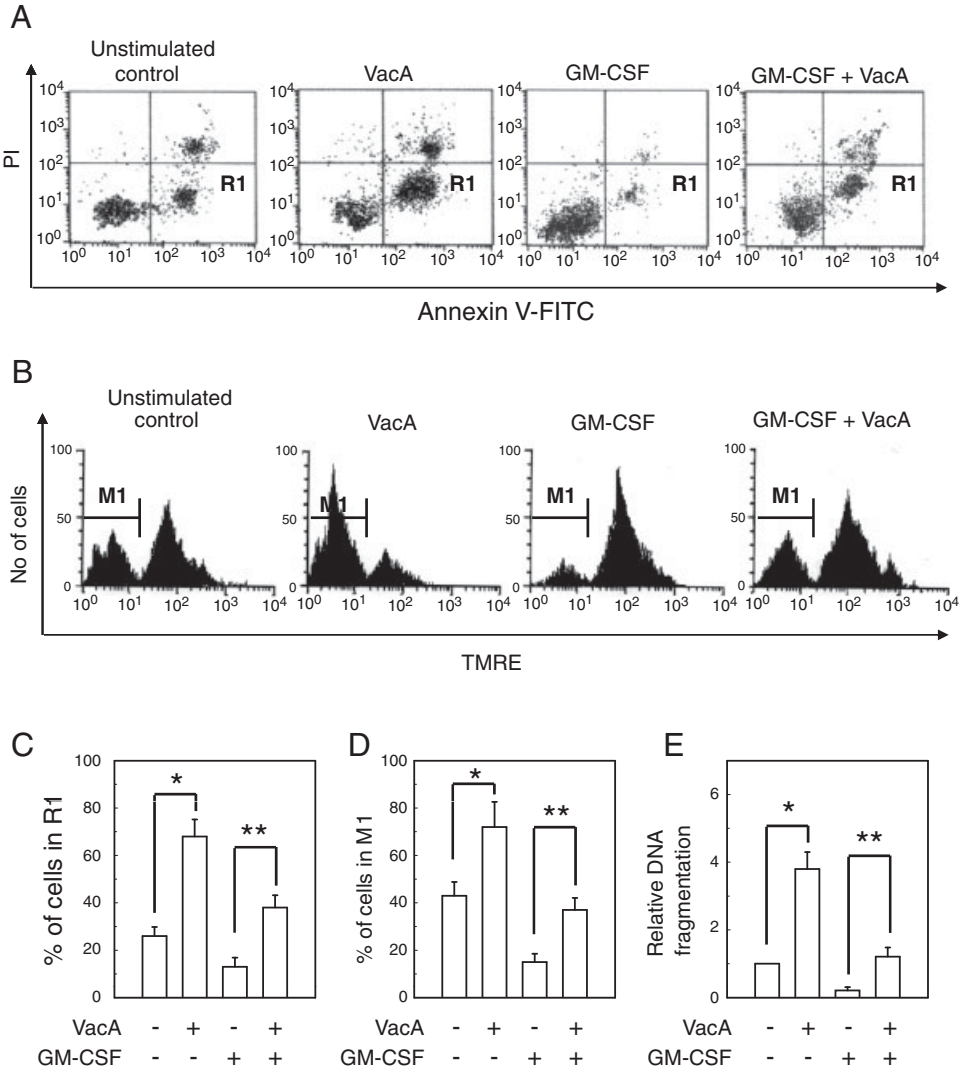

Figure 2. Flow cytometry analysis of human eosinophils stimulated with VacA and quantification of apoptotic cells. (A) Freshly isolated human eosinophils were left unstimulated as controls or stimulated with VacA (10 μg/mL), GM-CSF (1 ng/mL), or VacA+GM-CSF for 24 h. Cells were then incubated with FITC-conjugated annexin V and PI and analyzed by flow cytometry. Apoptotic cells have high FITC-annexin V and low PI staining (right lower quadrant, R1). Data are representative of three experiments. (B) Cells were incubated with 75 nM TMRE for 30 min at 37°C and analyzed by flow cytometry. The area marked as M1 contains the apoptotic cell population. Data are representative of three experiments. (C and D) Quantitative data for R1 and M1. Values are means+SEM ($n = 3$). (E) The cell death detection ELISA was measured as a fold increase in stimulated cells compared with each control (mean+SEM, $n = 3$). *$p<0.05$ *versus* untreated control; **$p<0.05$ *versus* GM-CSF alone.

with *H. pylori* VacA resulted in increased levels of cytochrome c in the cytosolic fraction, but decreased levels in the mitochondrial fraction. To confirm this result, immunohistochemistry and confocal microscopic analysis were performed. As shown in Fig. 3C, cytochrome c shows a diffuse distribution pattern in the cytoplasm of VacA-treated eosinophils. In contrast, cytochrome c was localized within the mitochondria of untreated cells. Eosinophils with diffuse cytoplasmic distribution pattern were observed in ~25% of the VacA-treated group, but in less than 5% of the untreated group. These results indicate that VacA promotes the release of cytochrome c from the mitochondria.

Time course of apoptosis in eosinophils stimulated with *H. pylori* VacA

An increase in eosinophil apoptosis was observed during the first 24 h after VacA stimulation, and eosinophil apoptosis continued to increase over the 48 h post-stimulation, as assessed by quantitative analysis of DNA fragmentation (Fig. 4A). Since we already demonstrated that VacA increases chemokine IL-8 expression in eosinophils [14], we next assessed the relationship between apoptosis and IL-8 expression in response to VacA stimulation. As shown in Fig. 4B, the numbers of IL-8 mRNA transcripts were

upregulated during the early period of stimulation relative to the rates of DNA fragmentation. Similar results were observed in VacA-stimulated cells, as assessed by flow cytometric analysis using annexin V and PI or TMRE (Fig. 4C and D).

Induction of c-IAP2 in human eosinophils is an early response to *H. pylori* VacA

We next assessed whether upregulation of c-IAP is an early response to VacA stimulation. To explore this hypothesis, we

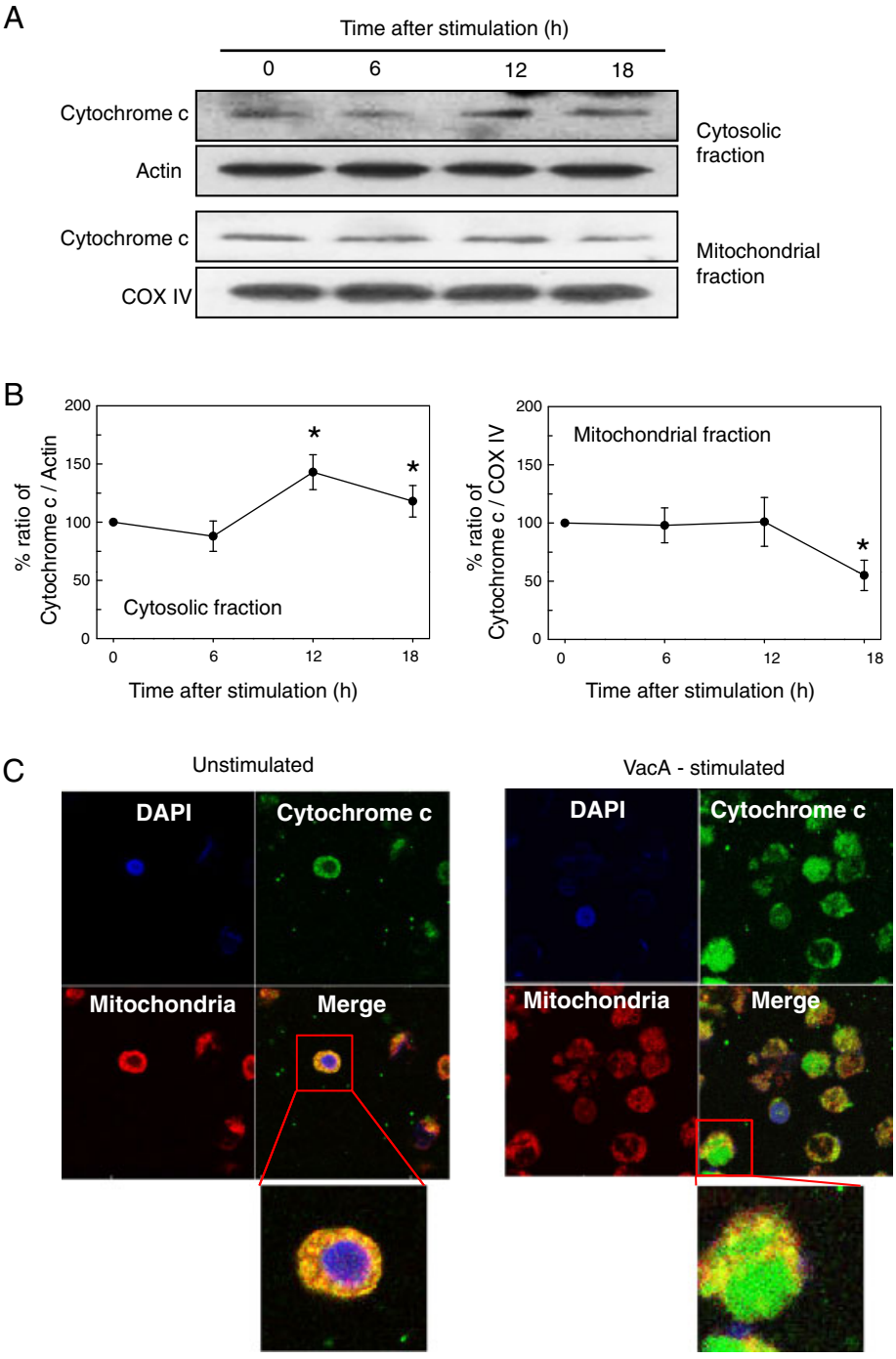

Figure 3. Cytochrome c release from VacA-stimulated human eosinophils. (A) Immunoblot analysis for freshly isolated human eosinophils stimulated with VacA (10 μg/mL). Data are representative of three or more independent experiments. (B) Densitometric analysis for the expressed proteins. The values represent percent relative densities of each protein compared with actin (cytoplasmic fraction) or COX IV (mitochondrial fraction) (mean ± SD, $n = 3$). *$p<0.05$ *versus* untreated control (0 h). (C) Freshly isolated human eosinophils were incubated with or without VacA (10 μg/mL) for 24 h, and immunocytochemistry and confocal analysis were performed. The cells were stained with anti-cytochrome c antibody (green), Mitotracker RED (red, mitochondria), and DAPI (blue, nucleus). The data are representative of at least five experiments.

analyzed the expression of c-IAP using quantitative RT-PCR and immunoblot analysis. Stimulation with VacA increased the expression of c-IAP2 mRNA within 3 h (Fig. 5A). However, expression of mRNA for c-IAP1 and XIAP was constitutive and was not affected by VacA (Fig. 5B and C). β-actin mRNA levels in VacA-stimulated cells remained relatively constant (Fig. 5D). Real-time PCR confirmed that stimulation with VacA for 3 h increased c-IAP2 mRNA levels by 9.6±2.5-fold as compared to unstimulated cells (mean±SEM, $n = 3$), whereas mRNA expression of c-IAP1 remained unchanged (c-IAP1, 1.3±0.7; mean± SEM, $n = 3$). Consistent with this finding, VacA increased c-IAP2 protein expression as determined by immunoblot analysis. Transfection with *c-IAP2* siRNA resulted in a significant decrease in c-IAP2 expression (Fig. 6A). We next explored whether c-IAP2 induced by VacA might be associated with eosinophil apoptosis. As shown in Fig. 6B and C, transfection with *c-IAP2* siRNA significantly augmented the caspase-3 activity and DNA fragmentation in EoL-1 cells stimulated with VacA. These results suggest that c-IAP2 is responsible for the suppression of apoptosis in the early period of VacA-treated eosinophils.

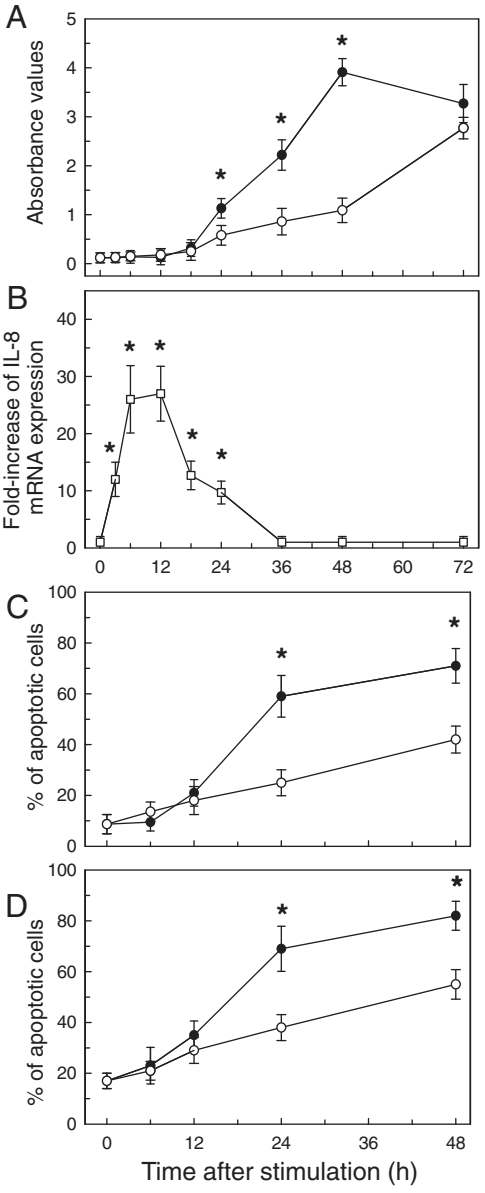

Figure 4. Time course of apoptosis and IL-8 mRNA expression in human eosinophils cells treated with VacA. Freshly isolated human eosinophils were stimulated with VacA (10 μg/mL) for the indicated time period. (A) Apoptosis was assessed using a cell death detection ELISA. Data represent absorbance values measured by ELISA reader (mean±SEM, $n = 5$). (B) Levels of IL-8 mRNA were quantified by RT-PCR using a standard RNA. The values are expressed as mean ± SD ($n = 5$). The β-actin mRNA levels in stimulated and unstimulated eosinophils remained relatively constant throughout the same period ($\sim 5 \times 10^6$ transcripts/μg of total RNA). (C) Apoptosis was assessed by flow cytometry using annexin V and PI, in which numbers of apoptotic cells were measured in the area of high FITC-annexin V and low PI staining (R1 area, mean ± SEM, $n = 3$). (D) Apoptosis was assessed by flow cytometry using TMRE. Numbers of apoptotic cells were calculated in M1 area (mean±SEM, $n = 3$). Open circles represent unstimulated controls and closed circles represent VacA-treated groups. *$p<0.05$ *versus* untreated controls.

H. pylori VacA induces mitochondrial trafficking of activated Bax

Bax activation results in initiation of the intrinsic apoptotic pathway by increasing mitochondrial permeability and release of cytochrome [25]. We had previously observed increases in cytosolic cytochrome c as well as increased release of cytochrome c from the mitochondria (Fig. 3). Consistent with this, Bax signals became concentrated within the mitochondrial fraction and were reduced in the cytosolic fraction after stimulation of human eosinophils with

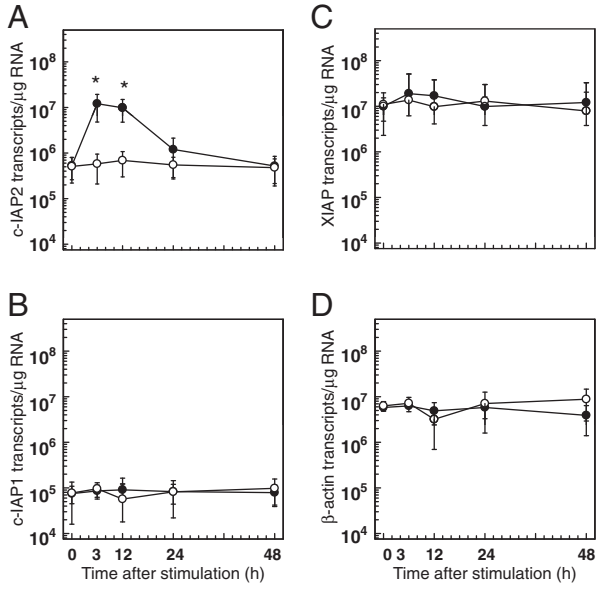

Figure 5. Induction of c-IAP2 mRNA expression in VacA-stimulated eosinophils. Freshly isolated human eosinophils were untreated (open circles) or treated with VacA (10 μg/mL, closed circles) for the indicated times. The mRNA levels of c-IAP2 (A), c-IAP1 (B), XIAP (C), and β-actin (D) were analyzed by quantitative RT-PCR using each standard RNA (mean±SD, $n = 5$). *$p<0.05$ *versus* untreated controls.

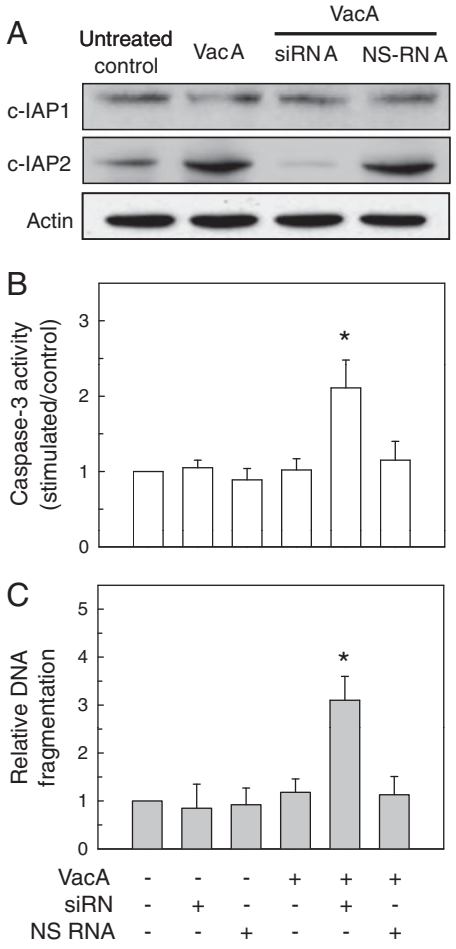

Figure 6. Suppression of c-IAP2 promotes caspase-3 activity and DNA fragmentation in VacA-stimulated eosinophils. (A) Immunoblot analysis for EoL-1 cells transfected with *c-IAP2*-siRNA. The results are representative of three independent experiments. (B and C) The transfected EoL-1 cells with each siRNA were either untreated or stimulated with VacA (10 μg/mL) for 6 h. (B) Caspase-3 activity in the cell extracts was assayed using a colorimetric assay kit and is expressed relative to untreated controls (mean+SEM, $n = 5$). (C) Oligonucleosome release into the cytoplasm was assayed by ELISA (mean+SEM, $n = 5$). *$p<0.05$ *versus* non-transfected cells treated with VacA.

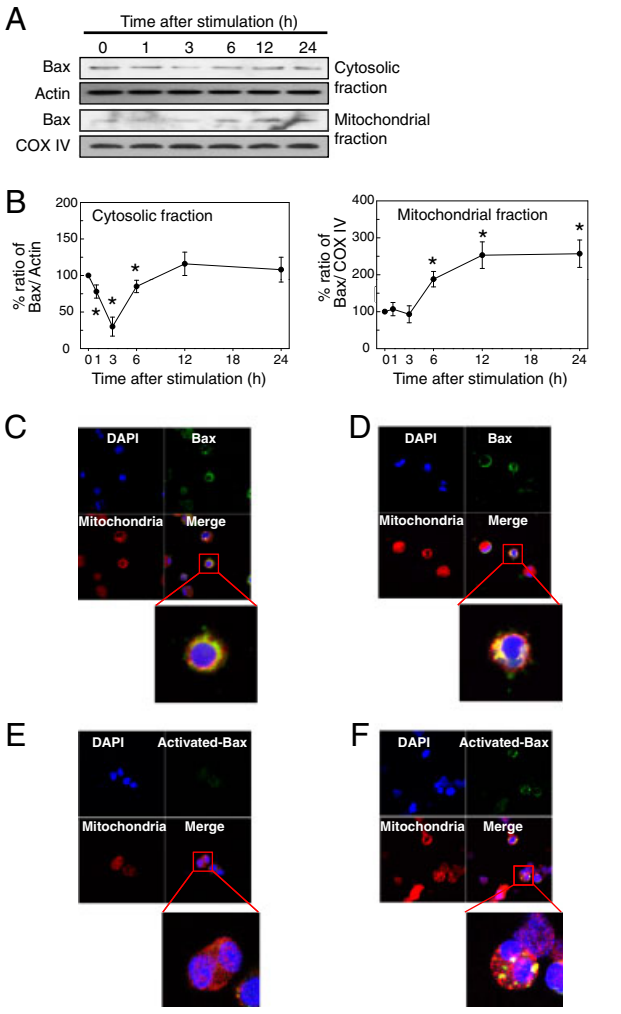

Figure 7. Translocation of cytoplasmic Bax to mitochondria in VacA-stimulated eosinophils. (A) Freshly isolated human eosinophils were treated with VacA (10 μg/mL) for the indicated period. Expression of Bax, actin (cytoplasmic fraction), and COX IV (mitochondrial fraction) was evaluated by immunoblot analysis. (B) Densitometric analysis for the expressed proteins. The values represent percent relative densities of each protein compared with actin (cytoplasmic fraction) or COX IV (mitochondrial fraction) (mean ± SD, $n = 3$). *$p<0.05$ *versus* untreated control (0 h). (C and D) Freshly isolated human eosinophils were (C) left unstimulated as controls or (D) stimulated with VacA for 18 h. Immunocytochemistry was performed using anti-Bax antibody (Santa Cruz ♯sc526). (E and F) Panel (E) represents unstimulated control and panel (F) represents VacA stimulation. Immunocytochemistry was performed using the active form-specific anti-Bax-NT antibody (Upstate ♯06-499). The data are representative of at least five experiments.

VacA (Fig. 7A and B). In addition, immunohistochemistry and confocal assay results that the distribution of Bax signals in response to VacA was limited to the mitochondria of VacA-stimulated human eosinophils (Fig. 7C and D). To confirm this finding, immunohistochemical analyses were performed using the active form of anti-Bax-NT antibody. The mitochondrial trafficking of activated Bax was confirmed in VacA-treated cells (Fig. 7E and F).

We next evaluated whether inhibition of Bax activity influences cytochrome c release and DNA fragmentation in VacA-treated eosinophils. In this experiment, Bax expression was suppressed in a human eosinophil cell line, EoL-1, by transfection with siRNA. As shown in Fig. 8A, cells transfected with siRNA against *Bax* showed significant decreases in cytochrome c release and DNA fragmentation compared to control cells. In this system, transfection with *Bax* siRNA significantly reduced the mitochondrial levels of phospho-Bax in VacA-treated cells (Fig. 8B).

p38 MAPK regulates Bax signals and apoptosis in eosinophils stimulated with *H. pylori* VacA

Purified *H. pylori* VacA strongly activated the phosphorylation of ERK1/2, p38, and JNK in eosinophils; the activation of ERK1/2 and p38 was sustained for a relatively long period of time compared to JNK (Fig. 9A). To explore the potential link between phosphorylation of MAPK and mitochondrial Bax trafficking in VacA-stimulated eosinophils, cells were pretreated with the

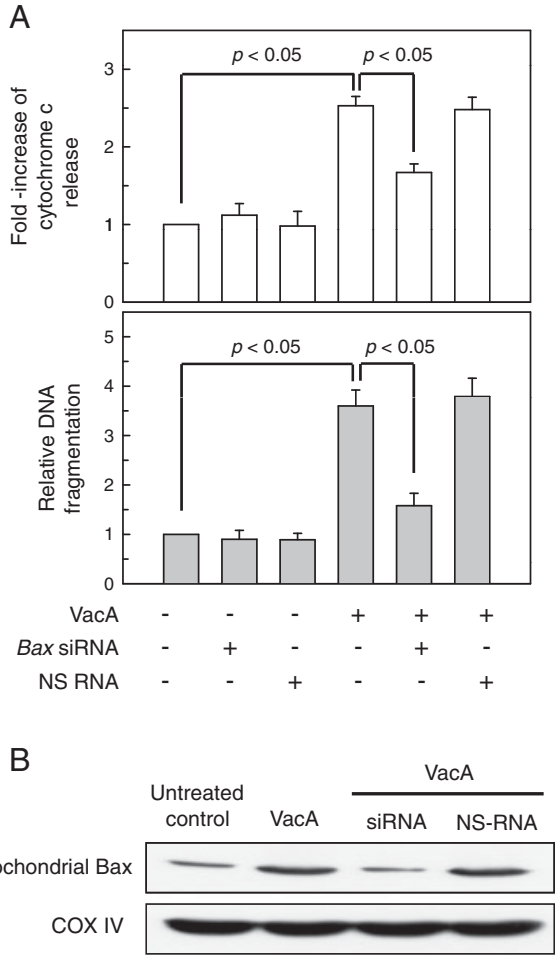

Figure 8. Cytochrome c release and DNA fragmentation in Bax-suppressed EoL-1 cells stimulated with VacA. (A) EoL-1 cells were transfected with *Bax*-specific siRNA or non-silencing control siRNA (NS RNA) for 48 h. The transfected cells were either untreated or stimulated with VacA (10 μg/mL) for 24 h. Cytosolic levels of cytochrome c and oligonucleosome were measured by each ELISA kit (mean fold-induction+SEM, $n = 5$). (B) Immunoblot analysis for the active form of Bax-NT was performed in the mitochondrial fractions of the transfected cells.

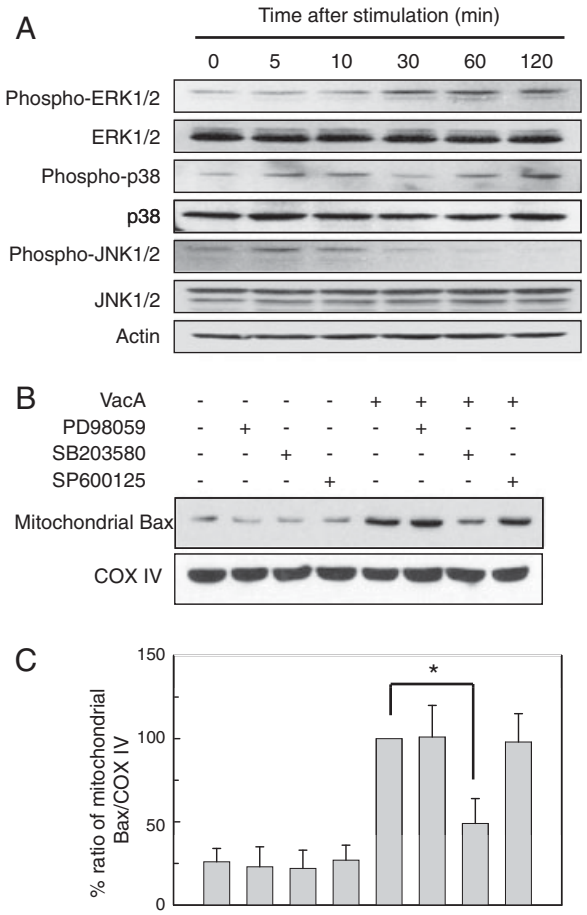

Figure 9. p38 MAPK is associated with the induction of Bax in VacA-stimulated human eosinophils. (A) Freshly isolated human eosinophils were stimulated with VacA (10 μg/mL) for the indicated periods of time. Phosphorylation of ERK1/2, p38, and JNK was measured by immunoblot analysis. Results are representative of three independent experiments. (B) Isolated human eosinophils were pre-incubated with PD98059 (40 μM), SB203580 (20 μM), or SP600125 (20 μM) for 30 min, and stimulated with VacA (10 μg/mL) for another 18 h. Immunoblot analysis for the active form of Bax-NT in the mitochondrial fractions was performed. The data are representative of three independent experiments. (C) Densitometric analysis for the expressed proteins. The values represent percent relative densities of each protein compared with COX IV (mean+SD, $n = 3$), in which VacA alone is 100%. *$p<0.05$ *versus* VacA alone.

following kinase inhibitors: PD98059 (an inhibitor of MEK1/2), SB203580 (an inhibitor of p38), and SP600125 (an inhibitor of JNK) [26, 27]. The results indicated that pretreatment with SB203580 significantly attenuated Bax expression in mitochondrial fractions from VacA-stimulated eosinophils. However, pretreatment with PD98059 or SP600125 did not change Bax expression levels (Fig. 9B and C). These results suggest that early signals such as p38 MAPK affect the late apoptosis of eosinophils. To confirm these results, transfection with recombinant adenoviruses containing dominant-negative mutants of p38 was performed. In this experiment, suppression of the p38 signal significantly decreased cytochrome c release and DNA fragmentation induced by VacA (Fig. 10A and B). The intracellular redistribution of Bax proteins was evaluated by immunoblot analysis of mitochondrial fractions using the active form of anti-Bax-NT antibody. VacA treatment showed a significant increase in Bax protein in normal cells. However, suppression of p38 signal by a dominant-negative mutant definitely attenuated the VacA-induced expression of active Bax in mitochondrial fractions (Fig. 10C). Phosphorylated activity of p38 were increased in eosinophils stimulated with VacA, as assessed by phospho-p38 ELISA kit (Active Motif, Carlsbad, CA, USA) (Fig. 10D).

Discussion

The apoptotic process is a natural physiological phenomenon. However, excessive apoptosis results in tissue damage. Since *H. pylori* infection induces apoptosis in gastric epithelial cells and

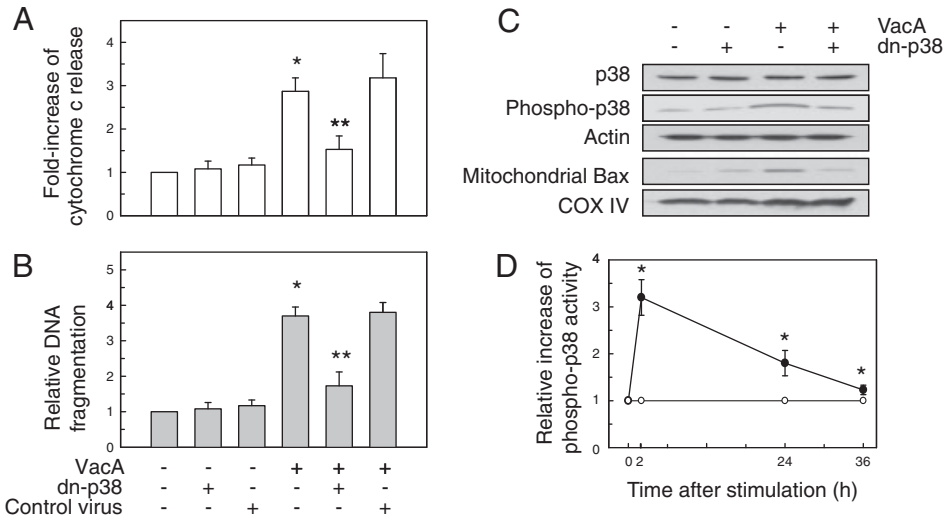

Figure 10. Effects of p38 suppression on apoptosis in EoL-1 cells stimulated with VacA. EoL-1 cells were transfected with recombinant adenovirus for 48 h. (A and B) Transfected cells were stimulated with VacA (10 μg/mL) for another 24 h. (A) Cytosolic levels of cytochrome c were measured by ELISA kit. Results are expressed relative to unstimulated controls (mean+SEM, $n = 5$). (B) Oligonucleosome release into the cytoplasm was assayed by ELISA (mean ± SEM, $n = 5$). $^{*}p<0.05$ compared with unstimulated control; $^{**}p<0.05$ compared with VacA alone. (C) Transfected EoL-1 cells were treated with VacA (10 μg/mL) for 3 h (MAPK) or 18 h (Bax). Immunoblot analysis was performed to detect p38, phospho-p38, and actin in whole cell lysates. The active forms of Bax-NT and COX IV were detected in the mitochondrial fractions. The data are representative of three independent experiments. (D) Freshly isolated human eosinophils were stimulated with VacA (10 μg/mL). Phosphorylated activity of p38 was measure by ELISA kit (mean ± SEM, $n = 3$). $^{*}p<0.05$ compared with unstimulated control. Closed circles, VacA; open circles, untreated control.

immune cells such as T lymphocytes and macrophages [28], the apoptotic process may play an important role in the pathogenesis of *H. pylori* infection. In the present study, VacA-exposed human eosinophils induced upregulation of c-IAP2 molecules as an early response to treatment with *H. pylori* VacA, and apoptosis as a relatively late response, with apoptosis involving activation of p38 MAPK, Bax translocation, and release of cytochrome c.

At the molecular level, classical apoptosis is caused by the activation of a family of cysteine proteases known as caspases that cleave their target proteins at specific aspartic acids [29]. Two major apoptotic pathways have been defined: (i) the extrinsic pathway, in which activation of a death receptor (*e.g.* Fas/CD95 and TNFRI/CD120a) by a ligand, such as TNF-α or Fas ligand, leads to the activation of initiator caspase-8; and (ii) the intrinsic pathway, which is a consequence of cellular stress and cytochrome c release from mitochondria, and which leads to caspase-9 activation. There is crosstalk between the mitochondrial-mediated and receptor-mediated pathways. Both pathways culminate in the activation of downstream executioner caspase-3. The activation of caspase-3 results in a series of events, such as cleavage of cytoskeleton proteins, activation of nucleases, and subsequent DNA fragmentation [30]. The results of the present study demonstrate that VacA induces cytochrome c release from the mitochondria into the cytoplasm in eosinophils. Of note, immunohistochemistry and confocal microscopy revealed diffuse cytoplasmic distribution of cytochrome c in VacA-treated eosinophils and a localized mitochondrial distribution in untreated cells. Interestingly, apparent apoptotic phenomena were observed as relatively late responses to VacA stimulation in eosinophils compared to proinflammatory responses such as IL-8 expression. Therefore, it is possible that the ability of VacA to inhibit apoptosis may result from the induction of anti-apoptotic pathways during early response. The present study used pharmacologic doses of VacA to induce eosinophil apoptosis. Since the *in vitro* pharmacologic dose appears to be higher than physiologic concentrations in toxigenic *H. pylori*-infected stomachs, further study is needed to investigate the relationship between *in vitro* pharmacologic doses and *in vivo* physiologic concentration of VacA.

IAP are ubiquitous intracellular proteins that may function as inhibitory signals of apoptosis. Thus, IAP induction may inhibit apoptosis in several cells [21, 31, 32]. Because of its ubiquitous distribution and inducible properties, the IAP family serves as cytoprotective machinery under bacterial toxin-stimulated pathophysiological situations [21]. In the present study, stimulation of eosinophils with VacA induced the over-expression of c-IAP2. In addition, the inhibition of c-IAP2 directly augmented caspase-3 activity and DNA fragmentation in VacA-treated cells. These observations suggest that the ability of VacA to suppress apoptosis in the early period of stimulation may have a molecular basis. However, c-IAP2 expression decreased to baseline between 24 and 48 h after stimulation, indicating that prosurvival signaling c-IAP2 was antagonized by VacA during the late period. Therefore, upregulated c-IAP2 is probably unable to reduce eosinophil persistence in stomachs infected with VacA-producing *H. pylori*.

The apoptotic processes that occur before cytochrome c release require a variety of effector molecules, including Bcl-2 family proteins, which are important regulatory molecules [33–36]. The Bcl-2 family is composed of two subfamilies: one consisting of anti-apoptotic proteins (*e.g.* Bcl-2, Bcl-X_L, Bcl-w, Bfl-1, Brag-1, and Mcl-1) and the other of pro-apoptotic proteins

(*e.g.* Bax, Bak, Bcl-X_S, Bad, Bid, Bik, and Hrk) [37]. Some pro-apoptotic Bcl-2 family proteins are localized in the cytoplasm in the normal state; however, during the apoptotic process, they translocate into the mitochondria, forming homo- and hetero-oligomers to regulate efflux of apoptosis-executioner proteins, including cytochrome c and Smac/DIABLO. Bax is known to be an important regulator of mitochondrial membrane permeabilization [33–35]. In normal cells, Bax is largely located in the cytoplasm as a monomeric protein. During apoptosis, it undergoes conformational changes near the amino and carboxyl termini to expose a functionally crucial Bcl-2 homology domain 3 and translocates to the mitochondrial outer membrane. Activated Bax undergoes homo-oligomerization and may participate in the formation of a large mitochondrial transition pore complex that facilitates cytochrome c release [36]. Recently, a paper demonstrated that Bax was activated and localized to mitochondria in gastric epithelial cells exposed to *H. pylori* VacA [22]. Consistent with this observation, our results demonstrated the translocation of Bax protein to mitochondria in VacA-exposed eosinophils. This finding was confirmed by confocal microscopy using a conformation-specific anti-Bax-NT antibody. In addition, transfection with siRNA against *Bax* significantly reduced the levels of caspase-3 activity, cytochrome c release, and DNA fragmentation, suggesting that *H. pylori* VacA may utilize an activated Bax signal to induce apoptosis in eosinophils. Since quantification of confocal analysis regarding Bax activation and cytochrome c distribution is very subjective, immunoblot data may be useful to address these questions. The results of immunoblot analyses showed that VacA increased levels of activated Bax in the mitochondrial fraction, increased levels of cytochrome c in the cytosolic fraction, and decreased levels of cytochrome c in the mitochondrial fraction. Therefore, the results of confocal analyses are congruent with observations of Bax activation and cytochrome c distribution in response to VacA stimulation. Eosinophils are terminally differentiated, non-dividing cells. In contrast, EoL-1 cells are immortal. Therefore, extrapolations based on data from EoL-1 cells in the present study may not be relevant to *in vivo* situation. Careful *in vitro* models must be developed to address this problem.

In eosinophil apoptosis, GM-CSF induces activation of kinase Erk1/2 and phosphorylated Bax molecule, and then this facilitates new interactions between Bax and prolyl isomerase Pin1, suggesting that Pin1 is a mediator of prosurvival signaling and is a regulator of Bax function [24]. In the present study, VacA significantly increased apoptotic cell population and DNA fragmentation in GM-CSF-treated eosinophils, suggesting that VacA may affect this signaling pathway. However, further study is needed to clarify this hypothesis.

In the present study, *H. pylori* VacA activated MAPK signals and inhibition of p38 MAPK resulted in a significant reduction of Bax activity and apoptosis in eosinophils. In contrast, Nakayama *et al.* reported that p38 did not participate in VacA-induced cytochrome c release and Bax activation in gastric epithelial cells [38]. However, several studies have demonstrated that p38 MAPK activates Bax to induce mitochondrial cytochrome c release and apoptosis in neuroblastoma cells [39], keratinocytes [40], and lens epithelial cells [41]. Therefore, the mechanisms for p38-associated cytochrome c release and Bax activation in eosinophils appear to be cell-specific and different from those in gastric epithelial cells.

In summary, this study demonstrates that the exposure of eosinophils to VacA results in the activation of intrinsic apoptotic signaling events, including p38 activation, Bax translocation, cytochrome c release, and DNA fragmentation. Apoptotic events are apparent during late periods after stimulation because the suppressor gene c-IAP2 is induced during early periods. Based on these findings, we propose that the survival advantage observed during the early period allows VacA-exposed eosinophils to accumulate proinflammatory signals, leading to an inflammatory response in gastric mucosal layers infected with VacA-producing toxigenic *H. pylori*.

Materials and methods

Reagents

Histopaque solution (density, 1.083 g/mL) and RBC lysis buffer were obtained from Sigma Chemical (St. Louis, MO, USA). Anti-CD16 antibody-conjugated with magnetic particles was obtained from Miltenyi Biotech (Bergisch Gladbach, Germany). LPS-free FBS, antibiotics, L-glutamin, Trizol, and RPMI 1640 were obtained from GIBCO BRL (Gaithersburg, MD, USA). Peroxidase-conjugated anti-rabbit and anti-mouse IgG were obtained from BD Pharmingen (San Diego, CA, USA). Antibodies against cytochrome c, pan-ERK1/2 (p44/p42), phospho-ERK1/2, pan-JNK (p54/p46), phospho-JNK, pan-p38, phospho-p38, cytochrome c oxidase subunit IV (COX IV), and actin were obtained from Cell Signaling Technology (Beverley, MA, USA). PD98059, SB203580, SP600125, and Hoechst 33258 were acquired from Calbiochem (La Jolla, CA, USA). Mitotracker RED CMXRos and Alexa fluor 488-conjugated secondary antibody against rabbit IgG were purchased from Invitrogen-Molecular Probes (Eugene, OR, USA). Anti-human Bax antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The active form of specific anti-Bax-NT was from Upstate Biotech (Temecala, CA, USA) and Vectashield mounting medium with DAPI was obtained from Vector Laboratories (Burlingame, CA, USA).

Purification of *H. pylori* VacA

A VacA-producing *H. pylori* strain 60190 (ATCC 49503) was grown in sulfite-free Brucella broth containing 5% FBS at 37°C under microaerophilic conditions. VacA was purified from broth culture supernatants according to our previously published procedures [14]. The purification of VacA protein was kindly supported by Professor Patrice Boquet at Laboratoire de Bacteriologie, l'Hopital de l''Archet 2, France [42]. The purified

protein was activated immediately before use on cells; 250 mM HCl was added to the purified VacA until a pH of 2.0 was reached [43]. NH_4Cl (5 mM) was also added to the medium to enhance the VacA activity [22].

Isolation of PBE and cell cultures

Eosinophils were isolated from the peripheral blood of volunteers using a magnetic cell separation system (Miltenyi Biotec), as described previously [14]. The Hanyang University College of Medicine Review Board approved the protocol used to obtain blood from volunteers. The purity of eosinophils counted by Randolph's stain was >98%. Purified eosinophils were used immediately for experiments using RPMI 1640 medium. The human eosinophil cell line, EoL-1, was grown (10^6 cells/mL) in RPMI 1640 medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) [14].

Analysis of apoptosis

For morphologic assessment of apoptotic cells, cells were stained with the DNA dye Hoechst 33258 (5 μg/mL) and examined by epifluorescence microscopy (Axiophot, Carl Zeiss, Oberkochen, Germany) [18]. TEM was conducted using JEOL JEM-100 CX, as described previously [44]. A mitochondrial staining kit (CS0390, Sigma) was used to detect morphologic changes of the mitochondrial inner-membrane electrochemical potential [21].

To assess numbers of cells undergoing apoptosis, eosinophils were incubated with FITC-conjugated annexin V and PI (Apoptosis Detection Kit; R&D Systems, Minneapolis, MN, USA). They were then analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA), as previously described [21]. The mitochondrial transmembrane potential was assessed using TMRE and flow cytometry, as previously described [23]. To assess DNA fragmentation, oligonucleosome release into the cytoplasm was assayed with the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany), as described previously [21]. Caspase-3 activity was assayed with a colorimetric assay kit according to the manufacturer's instruction (R&D Systems) [21]. The amount of cytosolic cytochrome c in the cell lysates was quantified using an ELISA-based cytochrome c assay kit according to the manufacturer's instructions (Quantikine, R&D Systems).

Quantitative RT-PCR and real-time PCR

Cells were stimulated with VacA, after which total cellular RNA was extracted using Trizol. Reverse transcription and PCR amplification were performed as previously described [45]. The sequences of each primer, annealing temperatures, cycle numbers, and PCR product sizes have also been previously described [21, 46]. Real-time PCR for c-IAP1 (GenBank accession number: NM_001166) and c-IAP2 (GenBank accession number: NM_001165) was performed using SYBR green PCR mix and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) [47].

Immunoblot analysis

Cell lysates from VacA-treated cells were obtained using lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 0.1% Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin). For the preparation of cytosolic and mitochondrial protein, cells were collected and washed twice in ice-cold PBS, resuspended in buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1.9 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, mixture of protease inhibitors), and incubated on ice for 20 min. The cells were then homogenized with a 26-G needle syringe for 50 strokes. Cell homogenates were spun at $1000 \times g$ to remove unbroken cells, nuclei, and heavy membranes. The supernatant was spun again at $14\,000 \times g$ for 30 min to collect the mitochondria-rich (pellet) and cytosolic (supernatant) fractions. The mitochondria-rich fraction was washed once with the extraction buffer, followed by a final resuspension in buffer B (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, mixture of protease inhibitors) for immunoblot analysis, as described previously [48, 49].

Transfection

A *Bax*-specific siRNA was constructed as described by Yamasaki *et al.* [22]. siRNA against *c-IAP2* (Birc3) was purchased from Gibco-Invitrogen (Stealth Select RNAi^{TM} siRNA, HSS100562, Carlsbad, CA, USA) [50]. Transfection was performed according to the procedure described previously [27, 51].

pGL3-c-IAP2-WT-Luc (1931 to +27) and pβ-actin-luciferase transcriptional reporter were supported by Dr. Shinae Kizaka-Kondoh, Department of Molecular Oncology, Kyoto University Graduate School of Medicine, Kyoto, Japan [46],] and by Dr. Kagnoff of the University of California, San Diego, respectively. Luciferase assay was performed in accordance with the manufacture's instructions (Tropix, Bedford, MA, USA) [14, 21]. Recombinant adenovirus containing a dominant-negative p38α mutant (ADV-105) was obtained from Cell Biolabs (San Diego, CA, USA) [26].

Confocal microscopy for Bax and cytochrome c

Freshly isolated eosinophils or EoL-1 human eosinophil cell lines were seeded (5×10^4 cells in 0.2 mL of RPMI 1640/well) on 8-well poly-D-lysine-coated culture microslides (Santa Cruz) overnight. After treatment with VacA for the indicated time period, cells were incubated with 100 nM Mitotracker RED CMXRos for 15 min at 37°C and fixed with 4% paraformaldehyde in PBS. For permeabilization, cells were treated with 0.2% Triton X-100 in PBS for 10 min at room temperature and blocked with

5% normal goat serum in PBS/Triton X-100 for 1 h, after which the cells were incubated with anti-Bax, the active form of specific anti-Bax-NT, or anti-cytochrome c antibodies for 3 h. Cells were then treated with Alexa fluor 488-conjugated secondary antibody against rabbit IgG for 1 h. Vectashield mounting medium with DAPI was applied to the cells and images were captured using a Confocal Laser Scanning Microscope LSM 5 Exciter (Carl Zeiss) independently with 488 (green)- and 543 (red)-nm lasers.

Statistical analyses

A Wilcoxon's rank sum test was used for statistical analysis and *p*-values less than 0.05 were considered statistically significant.

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Abbreviations: **c-IAP:** cellular inhibitor of apoptosis protein · **JC-1:** 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethyl benzimidazlcarbocyanine iodide · **PBE:** peripheral blood eosinophil · **TEM:** transmission electron microscopy · **TMRE:** tetramethylrhodamine ethyl ester · **VacA:** vacuolating cytotoxin

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