RELATIONSHIP BETWEEN VAC A TOXIN AND AMMONIA IN HELICOBACTER PYLORI-INDUCED APOPTOSIS IN HUMAN GASTRIC EPITHELIAL CELLS

VacA toxin is one of the most important virulence factors produced by H. pylori even though neither its role nor its action mechanisms are completely understood. First considered as a toxin inducing only cell vacuolation, VacA causes apoptosis of gastric epithelial cells by targeting mitochondria. A hotly debated question about VacA action is its relationship with ammonia, which is produced in vivo by H. pylori urease. While ammonia is strictly required for VacA-dependent vacuolation, its role in VacA-induced apoptosis is much less defined. This study was thus aimed to investigate the relationship between VacA toxin and ammonia in H. pylori-induced mitochondrial damage and apoptosis of human gastric epithelial cells in culture by means of flow cytometry. Our results show that, unlike cell vacuolation, in MKN 28 cells neither apoptosis nor dissipation of mitochondrial transmembrane potential induced by VacA require ammonia. Nevertheless, ammonia significantly potentiates both these VacA-induced effects, but independently of the swelling of VacA-containing endosomes (i.e., vacuolation). Our findings make unlikely the hypothesis that ammonia-dependent swelling and rupture of endosomal vesicles in which VacA is sequestered after cell internalization may allow the toxin to reach mitochondria and trigger apoptosis.

Key words: Helicobacter pylori, VacA toxin, apoptosis, gastric epithelial cells, gastric infection

INTRODUCTION

Helicobacter pylori (H. pylori) is a gram-negative microaerophilic spiral bacterium that colonizes the stomach of approximately half of the world’s population. Colonization of the stomach by H. pylori causes chronic gastritis that, during the decades that follow initial infection, can remain silent or evolve into more severe diseases, such as atrophic gastritis, peptic ulcer, lymphoma of the mucosa-associated lymphoid tissue (MALT) or gastric adenocarcinoma (1–4). The strong association between H. pylori infection and gastric cancer led the World Health Organization to classify H. pylori as a class 1 carcinogen (5). Cancer risk is believed to be related to differences among H. pylori strains and inflammatory responses governed by host genetics (3, 4). In particular, specific interactions between host factors that modulate the response to the infection, and bacterial virulence factors that can directly cause tissue damage seem to have a major pathogenic role in the development of gastric cancer (3, 4).

Among several virulence factors produced by the bacterium, a pivotal role in H. pylori pathogenic action is played by the vacuolating toxin VacA (reviewed in 3, 6, 7). VacA is a protein toxin, formed by monomers of ~90 kDa, able to induce cytoplasmic vacuoles in eukaryotic cells in culture (6, 7–9). Cytoplasmic vacuoles are also present in vivo in the gastric epithelium of H. pylori-colonized patients (10-12). When given to mice, purified VacA causes gastric epithelial damage closely resembling that found in H. pylori-colonized patients (13). After cell internalization, VacA localizes in the endocytic-endosomal compartment from which vacuoles originate (14–16). Vacuole development is strictly dependent on the presence in the incubation medium of weak bases like ammonia (which can be generated by urease activity, a hallmark of H. pylori) (15, 17). It has been reported that VacA may act as a channel-forming toxin and it has also been proposed that VacA channels play a direct role in cell vacuolation. Endocytosed VacA channels could stimulate the turnover of endosomal V-ATPase by increasing the permeability of the endosomal membrane to anions (reviewed in 6, 9). This would lead to the accumulation of osmotically active species (such as NH₄⁺) causing an osmotic imbalance of late endosomes with subsequent vacuole formation. However, despite about 20 years of research, it is yet unknown the role of VacA-induced vacuolation as well as the main function of VacA toxin in the overall virulence of H. pylori. It has been indeed proposed that VacA acts as a multifunctional toxin that can have pleiotropic effects on mammalian cells and tissues modulating both several epithelial cell functions and immune cell functions (3, 7). A few years ago, our group demonstrated that VacA (both the whole molecule and its N-terminal moiety) can reach mitochondria, leading to epithelial cell apoptosis through cytochrome c release (18). In this respect, it is worth noting that one mechanism by which H. pylori increases the risk for carcinogenesis is by altering cell turnover and mounting evidence suggests that H. pylori is associated with an increased
level of apoptosis in vivo (19-22). Interestingly, Cover et al. (22) demonstrated that, in AGS gastric epithelial cells in culture, VacA-induced apoptosis was strictly dependent on the presence of ammonia (at a concentration not apoptogenic by itself) in the culture medium and thus closely associated with the development of VacA-induced vacuoles. This observation raised the hypothesis that the requirement of ammonia (and thereby of vacuolation) for VacA to elicit apoptosis might be due to the need for VacA to escape from endosomes for reaching the cytosol and then the mitochondria where it triggers cytochrome c release (6). A simple mechanism for VacA release from an internal vesicular compartment would be indeed to induce swelling of the vesicles until they rupture. Nevertheless, Willhite et al. (23) suggested that cell vacuolation and mitochondrial damage were independent outcomes of VacA toxin activity, even though vacuolation temporally preceded cytochrome c release, both dependent on the formation of anion-selective membrane channels by the toxin at the endosomal or mitochondrial level, respectively. Recently, using AZ-521 gastric cells in culture, Yamasaki et al. (24) suggested however that VacA induces epithelial cell apoptosis via the mitochondria-dependent pathway not directly acting on mitochondria but rather activating the proapoptotic Bcl-2 family protein Bax. Ammonia would not be necessary for VacA-induced Bax activation and apoptosis, but would increase VacA action in a vacuolation-independent fashion (24).

This study was thus designed to further investigate the relationship between VacA toxin and ammonia in H. pylori-induced mitochondrial damage and apoptosis of human gastric epithelial cells in culture by means of flow cytometry.

MATERIAL AND METHODS

H. pylori growth and VacA toxin purification

VacA-producing (s1a/m1 vacA genotype) H. pylori strain 60190 (ATCC 49503) was grown in Brucella broth (Difco, Detroit, MI) supplemented with 1% Vitox (Oxoid, Basingstoke, UK) and 0.2% β-cyclodextrins (Sigma-Aldrich, St. Louis, MO) for 36-48 h at 37°C under microaerobic conditions and continuous shaking. VacA was purified from broth culture supernatant by ammonium sulphate precipitation and gel filtration chromatography using a Superose 6 10/300 GL column (Amersham Biosciences, Umeå, Sweden) in accordance with Cover et al. (25) and its concentration was determined using the DC protein assay kit (Bio-Rad, Heracles, CA). Purified VacA was stored in melting ice and, immediately before use, was activated by dropwise acidification to pH 3.0 with 0.2 N HCl (25, 26).

Human gastric epithelial cells in culture

We used the MKN 28 cell line, derived from a human gastric tubular adenocarcinoma, which is known to retain gastric type differentiation (14, 27, 28) and to provide a suitable model for the study of the response of gastric epithelial cells to H. pylori (14, 15, 26, 29-31). MKN 28 cells were grown in DMEM/Ham’s nutrient mixture F-12 (1:1) (Sigma) supplemented with 10% foetal calf serum (FCS; from Cambrex BioScience, Verviers, Belgium) at 37°C in a humidified atmosphere of 5% CO₂ in air and used at 30-40% confluency.

Experimental design

Cell monolayers were washed twice with Hank’s balanced salt solution (HBSS) and incubated at 37°C for different times (ranging from 1 to 24 h) with different doses (ranging from 2 to 50 nM) of activated purified VacA, diluted in HBSS in the absence or presence of 5 mM NH₄Cl, or, in some experiments, of 0.1 mM chloroumine. Equivalent volumes of acid-treated buffer alone, diluted in HBSS in the absence or presence of 5 mM NH₄Cl, were used as controls. At various time points, variously treated cell monolayers were then analyzed for cell apoptosis and loss of mitochondrial transmembrane potential (MTP) by flow cytometry. In some experiments, cells were preincubated for 1 h with 30 nM bafilomycin A₁, (BAFA1, a specific inhibitor of the endosomal V-ATPase) which was maintained during the subsequent incubation period with VacA or control. Morphologic appearance of variously treated cell monolayers in the absence or presence of BAFA1 was also evaluated using a phase-contrast inverted microscope (Diaphot 300; Nikon, Tokyo, Japan) equipped with a photocamera; representative microscopic fields were photographed by a technical assistant (V. Necchi) unaware of the treatment.

Assessment of apoptosis

To measure the degree of apoptosis of variously treated cell monolayers we used two different assays, the annexin V-labelling assay (32, 33) and the uptake of the vital DNA dye Hoechst 33342 (34, 35).

The binding of fluorescent annexin V is a widely used approach for the detection of phosphatidylserine exposure on the cell surface. Indeed, loss of plasma membrane asymmetry is an early event in apoptosis, independent of the cell type, resulting in the exposure of phosphatidylserine residues at the outer plasma membrane leaflet. Annexin V was shown to interact strongly and specifically with phosphatidylserine and can thus be used to detect apoptosis (32, 33). Phosphatidylserine exposure seems to last from the early execution phase of apoptosis until the final stage, at which the cell has broken up into apoptotic bodies (32, 33). When the plasma membrane looses its integrity, the cell becomes positive for both annexin V and non-vital DNA dyes such as propidium iodide, indicating a late apoptotic/necrotic stage of the cell.

For annexin V-labelling assay, we used the annexin V-Fluos staining kit from Roche (Penzberg, Germany) according to manufacturer’s instructions. Briefly, at the end of incubation time, the incubation medium was collected and the cell monolayer was split by means of TrypLE Express (Invitrogen, Carlsbad, CA), cell suspension was added to the corresponding incubation medium and centrifuged at 150 g for 5 min. Cell pellet was then incubated at RT for 15 min with 0.1 ml of annexin V-FITC-containing HEPES buffer. After dilution with 0.9 ml of HEPES buffer, each sample was immediately analyzed by flow cytometry.

Another specific and sensitive apoptosis assay for unfixed cells is based on the observation that, owing to changes in membrane permeability, early apoptotic cells show an increased uptake of the vital DNA dye Hoechst 33342 compared with non-apoptotic live cells, when stained with the dye at low concentration for a short time (34, 35). Co-staining with a non-vital DNA dye such as propidium iodide or 7-amino-actinomycin D allows discrimination of either late apoptotic or necrotic cells from early apoptotic cells (35). Indeed, late apoptotic cells, which have completely lost membrane integrity, take up the non-vital DNA dye as well, while necrotic cells lose membrane integrity early and become positive for the non-vital DNA dye without passing through a stage of increased uptake of Hoechst 33342 (35).

For the uptake of Hoechst 33342 dye, cell monolayers were processed as above, each cell pellet was incubated at 37°C for 10 min with 1 ml PBS containing 1 µg/ml Hoechst 33342 and 0.1
µg/ml propidium iodide, and then immediately analyzed by flow cytometry.

Measurement of loss of mitochondrial transmembrane potential

The uptake of the potential-sensitive dye tetramethylrhodamine ethyl ester perchlorate (TMRE) was used to measure relative MTP (36). TMRE is a positively charged, lipophilic fluorescent dye commonly used to make qualitative and quantitative measurements of MTP (37). Lipophilic cations accumulate in the mitochondrial matrix, driven by the MTP according to the Nernst equation, which states that at 37°C a hyperpolarization by 61.5 mV corresponds to a 10-fold increase in the intramitochondrial concentration of monovalent cations (38). Since in physiological conditions the MTP ranges from 120 to 180 mV (the intramitochondrial side being electronegative), the concentration of such cations is normally 2-3 logs higher in the mitochondrial matrix than in the cytosol (38). Thus a membrane-permeant cationic fluorochrome can be used to measure MTP, whose dissipation is a hallmark of early apoptosis in vitro and ex vivo (39).

Briefly, at the end of various incubation times, TMRE (from Molecular Probes, Eugene, OR; final conc.: 50 nM) was added to each cell monolayer which was incubated at 37°C for 5 min. After resuspension in 1 ml of its own supernatant, each sample was immediately analyzed by flow cytometry.

Flow cytometry

For each sample, at least 20,000 events were analyzed by a PAS II flow cytometer (Partec, Münster, Germany) equipped with two excitation sources: 1) an argon ion laser with 20 mW output power at 488 nm, and 2) a 100W mercury arc lamp used for UV excitation. Light emission was measured in the following channels: a) FL1 (510-535 nm bandpass filter) for annexin V-FITC, b) FL2 (540-590 nm bandpass filter) for TMRE, c) FL3 (600-650 nm bandpass filter) for propidium iodide, and d) FL4 (430-480 nm bandpass filter) for Hoechst 33342.

Data were recorded and analyzed using the FlowMax software from Partec.

Statistics

Results are expressed as means ± SEM of at least 3 independent experiments. The statistical significance of the differences was evaluated by analysis of variance (ANOVA) followed by Newman-Keuls's Q-test; significance was set at $P<0.05$.

RESULTS

Flow cytometry permits rapid, quantitative, cell-based determination of apoptosis compared with other classic techniques, such as DNA ladder formation by gel electrophoresis and morphological examination by electron or light microscopy (35). Many different flow cytometric staining methods that target various cellular attributes of the apoptotic pathway have been developed (40).

By using annexin V-labelling assay, in preliminary experiments we found that MKN 28 cells underwent VacA-induced apoptosis in a time- and dose-dependent fashion with the peak occurring at 24 h of incubation with 50 nM acid-activated purified VacA (Fig. 1A, B). Therefore, we focused our subsequent analyses on events occurring at this time point with the aforementioned toxin concentration. Figs. 1A, B and 2A, C show that VacA induced a statistically significant apoptosis even in the absence of ammonium chloride. Nevertheless, the addition to the incubation medium of 5 mM ammonium chloride (i.e., a concentration well within the in vivo range for H. pylori-infected patients (41) and which is the most commonly used to reveal ammonia-dependent effects of purified VacA on cultured epithelial cells (22-24)) did not induce apoptosis by itself in our cellular model, but significantly increased the apoptotic action of VacA (Fig. 2A, C). To assess whether ammonia-dependent increase in apoptosis was accounted for by ammonia-dependent vacuole development, we carried out another set of experiments in which vacuole formation was blocked by treating cells with a specific inhibitor of the endosomal V-ATPase (i.e., BAFA1). As shown in Fig. 2B, C, ammonia exhibited an increasing action on VacA-induced apoptosis also in BAFA1-treated cells in which no vacuolation occurred under phase-contrast microscopy evaluation (Fig. 3).

By using Hoechst 33342/propidium iodide-labelling assay, we found that MKN 28 cell monolayers treated with VacA for 24 h in the absence of ammonium chloride exhibited a statistically significant increase in both early and late apoptotic cells compared to control cells (Fig. 4A, C). When MKN 28 cell monolayers were treated with VacA for 24 h in the presence of 5 mM ammonium chloride, we found an increased percentage of early apoptotic cells compared to monolayers treated with VacA alone, while we found no difference in the percentage of late apoptotic cells (Fig. 4A, C). The same behaviour was observed also in BAFA1-treated cells (Fig. 4B, C) further confirming that the potentiating action of ammonia on VacA-induced apoptosis was not accounted for by ammonia-dependent vacuole development.
Taking that it is now widely accepted that VacA causes apoptosis through the intrinsic (i.e., mitochondria-dependent) pathway (6, 18, 24, 36), we finally investigated the effect of VacA and ammonia on the MTP of MKN 28 cells in culture by measuring TMRE uptake in variously treated cell monolayers. By flow cytometry evaluation of TMRE uptake, we found that MKN 28 cell monolayers treated with VacA for 24 h in the absence of ammonium chloride exhibited a statistically significant decrease in MTP compared to control cells (Fig. 5A, C). Nevertheless, the addition to the incubation medium of 5 mM ammonium chloride, a concentration not significantly affecting MTP by itself in our cellular model, significantly potentiated the VacA-induced decrease in MTP (Fig. 5A, C). Once again, the potentiating action of ammonia was not accounted for by ammonia-dependent vacuole development since it was completely preserved in BAFA1-treated cells (Fig. 5B, C).

![Fig. 2. Flow cytometry evaluation of FITC-labelled annexin V binding to MKN 28 cells incubated for 24 h with either acid-treated buffer diluted in HBSS (control) or 50 nM VacA, in the absence or presence of 5 mM ammonium chloride. R1: non-apoptotic cells. R2: apoptotic cells. A: Representative experiment carried out without BAFA1 treatment. B: Representative experiment carried out with BAFA1 treatment (see Methods). C: Statistical significance of the differences in R2 data was evaluated. Means ± SEM of 3 independent experiments. *, P<0.05 versus control; **, P<0.05 versus VacA.](image)

![Fig. 3. Phase-contrast microphotographs of variously treated subconfluent MKN 28 cell monolayers in the absence (A-D) or presence (E-H) of BAFA1 (see Methods). A, E: acid-treated buffer diluted in HBSS (control). B, F: acid-treated buffer diluted in HBSS plus 5 mM ammonium chloride. C, G: 50 nM acid-activated purified VacA in HBSS. D, H: 50 nM acid-activated purified VacA in HBSS plus 5 mM ammonium chloride. Note that VacA was causing cell vacuolation only in the presence of ammonium chloride (which by itself caused the development of a few small vacuoles only). BAFA1 was able to completely inhibit cell vacuolation (induced either by ammonium chloride alone or by VacA in the presence of ammonium chloride) without causing, by itself, any apparent cell damage. Original magnification: x100.](image)
To investigate whether the potentiating activity of ammonia on VacA-induced action was might accounted for by a specific effect of this substance or, on the contrary, also other weak bases may exert a similar action, we performed a set of experiments using 0.1 mM chloroquine instead of 5 mM ammonium chloride. Chloroquine is a weak base known to have an action similar to that of ammonia in the development of VacA-dependent cell vacuolation (17, 42). We found that chloroquine as well was able to potentiate both apoptosis and MTP decrease induced by VacA, causing, compared to cells treated with VacA in the absence of chloroquine, a) a statistically significant (P<0.05) increase in annexin V labelling either in the absence or presence of BAFA1 treatment (30 ± 4 % and 28 ± 4 %, respectively), and b) a statistically significant (P<0.05) decrease in TMRE uptake either in the absence or presence of BAFA1 treatment (32 ± 5 % and 35 ± 4 %, respectively).

**DISCUSSION**

*H. pylori* is a main causative agent of gastric cancer that, despite its declining incidence, remains the fourth most common cancer, the second leading cause of cancer-related death, and the fourteenth most common cause of death overall worldwide, killing more than 700,000 people each year (2, 3). It is thus of highest interest to better understand the molecular mechanism by which this microorganism interacts with its human host so as to trigger potentially harmful sequelae. It is now widely accepted that the exotoxin VacA is one of the most important virulence factors produced by *H. pylori* even though neither its role nor its molecular mechanisms of action are completely understood (3, 6, 7, 9). Our group demonstrated that VacA, first considered as a toxin inducing only cell vacuolation, was in fact promoting apoptosis of gastric epithelial cells by targeting mitochondria (18).
The apoptotic activity of VacA via mitochondria has been confirmed by several groups and is now widely accepted as a main activity of this toxin (7, 22, 43). Nevertheless, it is still unclear how VacA exerts its apoptotic action. For instance, it is completely unknown how VacA reaches mitochondria where it could trigger apoptosis by inducing mitochondrial membrane changes correlated to toxin channel activity (6, 18, 36, 43). In this respect, confirming our previous finding (15) that most of VacA was localized to vacuoles rather than mitochondria, Yamasaki et al. (24) suggested that VacA might trigger mitochondria-dependent apoptosis without directly reaching mitochondria but rather activating the proapoptotic Bcl-2 family protein Bax through a yet unidentified mechanism. However, it is worth noting that a common feature of bacterial toxins is that only a small fraction of the overall amount of toxin internalized by the cell is sufficient to fully exert the toxic action; for instance, in the case of diphtheria toxin, one molecule escaping endosomes and reaching the cytosol is sufficient to kill the cell (44). A hotly debated question about VacA cytotoxic action is its relationship with ammonia, which is produced in vivo by H. pylori urease. It is widely accepted that ammonia is strictly required for the development of VacA-dependent vacuolation (15, 17); on the contrary, its role in VacA-induced apoptosis is much less defined. The observation of Cover et al. (22) that also VacA-induced apoptosis was strictly dependent on the presence of ammonia raised the intriguing hypothesis that H. pylori might have developed a novel, highly ingenious strategy to allow its toxin to reach, after cell internalization by endocytosis, its ultimate cell target (i.e., the mitochondrion): ammonia-dependent swelling and rupture of endosomal vesicles in which VacA is sequestered. Nevertheless, it was found that vacuolation was not a prerequisite for VacA-induced mitochondrial damage (23), even though ammonia exerted a potentiating action on VacA-induced apoptosis (24).

The results here presented clearly show that, unlike cell vacuolation, in MKN 28 cells neither apoptosis nor MTP dissipation induced by VacA require ammonia. Nevertheless, ammonia significantly potentiates both these VacA-induced effects, but independently of the swelling of VacA-containing endosomes (i.e., vacuolation) since ammonia-dependent potentiation is present also in BAFA1-treated cells in which vacuole genesis is completely abolished. In addition, we found that the potentiating activity of ammonia on VacA-induced action is not accounted for by a specific effect of this substance taking that other weak bases (like chloroquine) may exert a similar action either in the absence or presence of BAFA1 treatment and thus independently of vacuole genesis. Thus our findings make unlikely the vesicle rupture model as an escape mechanism for VacA to reach mitochondria. Ammonia (and other weak bases) might act either causing an overall cell stress that favours Bax recruitment to mitochondria or directly sensitizing mitochondria to VacA-induced damage (45).

Interestingly, our group has recently demonstrated that dynamic comet-like F-actin structures play a pivotal role in intracellular trafficking of VacA connecting the GPI-anchored protein-enriched early endosomal compartment, where VacA is initially endocytosed, to late endosomes where vacuolation occurs.

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A possible scenario could thus envisage an early delivery of some VacA molecules to mitochondria by direct interaction between these organelles and the highly motile VacA-containing vesicles propelled by dynamic F-actin tails. In this respect, it is worth noting that organelle to organelle contact between iron-containing early endosomes and mitochondria has been reported as a likely mechanism to replenish mitochondrial iron by direct transfer (47). This early arrival of a small amount of VacA molecules could trigger an initially limited mitochondrial damage (with decreased MTP and cytochrome c release) but sufficient to cause further mitochondrial damage. Ammonia could play a role (even though yet undefined) in this amplification loop. Our recent observation (Oldani et al., manuscript in preparation), using the ratiometric potential-sensitive dye JC-1 and confocal microscopy, of focal MTP decrease induced by VacA in MKN 28 cells as early as 1 hour after toxin exposure and not affected by the presence or absence of ammonia further supports this hypothesis. This model would reconcile previous findings suggesting the need for VacA-induced apoptosis of a direct damaging action of VacA on mitochondria (18, 36) with those stressing the role of Bax recruitment (24).

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Author’s address: Prof. V. Ricci, Department of Physiology, Human Physiology Section, Via Forlanini 6, 27100 Pavia, Italy. Phone: +39-0382-987254; Fax: +39-0382-987254; e-mail: vricci@unipv.it