

Apoptotic effect of *Vibrio vulnificus* cytolysin on A549 human lung adenocarcinoma cells

JIACHUN SUN, JING ZHENG, GUIMING WANG, YAN LI and HONG SHEN

Department of Pathology, Southern Medical University, Guangzhou, Guangdong 510515, P.R. China

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Abstract. *Vibrio vulnificus* cytolysin (VVC) has a very strong cytotoxic effect on various types of mammalian cells. However, the inhibitory effect of VVC on the proliferation of human lung cancer cells has scarcely been reported. This study aimed to analyze the effects of recombinant VVC (rVVC) on the A549 human lung adenocarcinoma cell line and to investigate the underlying molecular mechanisms governing these effects. This study showed that rVVC inhibited the proliferation of A549 cells in a concentration- and time-dependent manner (as measured by the MTT assay). rVVC failed to induce the release of intracellular lactate dehydrogenase (LDH) from the target cells suggesting that osmotic lysis may not contribute to its cytotoxicity. Treatment of A549 cells with an IC_{50} (concentration of drug that inhibits cell growth by 50%) of rVVC resulted in morphological changes and blebbing typical of apoptosis. Annexin-V FITC analysis by FCM indicated that rVVC-induced apoptosis in A549 cells occurs in a dose-dependent manner. A DNA fragmentation assay was utilized to investigate the apoptosis induced by rVVC. The pro-apoptotic activity of rVVC was attributed to its ability to modulate, in a concerted manner, the expression of Bcl-2 and Bax proteins, which were down- and up-regulated, respectively. Caspase-9 and -3 were subsequently activated, however caspase-8 was not. These results prove that rVVC effectively induces programmed cell death and suggests that rVVC-induced apoptosis in the A549 cell line is mediated by the regulation of Bcl-2 protein expression and the activation of caspase-9 and -3.

Introduction

Vibrio vulnificus is a gram-negative, halophilic bacterium that inhabits marine and estuarine areas. It may cause wound infection, gastroenteritis and primary septicemia in humans. The mortality rates reach as high as 55% in primary septicemia

(1-3). Previous studies have demonstrated that various components and metabolites of *Vibrio vulnificus* such as its cytotoxin, toxic cytokines, MMPs and so on, may serve as toxic factors and play an important role in pathogenesis. Among these various pathogenic factors, *Vibrio vulnificus* cytotoxin (VVC) attracted our interest, since it perforates the cell membrane and exerts cytotoxic effects on mammalian cells.

VVC, a polypeptide with a molecular weight of 50851, is encoded by the *vvhA* gene. It is produced by most pathogenic strains, is water-soluble and demonstrates thermal instability. VVC is an extremely active exotoxin and is characteristic of the *Vibrio vulnificus* species. In addition to hemolytic activities (4-5), VVC destroys various other mammalian cells including mast (6,7), macrophages (8) and endothelial cells (9). Studies have shown that VVC perforates the target cell membrane by binding to cholesterol in the host cell membrane and plays an important role in the dissolution of cells (10,11). Rho *et al* found that a low concentration of VVC induced apoptosis in CAPE (12). Kwon *et al* studied the effects of VVC in EVC304 cells. The results from this study suggested that cell apoptosis induced by VVC was triggered by superoxide anion generation, cytochrome c release, caspase-3 activation, ADP ribose polymerase degradation and DNA cleavage (13). Recently Zhao *et al* also found that VVC induced apoptosis in HUVEC, SGC-7901 and SMMC-7721 cells through the activation of the caspase-9 and -3 pathway (14). Literature studies show that VVC produces potent cytotoxicity in various mammalian cells. However, few studies have been conducted to investigate the cytotoxic effects of VVC on lung cancer cells to date.

In this study, we analyzed the cytotoxic effects of recombinant (r)VVC on A549 cells (human lung adenocarcinoma cells) *in vitro* and investigated the possible mechanisms involved. Our results suggest that rVVC may down-regulate Bcl-2 while up-regulating Bax, thereby triggering apoptotic signals leading to the death of A549 cells.

Materials and methods

Culture of the bacterial strain and cell lines. *Vibrio vulnificus* ATCC27562 was purchased from the China General Microbiological Culture Collection Center, and was cultured in 2.5% NaCl heart infusion (HI) medium (Difco, USA) at 37°C. A549 cells were purchased from the Cell Bank of the Institute of Cellular Biology in Shanghai (Chinese Academy of Science) and were cultured in RPMI-1640 medium (Gibco,

Correspondence to: Professor Hong Shen, Department of Pathology, Southern Medical University, No. 1838 North Guangzhou Avenue, Guangzhou, Guangdong 510515, P.R. China
E-mail: shenhong20101681@163.com

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USA) supplemented with 10% FBS (Gibco) in humidified air with 5% CO₂ at 37°C.

Purification of rVVC. Genomic DNA of *Vibrio vulnificus* strain ATCC27562 was extracted by the phenol-chloroform method. The entire *vvhA* gene was amplified by PCR with specific primers: 5'-C-GGATCC-CAAGAATATGTGCCGATTGT-3' *Bam*HI (forward), and 5'-CCG-CTCGAG-CTAGAGTTTGACTTGTGT-3' *Xho*I (reverse). The PCR was initiated by incubation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec/cycle, 52°C for 30 sec and 72°C for 120 sec to amplify the *vvhA* gene. Finally, the PCR products were incubated at 72°C for 10 min. The PCR products were cloned into the pGEM-T vector (Takara, Japan) for sequencing. Having been confirmed by sequence analysis (Takara), the cloned *vvhA* gene was subcloned into the pET32a vector (Novagen, USA) by the *Bam*HI-*Xho*I sites to form pET32a-*vvhA*, and then the recombinant plasmid was transformed into competent *E. coli* BL21 (DE3) (Novagen, USA). rVVC was expressed by *E. coli* BL21 (DE3) pET-32a-*vvhA* under the induction of 1 mmol/l IPTG (Merck, Germany) at 30°C. After ultrasonication and centrifugation, the expression of rVVC was analyzed by SDS-PAGE Plus Bio-Rad Gel Image Analyzer (Bio-Rad, USA). Afterwards the cytolysin with His-tag in the cell lysate was purified by binding with Ni-IMAC resin (GenScript, USA).

Hemolysis assay. The hemolytic activity of rVVC against mouse erythrocytes was investigated according to the procedure described by Bernheimer and Schwartz (15). Briefly, the purified rVVC was diluted with 0.1% BSA-PBS (pH 7.4) to different concentrations, and incubated with the same volume of a 0.5% mouse erythrocyte suspension for different periods of time at 37°C. After centrifugation at 2000 rpm for 5 min, the absorbance values of each supernatant were assayed at 545 nm.

Cell viability assay. A549 cells in the logarithmic growth phase were plated at a density of 3×10^4 cells/ml in 96-well plates. Twenty-four hours later, 5, 10, 15, 20, 25 and 30 µg/ml of rVVC was added, respectively. After 24, 48 and 72 h of incubation, the culture medium was discarded and 20 µl MTT solution (5 mg/ml PBS) was added to each well. Then the plates were incubated for another 4 h at 37°C, the supernatant was removed and 200 µl of DMSO was added to each well. Finally, the optical density (OD) was assayed at a 570-nm wavelength using a Bio-Rad Model 680 microplate reader (Bio-Rad). Cell inhibition ratio (I%) and IC₅₀ were then calculated (16).

Detection of cellular LDH. A549 cells were seeded in 6-well plates at a density of 3×10^5 cells/ml and incubated for 24 h. The cells were subsequently incubated with 10 µg/ml rVVC at 37°C for 0.5, 2, 4 and 8 h, respectively. Normal controls were untreated with rVVC. The cells were harvested and after transient centrifugation, the supernatants were collected. The OD500 value was measured to examine the activity of LDH in the supernatants by dinitro-phenyl-hydrazine (DPNH) colorimetry.

Cell morphological assessment. A549 cells were seeded in 6-well plates and treated with 10 µg/ml rVVC for 24 h. At the end of the incubation, cell morphology was observed and

photographed using an inverted light microscope. Afterwards, the cells were collected and fixed with 4% formaldehyde in PBS for 15 min and stained with Hoechst 333258 (10 mg/ml) at room temperature for 10 min. Finally, after the cells were washed with PBS, the morphology of the cells was monitored under a fluorescence microscope and photographed at x300 magnification.

Cell ultrastructure observation. A549 cells were treated with 10 µg/ml rVVC at 37°C for 24 h, and then collected and washed with PBS, followed by fixation with 2.5% glutaral solution at 4°C and embedding in paraffin. Sections of the cell samples were observed and photographed by using transmission electron microscopy (Philips, Holland).

Annexin V-FITC/PI analysis. A549 cells were seeded in 6-well plates at a density of 3×10^5 cells/ml and incubated for 24 h. Then they were incubated with concentrations of 5, 10 and 20 µg/ml of rVVC for 24 h. Afterwards, they were harvested and treated according to the manufacturer's instructions for both the samples described above. Data acquisition and analysis were performed on a Becton Dickinson FACSCalibur flow cytometer using the Cell Quest software (Franklin Lakes, NJ, USA).

Measurement of DNA fragmentation. A549 cells were grown in 6-well plates at a density of 3×10^5 cells/ml and were incubated without rVVC and with 5, 10 and 20 µg/ml rVVC for 24 h. Then cells were harvested, washed, and lysed in buffer (10 mM Tris-HCl pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6% SDS) at 55°C for 3 h, followed by the addition of RNase (4 mg/ml), and further incubation at 55°C for 18 h. The lysates were centrifuged at 10,000 x g for 20 min. The genomic DNA in the supernatant was electrophoresed on a 1.5% agarose gel at 100 V for 2 h and analyzed. The fragmented inter-nucleosomal DNA was visualized using a UV trans-illuminator.

Western blot analysis. A549 cells were treated with rVVC at different concentrations (0, 5, 10 and 20 µg/ml) for 24 h. Then the cells were re-suspended in cell lysis buffer (1% Triton X-100, 0.015 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 10 µg/ml of each leupeptin and pepstatin A) and then incubated on ice for 30 min. The cell lysates were centrifuged at 12000 x g for 15 min at 4°C. One part of the supernatant was mixed with four parts of SDS sample buffer, boiled for 5 min, then separated through a 12% SDS-PAGE gel. After electrophoresis, the proteins were transferred to nylon membranes following the manufacturer's instructions (Bio-Rad). The membranes were blocked in 5% dry skimmed milk (1 h), rinsed, and incubated with primary and secondary antibodies (diluted 1:2000) in TBS at room temperature for 2 h. Finally each protein was detected using an enhanced chemiluminescence system (GE Healthcare, USA).

Caspase activity detection. After incubation with 10 µg/ml rVVC for 24 h, A549 cells (5×10^6) were collected and washed with PBS. The collected cells were treated according to the protocols described in the Caspase-3, -8 and -9 fluorometric assay kits (Biovision, USA). The activities of caspase-3, -8 and -9 were measured using a spectrofluorometer (Hitachi,

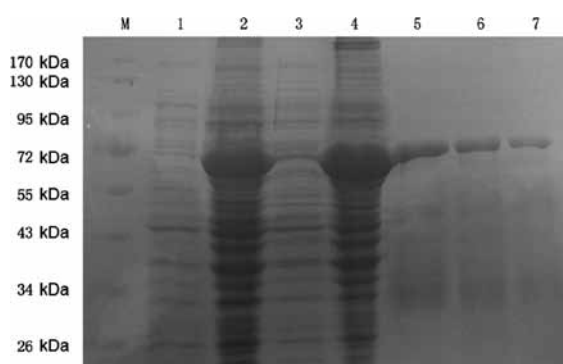


Figure 1. Expression and purification of rVVC. rVVC was expressed by *E. coli* BL21(DE3)pET-32a-vvhA (2) under the induction of 1 mmol/l IPTG at 37°C and (1) control bacteria were left un-induced. (3) Expressed rVVC was present in the supernatant and (4) precipitant of the lytic bacteria, and (5-7) the purified rVVC presented a single protein fragment.

Japan) with an excitation and an emission wavelength of 400 and 505 nm, respectively.

Statistical analysis. Data are expressed as the means \pm SD. Statistical analysis was performed with ANOVA using SPSS 13.0 software. Differences were considered significant when P-value was <0.05 .

Results

Purification of rVVC. rVVC (~70 kDa) was expressed by *E. coli* BL21(DE3)pET-32a-vvhA in LB medium under the induction of 1 mmol/l IPTG, and its yield amounted to approximately 58% of the total bacterial protein (Fig. 1). Since the recombinant protein which we obtained was expressed in insoluble (inclusion body) forms, we used the method of dialysis to refold the diluted purified protein using a refolding medium with 15% glycerol, thus ensuring stability of the protein.

Hemolytic activities of rVVC towards mouse erythrocytes. The purified rVVC was confirmed to possess hemolytic activity by a hemolysis test. After incubation with mouse erythrocytes for different periods of time and at different concentrations, VVC showed hemolytic activity that was both incubation time- and dose-dependent (Fig. 2A).

Cell viability assay. An MTT assay showed that rVVC significantly inhibited the proliferation of A549 cells (Fig. 2B). Cells were incubated in the absence and presence of different rVVC concentrations for 24, 48 and 72 h. rVVC had a significant growth-inhibiting effect on the A549 cell line. The inhibitory effects of rVVC on A549 cells was dose- and time-dependent. The IC_{50} value (24 h) for rVVC treatments was 8.59 μ g/ml.

Detection of cellular LDH. As shown in Fig. 2C, after treatment with 10 μ g/ml rVVC at 37°C for different periods of time, there was no significant change in LDH activity in all supernatants of the rVVC-treated cells ($P>0.05$).

Cell morphological assessment. Fluorescence microscopic analysis showed marked morphological features of apoptosis

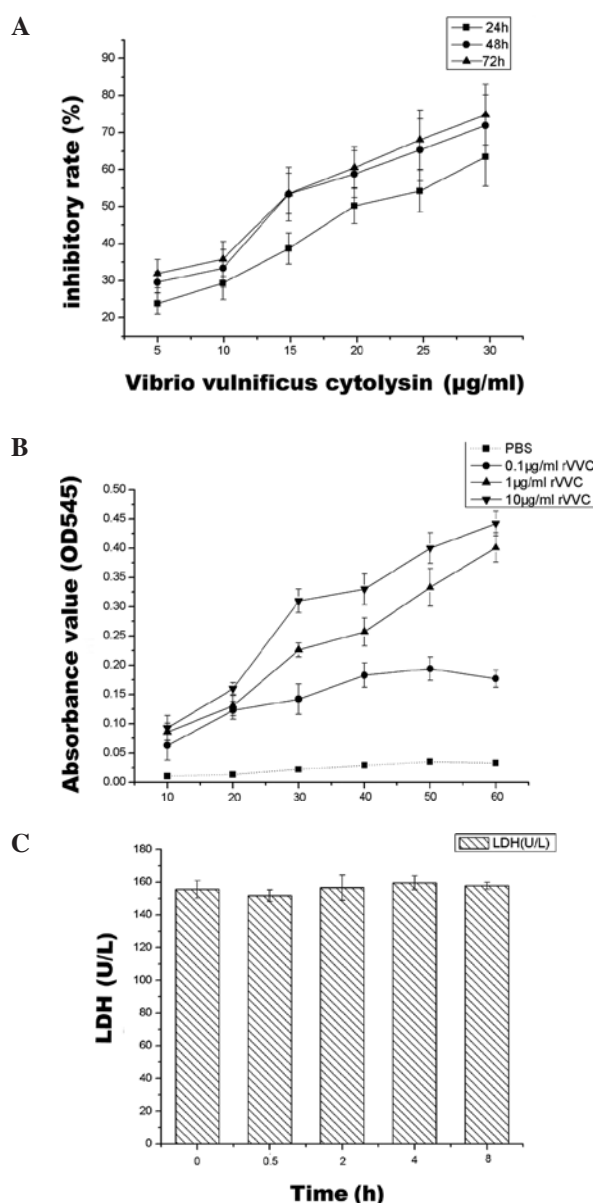


Figure 2. (A) Hemolytic activity of the rVVC towards mouse erythrocytes (mean \pm SD, $n=3$). Mouse erythrocytes were incubated with 0.1, 1 and 10 μ g/ml rVVC for various time intervals, and the OD545 in supernatants was measured to reflect the hemolytic activity of rVVC. (B) Growth-inhibitory effects of rVVC on A549 cells. Cells were treated with various concentrations of rVVC as indicated. (C) Effects of rVVC on LDH activity in A549 cells. A549 cells were treated with 10 μ g/ml rVVC for 0.5, 2, 4 and 8 h and LDH activity was detected by dinitro-phenyl-hydrazine (DPNH) colorimetry. Data are shown as the mean \pm SD ($n=3$); $P>0.05$ vs. the untreated controls.

in A549 cells such as brighter nuclei and nuclear condensation when treated with rVVC (10 μ g/ml) for a period of 24 h (Fig. 3B). A549 cells were incubated with 10 μ g/ml rVVC for 24 h and typical apoptotic characteristics including crescent-shaped nuclei and chromatin condensation were found under transmission electron microscopy (Fig. 3C).

Apoptosis assessment by Annexin-V and PI double-staining assay. The percentage of apoptotic cells (Annexin-V⁺/PI⁺ and Annexin-V⁺/PI⁻) in the control group was 7.01%. After treatment with 5, 10 and 20 μ g/ml rVVC for 24 h, the percentages

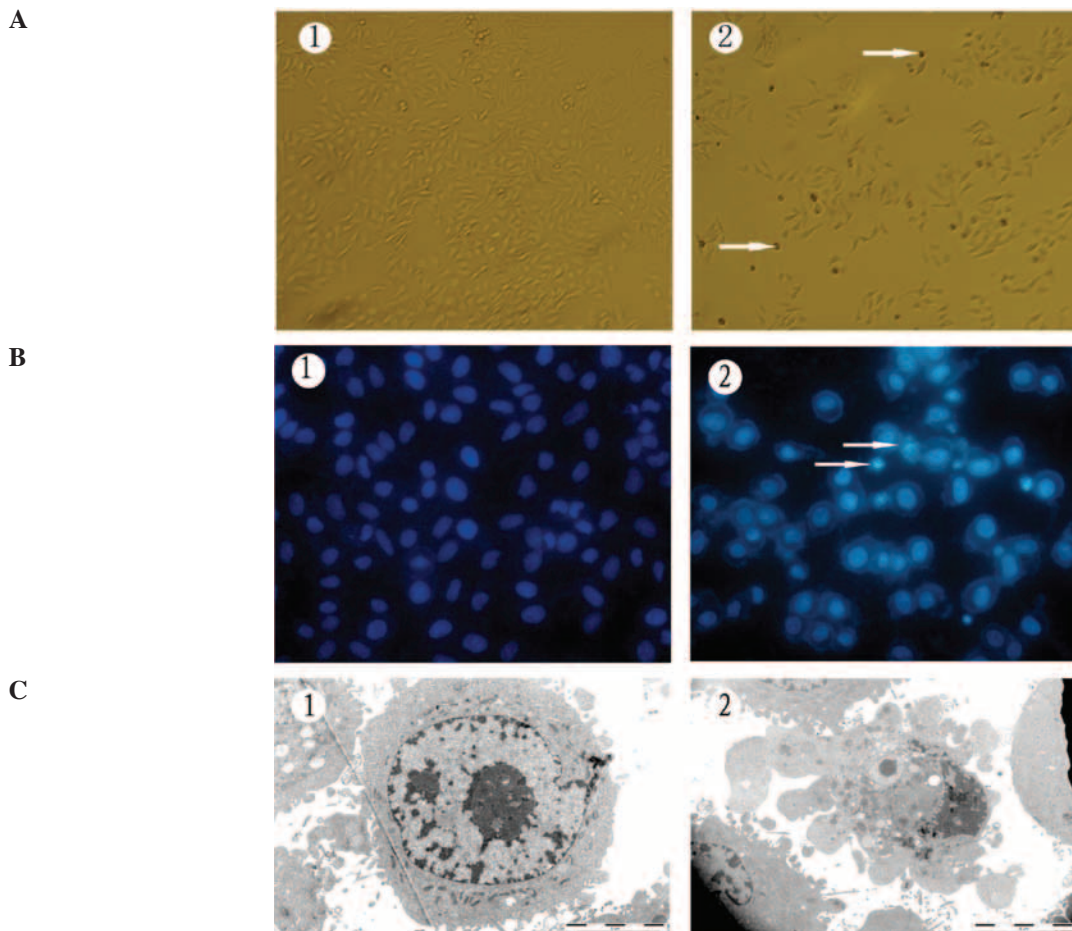


Figure 3. Morphologic changes of A549 cells treated with 10 $\mu\text{g/ml}$ rVVC for 24 h compared with the untreated cells (control). Cells were grown in 6-well plates and treated in the presence or absence of rVVC for 24 h. Exposure of A549 cells to 10 $\mu\text{g/ml}$ rVVC provoked significant morphological changes: (A1-2) Cells had shrunk and retracted from their neighbors, floating cells appeared in the culture medium and most of the A549 cells lost their flat, polygonal shape. (B1-2) Nuclear morphology was also observed by Hoechst 333258 staining. Arrows identify apoptotic cells with bright, nuclear condensation. Cells were incubated with 10 $\mu\text{g/ml}$ rVVC for 24 h, and photographed under transmission electron microscopy at x6200 magnification. (C1-2) Typical apoptotic characteristics were observed in A549, compared with the untreated A549.

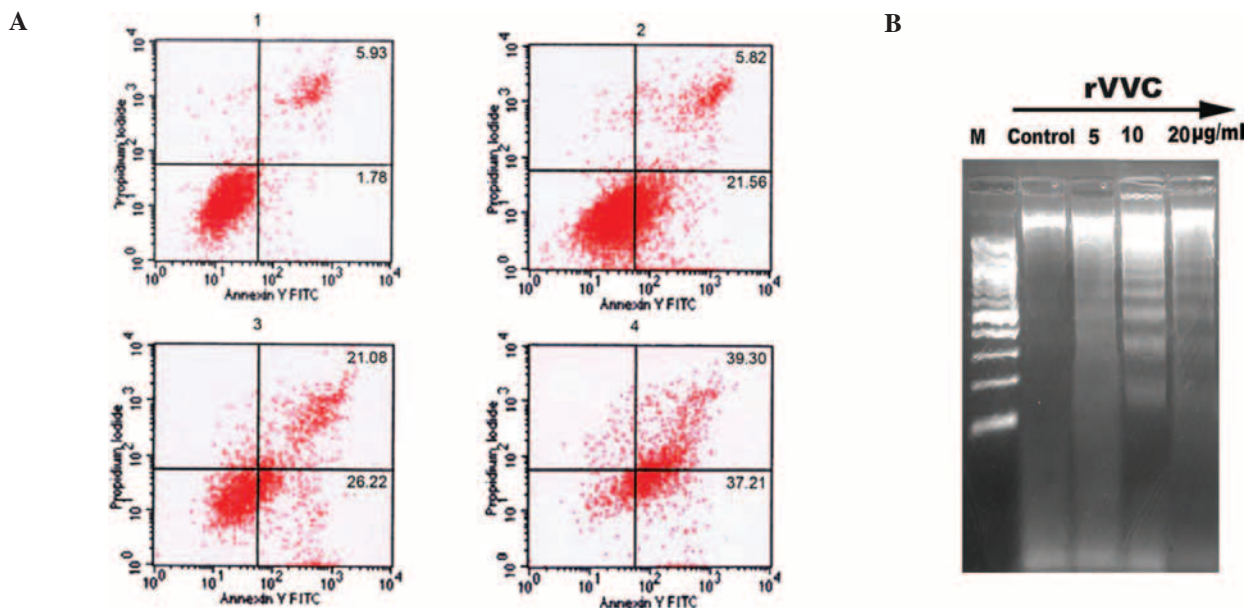


Figure 4. (A) Dosage-dependent apoptosis in rVVC-treated cells detected by flow cytometry. Cells were incubated with various concentrations of rVVC for 24 h and apoptosis was detected by flow cytometric analysis after staining with Annexin V-FITC and propidium iodide (PI). Numbers 1-4 represent the normal control, and the cells treated with 5, 10 and 20 $\mu\text{g/ml}$ rVVC for 24 h, respectively. (B) Analysis of DNA integrity in the rVVC-treated A549 cells by agarose electrophoresis. A549 cells were treated with different concentrations (5, 10 and 20 $\mu\text{g/ml}$) of rVVC for 24 h, DNA from the cells was extracted and electrophoresed on a 1.5% agarose gel and visualized using a UV trans-illuminator.

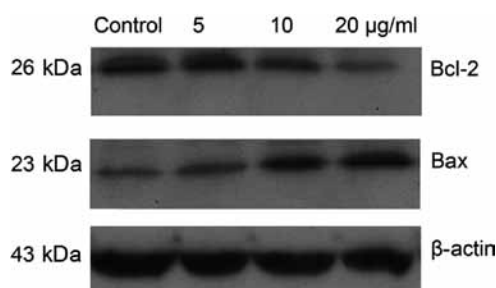


Figure 5. Bax and Bcl-2 expression. Whole cell lysate was obtained and used for Western blot analysis with Bax and Bcl-2 antibodies. β -Actin was used as an equal loading control.

of apoptotic cells were 27.38, 47.30 and 76.51%, respectively (Fig. 4A).

DNA fragment assay. Chromosomal DNA from untreated and A549 cells treated with rVVC (5, 10 and 20 μ g/ml) for 24 h, was isolated, extracted and resolved using agarose gel electrophoresis. Results of the DNA integrity analysis showed that rVVC (5, 10 and 20 μ g/ml) caused the digestion of genomic DNA into ladders as was expected (Fig. 4B).

Expression of Bcl-2 and Bax proteins. Western blot analysis indicated that the expression of Bcl-2 protein decreased slightly, while the expression of Bax protein dramatically increased when compared to the control. This resulted in a large change in the Bcl-2/Bax ratio (Fig. 5).

Activation of caspase-9 and caspase-3 during rVVC-induced apoptosis. As shown in Fig. 6, there was an increase in caspase-3 activity in the rVVC-treated A549 cells (Fig. 6A). Caspase-9 activity also exhibited an increase (Fig. 6B). However, no alteration in caspase-8 activity was found in all of the cells tested (Fig. 6C). These data indicate that the apoptosis induced by rVVC was mediated by caspase-9 activation rather than caspase-8 activation.

Discussion

Although VVC is an exotoxin that is characteristic of *Vibrio vulnificus*, the yield of secretion of natural cytotoxin from *Vibrio vulnificus* culture is relatively low (3). It is very difficult to attain sufficiently high purity of VVC by routine biochemical methods. Therefore, we constructed the pET32a (+)-vvhA expression vector and also succeeded in optimizing the conditions required for the expression, purification and renaturation of the toxin. This has laid the foundation for further studies on the biological activity of rVVC.

It has been demonstrated that other pore-forming toxins such as staphylococcal-toxin (17), streptolysin-O (18) and *Vibrio cholerae* E1 Tor cytotoxin (19) possess cytotoxicity toward nucleated cells. Their mechanism of the cytotoxic activity mainly involves 'punching' holes in the cell membrane and causing the dissolution of target cells. It was reported that a member of the pore-forming toxin family, VVC, binds to the surface of target cell membranes, forms oligomers and creates holes in the cell membrane. The pore-mediated osmotic

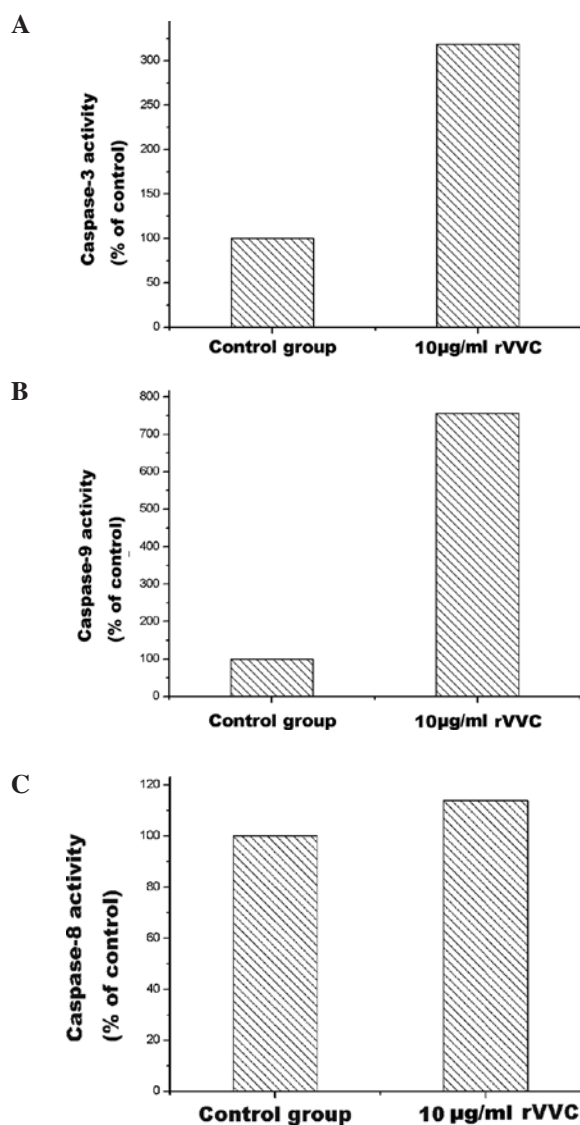


Figure 6. Changes in caspase-3, -8 and -9 activities in the rVVC-treated cells. Cells were incubated with 10 μ g/ml rVVC for 24 h. Cytosolic extracts were assayed for caspase-3, -8 and -9 activities based on the detection of fluorescence of AFC from cleavage of their specific substrates. Comparison of the fluorescence from apoptotic samples with un-induced controls allows determination of the increase in caspase activity.

lysis was always accompanied by the quick leaking out of cytoplasmic LDH. Thus, LDH activity in cell culture media may indirectly reflect the condition of cell injury. However, we discovered that after treatment with rVVC (10 μ g/ml) for different periods of time, the LDH activity in the culture media of A549 cells showed no obvious change when compared with the control groups ($P > 0.05$). Therefore, we speculated that the inhibition of proliferation in A549 cells by rVVC may not be through the induction of pore-formation and dissolution.

In order to explore the mechanism of inhibition of the proliferation of A549 cells by rVVC, we studied the effect of rVVC on apoptosis in A549 cells. Apoptosis is a mode of programmed cell death that is an important physiological process responsible for maintaining homeostasis (20). A dysregulation of apoptosis plays a central role in aberrant cell survival and tumorigenesis (21). Apoptosis is characterized by chromatin condensation, DNA fragmentation, membrane blebbing, and/

or apoptotic bodies (22). In our study, both Annexin-V FITC analysis by FCM and Hoechst 333258 staining assay *in vitro* displayed higher percentages of apoptotic cells in A549 cells treated with rVVC. When the A549 cells were treated with 10 $\mu\text{g/ml}$ rVVC for 24 h, marked morphological changes were clearly observed by using a transmission electron microscope. To further confirm the rVVC-induced apoptotic responses in A549 cells, chromosomal DNA from rVVC-untreated and rVVC (5, 10 and 20 $\mu\text{g/ml}$)-treated cells was analyzed by DNA fragmentation analysis. rVVC treatment resulted in inter-nucleosomal DNA cleavage in A549 cells as indicated by DNA laddering, while the inter-nucleosomal DNA from untreated cancer cells remained intact. The above results indicate that rVVC induced A549 cell apoptosis and indirectly inhibited A549 cell growth.

Apoptosis is tightly regulated by multiple pathways and proteins. Among the numerous proteins and genes involved, the members of the Bcl-2 family play a critical role in inhibiting or promoting apoptosis. The Bcl-2 family is composed of both anti-apoptotic molecules (Bcl-2, Bcl-W, Bcl-XL, A1) and pro-apoptotic molecules (Bax, Bcl-Xs, Bad, Bak, Bid, Bim, Bik) (22,23). Although an important anti-apoptosis protein, Bcl-2, may take part in the formation of mitochondrial PT (permeability transition) holes, however, only at a high pH. On the other hand Bax may form holes at a large range of pHs, and is considered pro-apoptotic. Bax permits some ions and small molecules such as cytochrome C to penetrate the mitochondrial membrane and enter the cytoplasm, causing cell apoptosis. However the function of Bcl-2 is the opposite. It negates the hole-forming activity by Bax and prevents small molecules from penetrating freely, thus protecting cells from apoptosis (23-27). Thus, the ratio between the two subsets helps determine, partly, the susceptibility of cells to a death signal. Our study detected a slight decrease in Bcl-2 and an increase in Bax protein in rVVC-treated A549 cells, resulting in a decrease in the Bcl-2/Bax ratio. Therefore, we propose that Bcl-2 protein primarily participates in rVVC-induced apoptosis in A549 cells.

Caspases, intracellular cysteine proteases, may cause apoptosis by two major pathways, namely FADD-caspase-8/3 and cytochrome C (CytC)-caspase-9/3. The FADD-caspase-8/3 route is triggered by Fas/FasL, and the CytC-caspase-9/3 route is initiated by mitochondrial injury. Finally, caspase-3, an endogenous endonuclease, is activated, which causes irreversible cell apoptosis (28-30). We measured the changes in caspase-3, -8 and -9 activity by colorimetric assay. Our results showed that caspase-3 and -9 activities in rVVC-treated A549 cells were markedly elevated, whereas caspase-8 activity was not affected by rVVC. These data strongly suggest that the apoptosis caused by rVVC was mitochondrion-dependent, and not related to the Fas/FasL and FADD-caspase-8 route. This implies the possibility that the mitochondrion-associated CytC-caspase-9/3 route may be responsible for rVVC-induced apoptosis.

In summary, our study conclusively demonstrated for the first time that rVVC inhibits proliferation and induces apoptosis in the A549 cell line. The mechanism of apoptosis involves down-regulation of Bcl-2 and up-regulation of Bax protein expression. Furthermore, the apoptosis is mediated by the mitochondrion-associated caspase 9/3 pathway. This means that the mechanism of 'punching' holes in the cell

membrane does not accurately describe the pathogenic effects of this toxin. However, the elucidation of precise mechanisms underlying these effects needs further study.

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