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Original article

Vibrio vulnificus RTX toxin plays an important role in the apoptotic death of human intestinal epithelial cells exposed to *Vibrio vulnificus*

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Abstract

During *Vibrio vulnificus* infection, *V. vulnificus* reaches the intestine and then invades the bloodstream by crossing the intestinal mucosal barrier of the host, which results in systemic septicemia. Previously, we reported that the RtxA toxin secreted through the RtxE transporter contributes to the cytotoxicity of *V. vulnificus* against intestinal epithelial cells. Here, we used gene mutants of *rtxE* and *rtxA* to determine the role that *V. vulnificus* RtxA toxin plays in the apoptotic death of human intestinal epithelial cells. The levels of DNA fragmentation were lower in human epithelial cells infected with an *rtxE* mutant of *V. vulnificus* than in those that were infected with the wild type. In addition, the *rtxE* mutant was found to induce lower levels of TUNEL positive cells and cell cycle arrest at the subG$_1$ than the wild type *V. vulnificus*. Furthermore, the decreased levels of DNA fragmentation, TUNEL positive cells and subG$_1$ arrest by the *rtxE* gene mutation were restored by the complementation of an *rtxE* gene into the *rtxE* mutant *V. vulnificus*. Finally, the *rtxA* mutant induced significantly lower levels of apoptotic cell death than the wild type. The levels of the PARP, cytochrome *c*, caspase-3, and mitochondrial membrane depolarization were lower in human epithelial cells infected with the *rtxE* and *rtxA* mutants, compared with the wild type and *rtxE* gene-complemented strains of *V. vulnificus*. Taken together, these results indicate that *V. vulnificus* RtxA toxin induces the apoptotic death through a mitochondria-dependent pathway in human intestinal epithelial cells exposed to *V. vulnificus*.

Keywords: *V. vulnificus*; RTXA toxin; Apoptotic cell death; Human intestinal epithelial cells

1. Introduction

Vibrio vulnificus, a Gram-negative bacterium, causes septicemia in humans who suffer from liver cirrhosis, haemochromatosis, immunocompromised conditions and diabetes [1,2]. Mortality from *V. vulnificus* infection exceeds 50%, and increases to more than 90% in patients who go into shock shortly after admission to the hospital. The majority of fatal cases are caused by a septic shock, which results from a variety of virulence factors produced by *V. vulnificus*, including capsular polysaccharide [3,4], siderophores [5], haemolysin [6], matrix metalloproteinase, flagella [7], and RtxA toxin [8–10].

The RTX toxin is a member of the RTX family of bacterial protein toxins. RTX toxins are pore-forming protein toxins that are produced by a broad range of pathogenic Gram-negative bacteria. For examples, bacterial pathogens of humans and animals that produce structurally similar RTX toxins include *V. cholerae* (RTX) [11–14], *A. pleuropneumoniae* (leukotoxin; LTX) [15,16], *A. actinomycetemcomitans* [17], and *M. morganii* (hemolysin/cytolysin) [18]. Most RTX toxins are proteins with a molecular mass of 100–200 kDa that are post-translationally activated by acylation *via* a specific activator protein [19]. The RTX toxins comprise four genes of two *rtx* operons: *rtxA* which encodes the toxin, *rtxC* which encodes an essential acylase of RtxA,

Abbreviations: MOI, multiplicity of infection; PARP, poly(ADP-ribose)-polymerase; RTX, repeat in toxin; TISS, type I secretion system; TUNEL, terminal deoxynucleotidyl transferase-mediated fluorescence dUTP-nick and labeling.

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rtxB/*rtxE* which encodes an ATP-binding cassette transporter of RtxA, and *rtxD* which has no clearly known function [13]. The RTX toxins exhibit a cytotoxic and often hemolytic activity *in vitro*, and their cytotoxicity results in damage to the membrane, osmotic swelling and cell lysis [20]. Furthermore, the cytotoxicity of RTX toxins in host cells leads to necrosis and apoptosis, although their underlying mechanisms are still not fully understood. For example, lymphocytes and natural killer cells that are exposed to relatively high doses of *A. actinomycetemcomitans* (LTX; like RTX toxin) exhibit a decrease in cell size, chromatin condensation and DNA fragmentation, all of which are indicative of an alternative mechanism of cell death and apoptosis [21–23].

Bacterial pathogenicity depends on the secretion of virulence factors out of the cell from the cell surface [24]. Gram-negative bacteria contain several different types of secretion systems [25], including the type I secretion system (TISS). The TISS is composed of three cytoplasmic membrane components, a specific outer membrane protein (OMP), an ATP-binding cassette (ABC) and a membrane fusion protein (MFP) [26]. The *V. cholerae* RtxA toxin is known to be the most potent cytotoxic toxin with actin cross-linking activities, and secreted out of the cell *via* the TISS consisting of RtxB (ABC), RtxD (MFP), RtxE (ABC), and TolC (OMP) [13]. Therefore, the TISS plays direct and/or indirect roles in the export of bacteria toxins [26,27]. Previously, we constructed a *V. vulnificus* null mutant in which the *rtxA* gene was inactivated by allelic exchanges, and found that *V. vulnificus* RtxA was involved in *V. vulnificus* pathogenicity [8–10]. In addition, we reported that an *rtxE* gene mutant of *V. vulnificus* was relatively defective in the cytotoxicity and lethality, *in vitro* and *in vivo* [9]. These results indicate that RtxA toxin secreted through the RtxE transporter of *V. vulnificus* contributes to the cytotoxic activity and cell death of *V. vulnificus* disease.

In this study, *rtxE* and *rtxA* gene mutants of *V. vulnificus* were used to investigate the role that the RtxA toxin plays in the apoptotic cell death of human intestinal epithelial cells. Here, we demonstrate for the first time that *V. vulnificus* RtxA toxin is crucial for inducing the apoptotic death of human epithelial cells that are exposed to *V. vulnificus*.

2. Materials and methods

2.1. Cell culture

The human intestinal epithelial INT-407 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained at 37 °C in 5% CO_2 in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and antibiotics (10 unit/ml penicillin G and 10 μg/ml streptomycin) (growth medium).

2.2. Bacterial strains and growth conditions

V. vulnificus strain MO6-24/O wild type used in this study was isolated from the patients, and the *rtxA* mutant (MW064), *rtxE* mutant (MW061) and *rtxE* complementation MW061(pMW0612) strains were previously described [8,9]. For the infection experiments, the bacteria strains were grown overnight at 30 °C in Luria–Bertani medium supplemented with 2.0% NaCl LBS medium, and then diluted to approximately 6×10^8 CFU/ml in LBS. The cells were then centrifuged and resuspended in antibiotic-free MEM medium prior to infection into epithelial cells. Bacterial concentrations were then confirmed *via* viable cell counts on LBS agar.

2.3. Infection protocol

Human intestinal epithelial INT-407 cells were infected with *V. vulnificus*, as previously described [8,9,28]. Briefly, intestinal epithelial cells were grown in culture T-75 flasks at 37 °C in a 5% CO_2 incubator. The cells were then seeded onto 24- or 96-well plates, or 6 cm tissue culture dishes and cultured for 24 h in antibiotic-free growth medium. Prior to infection, the bacteria were centrifuged for 3 min at $2500 \times g$, resuspended, and then adjusted to approximately 6×10^8 CFU/ml in antibiotic-free MEM medium. The bacterial suspensions were then added to epithelial cells at various multiplicities of infection (MOI; ratio of bacteria number to epithelial cell number), after which the infected cells were incubated in a 5% CO_2 incubator at 37 °C in antibiotic-free growth medium.

2.4. Cytotoxicity assay

The bacterial infected INT-407 cell cultures were prepared in a 96-well tissue culture plate (Nunc, Roskilde, Denmark) as previously described [8,9]. The cytotoxicity was then determined by measuring the activity of lactate hydrogenase (LDH) in the supernatant using a cytotoxicity detection kit (Roche, Mannheim, Germany) and expressed using the total LDH activity of the cells that were completely lysed by 1% Triton X-100 as 100% [8,9].

2.5. Treatment of epithelial cells with the culture supernatants of V. vulnificus

INT-407 cells were cultured in a 24-well plate (2×10^4 cells/well), and incubated for 12 h with each of the culture supernatants of *V. vulnificus*. The culture supernatants of *V. vulnificus* were prepared from 6×10^8 CFU/ml *V. vulnificus* in antibiotic-free MEM medium. The cell viability was assessed by the trypan blue exclusion as previously described [29]. Viable cells were quantitated under bright field microscopy.

2.6. Morphological study

INT-407 (2×10^4 cells/well) cells were either incubated with bacteria in a 24-well plate for 2 h at MOI 10 or washed twice with PBS and post-incubated for 8 h in MEM medium containing antibiotics including penicillin G (10 unit/ml), streptomycin (10 μg/ml), and gentamicin (50 μg/ml). The

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culture plates were then centrifuged at 3000 × *g* and washed twice with pre-warmed PBS (pH 7.4), fixed with 4% para-formaldehyde (Sigma) for 10 min at room temperature, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Next, the cells were washed twice with PBS, after which they were stained with rhodamine phalloidin (Molecular Probe) for 1 h at room temperature. Fluorescence images of specimens mounted with a Dabco reagent (Sigma) were then acquired using a fluorescence microscope (Olympus IX 71, Japan).

2.7. DNA fragmentation assay

DNA fragmentation was analyzed as described previously, with slight modification [30]. INT-407 (1×10^6 cells/well) cells were incubated with bacteria in 6 cm tissue culture dishes for 2 h at MOI 10, after which the cells were washed twice with PBS and then post-incubated for 8 h in MEM medium containing antibiotics. The cells were then detached and centrifuged, after which the cell pellets were suspended in lysis buffer (10 mM Tris/HCl, pH 7.6; 15 mM EDTA; 0.5% Triton X-100). Afterwards, the samples were centrifuged for 15 min at 14,000 × *g*, and the supernatant containing fragmented DNAs was digested for 1 h with 100 μg/ml RNase (Sigma) at 37 °C. The DNAs were then extracted twice with phenol/chloroform, after which it was precipitated overnight using isopropyl alcohol. The DNA fragments were then separated on 1.8% agarose gels, visualized with ethidium bromide, and photographed.

2.8. TUNEL assay and cell cycle analysis

Apoptotic cells were measured by a TUNEL [terminal deoxynucleotidyl transferase-mediated fluorescence dUTP-nick and labeling] assay using an *in situ* Cell Death Detection kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's instruction. INT-407 (1×10^6 cells/well) cells were incubated with bacteria in tissue culture dishes for 2 h at MOI 10, and then post-incubated for 8 h. Next, the cells were centrifuged and washed with PBS, after which they were fixed for 30 min with 2% para-formaldehyde on ice. The cells were washed twice, permeabilized by treatment with cold 70% ethanol at −20 °C for 30 min and then washed again. The samples were then resuspended in labeling reaction solution that contained terminal deoxynucleotidyl transferase and fluorescence dUTP, and then incubated for 1 h at 37 °C.

For analysis of the cell cycles, cells were centrifuged, fixed with 70% methanol, and wash twice with PBS. Next, the cells were stained with 50 μg/ml propidium iodide for 1 h and 100 μg/ml RNase at room temperature. Afterwards, the cells were washed twice and resuspended in PBS for flow cytometric analysis in a Becton Dickinson Facstar^{Plus} flow cytometer.

2.9. FITC-conjugated annexin-V binding assay

Translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane has been observed in many cell types undergoing apoptosis. To determine whether *V. vulnificus* RtxA toxin-mediated death of INT-407 cells involved a similar mechanism, perturbation, the translocation of phosphatidylserine was measured by binding FITC-conjugated annexin-V to *V. vulnificus*-infected intestinal epithelial cells using an Apoptosis Detection Kit (R&D Systems, Minneapolis, MN, USA). Briefly, INT-407 (1×10^6 cells/well) cells were incubated with bacteria in tissue culture dishes for 2 h at MOI 10, after which they were post-incubated for 8 h. The cells were then colleted by centrifugation and resuspended in 500 μl of 1× binding buffer. The cells were stained for 20 min with 5 μl FITC-conjugated annexin-V and 5 μl propidium iodide at room temperature in the dark. The cells were then washed and fixed in 2% para-formaldehyde prior to being visualized under a Bio-Rad MRC-1024 laser scanning confocal system (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

2.10. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential was measured by a flow cytometry using the dye $DiOC_6$, as previously described [31]. INT-407 cells were plated at a density of 1×10^6 cells/well in 6-well plates, and incubated with bacteria for 2 h at MOI 10, followed by post-incubation for 8 h. The cells were collected in ice cold PBS and resuspended in 500 μl fresh MEM with the addition of 100 nM $DiOC_6$ and incubated for 15 min at 37 °C. The cells were then washed with PBS. Data were obtained and analyzed with a flow cytometry in a Becton Dickinson Facstar^{Plus} flow cytometer.

2.11. Western blot analysis

The cell lysates were prepared from human epithelial cells infected with each strain of *V. vulnificus*, as previously described [28]. Equal amounts (20 μg/ml) of whole cell lysates were subjected to10% (GAPDH and PARP) and 12% (cytochrome *c* and caspase-3) SDS-PAGE. The proteins were transferred onto a polyvinylidene fluoride membrane using a Semi-Phor (Hoefer Scientific Instrument). The membrane was then incubated with washing buffer (PBS solution containing 0.1% Tween 20) containing 2% bovine serum albumin for at least 1 h to block nonspecific protein binding. Afterwards, the membrane was, respectively, treated with rabbit anti-GAPDH (1:5,000 of goat polyclonal; Santa Cruz Biotechnology, Inc.), poly(ADP-ribose)polymerase (PARP; 1:2,500 of rabbit polyclonal; Upstate Biotechnology, Inc.), caspase-3 (1:500 of rabbit polyclonal; Upstate Biotechnology, Inc.), and cytochrome *c* (1:5,000 of goat polyclonal; Santa Cruz Biotechnology, Inc.) antibodies. After incubation with HRP-conjugated anti-rabbit or anti-goat antibody, immunoreactive proteins were detected with the ECL system (Amersham Biosciences, England).

2.12. Statistical analysis

Student's *t*-tests and one-way analysis of variance (ANOVA) followed by the Bonferroni method were employed

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if statistical differences existed between the values of the various experimental and control groups. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Mutation of the rtxE gene decreases the cytotoxicity of V. vulnificus against human intestinal epithelial cells

We reported that the RtxA toxin secreted through the RtxE transporter contributes to the cytotoxicity of *V. vulnificus* against intestinal epithelial cells. Therefore, we investigated the *V. vulnificus* RtxA toxin to determine its role in the apoptotic death of human intestinal epithelial cells using genetic mutants of *rtxE* and *rtxA* [8,9]. First, to determine the role that the *rtxE* gene plays in the cytotoxicity of *V. vulnificus* against human epithelial cells, the wild type (WT), *rtxE* mutant MW061 (*rtxE* MT), and *rtxE* complementation MW061(pMW0612) (*rtxE* CT), of *V. vulnificus* were infected into epithelial cells. The levels of LDH released into the cell culture were determined and compared. As shown in Fig. 1A and 1B, both the wild type and MW061 induced the release of LDH from the infected INT-407 cells at different extents. Importantly, the MW061 induced significantly less cytotoxicity at MOI up to 50 (Fig. 1A). The INT-407 cells were also infected at an MOI of 10, and the LDH activities from the cells that were incubated for different lengths of times were then compared (Fig. 1B). The *rtxE* mutant MW061 induced only $5.43 \pm 2.23\%$ release of LDH in intestinal epithelial cells that were infected for 2 h at MOI 10, whereas the wild type induced $37.9 \pm 7.12\%$ release of LDH. Furthermore, lower levels of LDH were released from the cells infected with MW061 than the cells infected with the wild type when the cells were subjected to bacterial infection for up to 3 h. In addition, the MW061 induced significantly lower levels of cell cytotoxicity than the wild type; however, these levels were restored in INT-407 cells that were infected with the MW061(pMW0612) *V. vulnificus* ($32.9 \pm 4.34\%$) ($P < 0.01$). The levels of cytotoxicity were approximately the same in INT-407 cells that were infected with the wild type and *rtxE* complementation MW061(pMW0612) *V. vulnificus* (Fig. 1A and 1B).

In addition, we observed that the wild type *V. vulnificus*-infected-INT-407 cells showed damage to their actin prior to their detachment from the bottoms of the culture plates. In order to determine if the cell detachment was related to damage to the actin, as reported for cells infected with *V. vulnificus*, actin was stained with rhodamine phalloidin and the cells were then observed with fluorescence microscopy [10]. The rhodamine phalloidin stained INT-407 cells exhibited marked cellular damage and cytoplasmic loss, when the cells were infected with the wild type and MW061(pMW0612) *V. vulnificus*. Conversely, the cells infected with MW061 exhibited a less-damaged surface and less cytoplasmic loss (Fig. 1C). These data clearly indicate that the *rtxE* gene of *V. vulnificus* plays an important role in inducing cell cytotoxicity when the intestinal epithelial cells are exposed to *V. vulnificus*.

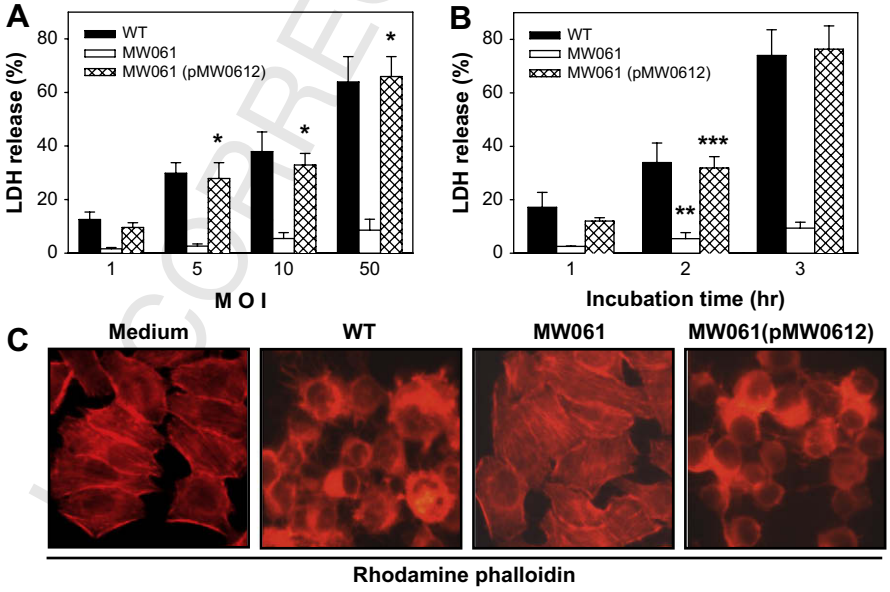

Fig. 1. Effects of the *rtxE* gene mutation on the cell cytotoxicity of *V. vulnificus* against intestinal epithelial cells. (A) INT-407 cells were infected for 2 h with the wild type, MW061, or MW061(pMW0612) *V. vulnificus* at various MOIs, and the cell cytotoxicity was then determined by the LDH release assay, as described in the Section 2. The data represent the means ± standard errors ($n = 3$). *, $P < 0.01$, relative to groups infected with the wild type at each MOI. (B) INT-407 cells were infected with the wild type, MW061, or MW061(pMW0612) at an MOI of 10 for various incubation times. The data represent the means ± standard errors ($n = 3$). **, $P < 0.01$, ***, $P < 0.01$, relative to a group infected with the wild type for 2 h. (C) INT-407 (2×10^4 cells/well) cells were incubated with bacteria in 24-well plates for 2 h at MOI 10. The culture plates were centrifuged and washed twice with pre-warmed PBS (pH 7.4), fixed with 4% para-formaldehyde. The cells were washed twice with PBS, and stained with rhodamine phalloidin. Fluorescence images of specimens mounted with a Dabco reagent (Sigma) were acquired using a fluorescence microscope.

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3.2. The rtxE mutant V. vulnificus induces significantly lower levels of apoptosis in intestinal epithelial cells than the wild type V. vulnificus

To characterize the cell cytotoxicity induced by *V. vulnificus*, INT-407 cells were infected with the wild type, MW061, or MW061(pMW0612) *V. vulnificus* for 2 h. Afterwards, the cells were post-incubated for 8 h, after which the apoptotic cells were evaluated by apoptosis detection assays, including the DNA fragmentation assay and cell cycle analysis. As shown in Fig. 2A, in the assay of DNA fragmentation, the wild type was found to significantly induce DNA laddering, whereas the MW061 did not. Importantly, the decrease in DNA laddering induced by the MW061 was restored by the complementation of the *rtxE* gene into the MW061. Infection with the MW061(pMW0612) induced levels of DNA fragmentation that were similar to those of DNA laddering induced by the wild type (Fig. 2A). Paclitaxel (2 μg/ml)-treated cells, which were used as a positive control, strongly exhibited DNA laddering. In addition, cell cycle analysis using propidium iodide revealed that infection with the wild type bacteria increased the populations of $subG_1$ cells (53.85%), which is indicative of apoptotic cell death. In contrast, only 2.49% of the INT-407 cells infected with the MW061 were $subG_1$ positive (Fig. 2B). The levels of cells in the $subG_1$ phase of the cell cycle were 42.83% restored by the complementation of the *rtxE* gene into the MW061 *V. vulnificus*.

To further examine the apoptotic cell death induced by *V. vulnificus* RtxE, the apoptotic cells were evaluated using a TUNEL assay and an FITC-conjugated annexin-V binding assay. Flow cytometric analysis revealed that the MW061 (mean intensity: 12.34) induced lower levels of TUNEL positive cells than the wild type (40.33), and that the decreased levels were significantly restored by the MW061(pMW0612) (31.11) (Fig. 3A). In addition, as shown in Fig. 3B, the assay of FITC-conjugated annexin-V revealed that the wild type and MW061(pMW0612) *V. vulnificus* significantly induced apoptotic cell death (green: annexin-V), whereas the MW061 did not (Fig. 3B). To examine the shape of the apoptotic or healthy cells, bacteria infected-INT-407 cells were stained with rhodamine phalloidin. As shown in Fig. 3C, INT-407 cells that were infected with the wild type and MW061(pMW0612) exhibited cell rounding and cytoplasmic loss, whereas the cells infected with MW061 did not (Fig. 3C). These results demonstrate that the *V. vulnificus* RtxE plays an important role in the induction of apoptotic cell death in intestinal epithelial cells.

3.3. V. vulnificus RtxA toxin plays an important role in the apoptotic cell death of human intestinal epithelial cells exposed to V. vulnificus

Previously, we found that *V. vulnificus* RtxA and RtxE were involved in *V. vulnificus* pathogenicity. In addition, secretion of RtxA toxin through the RtxE transporter of *V. vulnificus* is known to contribute to cytotoxicity and lethality in mice infected with *V. vulnificus* [8,9].

To determine if apoptotic cell death was induced by *V. vulnificus* RtxA toxin, INT-407 cells were infected with the wild type, MW061, MW061(pMW0612), or MW064 for 2 h.

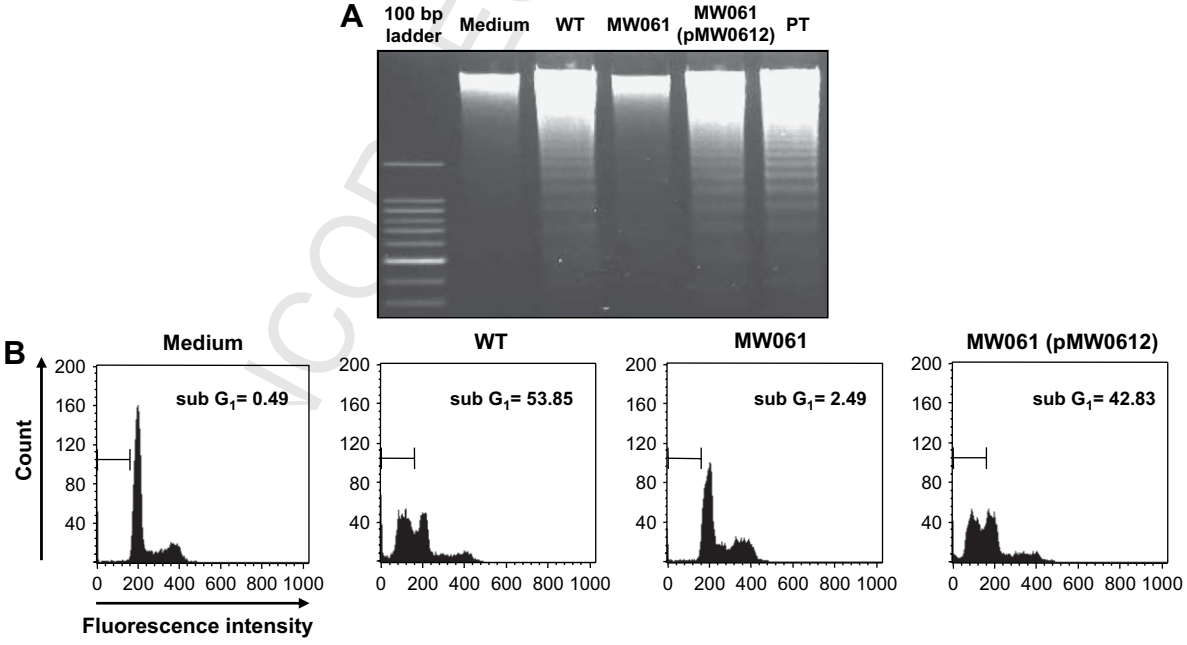

Fig. 2. Effects of the *rtxE* gene mutation on the DNA fragmentation and number of cells in the $subG_1$ of the cell cycle in intestinal epithelial cells. (A) INT-407 cells were infected for 2 h with the wild type, MW061, or MW061(pMW0612) *V. vulnificus* at MOI of 10. The cells were washed twice with PBS, after which they were post-incubated with gentamicin (50 μg/ml)-containing growth medium. After 8 h of post-incubation, the cells were collected and the low molecular weight DNA was isolated and resolved by 1.8% agarose gel electrophoresis. As a positive control, the cells were treated with paclitaxel (PT, 2 μg/ml) for 8 h. The data are representative of three independent experiments. (B) INT-407 cells were washed twice with PBS and then post-incubated for 8 h. The cells were then labeled by the propidium iodide and subjected to flow cytometric analysis to determine the population of the $subG_1$. The data represent the means ± standard errors ($n = 3$).

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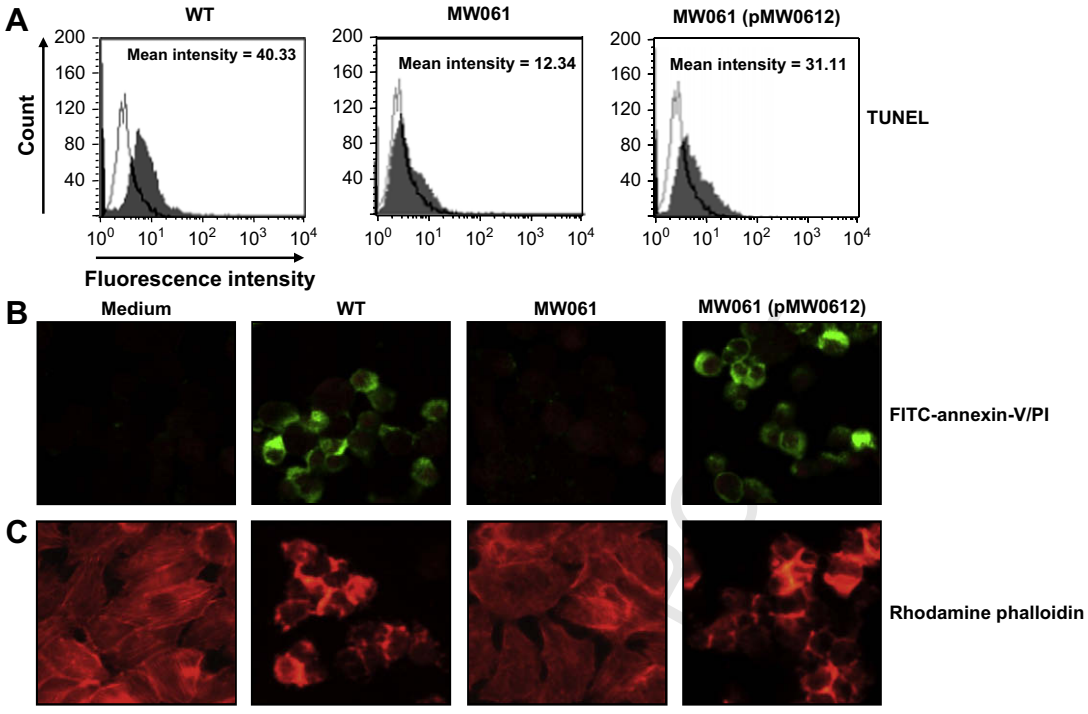

Fig. 3. Effects of the *rtxE* gene mutation on the TUNEL and FITC-conjugated annexin-V positive intestinal epithelial cells. (A) INT-407 (1×10^6 cells/well) cells were incubated with bacteria in a tissue culture dish for 2 h at MOI 10, and then post-incubated for 8 h. The cells were centrifuged and then fixed with 2% paraformaldehyde on ice. The cells were permeabilized, resuspended in labeling reaction solution containing terminal deoxynucleotidyl transferase and fluorescence dUTP. Afterwards, the cells were analyzed using a flow cytometer. (B) INT-407 (1×10^6 cells/well) cells were incubated with bacteria for 2 h at MOI 10, and then post-incubated for 8 h. The cells were detached from the culture plates, colleted by centrifugation and resuspended. The cells were then stained with FITC-conjugated annexin-V (green) and propidium iodide (red), after which they were fixed and observed using a laser scanning confocal system. (C) INT-407 (1×10^6 cells/well) cells were incubated with bacteria for 2 h at MOI 10, and then post-incubated for 8 h. The culture plates were centrifuged, washed, and fixed. The cells were stained with rhodamine phalloidin, and fluorescence images of specimens mounted with a Dabco reagent were acquired using a fluorescence microscope.

Afterwards, the cells were post-incubated for 8 h, after which the apoptotic cells were determined by a DNA fragmentation assay, TUNEL, and cell cycle analysis. As shown in Fig. 4A, in the assay of DNA fragmentation, the wild type and MW061(pMW0612) significantly induced DNA laddering, whereas the MW061 and MW064 did not. Importantly, infection with the MW064 induced similar levels of DNA fragmentation, as the MW061 (Fig. 4A). Also, in the TUNEL assay, the wild type (mean intensity: 85.49) and MW061(pMW0612) (71.80) induced higher level of TUNEL positive cells that the MW061 (54.35) and MW064 (58.70) (Fig. 4B). Moreover, cell cycle analysis revealed that infection with the wild type significantly increased the population of subG$_1$ cells (70.93%), which is characteristic of apoptotic cell death. In contrast, only 7.62% and 10.63% of the INT-407 cells infected with the MW061 and the MW064, respectively, were subG$_1$ cells (Fig. 4C).

To further confirm that the RtxA toxin plays an important role in apoptosis of *V. vulnificus*-infected intestinal epithelial cells, we examined the cell death by using the culture supernatants of wild type, MW061, MW061(pMW0612), MW064 *V. vulnificus* in human intestinal epithelial cells. The culture supernatant of wild type and MW061(pMW0612) significantly induced higher levels of the cell death than the MW061 and MW064 *V. vulnificus* (Fig. 4D). These results show that the secreted RtxA toxin through the RtxE transporter is very important role in *V. vulnificus*-induced apoptotic cell death.

To further examine the effect of *V. vulnificus* RtxA toxin on apoptosis in human intestinal epithelial cells, INT-407 cells infected with the wild type, MW061, MW061(pMW0612), or MW064 were stained using FITC-conjugated annexin-V and propidium iodide. As shown in Fig. 5A, the MW061 and the MW064 induced lower levels of FITC-conjugated annexin-V positive cells than the wild type (Fig. 5A). The shape of the infected INT-407 cells was observed by rhodamine phalloidin-actin staining. As shown in Fig. 5B, INT-407 cells exhibited cell rounding and cytoplasmic loss after infection with the wild type. In contrast, cells that were infected with the MW064 did not exhibit cell rounding and cytoplasmic loss (Fig. 5B). Taken together, we suggest that the *V. vulnificus* RtxA toxin induces the apoptotic cell death of human intestinal epithelial cells exposed to *V. vulnificus*.

3.4. V. vulnificus *RtxA toxin induces mitochondrial membrane depolarization and caspase-3-dependent apoptotic death in human intestinal epithelial cells*

To address the mechanism by which *V. vulnificus* RtxA induces the apoptotic effect on human epithelial cells, we first

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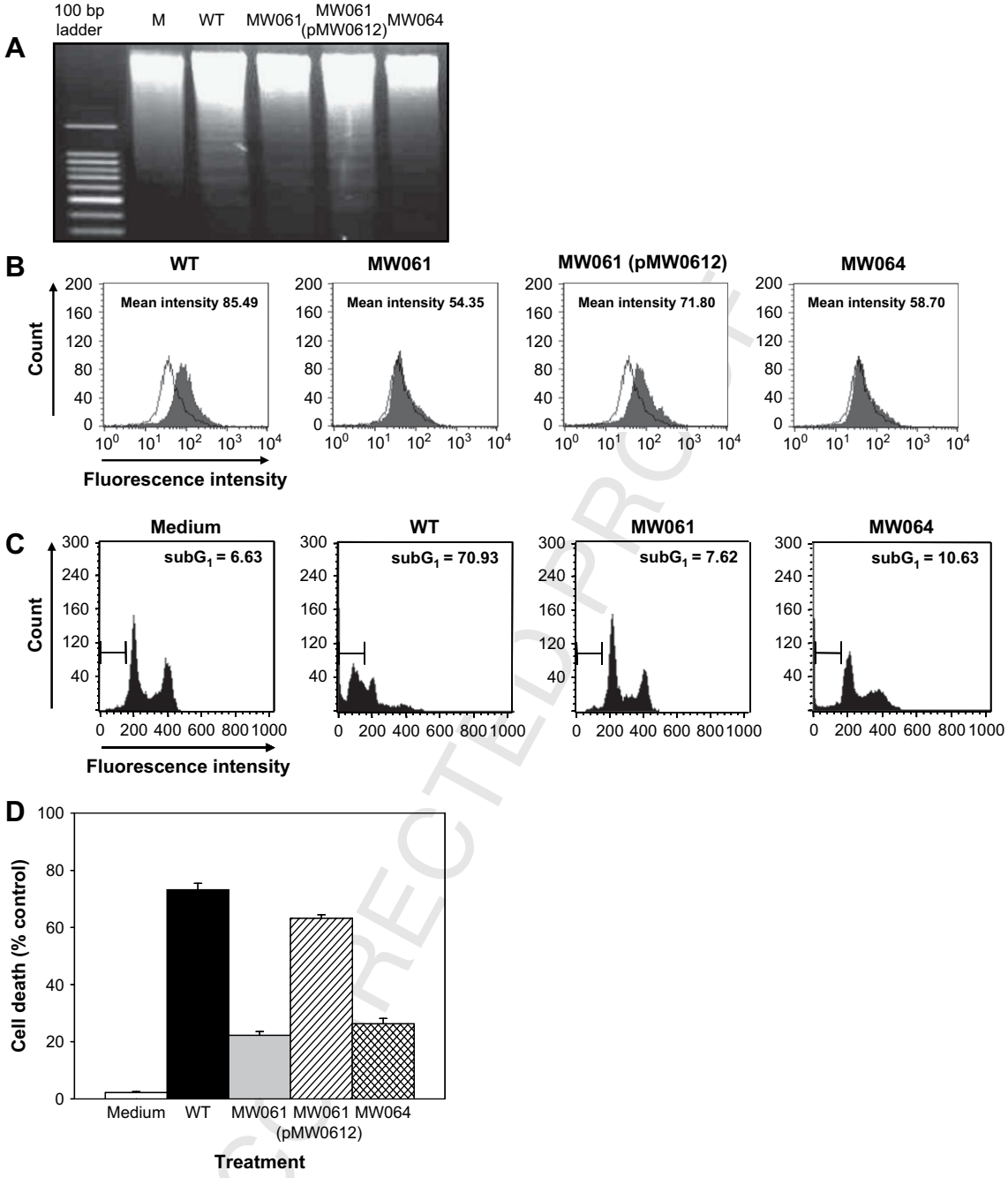

Fig. 4. Effects of RtxA toxin on DNA fragmentation, TUNEL positive, and cell cycle subG$_1$ in intestinal epithelial cells. (A) INT-407 cells were infected for 2 h with the wild type, MW061, MW061(pMW0612), or MW064 at MOI of 10. The cells were post-incubated for 8 h, after which the DNA was isolated and resolved by 1.8% agarose gel electrophoresis. (B) The cells were fixed, permeabilized, and resuspended in labeling reaction solution containing terminal deoxynucleotidyl transferase and fluorescence dUTP. Afterwards, the cells were analyzed using a flow cytometer. (C) INT-407 cells were labeled by propidium iodide for cell cycle subG$_1$ and then analyzed using a flow cytometer. (D) INT-407 cells were incubated for 12 h with each of the culture supernatants of wild type, MW061, MW061(pMW0612), or MW064. The cell death was determined by the trypan blue exclusion assay. The data are representative of three independent experiments.

analyzed the expression of apoptosis-related proteins in *V. vulnificus*-infected intestinal epithelial cells. Cytochrome *c* is a mitochondrial membrane protein that can activate caspases. Mitochondria are well known to play a central role in mediating 'intrinsic death signaling pathway' [32]. As shown in Fig. 6A, the wild type and MW061(pMW0612) significantly increased the expression of cytochrome *c*, PARP, and caspase-3, whereas the MW061 and MW064 did not. However, we cannot detect the activation of caspase-8 in *V. vulnificus*-infected intestinal epithelial cells (data not shown).

To further demonstrate the induction of apoptosis by *V. vulnificus* RtxA toxin, the mitochondrial membrane depolarization was examined in epithelial cells infected with each of *V. vulnificus* strains. As shown in Fig. 6B, the wild type and MW061(pMW0612) *V. vulnificus* markedly induced a depolarization of mitochondrial membranes to facilitate

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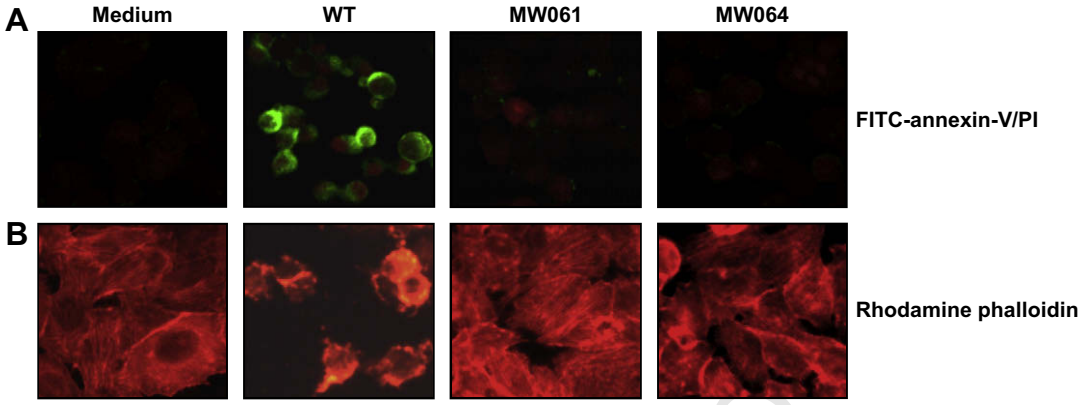

Fig. 5. Effects of *V. vulnificus* RtxA toxin on apoptotic cell death of intestinal epithelial cells. (A) INT-407 (1×10^6 cells/well) cells were incubated with the wild type, MW061, or MW064 for 2 h at MOI 10, and then post-incubated for 8 h. The cells were detached from culture plates, colleted by centrifugation and resuspended. The cells were stained with FITC-conjugated annexin-V (green) and propidium iodide (red), after which they were observed using a laser scanning confocal system. (B) INT-407 (1×10^6 cells/well) cells were incubated with bacteria for 2 h at MOI 10, and then post-incubated for 8 h. The culture plates were centrifuged, washed, and fixed. The cells were stained with rhodamine phalloidin, and fluorescence images of specimens mounted with a Dabco reagent were acquired using a fluorescence microscope.

cytochrome *c* release into cytosol, whereas MW061 and MW064 *V. vulnificus* did not. These results suggest that *V. vulnificus* RtxA toxin may induce apoptotic cell death by a mitochondria-mediated intrinsic pathway.

4. Discussion

V. vulnificus is a Gram-negative estuarine bacterium that is known as a significant human pathogen. When *V. vulnificus* is

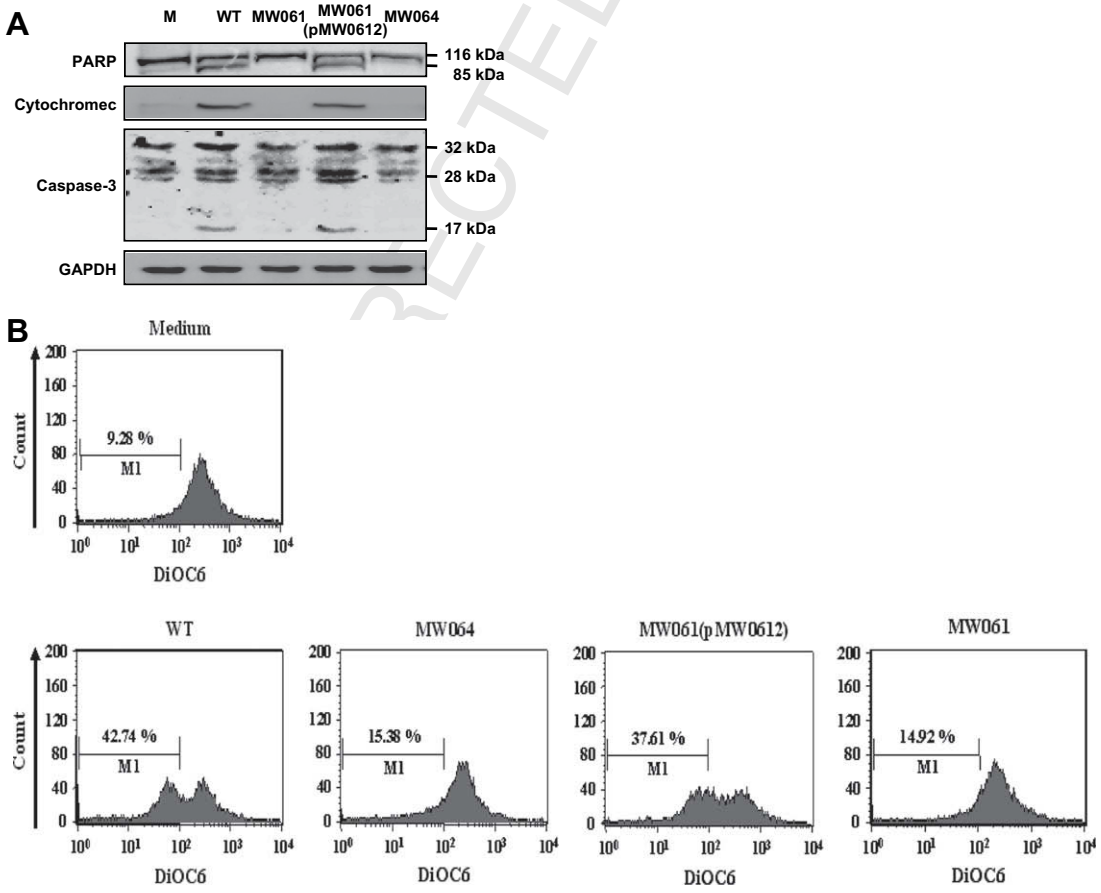

Fig. 6. Mitochondrial membrane depolarization and caspase-3 activation in *V. vulnificus*-induced apoptotic death of intestinal epithelial cells. (A) INT-407 cells were infected for 2 h with the wild type, MW061, MW061(pMW0612), or MW064 at MOI of 10. The cells were post-incubated for 8 h, after which the cell lysates were prepared. The levels of PARP, cytochrome *c*, caspase-3, and GAPDH were determined by Western blot analysis. (B) The cells were fixed, permeabilized, and resuspended in a labeling reaction solution containing terminal deoxynucleotidyl transferase and fluorescence dUTP. Afterwards, the cells were analyzed using a flow cytometer.

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ingested orally *via* contaminated shellfish, it enters the intestine and then invades the bloodstream by crossing the intestinal mucosal barrier of the host, resulting in systemic septicemia.

Apoptosis and necrosis of host cells are crucial in the pathogenesis of some pathogenic microorganisms. The bacterial pathogenicity depends on the secretion of virulence factors to the cell surface and into cell exterior [24]. Recently, many studies have shown that some bacterial pathogens induced cell cytotoxicity *via* pore-forming toxins (RTX toxins) [11–18]. In addition, several studies have shown that the RTX toxins produced by *P. haemolytica* and *A. actinomycetemcomitans* induced apoptosis in the bovine and human cell line [23,33,34]. For example, *V. cholerae* RtxA toxin is known to act as a potent cytotoxic toxin that has actin cross-linking activities, and is secreted into the cell exterior through the TISS consisting of RtxB, RtxD, RtxE, and TolC [13]. Previously, we showed that the *V. vulnificus* RtxA toxin, which is secreted through the RtxE transporter, plays an important role in the cell cytotoxicity and lethality in infected mice [8,9]. In addition, another group reported that the *V. vulnificus* RtxA1 toxin is multifunctional cytotoxin that plays an essential role in the pathogenesis of *V. vulnificus* infections [10]. In this study, we have demonstrated for the first time that *V. vulnificus* RtxA toxin secreted through RtxE transporter plays a role in inducing apoptotic cell death of intestinal epithelial cells exposed to *V. vulnificus*.

The MW061 ($5.43 \pm 2.23\%$) induces significantly lower levels of cell cytotoxicity than the wild type ($37.9 \pm 7.12\%$) and the MW061(pMW0612) ($32.9 \pm 4.34\%$) ($P < 0.01$) (Fig. 1A and 1B). Additionally, the actin-stained INT-407 cells that were infected with the wild type and MW061(pMW0612) exhibited marked cellular damage and cytoplasmic loss; however, the MW061 had no effect on the cell rounding and cytoplasmic loss (Fig. 1C).

Several experiments have provided evidence supporting an involvement of the *rtxE* gene in the apoptotic death of human epithelial cells exposed to *V. vulnificus* (Figs. 2–5). For example, intestinal epithelial cells exposed to the MW061 clearly showed little or no DNA fragmentation (Figs. 2A and 4A). In addition, the cell cycle analysis revealed that infection with the wild type increased the populations of $subG_1$ cells (53.85 or 70.93%), which is characteristic of apoptotic cell death. In contrast, the levels of $subG_1$ cells ranged from 2.49 to 7.62% in the MW061-infected intestinal epithelial cells (Figs. 2B and 4C). Importantly, the MW061(pMW0612) restored the levels of $subG_1$ cells (42.83 or 58.19%) that were reduced by the *rtxE* gene mutation (Fig. 2B). The restoration of the apoptotic cell death by the MW061(pMW0612) was confirmed by several assays, including determinations of DNA fragmentation and TUNEL positive cells, as well as an FITC-conjugated annexin-V binding assay (Figs. 2–4).

We found that the cell death induced by the MW061 and MW064 are significantly lower than with the wild type and MW061(pMW0612). Therefore, in order to determine whether the RtxA toxin is required for the apoptotic cell death by the *V. vulnificus* infection, the supernatants were collected from cultures of the wild type, MW061, MW061(pMW0612), and MW064. The culture supernatants of wild type and MW061(pMW0612) significantly induced higher levels of host cell death than MW061 and MW064 *V. vulnificus* (Fig. 4D). Previously, we detect the RtxA toxin in the supernatants of wild type and MW061(pMW0612), but not MW061 and MW064 [8,9]. Here, we found that the culture supernatants of wild type and MW061(pMW0612) *V. vulnificus* contain RtxA toxin, but not MW061 and MW064. Taken together, the apoptotic cell death of the *V. vulnificus*-infected intestinal epithelial cells was significantly decreased by gene mutation of the *rtxE* as well as *rtxA*, indicating that the *V. vulnificus* RtxA toxin secreted through RtxE transporter essential role for the *V. vulnificus*-induced apoptotic cell death in intestinal epithelial cells.

Recently, a protein toxin secreted by an ABC transporter system was reported to be cytotoxic only when the pathogen contacted with host cells. The supernatants obtained from the culture were *V. vulnificus* was evidently expressing RtxA1 could not cause cytotoxicity or cell rounding [10]. However, in our study the culture supernatants of MW061 and MW064 *V. vulnificus* induce the host cell death. We think that the induction of cell death by the culture supernatant of MW061 or MW064 *V. vulnificus* might be resulted from other secreted toxins, such as cytotoxins, haemolysin, and proteases.

The RtxA1 toxin of *V. vulnificus*-induced cytoskeletal rearrangement, plasma membrane blebs and hemolytic activity, which led to necrotic cell death [10]. However, our study showed that *V. vulnificus* RtxA toxin induced mitochondrial membrane depolarization and caspase-3-dependent apoptotic death in human intestinal epithelial cells (Fig. 6), indicating that the difference between necrotic and apoptotic cell death induced by *V. vulnificus* may be related to the cell culture conditions, such as infection time, number of bacteria, and toxin types. Therefore, the RtxA toxin of *V. vulnificus* can induce cell death *via* necrosis and apoptosis.

These results indicate that acute cell cytotoxicity, loss of cell–cell interaction as a result of cellular damage, and apoptotic cell death by the *V. vulnificus* RtxA toxin contributes significantly to the invasion and pathogenesis of *V. vulnificus*.

Many bacterial toxins target small Rho GTPases in order to manipulate the actin cytoskeleton. Recently, the *V. cholerae* RTX toxin is known to induce the cell rounding by the inactivation of the small Rho GTPase. A Rho GTPase-inactivation domain (RID) identified in *V. cholerae* RTX toxin is also conserved in *V. vulnificus* RTX toxin [35]. This domain in RTX toxins plays an important role in the actin cytoskeleton modification and cell rounding of *V. cholerae*-infected host cells. Therefore, the RID in *V. vulnificus* RtxA toxin may be also closely related with the cytoskeleton modification, actin cross-linking, and cell rounding in *V. vulnificus*-infected intestinal epithelial cells.

The attenuated virulence of the *rtxE* gene mutation is associated with a defect in its ability to transport *V. vulnificus* RtxA. This is because the *rtxE* gene encodes an ATP-binding cassette transporter of RtxA, which is an important virulence factor in the pathogenesis of *V. vulnificus*. As little is known

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about the cytotoxic mechanism of RtxA toxin in host cells, further studies should be conducted to investigate their underlying mechanisms of cytotoxic activity in intestinal epithelial cells exposed to *V. vulnificus* and also to determine whether *V. vulnificus* RtxA toxin induces apoptotic cell death *in vivo*.

Taken together, the results of this study demonstrate that the *rtxA* and *rtxE* genes of *V. vulnificus* play an important role in induction of apoptotic cell death through a mitochondria/caspase-3-dependent pathway in human intestinal epithelial cells. In addition, these results indicate that the RtxA toxin may be a new chemotherapeutic or molecular target in the treatment of *V. vulnificus* infectious disease.

Acknowledgement

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