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VvpM Induces Human Cell Death *via* Multifarious Modes Including Necroptosis and Autophagy

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VvpM, one of the extracellular metalloproteases produced by *Vibrio vulnificus*, induces apoptotic cell death *via* a pathway consisting of ERK activation, cytochrome *c* release, and activation of caspases-9 and -3. VvpM-treated cells also showed necrotic cell death as stained by propidium iodide (PI). The percentage of PI-stained cells was decreased by pretreatment with Necrostatin-1, indicating that VvpM-mediated cell death occurs through necroptosis. The appearance of autophagic vesicles and lipidated form of light-chain-3B in rVvpM-treated cells suggests an involvement of autophagy in this process. Therefore, the multifarious action of VvpM might be one of the factors responsible for *V. vulnificus* pathogenesis.

Keywords: *Vibrio vulnificus*, metalloprotease VvpM, necroptosis, autophagy

Vibrio vulnificus causes a fatal septicemia in humans *via* ingestion of *V. vulnificus*-enriched marine organisms or exposure to *V. vulnificus* in seawater [18]. This bacterium is able to destroy human blood cells and Jurkat T-cell lines in a necrotic manner, *via* NADH-oxidase-derived production of reactive oxygen species and a subsequent activation of MAPKs [8, 9]. One of the secreted enzymes responsible for this pathology is the elastolytic zinc-metalloprotease VvpE [6]. Another metalloprotease, VvpM, detected in the culture supernatants of *V. vulnificus* [11], induces apoptotic cell death *via* a pathway consisting of ERK activation, cytochrome *c* release, and activation of caspases-9 and -3 [12]. Interestingly, only 30~40% of the VvpM-treated cells demonstrated the apoptotic characteristics. Therefore, the present study has been aimed to define the other pathways involved in the VvpM-induced cell death.

Recombinant VvpM (rVvpM) was prepared from the culture supernatant of *V. vulnificus* as previously described [12]. A human colorectal carcinoma cell line, HCT-116 (10247, Korean Cell Line Bank), was grown at 37°C in RPMI-1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G, 100 mg/ml streptomycin, 25 mM sodium bicarbonate, and 25 mM HEPES. The HCT-116 cells were seeded in a 6-well plate (1×10^6 cells per well) and incubated for 24 h. Prior to treatment with rVvpM, HCT-116 cells were incubated for 1 h in serum-free RPMI-1640. The cells were treated with 0.5 μM rVvpM for 6 h, and stained with Annexin V and propidium iodide (PI) using a GFP-certified Apoptosis/Necrosis Detection kit (Enzo Life Science) according to the manufacturer's instructions. The cells were stained with 4,6′-diamino-2-phenylindole (DAPI; Sigma), rinsed with phosphate-buffered saline, and mounted with anti-fade mounting medium. They were then observed under a Zeiss LSM 510 laser scanning confocal microscope. The control cells were rarely stained with PI and Annexin V (Fig. 1A). rVvpM-treated cells were stained with both Annexin V and PI, which appeared as yellow and red cells, respectively (Fig. 1B). This observation indicated that rVvpM induced cell death in both an apoptotic and a necrotic manner.

In subsequent experiments, the human cell lines were incubated for 6 h with various amounts of rVvpM and the mortality of these cells was monitored by staining with PI (Figs. 2A and 2B). A human colonic carcinoma cell line, HT-29 (HTB-38; American Type Culture Collection), was

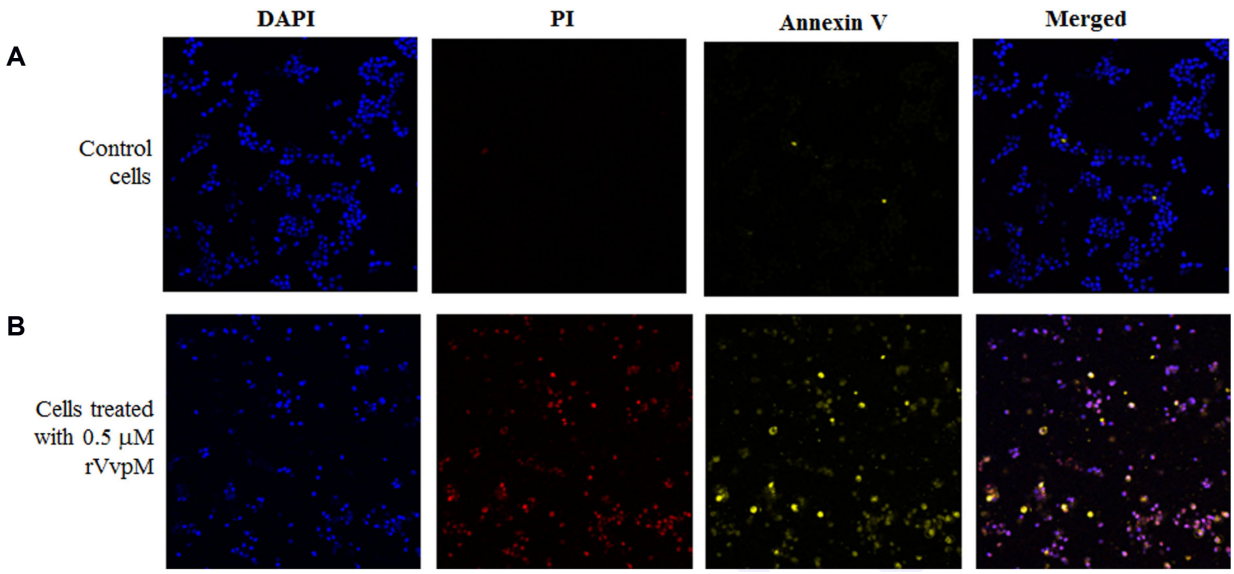

Fig. 1. Staining of the rVvpM-treated HCT-116 cells with Annexin V and PI.

HCT-116 cells were treated with 0.5 μM rVvpM at 37°C for 6 h, and then stained with Annexin V-FITC (yellow) and PI (red) using a GFP-certified Apoptosis/Necrosis Detection kit. The cells were then reacted with DAPI (blue), mounted with anti-fade mounting medium, and observed under a laser scanning confocal microscope. As a control, HCT-116 cells not treated with rVvpM were included in this assay.

added in this assay, which was cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. HCT-116 or HT-29 cells were seeded in a 12-well plate (5×10^5 cells per well) and incubated for 24 h. After washing with FBS-free medium, the cells were treated with various concentrations of rVvpM (from 0.01 to 0.5 μM) for 3 h. As a positive control for cell death, each cell line was treated with 10 mM H_2O_2 for 6 h. Cells were then stained with 0.5 μg/ml PI, and the degree of dye-binding was assessed using fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson). Statistical analysis for pairwise comparison was performed using Student's *t*-test (SYSTAT program, SigmaPlot ver. 9). Seven percent of HCT-116 cells were stained with PI in the absence of rVvpM, whereas 33% of the cells were stained with PI after treatment with 0.5 μM rVvpM ($p = 0.0022$). In the same manner, less than 8% of HT-29 cells were stained with PI without rVvpM treatment, but 27% of HT-29 cells were stained by PI after treatment with 0.5 μM rVvpM ($p = 0.0001$).

Apoptosis and necrosis have been considered as two representative pathways for cell death. In contrast to the apoptotic pathway involving the sequential activation of caspases [5, 24], necrotic cell death occurs drastically, showing cytoplasmic swelling due to plasma membrane permeabilization. It has been recently reported that necrosis could occur in a tightly programmed fashion named necroptosis [4]. Necroptosis requires the interaction of receptor-interacting serine-threonine kinase 1 (RIP1) with another kinase, RIP3 [2, 5]. To investigate whether rVvpM-mediated cell death is related to necroptosis [20], necrostatin-1 (Nec-1), a chemical compound inhibiting the kinase activity of RIP-1 [3], was treated to cells prior to rVvpM-challenge (Fig. 2C). HCT-116 or HT-29 cells were seeded in a 12-well plate and incubated for 24 h. The cells were pre-treated with Nec-1 at 50 μM for 1 h, and then exposed to 0.5 μM rVvpM for 6 h. As a control, the cells were incubated with 1% DMSO for 1 h before challenging with rVvpM. Degree of cell death was monitored by PI staining and subsequent FACS analysis. Pre-treatment of HCT-116 or HT-29 with Nec-1 reduced the degree of PI staining of rVvpM-treated cells, which was estimated to be 83% ($p = 0.0082$) or 67% ($p = 0.0022$) of the control, respectively. This result indicates that RIP1-mediated necroptosis is one of the pathways involved in the rVvpM-initiated cell death. However, the degree of reduction in PI staining of cells pre-treated with necrostatin-1 was only <35%, suggesting that death of rVvpM-treated cells also occurred *via* RIP-1-independent mechanisms.

When HT-29 cells treated with rVvpM were observed under a transmission electron microscope, these cells showed the morphological changes indicating apoptosis, as previously described [12]. Interestingly, apparent autophagosome-like structures, in addition to the morphological changes derived

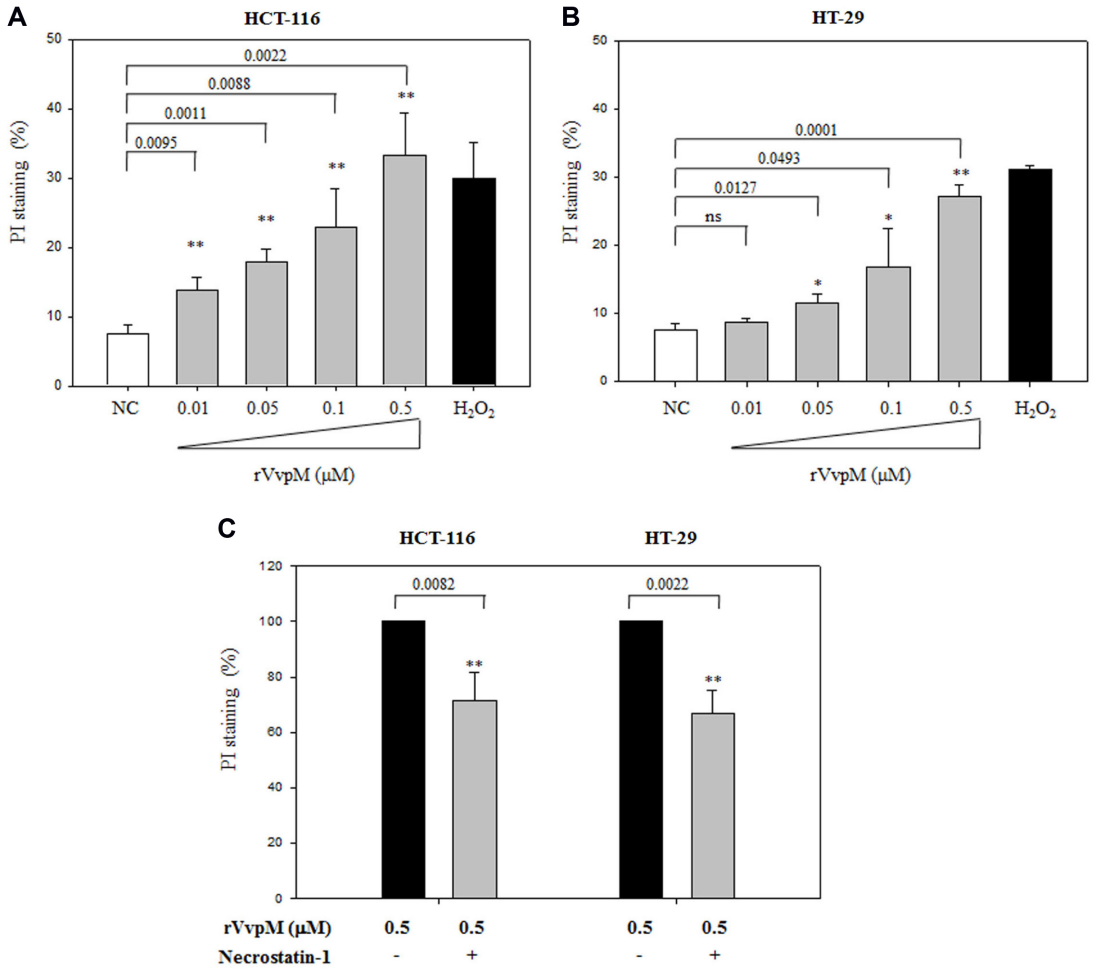

Fig. 2. PI staining of the rVvpM-treated HCT-116 and HT-29 cells, and the effect of Nec-1 on PI staining.

(**A**, **B**) HCT-116 or HT-29 cells (5×10^5 cells per well) were treated with various concentrations of rVvpM (from 0.01 to 0.5 μM) at 37°C for 6 h. The cells treated with 10 mM H_2O_2 or medium only (NC) were included as a positive and a negative control, respectively. Cells were then stained with PI at a concentration of 0.5 μg/ml, and the degree of binding was assessed using fluorescence-activated cell sorting (FACS) analysis. FACS analyses were performed on at least 10,000 cells per sample with a FACScan (Becton Dickinson). (**C**) HCT-116 and HT-29 (5×10^5 cells per well) pre-treated with 1% DMSO solution containing 50 μM Necrostatin-1 for 1 h were exposed to 0.5 μM rVvpM for 6 h at 37°C. As a control, the cells pre-treated with 1% DMSO were challenged with rVvpM. Degree of cell death was monitored by PI staining and subsequent FACS analysis as described above. Results were expressed as the averages ± standard deviations from at least three independent experiments. The *p*-values were derived from comparison with the control: A *p*-value <0.05 was considered statistically significant and the *p*-values are presented in the corresponding figures with a single asterisk (*) for $0.01 < p < 0.05$ or double asterisks (**) for $p < 0.01$. ns, non-significant difference.

from the apoptosis, were observed in the transmission electron micrograms of VvpM-treated cells (Fig. 3). Autophagy is a cellular mechanism by which cytoplasmic components are degraded under stress conditions [13]. Recently, autophagy has been considered as one of the host defense mechanisms to respond to bacterial invasion by delivering intracellular pathogens to lysosomes [15]. Representative markers for autophagic events include beclin-1 for the nucleation process [1], light-chain-3 (LC3) involved in autophagosome formation and elongation [22], and scaffold protein p62 delivering ubiquitinated proteins to the autophagosome [14].

Thus, we examined whether VvpM acted to elicit a characteristic of autophagy by monitoring for LC3B [10]. LC3B involving the autophagosome formation [16] could be present in either the cytoplasmic LC3B-I or the lipidated LC3B-II associated with the autophagosomal membrane [7]. HCT-116 or HT-29 cells were seeded in a 12-well plate and incubated for 24 h. Before treatment with rVvpM, one set of cell cultures was pre-treated with 1% DMSO/2%

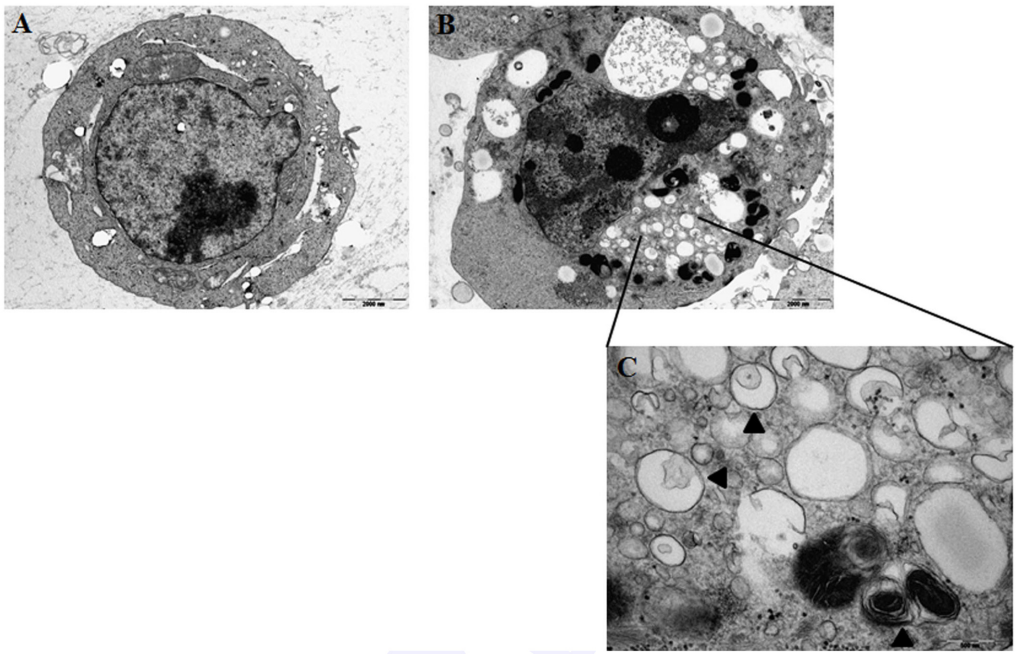

Fig. 3. Transmission electromicrographs of the control HT-29 (**A**) and the rVvpM-treated HT-29 cells (**B**, **C**).
HT-29 cells were cultured for 6 h in the absence (**A**) or presence (**B**, **C**) of 0.1 μM rVvpM. Autophagic vesicles are indicated by arrowheads. The scale bars in panels A and B are 2,000 nm, whereas the bar in panel C is 500 nm.

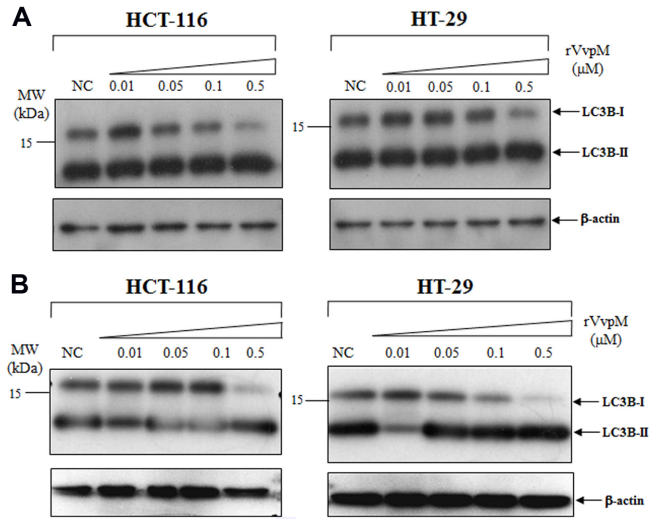

Fig. 4. Formation of LC3B-II in the rVvpM-treated HCT-116 and HT-29 cells.
Effects of rVvpM-treatment on the conversion to LC3B-II were examined in HCT-116 and HT-29 cells. Before treatment of various concentrations of rVvpM (from 0.01 to 0.5 μM), one set was treated with 1% DMSO/2% ethanol (**A**) and the other set was treated with E64d and pepstatin (each 10 μg/ml of 1% DMSO/2% ethanol) for 3 h (**B**). Twenty micrograms of the protein extracts separated on 15% SDS-PAGE was probed with anti-LC3B monoclonal antibodies (Cell Signaling Technology). The level of β-actin in the same samples was monitored as a loading control (the lower panels).

ethanol, and the other set was pre-treated with 1% DMSO/2% ethanol solution containing protease inhibitors (10 μg/ml E64d and 10 μg/ml pepstatin). After 3 h of incubation, they were added with various concentrations of rVvpM (0.01 ~ 0.5 μM) and further incubated for 6 h. Twenty micrograms of the cellular extracts were separated by 15% SDS-PAGE and probed with anti-LC3B monoclonal antibodies (Cell Signaling Technology). For a loading control of the western blot, anti-β-actin antibodies were used. In both cell lines, the levels of LC3B-I were gradually decreased as the added concentrations of rVvpM were increased, suggesting the occurrence of conversion of LC3B-I to LC3B-II. The apparent increase in the LC3B-II levels, however, were not observed in the same cells (Fig. 4A). Since it is possible that the LC3B-II formed might be degraded during the subsequent step(s) of autophagy, such as lysosomal degradation [19], the LC3B levels were also monitored after a pre-treatment with protease inhibitors. Formation of LC3B-II was evidently observed in rVvpM-treated cells incubated with E64d and pepstatin (Fig. 4B), indicating that this rVvpM-induced cell death was related to autophagy.

The co-occurrence of apoptosis and autophagy in rVvpM-treated cells [12] might indicate the presence of a connection between autophagy and apoptosis, possibly *via* the association of another autophagy marker, beclin-1, with

a key member for the apoptotic process, such as the Bcl-2 family member, as reviewed by Wang [21]. It has been previously described that some substances, such as calcium ions and sphingolipids, lead to a co-occurrence of autophagy and apoptosis [17, 23].

This study demonstrates that rVvpM-induced cell death occurs *via* multiple pathways, including necroptosis and autophagy in addition to apoptosis. The implication of this multifarious action of VvpM in the pathogenesis of *V. vulnificus* should be evaluated.

Acknowledgments

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