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Review article

Cytotoxins of *Vibrio vulnificus*: Functions and roles in pathogenesis

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ABSTRACT

Vibrio vulnificus, a marine Gram-negative bacterium, may cause systemic infections in humans and eels. In humans, particularly those with chronic liver disorders, this organism produces severe soft tissue damage via infecting a wound and fulminant sepsis with a high mortality rate in those who ingest contaminated shellfish. The ability of *V. vulnificus* to invade the bloodstream from an intact gastrointestinal tract and cause hemorrhagic necrosis of soft tissue, the most prominent features of its infectious disease, has been the focus of studies on the pathogenesis mechanism. This organism is cytotoxic to a variety of host cells, and this property may be associated with its invasiveness and tissue damaging ability. Two cytotoxins, VvhA and $MARTX_{Vv}$, have been identified. The former exhibits pathological effects resembling those observed in patients, however, it plays only minor roles in cytotoxicity to cocultured host cells and virulence in a mouse model. $MARTX_{Vv}$ has a predicted molecular weight of 556 kDa, and neither the full-length protein nor processed peptides have been purified. This has hampered research on its functions and mechanism of cytotoxicity. Nevertheless, by characterizing the $MARTX_{Vv}$-deficient mutants, this cytotoxin has been demonstrated to be required for survival of *V. vulnificus* at the primary infection site and internal organs as well by preventing the organism from engulfment by the phagocytes. The identification of the functional domains in $MARTX_{Vv}$ should facilitate future studies to reveal how this toxin blocks phagocytosis and causes cell lysis.

1. Introduction

Vibrio vulnificus is a marine bacterium that causes infectious diseases worldwide, with strikingly high mortality rates, mostly due to fulminant septicemia in persons with underlying conditions, particularly liver cirrhosis and hepatoma [1,2]. This bacterium has also caused systemic infection, called vibriosis, in the cultured eels, and has resulted in economical losses in Japan and Europe [3,4]. In studies that have aimed at understanding how this bacterium causes such serious infectious diseases in both hosts, several factors potentially attributing to virulence have been proposed. Some of them, including the capsule, iron-acquisition ability, and cytotoxicity, have been demonstrated as the important

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virulence determinants of *V. vulnificus* in mice, an animal model for human pathogens, and the eel [5–9]. Interestingly, different sets of virulence factors are used in these two animals [10].

Two cytotoxins, the cytolysin/hemolysin and multifunctional autoprocessing repeats in toxin (MARTX), have been identified in *V. vulnificus*. The cytolysin/hemolysin, designated VvhA, has been purified and its biological activities observed in the animals suggest that it might be associated with the invasiveness of the organism and the formation of skin lesions observed in most patients [11,12]. However, a mutant deficient in VvhA is as virulent as the wild-type strain in mice, indicating that VvhA is dispensable for virulence in this animal [13]. In contrast, the MARTX cytotoxin has been recently demonstrated to be required for the virulence of *V. vulnificus* in both mice and eels [6,7,14]. This article reviews and discusses the studies on the functions of these two cytotoxins and their roles in the pathogenesis of systemic infections caused by *V. vulnificus* in mice.

2. *V. vulnificus* and infectious diseases

The identification of unnamed lactose-positive *Vibrio* isolates from patients' blood and wounds was first reported by the US Centers for Disease Control in 1976 [15]. This new species, which could be differentiated from *Vibrio parahaemolyticus* by a lower tolerance for NaCl and fermentation of lactose, was later given a new species name, *vulnificus*, which means "wound" in Greek to reflect one of its infection types. *V. vulnificus* is distributed worldwide, either freely in estuarine water or as a commensal organism in a variety of fish or shellfish, with the highest density in oysters [16,17]. It was estimated that about 50% of *V. vulnificus* environmental isolates are potentially pathogenic to humans [17]. This organism has been shown to enter a viable but nonculturable condition at low temperature [18], and this may explain why this bacterium is almost undetectable in seawater at a temperature below 15°C during the winter in temperate zones. Cells in a viable but nonculturable condition are capable of resuscitation as the water temperature rises to above 15°C [18].

Strains of this species are divided into three biotypes, BT1, BT2, and BT3, based on the biochemical trait, host range, and epidemiological pattern [3,19,20]. All biotypes have been isolated from human cases, but only the BT2 strains are pathogenic to eels [3]. The BT1 strains comprise most of the clinical and environmental isolates collected worldwide and are genetically heterogeneous [17,20], whereas the BT3 strains are isolated only in Israel and are genetically homogeneous [20]. The eel-pathogenic BT2 strains possess a common plasmid that is absent from other biotypes [10]. This plasmid carries the genes that are required for bacterial survival in the eel serum and virulence in the eels [10]. Nevertheless, the common plasmid-cured BT2 strain, although it loses virulence in eels, remains fully virulent in mice indicating that the virulence genes for eels in this plasmid are dispensable in rodents [10]. This might be the first example to show that a microbial species could utilize distinctive survival strategies in response to the different defense systems encountered in different hosts.

Although *V. vulnificus* infection in humans was first reported in 1976, it was suspected that an acute, fatal illness with a swollen reddish foot, high fever, and delirium in a man who lived on an island in the Aegean Sea described by Hippocrates in the fifth century BC was caused by this organism [21]. Infection by *V. vulnificus* is acquired mainly via two routes: ingestion of contaminated seafood, typically raw oysters, and exposure of a wound, pre-existing or newly formed, to substances carrying this organism [1,2]. Individuals with underlying conditions, particularly those with liver cirrhosis and hepatoma that are usually accompanied by elevated iron levels and impaired immune response, are highly susceptible [22]. Patients, whether infected via the mouth or a wound, may develop skin lesions with bullae and hemorrhagic necrosis, which may turn into necrotizing fasciitis, and/or fulminant septicemia [1,23]. The mortality rate of primary septicemia acquired *per os* can be >50%, depending on the timing of treatment relative to the appearance of signs of bacteremia, while that of secondary septicemia developed after wound infection is about 25% [1,2].

Intriguingly, the BT3 strains, which are genetically homogeneous and geographically restricted to Israel, exhibit clinical characteristics different from those of the BT1 strains. About 95% of BT3 infections are associated with percutaneous exposure to fish, typically tilapia, while people are purchasing or preparing fish for cooking, selling fish, or cleaning fish ponds. In addition, most of the BT3 victims have no underlying diseases, and < 20%, even for the fatal cases, have liver disorders [20]. Therefore, BT3 strains are considered more virulent than BT1 and BT2 strains for humans.

The BT2 strains rarely cause human infection, but they produce lesions, including skin ulcer, hemorrhagic fins, protrusion of the rectum, and hemorrhages of the internal organs, in an infected eel [4] similar to those observed in human cases.

3. Virulence mechanism of *V. vulnificus*

As described above, *V. vulnificus* infection is characterized by high invasiveness, rapid progression to septicemia, and formation of severe necrotic skin lesions. These features have been the subjects of studies aiming to reveal the virulence mechanism of this pathogen. Although several potential virulence determinants have been proposed, it is only recently that the mechanisms of how *V. vulnificus* invades from local infection sites into the bloodstream to cause systemic infection and how it defends against immune attack have been better understood.

Normal mice or those with iron overload have been used as an animal model for *V. vulnificus*, because mice receiving a clinical isolate via intraperitoneal, subcutaneous, or intravenous injection, or by force feeding can also develop fulminant sepsis [5,24,25]. So far, the capsular polysaccharides, iron-acquisition ability, flagella, type IV pili, non-pilus adhesins, and cytotoxins have been demonstrated as important virulence factors in mice.

The capsular polysaccharides of most, but not all, strains protect the bacterial cells from phagocytosis and complement-mediated bactericidal activity of human serum [5], and can

induce type-specific protective antibodies in mice [26]. Vulnibactin, a siderophore, and its receptor as well as a heme receptor, HupA, are induced under iron-limited conditions, and they are required for bacterial virulence in mice [9,27]. The genes involved in the utilization of two heterologous siderophores, aerobactin and ferrioxamine B, have also been identified in *V. vulnificus* [28,29], but their associations with virulence have not been determined. The flagellum is involved in bacterial adherence to the host cells and virulence in mice [30], and the type IV pili are associated with biofilm formation, adherence to host cells, and virulence in mice with iron overload [31]. The most abundant outer membrane protein in *V. vulnificus*, OmpU, interacts with host extracellular matrix proteins including fibronectin, and is associated with adherence and toxicity to the epithelial cells [32]. A membrane-bound lipoprotein, IlpA, is involved in adherence to as well as cytotoxicity of the host cells and induction of cytokines [33]. Lysine decarboxylase breaks down lysine to form cadaverine, which may neutralize the highly acidic environment and act as a superoxide scavenger when the bacteria are ingested into the stomach with raw shellfish [34].

V. vulnificus also secretes several enzymes such as the metalloprotease, Vvp [35], phospholipases [36], and nuclease [37]. Purified Vvp has been shown to cause manifestations in animals similar to those observed in humans [38,39]. However, none of these extracellular products is needed for *V. vulnificus* virulence in mice [25,37,40,41].

V. vulnificus is cytotoxic to a variety of cells such as epithelial cells, phagocytes, and endothelial cells by producing two main cytotoxins, VvhA and $MARTX_{Vv}$. VvhA has long been identified in the culture supernatant of *V. vulnificus* [42,43], and the purified recombinant protein has been used to examine its biological activities in animals. It was not until the *vvhA* gene was knocked out that the existence of a second cytotoxin, $MARTX_{Vv}$, was recognized. It was later found that these two cytotoxins were not equally important for the virulence of *V. vulnificus* in mice. Although VvhA seems to be dispensable for bacterial virulence in mice [13], $MARTX_{Vv}$ is required for the colonization of *V. vulnificus* at the infection sites and in the internal organs [6,44], by preventing the bacteria from engulfment by the phagocytes [6].

Based on these findings, a model for the pathogenesis of *V. vulnificus* (Fig. 1) is proposed, in which various virulence factors that may be involved in different stages of the infectious disease are indicated. In the following discussion, the functions and roles of VvhA and $MARTX_{Vv}$ in pathogenesis are summarized.

4. Functions of VvhA and its role in pathogenesis

Kreger and Lockwood were the first to report the cytolytic activity of VvhA against mammalian erythrocytes and Chinese hamster ovary cells in the mid-log-phase culture filtrate of a virulent strain [43]. This culture filtrate also conveyed vascular permeability enhancing activity in guinea pig skin as well as lethal activity for mice [43]. Gel filtration of the culture filtrate revealed two peaks of about 38.5 kDa (major peak) and >150 kDa (minor peak), with the four activities [43]. It was not examined whether the molecule in the minor peak represented another cytotoxin or a multimer of the molecule in the major peak. They also noted in a kinetic assay that the cytolytic activity decreased simultaneously with the appearance of extracellular protease activity in the culture, suggesting that the cytolysins may be degraded by the extracellular protease. Consistent with this observation, a mutant deficient in this protease exhibits increased and prolonged cytolysin activity in the culture supernatant [40]. However, in another study, Shin et al demonstrated in a protease-deficient mutant of another strain that the oligomerization, but not degradation, of VvhA by an unknown mechanism increased with the concomitant loss of hemolytic activity in the late growth phase [45]. A protein of 56 kDa with the four activities of cytolysin was further purified from the culture supernatant [42]. The gene encoding this cytolysin, designated *vvhA*, was later cloned and sequenced [46]. This gene resembles the *V. cholerae* El Tor hemolysin gene, *hlyA*, in the amino acid sequences of two short regions as well as the arrangement of cystine residues that may relate to critical domains for cytolytic activity [46]. From the DNA sequence of *vvhA*, a pair of *V. vulnificus*-specific primers has been designed

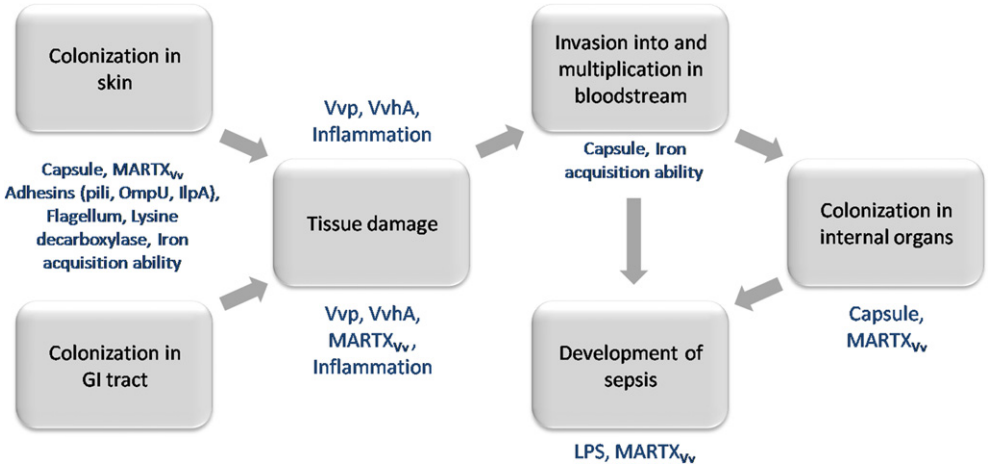

Fig. 1 – Model for the pathogenesis of *Vibrio vulnificus* in mice. Progression of systemic infection leading to sepsis is divided into five stages (in boxes). The contributing factors in each stage are indicated above or below the box. GI = gastrointestinal tract.

and shown to be useful for the identification of this pathogen by polymerase chain reaction [47].

Kim et al studied the hemolytic mechanism of VvhA and found that the hemolysis induced by a high dose of recombinant VvhA (rVvhA) was accompanied by the conversion of membrane-bound cytolysin into a tetramer. Moreover, cholesterol could inactivate VvhA by converting the active monomer into inactive oligomer [48]. These results suggest that VvhA may lyse the erythrocytes by cholesterol-mediated oligomerization that leads to formation of small pores on the erythrocyte membrane. Nevertheless, it was later shown that treatment of human endothelial cells and cancer cells with a low dose of rVvhA induced a rapid efflux of intracellular K^+ without lysing the cells. Instead, these rVvhA-treated cells underwent apoptosis via the mitochondrion-dependent caspase-9/3 signaling pathway [49,50]. Collectively, VvhA is cytotoxic to a variety of host cells by causing necrotic cell death or apoptosis, depending on the amount of toxin to which a cell is exposed. Other than cytotoxicity, VvhA can also activate the calcium/calmodulin signaling pathway, which results in stress fiber formation and phosphorylation of myosin in the endothelial cells, and this effect may lead to blood vessel hyperpermeability [51].

The biological effects of rVvhA have been extensively studied in animals. This cytotoxin is lethal to mice at a submicrogram level [11], and is capable of inducing hemoconcentration, increasing pulmonary vascular permeability [11], and causing acute cellulitis in mice [12]. It can also dilate the thoracic aorta, leading to hypotension and tachycardia [52], and damage the mast cells, resulting in release of histamine [53] in rats. Collectively, the cytolysin was thought to be important for the pathogenesis of *V. vulnificus*. The presence of VvhA in the skin lesions and sera of *V. vulnificus*-infected mice [54] and the detection of antibodies against VvhA in the sera of mice and a human that survived *V. vulnificus* disease [55] further support the role of cytolysin in disease development. However, the mutants deficient in VvhA are as virulent as the wild-type strains in mice [13,25], indicating that this cytotoxin is dispensable for bacterial virulence. Surprisingly, mutants deficient in VvhA alone or both VvhA and the extracellular protease Vvp, although they completely lose the cytolytic activity in culture supernatant, exhibit a wild-type cytotoxicity when the bacteria are co-incubated with the host cells [25]. This result implies the presence of at least an unidentified cytotoxin, whose activity is not detectable in the culture supernatant of *V. vulnificus*. Thus, whether it is because VvhA indeed plays no role or the unknown cytotoxin may compensate for loss of VvhA, so that the VvhA-deficient mutant remains virulent, cannot be known unless this novel cytotoxin is identified.

5. Functions of $MARTX_{Vv}$ and its role in pathogenesis

5.1. *Identification of* $MARTX_{Vv}$

Six years after the report of detecting the activity of an unidentified cytotoxin in the VvhA-deficient mutant, two papers on the identification of a homolog of MARTX cytotoxin in *Vibrio cholerae* ($MARTX_{Vc}$) as an important virulence factor were published. By screening a mutant library generated by transposon mutagenesis, Lee et al obtained a mutant with greatly reduced cytotoxicity for the enterocyte cell line INT-407. In this mutant, the transposon was found inserted in a homolog of *V. cholerae rtxE*, which is involved in the secretion of $MARTX_{Vc}$. A mutant with deletion of *rtxA*, which encodes $MARTX_{Vv}$, a homolog of $MARTX_{Vc}$, was isolated and shown to be impaired for both cytotoxicity to INT-407 cells and virulence in mice infected intraperitoneally [14]. In parallel, Liu et al compared the gene expression profiles of a wild-type strain and an isogenic mutant with deletion of *hlyU* (Δ*hlyU* mutant). HlyU was previously identified as an antigen recognized by the pooled convalescent sera of *V. vulnificus* septicemia patients and shown to regulate positively expression of VvhA [56]. The *rtx* gene cluster, in addition to *vvhA*, was downregulated in the Δ*hlyU* mutant. They further demonstrated that a *V. vulnificus* mutant with deletion of *rtxA1* (formerly *rtxA*) lost its cytotoxicity for HeLa cells and was less virulent by three orders in mice with iron overload [57].

5.2. *Structure and function of* $MARTX_{Vv}$

The MARTX cytotoxins, together with other RTX toxins produced by many Gram-negative bacteria, contain a GD-rich tandem nonapeptide repeat near the C terminus as a common feature [58]. The production and secretion of $MARTX_{Vv}$ are executed by the genes in two divergent operons, *rtxC-rtxA1* and *rtxB-rtxD-rtxE*, which show high homology in nucleotide sequence and gene organization with those of *V. cholerae* [14,57]. The *rtxA1* gene encodes $MARTX_{Vv}$ itself, whereas *rtxB*, *rtxD*, and *rtxE* encode the proteins that form a complex required for the translocation of $MARTX_{Vv}$ across the cytoplasmic membrane. Although the *rtxC* gene is required for activation of $MARTX_{Vc}$ by acylation, it is dispensable for the cytotoxicity of $MARTX_{Vv}$ [57,59].

The unprocessed $MARTX_{Vv}$ predicted from the nucleotide sequence of *rtxA1* is 556 kDa, the largest among the RTXs known to date. Nevertheless, western blotting analyses with antibodies against various regions in this toxin always detect multiple peptides with sizes ranging from about 75 kDa to 300 kDa in the concentrated supernatant or total cell lysate of the bacteria cocultured with host cells [14,60,61]. Both $MARTX_{Vc}$ and $MARTX_{Vv}$ contain a conserved domain, CPD, which exhibits cysteine protease activity and is involved in autoprocessing and cytotoxicity of $MARTX_{Vc}$ [62]. The recombinant CPD of $MARTX_{Vv}$ has also been shown to undergo autoprocessing [63]. However, the $MARTX_{Vv}$ mutant with deletion of CPD is similarly processed into smaller peptides and is as cytotoxic as the wild-type [64], suggesting that $MARTX_{Vv}$ may also be processed by some cytoplasmic proteases in *V. vulnificus*.

Unlike *V. cholerae*, which causes only cell rounding of the epithelial cells in the presence of $MARTX_{Vc}$ [65,66], *V. vulnificus*, either BT1 or BT2, forms pores on the cell membrane and causes cell lysis in the presence of $MARTX_{Vv}$ [10,61]. This functional discrepancy may be attributed to the difference in the nucleotide sequence of *rtxA*. Comparison of the modular structures of $MARTX_{Vc}$ and $MARTX_{Vv}$ produced by BT1 and BT2 strains is shown in Fig. 2. These MARTXs contain two conserved modules with the repeated motifs at the N and C

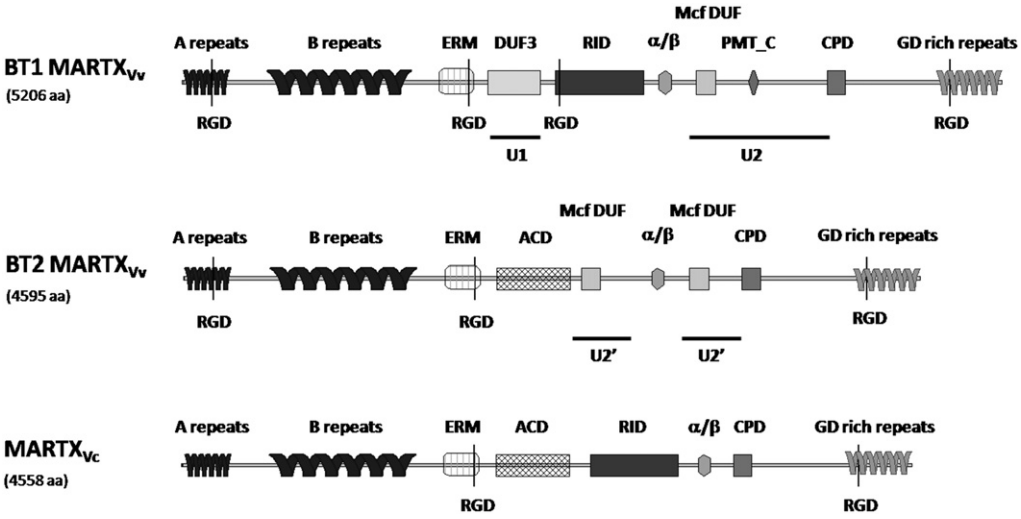

Fig. 2 – Modular structures of the MARTXs produced by *Vibrio cholerae*, *Vibrio vulnificus* biotype 1 and *V. vulnificus* biotype 2. ACD = actin cross-linking domain; α/β = α/β hydrolase; CPD = cysteine protease domain; DUF = domain with unknown function; ERM = ezrin/radixin/moesin domain for actin binding; Mcf = *Photorhabdus luminescens* Mcf toxin; PMT_C = *Pasteurella* mitogenic toxin C terminus; RGD = Arg-Gly-Asp motif; RID = rho GTPase inactivation domain; U1 = biotype 1 $MARTX_{Vv}$ unique sequence 1; U2 = biotype 1 $MARTX_{Vv}$ unique sequence 2.

termini, and one variable internal module containing different functional domains [67]. Interestingly, differences in the internal modular structure are seen not only between $MARTX_{Vc}$ and $MARTX_{Vv}$ but also between the BT1 and BT2 $MARTX_{Vv}$. The BT2 $MARTX_{Vv}$ shows a mosaic structure composed of regions from those of $MARTX_{Vc}$ and BT1 $MARTX_{Vv}$. It is suspected that the ability to lyse host cells may reside in the N terminus (359 amino acids) of U2; the only region of substantial length that is absent from $MARTX_{Vc}$, but is present in both BT1 and BT2 $MARTX_{Vv}$. However, the cell lytic ability of a mutant with deletion of the entire U2 region in BT1 $MARTX_{Vv}$ is not impaired (our unpublished data), suggesting that sequences other than U2 may be responsible for cell lysis. Alternatively, an unknown factor that is absent in *V. cholerae* but present in *V. vulnificus* may function in concert with a region that is shared by $MARTX_{Vc}$ and $MARTX_{Vv}$ to lyse the host cell. Notably, it has recently been reported that $MARTX_{Vc}$ can also mediate cell lysis, although with low activity, in mouse bone-marrow-derived macrophages [68], meaning that this toxin may exert different effects in different cells. In this case, the ability to lyse cells is possibly determined by sequences shared by these two toxins.

Several studies have been conducted to uncover the mechanism of cell death caused by $MARTX_{Vv}$. Toma et al have proposed that this toxin may cause caspase-1-mediated necrotic death in mouse macrophages [68]. However, Lee et al noticed that the human intestine epithelial cells exposed to the wild-type, but not $MARTX_{Vv}$-deficient mutant, can undergo apoptosis via a mitochondrion-dependent pathway [69]. In another study, it has been demonstrated in the murine intestine epithelial cell line, CMT-93, that $MARTX_{Vv}$ may activate NAD(P)H oxidase to induce the generation of reactive oxygen species, which kill the host cell, by modulating the small GTPase Rac2 [70]. Although more studies are needed to reveal how $MARTX_{Vv}$ results in cell death, it is possible that this toxin exerts different effects depending on the target cell, multiplicity of infection (MOI) and incubation period.

5.3. *Role of $MARTX_{Vv}$ in pathogenesis*

In mice infected subcutaneously with a MARTX-deficient *V. vulnificus* mutant, Lo et al found that colonization at the infection site and subsequent spread into the bloodstream were impaired [6]. Phagocytes play important roles in clearance of invading bacteria, therefore, association of $MARTX_{Vv}$ with bacterial resistance to clearance and phagocytosis by the phagocytes were investigated. It was shown that the defects of the $MARTX_{Vv}$-deficient mutant in colonization were restored either in mice depleted of neutrophils or when this mutant was administered into normal mice with equal numbers of wild-type strain. Furthermore, compared to the wild-type strain, this mutant was more readily cleared from the macrophage-rich mouse peritoneal cavity. In addition, at a low MOI, under which the macrophages remained viable throughout the experiment, the number of $MARTX_{Vv}$-deficient mutant detected in murine macrophages was significantly higher than that of the wild-type strain. Nevertheless, the survival rates of the internalized bacteria were similar between the wild-type and mutant strains [6]. These results suggest that $MARTX_{Vv}$ is required for *V. vulnificus* survival during infection by protecting the organism from engulfment by phagocytes.

The role of $MARTX_{Vv}$ in pathogenesis of *V. vulnificus* was also investigated by Jeong et al in mice orally infected with a different strain, and they concluded that this toxin contributes to rapid *in vivo* growth of bacteria in the gut [44]. However, they did not examine whether the rapid bacterial

colonization was associated with reduced phagocytosis in the presence of $MARTX_{Vv}$.

Whether $MARTX_{Vv}$ promotes bacterial spread into the bloodstream and internal organs by causing tissue damage is controversial. Lo et al have found that in neutropenic mice infected subcutaneously, the number of MARTX-deficient mutants in the bloodstream reached a wild-type level, and this mutant resulted in severe tissue damage at the primary infection site. These results imply that $MARTX_{Vv}$ is dispensable for tissue destruction [6]. In contrast, Jeong et al have observed only minor damage in the guts of mice infected orally by the $MARTX_{Vv}$-deficient mutant at a dose high enough to reach a concentration in the lumen 8 hours after infection, at which the wild-type strain caused severe tissue damage [44]. This suggests that $MARTX_{Vv}$ may also contribute to tissue damage, which facilitates bacterial dissemination. As mentioned above, *V. vulnificus* produces a variety of tissue-damaging factors, such as Vvp and VvhA, other than $MARTX_{Vv}$. It is plausible that the environmental factors affecting the expression levels of these factors at different anatomical sites might not be the same. As such, $MARTX_{Vv}$ might not be equally required in causing tissue damage at the primary infection sites in mice infected via different routes.

$MARTX_{Vv}$ has also been shown to be involved in the production of interleukin-1β by bone-marrow-derived macrophages after infection by *V. vulnificus* via activation of the NLRP3 inflammasome [68]. As such, $MARTX_{Vv}$ may also contribute to the induction of inflammation after bacterial infection and, consequently, the formation of severe skin lesions and the development of septicemia.

6. Additive effects of VvhA and $MARTX_{Vv}$ on cytotoxicity and virulence

By comparing the cytotoxicity of various *V. vulnificus* mutants deficient in either one or both of VvhA and $MARTX_{Vv}$, several laboratories have shown that both cytotoxins contribute, although not equally, to the cytotoxicity observed in a bacterium–host co-culture system. The Δ*vvhA* mutant shows a wild-type level of cell lysis; Δ*rtxA1* mutant exhibits residual cytotoxicity detected only at high MOI or after prolonged incubation; and the Δ*rtxA1*Δ*vvhA* double mutant does not lyse cells [44,68,71]. Consistently, VvhA exerts less impact on bacterial *in vivo* growth and progression of infection than $MARTX_{Vv}$, and the double mutant is essentially avirulent in mice infected orally [44]. Therefore, although $MARTX_{Vv}$ is no doubt required for the virulence of *V. vulnificus* in mice [6,44], the effect of VvhA, although small, may not be overlooked.

7. Conclusions and future directions

Although rarely reported, *V. vulnificus* still causes life-threatening infections worldwide. Understanding the pathogenetic mechanism of infectious diseases caused by this organism would help us better control or prevent severe outcomes, such as necrotizing fasciitis and sepsis, for example, by developing effective vaccines or therapeutic agents. The cytotoxins, VvhA and $MARTX_{Vv}$, produced by *V. vulnificus* have both been shown to result in host cell lysis by forming pores on cell membranes and participating in pathogenesis. Despite the clinical manifestations resembling local and systemic pathological effects that it produces in rodents, VvhA appears to play a minor role at most in the pathogenesis of *V. vulnificus* systemic infection in mice via involvement in tissue destruction. In contrast, $MARTX_{Vv}$ alone is potent enough to abolish bacterial engulfment by phagocytes and cause host cell lysis. $MARTX_{Vv}$-deficient mutants are readily cleared from the primary infection site, therefore, inactivation of this toxin in the early stage of infection may impede the colonization and, subsequently, spread of the organism to result in systemic infection.

In contrast to VvhA, which is 56 kDa in size and can be purified as a recombinant protein, $MARTX_{Vv}$ is huge, 556 kDa if unprocessed, and therefore is extremely difficult to clone or purify. As such, although the biological effects of VvhA are mostly determined with the recombinant proteins, those of $MARTX_{Vv}$ can only be deduced from the phenotypes of a gene knockout mutant compared to those of the wild-type strain. Moreover, further explorations to disclose the molecular mechanisms of $MARTX_{Vv}$ in cytotoxicity and blocking phagocytosis are hampered. The identification of functional domains by characterizing either the cloned domain peptides or mutants deleted of various domains is therefore anticipated to solve this problem. Our laboratory has already found that several regions are not required for the function of $MARTX_{Vv}$. Hopefully, a functional, shortened form of this toxin could be generated, with which studies on the functions of $MARTX_{Vv}$ can be accelerated.

It is plausible that upon interaction with the host cell, the processed $MARTX_{Vv}$ may interfere with signaling for actin rearrangement to block the internalization of bacteria. Our laboratory has identified the signaling pathway affected by $MARTX_{Vv}$ in mouse macrophages. Exactly how this cytotoxin causes the alteration of signaling status awaits further studies.

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