Inhibition of Binding of *Vibrio vulnificus* Hemolysin (VVH) by M β CD

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ABSTRACT. *Vibrio vulnificus* secretes a pore-forming toxin called *Vibrio vulnificus* hemolysin (VVH). In this study, we showed that methyl-beta-cyclodextrin (M β CD), an oligosaccharide, decreased binding of VVH to Chinese hamster ovary (CHO) cells, resulting in inhibition of its cytotoxicity. When the VVH was incubated with M β CD, cytotoxicity of the toxin was inhibited from $100.3 \pm 7.2\%$ to $19.6 \pm 5.3\%$. Binding analysis showed that the amount of VVH on the cells was decreased from $101.4 \pm 9.2\%$ to $18.1 \pm 8.0\%$ only when M β CD was present in the culture media. Our results indicate that the inhibition of cytotoxicity of VVH by M β CD was due to a decrease in the amount of toxin binding to CHO cells.

KEY WORDS: cytotoxicity, hemolysin, methyl-beta-cyclodextrin, *Vibrio vulnificus*.

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Vibrio vulnificus, which is a Gram-negative bacterium, typically infects humans suffering from liver cirrhosis, alcoholism, hemochromatosis, immunocompromised conditions or diabetes, resulting in septicemia [5, 9, 10, 17]. Primary septicemia in *V. vulnificus* infection is caused by the ingestion of contaminated seafood or through wound infection resulting from exposure to contaminated seawater or marine products [10, 17]. *Vibrio vulnificus* hemolysin (VVH) is a possible virulence factor which is produced by *V. vulnificus* [3, 12]. This toxin has been reported to cause cytolysis of various eukaryotic cells, as well as erythrocytes [6, 12, 20]. With regard to the hemolytic or cytotoxic mechanism (s) of VVH, it has been reported that VVH monomer binds to cellular membranes to form oligomers, and that these oligomers form ion-permeable pores which exert hemolysis or cytotoxicity via colloid osmotic shock [8, 19].

Methyl-beta-cyclodextrin (M β CD) is an oligosaccharide with a ring-shaped structure, which sequesters cholesterol from cellular membrane, and inhibits formation of lipid rafts by decreasing the contents of membrane cholesterol. Therefore, M β CD is used to analyze the function of lipid rafts which are cholesterol-rich membrane domains. In the process of investigation into the localization of VVH on the cellular membrane using M β CD, when the cells were not washed after M β CD treatment, we found that the cytotoxicity of VVH was inhibited. On the other hand, when the cells were washed to remove the M β CD in the culture media, the cytotoxicity of VVH was not affected [18]. Here, the present study investigated the mechanism by which M β CD inhibits the cytotoxicity of VVH.

First, we reconfirmed that the cytotoxicity of VVH was

inhibited in CHO and Caco-2 cells by M β CD, when the M β CD was not washed out. These cell lines were cultured as previously described [18], and the purified VVH, prepared by the method of Oh *et al.* [14], was used in this study. Cytotoxicity was evaluated by measuring a lactate dehydrogenase (LDH) release as previously described with slightly modification [18]. At 1 hr after the culture media were replaced with DMEM containing 8 mM M β CD, the cells were washed or not washed with DMEM. In a pilot study, we confirmed that there was no difference in cytotoxicity of VVH between washing the cells with DMEM containing 8 mM M β CD and not washing (data not shown), indicating that the mechanical impact could not affect the cytotoxicity. Therefore, we chose without washing the cells in this study. The LDH release by M β CD in M β CD-untreated CHO and Caco-2 cells was $74.9 \pm 4.2\%$ or $71.3 \pm 4.2\%$, respectively (Fig. 1). This LDH release in CHO or Caco-2 cells was $11.5 \pm 5.6\%$ or $20.4 \pm 6.9\%$ in the presence of M β CD, whereas that in CHO or Caco-2 cells without M β CD was $72.1 \pm 5.2\%$ or $70.4 \pm 6.9\%$, respectively (Fig. 1). It was revealed that the M β CD only in culture medium can inhibit the cytotoxicity of VVH in these cell lines. These data gave us the idea that the M β CD in culture media could inhibit the cytotoxicity of VVH without the interaction with cells, i.e. the M β CD would inhibit the cytotoxicity of VVH by binding to VVH directly.

Next, we investigated the direct binding of M β CD with VVH. VVH was pre-incubated with 8 mM M β CD at 37°C for 1 hr, and the culture media in these cell lines were replaced with this mixture. As shown in Fig. 2, the cytotoxicity of VVH that was pre-incubated with M β CD in CHO or Caco-2 Cells was depressed to $13.6 \pm 3.5\%$ or $23.1 \pm 5.8\%$, whereas it was 80.1 ± 8.3 or $71.3 \pm 4.2\%$ in these cell lines incubated without M β CD, respectively. The results of Fig. 2 suggest that the M β CD inhibits the cytotoxicity of VVH by binding to VVH directly before interacting with cellular membranes. Although M β CD may inhibit the cytotoxicity of VVH by binding to VVH directly, the cells might be exposed

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Fig. 1. Cytotoxicity of VVH was inhibited by M β CD in culture media.

CHO and Caco-2 cells were incubated with or without (closed bar) 8 mM M β CD at 37°C for 1 hr. After washing (open bar) or not washing (gray bar), these cells were incubated with 1 μ g/ml of VVH for 1.5 hr at 37°C. The cytotoxicity of VVH in these cells was assayed by the release of lactate dehydrogenase (LDH). Data are represented as the mean $\pm$ SD and represent more than three independent experiments, each in triplicate wells. *, analysis of variance and Tukey's test, $P < 0.01$.

Fig. 2. Cytotoxicity of VVH was inhibited by pre-incubation with M β CD.

VVH was pre-incubated with (gray bar) or without (closed bar) 8 mM M β CD at 37°C for 1 hr. After incubation, the mixture of VVH and M β CD was exposed to CHO or Caco-2 cells. Cytotoxicity of VVH was measured by LDH release assay. Data are represented as the mean $\pm$ SD and represent more than three independent experiments, each in triplicate wells. *, $P < 0.01$.

to the unreacted M β CD in the mixture and these M β CD might decrease the contents of cellular cholesterol, followed by the inhibition of cytotoxicity. It has been reported that the cytotoxicity of several pore-forming toxins was inhibited by M β CD decreasing the cellular cholesterol [2, 11]. However, in our previous study, the cytotoxicity of VVH was

not inhibited even in the M β CD-treated CHO cells in which cellular cholesterol decreased to $57.2 \pm 8.8\%$ [18]. In addition, there is no difference in the percentages of cytotoxicity inhibition of VVH between adding VVH to medium containing M β CD and pre-incubating VVH with M β CD from the data of Figs. 1 and 2. Therefore, the result of Fig. 2 strongly

Fig. 3. MβCD induces oligomerization of VVH.

VVH was incubated with (shade bar) or without (gray bar) 8 mM MβCD at 37°C for 1 hr. After incubation, the mixture of VVH and MβCD was loaded onto SDS-PAGE, and transferred to an Immobilon-P transfer membrane. VVH was detected by western blotting using anti-VVH polyclonal antibody. The band intensity of the protein was measured using NIH Image J software.

*, $P < 0.01$.

suggested that MβCD directly interacted with VVH, and the unreacted MβCD did not show any remarkable influence in this experiment.

It is well known that cholesterol induced the conversion of monomer to oligomer in VVH, and that oligomerized VVH could not bind to target cells anymore [7, 8, 19]. In our study, cytotoxicity of VVH pre-incubated with MβCD was significantly decreased (Fig. 2). These facts raise the possibility that the MβCD induced oligomerization of VVH by direct binding to VVH, resulting in decrease of VVH bound to cellular membranes. In Fig. 3, we examined this conversion of VVH by MβCD. VVH was incubated with or without 8 mM MβCD for 1 hr at 37°C, and the mixture of VVH and MβCD was loaded onto SDS-PAGE without exposing the mixture to CHO cells. As a result, the VVH oligomer was detected in the mixture by western blotting using anti-VVH polyclonal antibody. As shown in Fig. 3, the amount of oligomer was increased about 13.5-fold in the VVH incubated with MβCD compared with that incubated without MβCD, indicating that MβCD clearly induced oligomerization of VVH by binding to VVH directly.

As described above, it had been reported that VVH oligomer cannot bind to cellular membranes. Next, we examined whether the binding of VVH to cellular membranes is inhibited by MβCD or not. Binding amount of VVH to CHO cells was measured by binding assay which modified oligomerization assay as previously described [18]. Briefly,

Fig. 4. Binding amount of VVH decreased in an MβCD-concentration-dependent manner.

CHO cells were incubated with indicated concentrations of MβCD for 1 hr at 37°C. The cells were exposed to VVH (1 μg/ml) without washout of MβCD, and further incubated for 1.5 hr at 37°C. After incubation, the cells were lysed with lysis buffer. Protein concentrations in the cell lysates were measured by Bradford assay. Equal amounts of proteins were transferred to a Hybond-C Extra membrane. VVH was detected using anti-VVH polyclonal antibody. The dot intensity of the protein was measured using NIH Image J software. *, $P < 0.05$. **, $P < 0.01$.

VVH was exposed to CHO cells without washing after incubating with the indicated concentration of MβCD. The cells were washed to remove VVH which does not bind to cellular membranes, and then the cells were lysed with lysis buffer. VVH in the lysate was detected by dot blotting, and the dot intensity was quantified by using Image J software. As shown in Fig. 4, the dot intensity of VVH was decreased by MβCD in a concentration-dependent manner. Binding amount of VVH was decreased to $18.1 \pm 8.0\%$ (8 mM), $26.6 \pm 6.1\%$ (4 mM) and $57.5 \pm 6.9\%$ (2 mM) in CHO cells (Fig. 4). Thus, the MβCD inhibited the binding of VVH to the CHO cells in a concentration-dependent manner. Therefore, the inhibition of cytotoxicity of VVH by MβCD was due to a decrease in the amount of VVH binding to the CHO cells.

Ricin is a plant toxin composed of A- and B-subunits. The B-subunit includes a β-trefoil lectin domain that is involved in the binding of carbohydrate chains, and forms a β-trefoil structure consisting of 3 repeats, α-, β- and γ-repeat. There is a QxW motif in each repeat, and carbohydrate chains bind to the QxW motif in α-repeat especially [13, 16]. It was shown that VVH has a β-trefoil lectin domain, and that the QxW motif is also conserved within the lectin domain of this toxin [4]. The identity is 24% between VVH-lectin domain and ri-

cin-lectin domain [4]. It is likely that the VVH-lectin domain can recognize a carbohydrate chain. In fact, Kashimoto *et al.* have suggested that the VVH-lectin domain may be required to bind to cellular membranes [6]. In this study, M β CD decreased the binding efficiency of VVH to CHO cells (Fig. 4), and also inhibited the cytotoxicity of VVH in the cells (Fig. 1). These data suggest that M β CD may directly interact with the VVH-lectin domain via the QxW motif in the β -trefoil lectin domain of VVH. *Vibrio cholerae* cytotoxin (VCC) which is produced by *Vibrio cholerae* was composed of three domains, cytotoxin domain, β -prism lectin domain and β -trefoil lectin domain [1, 15]. After the β -prism lectin domain binds to carbohydrate chain on cellular membranes, resulting in induced rearrangement of this domain and pore formation [1]. In this process, VCC formed oligomer to pore formation. It is well known that the β -prism lectin domain was absent in VVH [15]. The β -trefoil lectin domain in VVH may substitute for the β -prism lectin domain in VCC, and therefore conformational change of this domain in VVH may facilitate oligomerization after binding to carbohydrate chain (s) on cellular membranes. However, further studies will be needed to confirm that the β -trefoil lectin domain of VVH can recognize carbohydrate chain (s).

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