主 論 文

Vibrio alginolyticus VepA Induces Lysosomal Membrane Permeability and Cathepsin-Independent Cell Death

(*Vibrio alginolyticus* の VepA 依存的なリソソーム膜透過性を伴う細胞死誘導)

Introduction

Vibrio alginolyticus is a halophilic, Gram-negative bacteria, motile, and naturally distributed in the marine and estuarine waters. This bacterium is an opportunistic pathogen for human and causes wound infection. Due to climate change issues, the incidence of *V. alginolyticus* infection is increasing. Therefore, a complete understanding of the virulence mechanism of *V. alginolyticus* is necessary to prevent future outbreaks.

V. alginolyticus type III secretion system (T3SS) is required for its cytotoxicity. A T3SS is a syringe like apparatus used by bacteria to introduce effector proteins into the cytoplasm of eukaryotic host cells. Although the role of T3SS in *V. alginolyticus* has been known in cytotoxicity, however, the effector of T3SS that is responsible for the cytotoxicity has been unclear.

In this study, we analyze the role of VepA in cytotoxicity of *V. alginolyticus* and cell death mechanism of VepA-dependent cytotoxicity.

Materials and Methods

Bacterial strains and eukaryotic cell

We used *V. alginolyticus* ATCC 17749 as the parent strain for construction of gene-deletion mutant. We used HeLa cells for the infection experiment.

Cytotoxicity assay

HeLa cells were infected with *V. alginolyticus* at a multiplicity of infection of 100 for 4 hours. We measured the release of lactate dehydrogenase (LDH) into the medium after 4 hr of infection to determine the cytotoxicity of *V. alginolyticus* toward HeLa cells.

AO relocation assay

HeLa cells were treated with 2 μg/ml acridine orange (AO) for 15 minutes, then infected to *V. alginolyticus* strain for 4 hr. AO-stained HeLa cells were analyzed by fluorescence microscopy.

Fluorescent-dextran translocation assay

HeLa cells were incubated in 100 μg/ml fluorescein isothiocyanate (FITC)-dextran for 16 hours before infected by *V. alginolyticus* or challenged with a lysosomal disruptor, L-Leucyl-L-Leucine methyl ester (LLOMe). We analyzed fluorescent dextran in HeLa cells by fluorescence microscopy.

Inhibition of cathepsin and reactive oxygen species (ROS), LMP induction, and iron chelation.

We used 100 μM E-64d to inhibit cathepsin. We used 3 mM LLOMe to induce LMP. To chelate ferrous ion, we used 100 μM 2,2'-bipyridyl (BIP). To inhibit ROS, we used 5 mM N-acetyl cysteine (NAC) or 250 μM Trolox.

Statistical analysis

The two-tailed Student's *t*-test was used for the statistical analyses.

RESULTS

VepA contributes to HeLa cell cytotoxicity in V. alginolyticus infection

vepA mutant showed significantly less cytotoxicity than the wild-type (WT) and *vepA* complimentary in *vepA* mutant completely restored the cytotoxicity (similar to WT). These results indicate that mainly VepA is responsible for the cytotoxicity of *V. alginolyticus*.

VepA in V. alginolyticus induced lysosomal membrane permeabilization (LMP)

Acridine orange (AO) relocation assay was used to determine LMP in HeLa cells. In uninfected HeLa cells, we observed small red fluorescent dots, which reflect an accumulation of AO in intact lysosomes. In the WT infection, the appearance of red fluorescent dots was disappeared. In *vepA* mutant infection, the appearance red dots was comparable to those in the uninfected cells. In the *vepA* complementation, the fluorescent pattern reverted to one similar to that obtained with the WT infection. These results indicate that *V. alginolyticus* infection induces the leakage of the lysosomal contents into the cytosol in a VepA-dependent manner.

The VepA-induced LMP in *V. alginolyticus* was not due to a lysosomal membrane rupture

To determine whether the molecules larger than AO (0.3 kDa) was released during *V. alginolyticus* infection, we observed lysosomal leakage by using larger molecules: FITC-dextran 10 or 4 kDa. Both in 10 and 4 kDa FITC-dextran-loaded HeLa cells showed diffuse green fluorescence in the cytosol when challenged with a lysosomal disruptor, LLOMe, indicating FITC-dextran were release from the lysosome. In contrast, the HeLa cells

infected with *V. alginolyticus* strains did not exhibit the fluorescent diffusion into the cytosol, but the cell did show fluorescent punctates. These results indicate that the VepA-mediated LMP allows the translocation of molecules < 4 kDa.

VepA-dependent cytotoxicity is not due to lysosomal cathepsin

Since *V. alginolyticus* VepA-induced LMP allowed the release of only molecules < 4 kDa, we hypothesized that cathepsins (approx. 30 kDa) could not pass through the lysosomal membrane. We used a broad-spectrum cathepsin inhibitor, E-64d to determine whether the VepA-mediated cell death is due to cathepsin release from lysosomes. The cytotoxicity induced by lysosomal disruptor (LLOMe) was reduced by E-64d treatment. However, E-64d could not protect the cells against WT infection. These results suggest that cathepsin is not the major causative factor of *V. alginolyticus* cytotoxicity.

Iron involved in VepA-dependent cell death

Since the lysosome is one of the iron-rich intracellular organelles, VepA might allow excessive iron release into the cytosol to induce cell death via reactive oxygen species (ROS) known as ferroptosis. To investigate the role of iron and ROS in cell death in *V. alginolyticus* infection, we compared their cytotoxic effects in the presence of an iron chelator (BIP), and an ROS scavenger (NAC or Trolox). In the presence of BIP, the cytotoxicity was significantly reduced. However, after the ROS scavenger treatment, the cytotoxicity was slightly reduced or not changed, respectively. These results indicate that iron is involved in VepA-dependent cell death without ROS activation.

Discussion

VepA induces cell death in *V. alginolyticus* infection and induces lysosomal damage that allows only molecule < 4 kDa release into cytosol. Cathepsin-dependent cell death induced by a lysosomal disruptor could be stopped by cathepsin inhibitor, however, VepA-dependent cell death in *V. alginolyticus* infection could not. These results indicate that VepA-dependent cell death is cathepsin-independent cell death. Since VepA induced only small molecules release from lysosomes and iron chelation reduced the cytotoxicity during *V. alginolyticus* infection, indicating that iron involve in VepA-dependent cell death. A cell death mechanism known as ferroptosis, involves the ROS activation by ferrous ion. In *V. alginolyticus* infection, ROS scavenger failed to protect the cell, indicating ROS does not involve in VepA-dependent cell death. We therefore propose that iron induces cell death without ROS accumulation in this infection model.

Conclusion

V. alginolyticus VepA induces cathepsin-independent cell death and size-specific LMP that allows only small molecules to be released into the cytosol. We propose that iron leakage from lysosome plays an important role in VepA-dependent cell death in *V. alginolyticus* infection. Further research is required to elucidate the underlying molecular mechanism, which might provide insights into the development of novel therapeutics that target the VepA-related cell death mechanism.