Note

Identification of Vibrio anguillarum Outer Membrane Vesicles Related to Immunostimulation in the Japanese Flounder, Paralichthys olivaceus

Gyeong-Eun Hong,† Dong-Gyun Kim,† Eun-Mi Park, Bo-Hye Nam, Young-Ok Kim, and In-Soo Kong

Department of Biotechnology, Pukyong National University, Busan 608-737, Korea
National Fisheries Research and Development Institute, Busan 619-902, Korea

Received August 22, 2008; Accepted September 24, 2008; Online Publication, February 7, 2009

We identified outer membrane vesicle (OMV) production in Vibrio anguillarum O1, a major fish pathogen that causes vibriosis, and characterized the OMVs. They were produced during normal growth, and were appeared as spherical vesicle fractions. The protein profile of the OMVs was similar to that of the outer membrane proteins, and the 38-kDa major protein band of OMV was identified as OmpU. The OMVs had enzyme activity of metalloprotease, hemolysin, and phospholipase, and stimulated the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 when injected into the flounder.

Key words: Vibrio anguillarum; outer membrane vesicle; pathogen; cytokine

Outer membrane vesicles (OMVs) are composed of outer membrane proteins (OMPs), lipopolysaccharides (LPSs), phospholipids, and periplasmic proteins, but lack inner membrane proteins and cytoplasmic components.1,2 They play an important role in initiating infection,2–4 because they deliver antigens and virulence factors into host cells.2,5 OMVs deliver virulence factors, including degrading enzymes, into the host cell and subsequently damage the cell. Production of OMVs by Gram-negative bacteria has been widely reported in Escherichia coli, Neisseria meningitides, Shigella flexneri, S. dysenteriae, Bacteroides gingivalis, B. saccharolyticus, and B. endodontalis.5 Kondo et al. reported that Vibrio cholerae and V. parahaemolyticus produce OMVs.2,3 However, the biochemical characteristics of these OMVs are still unknown. Furthermore, no studies of OMVs from V. anguillarum have been published. V. anguillarum, an agent that causes vibriosis in several fish species, occurs throughout the marine environment.6 In this study, we investigated the production of OMVs from V. anguillarum and characterized some of their biochemical properties.

To identify OMV production in V. anguillarum serotype O1, the bacterium was cultured overnight on BHI agar plates (25°C) and fixed. Thin sections were taken for transmission electron microscopy (TEM; JEM-1022EX2; JEOL, Tokyo). OMVs bud from the outer membranes of cell colonies grown on BHI agar and are then released from the outer membrane into the environment (Fig. 1A). V. anguillarum O1 produces OMVs that can encapsulate the periplasm of the bacterium (Fig. 1A). To purify OMVs in large quantities, cells were incubated in 1 liter of culture medium, and the OMVs were isolated from the culture supernatant using the methods of Zhou et al.9 Isolated OMVs were observed under TEM after a negative stain. The individual OMVs that were released from the cell body had a spherical form 150–300 nm in diameter (Fig. 1A inset). The protein profiles of the purified OMVs and OMPs from whole cells were compared by SDS–PAGE. OMPs of V. anguillarum O1 were prepared from the cell pellet as described by Chalcroft et al.10 OMPs and OMVs in equal amounts were loaded onto 12% acrylamide gel. The band patterns were similar, but the OMVs had fewer bands than the OMPs (Fig. 1B). For the OMVs, the largest band was an outer membrane protein of 38 kDa. It was identified as an OmpU protein by MALDI-TOF/MS analysis (data not shown). OmpU is the major outer membrane protein of V. anguillarum O1.11 V. anguillarum produces various virulence factors enhancing infection and secretes several exotoxins, such as hemolysins, proteases, metalloproteases, dermatotoxins, hemaglutinins, and cytoxins.12 OMVs obtained from other Gram-negative bacteria contain many virulence factors and exoenzymes associated with pathogenesis.13 We examined metalloprotease, hemolysin, and phospholipase activity in the OMVs. The substrates used for these enzyme types were 0.5% gelatin, 5% sheep blood, and 5% egg yolk, respectively. A 5-μg sample purified OMV or OMP was placed on a plate containing the appropriate substrate and incubated overnight at 25°C. Protease activity appeared as a clear zone around the OMV sample after the addition of Frazier solution to the appropriate substrate and incubated overnight at 25°C. Protease activity was markedly reduced by 10 mM EDTA (Fig. 2A).13 Protease activity was detected clearly on 5% sheep blood agar and 5% egg yolk plates respectively (Fig. 2C, D). However, the purified OMP samples showed no definite enzyme activity. These results suggest that OMVs from V. anguillarum O1 encapsulate enzymes such as hemolysin, metalloprotease, and phospholipase that can damage the host cell through virulence factors liberated into the target tissue.

† To whom correspondence should be addressed. Tel: +82-51-629-5865; Fax: +82-51-629-5863; E-mail: iskong@pknu.ac.kr
* These authors contributed equally to this paper.
Fig. 1. TEM Images of V. anguillarum O1 and SDS–PAGE Analysis of the Isolated Outer Membrane Vesicles (OMVs). A, Thin section of a V. anguillarum cell showing the formation of vesicles and freed OMVs. Vesicles bud from the outer membrane. Bar, 100 nm. The inset is a TEM image of negatively stained OMVs isolated from V. anguillarum O1. Vesicles are uniform spheres of various sizes, ranging from 150 to 300 nm. Bar, 1 μm. B, SDS–PAGE profiles of outer membrane proteins (OMPs) and outer membrane vesicles (OMVs) from cells of V. anguillarum O1. Lane M, molecular mass standard (molecular masses are indicated on the left); lane 1, OMPs; lane 2, OMVs.

Fig. 2. Enzyme Activity of OMVs from V. anguillarum. A, Protease activities of OMPs and OMVs, and B, metalloprotease activities of OMVs. A1, OMPs; A2, OMVs; B1, untreated OMVs; B2, 10 mm EDTA treated OMVs; A2, Protease activity appeared around the OMVs; B2, protease activity of EDTA-treated OMVs was reduced. Hemolytic and phospholipase activities of OMPs and OMVs; C and D, C1 and D1, OMPs; C2 and D2, OMVs.

Fig. 3. Semi-Quantitative RT-PCR Analysis of the Expression of Flounder TNF-α, IL-1β, and IL-6 after Injection with Outer Membrane Vesicles (OMVs) of V. anguillarum O1. A, Total RNA was extracted from the spleen. Each lane shows the PCR products of total RNA from PBS-injected spleen; 3 and 5 μg refer to the amounts of OMV protein injected. B, Total RNA was extracted from the spleen, kidney, liver, and gill at 1, 3, 6, 12, 24, and 48 h post-injection with 5 μg of OMVs. C, PCR products detected in spleen tissue sampled at 1, 3, 6, 12, 24, and 48 h post-injection.

Lapinet et al. reported that OMVs isolated from group B N. meningitidis stimulate the expression and production of proinflammatory cytokines in human neutrophils.14 There are many reports to the effect that vaccination in humans and mice induces immunological memory and increases the immune response.15–17 Cytokines are important signaling molecules that mediate interactions among cells in the immune system. In fish, TNF-α and IL-1β are key components in the inflammatory response of the innate immune system, which is the first line of inducible host defense against bacterial, fungal, and viral pathogens.18,19 IL-6 is a multifunctional cytokine that plays major roles in regulating immune responses, acute-phase reactions, hematopoiesis, inflammation, and nervous system responses.20 We injected OMVs into fish and examined the transcriptional levels of major cytokine genes using the RT-PCR analysis of Park et al.21 to study the immuno-relationships of OMVs.

Purified OMVs (3 or 5 μg) were injected into the abdominal cavity of healthy juvenile (9.1 ± 1.14 g) flounder, Paralichthys olivaceus, obtained from the National Fisheries Research and Development Institute of Korea. Total RNA was extracted from the injected fish tissues using Trizol reagent (Invitrogen, Carlsbad, CA). Subsequently, first-strand cDNA synthesis was carried out using a Transcriptor First-Strand cDNA Synthesis kit (Roche, Basel, Switzerland). Cytokine expression was detected by RT-PCR with primers TNF-α-F (5′-ATGTTGCAAATACACAAGTGCA-3′), TNF-α-R (5′-TCAAAGTGCAAAGACACCAGA-3′), IL-1β-F (5′-ATGGAACTCAAGATGAAATGC-3′), IL-1β-R (5′-TAACTCTGATGATGAGTTGTA-3′), IL-6-F (5′-ATACTTCACAAACAAGTGCG-3′), IL-6-R (5′-TTTCCTTTCTGCTTGTCTGCTG-3′), and β-actin-R (5′-GGGCTCTCTCTGCCTCGTGTA-3′).22 The PCR product of β-actin was used as an internal control, and it showed the expected size in all samples, confirming that the cDNA was intact and that the PCR conditions were suitable for amplification. The PCR program included initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 20 s, and extension at 72°C for 30 s, and a final extension at 72°C for 7 min. Three infection experiments were performed. In the first experiment, we injected OMVs into the abdominal cavity of healthy flounder to determine whether OMVs would induce cytokine expression in the fish. The spleen was removed 1 h post-injection for RT-PCR targeting of the TNF-α, IL-1β, and IL-6 genes. Treatment with 5 μg of OMVs resulted in TNF-α and IL-1β expression in the spleen (Fig. 3A). In the second experiment, we examined the effect of OMVs on the expression of TNF-α in the spleen, kidney, liver, and gills using 5 μg of OMVs. The organs were removed from the fish at 1, 3, 6, 12, 24, and 48 h post-injection. After extracting the total RNA from the organs, PCR was carried out targeting the TNF-α gene. In the spleen, TNF-α transcription increased markedly at 3 h post-injection, and the RT-PCR products were clearly detected until 12 h post-injection (Fig. 3B). TNF-α expression in the kidney was observed at 3 h post injection, and decreased dramatically 6 h post-injection (Fig. 3B). TNF-α expression was not detected in the liver or gills. To assess the effect of OMVs in stimulating the
expression of other cytokines in the spleen of the injected fish, 5 μg of OMVs was injected into the flounder intraperitoneally, and cytokine expression in the spleen was determined. The spleen was extracted from the fish at 1, 3, 6, 12, 24, and 48 h post-injection. Major stimulation of TNF-α gene transcription was observed at 3 h post-injection, and peaked at 6 h post-injection (Fig. 3C). IL-1β expression was markedly induced at 3 h post-injection, and strong induction was observed at 6 h post-injection (Fig. 3C). IL-6 expression was detected at 6 h post-injection, but the signal was indistinct. The protruded OMVs of V. anguillarum O1 were surrounded by the outer membrane (Fig. 1A). Because one of the major constituents of the outer membrane fraction isolated from V. anguillarum O1 is a lipopolysaccharide, we expected that the purified OMVs are an important lipopolysaccharide reservoir. Many factors can induce immune responses when LPS is delivered to host animal cells. Therefore, our RT-PCR results suggest that the OMVs of V. anguillarum O1 markedly stimulate immune-related genes in the spleen of fish, but just how all of the components in the OMVs function in the immune response needs to be clarified.

In summary, this is the first report of OMVs of V. anguillarum serotype O1. V. anguillarum O1 produces OMVs during normal growth, and purified OMVs have metalloprotease, hemolysin, and phospholipase activity. The expression of cytokine genes in OMV-injected flounder suggests that the OMVs of V. anguillarum O1 contribute to the immune response in the host. Hence purified OMVs can be useful as potential vaccine candidates to protect against vibriosis. Further studies are needed to determine the toxicity of OMVs from V. anguillarum O1 and their possible role in defense against virulent bacterial challenge in the form of a vaccine.

Acknowledgments

This research was supported by a grant (M2007-06) from Marine Bioprocess Research Center of the Marine Bio21 Project funded by the Ministry of Land, Transport and Maritime, Republic of Korea. We thank Dr. Holmstrøm, Biotechnological Institute, Department of Molecular Characterization, Denmark, for providing V. anguillarum serotype O1.

References