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Short communication

Effect of G gene-deleted recombinant viral hemorrhagic septicemia virus (rVHSV-ΔG) on the replication of wild type VHSV in a fish cell line and in olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

In an earlier study, we generated a replicon viral hemorrhagic septicemia virus (VHSV) particle that was lacking the G gene in the genome (rVHSV-ΔG), and proved the potential of it as a protective vaccine through the immunization of olive flounder (*Paralichthys olivaceus*) fingerlings. Safety is the most important preconsideration for the development of recombinant live vaccines, and a major concern of propagation-incompetent viral particles would be the possible harmful effect to hosts through the interaction with wild-type viruses. Thus, in the present study, we analyzed the replication of rVHSV-ΔG in the presence of wild-type VHSV and the effect of rVHSV-ΔG on the replication of wild-type VHSV in *Epithelioma papulosum cyprini* (EPC) cells and in olive flounder fingerlings. The replication of wild-type VHSV in EPC cells was severely suppressed when the MOI of rVHSV-ΔG was 0.1 or 0.01, on the other hand, the titers of rVHSV-ΔG were not increased and stayed in a relatively constant according to time lapse. Furthermore, the replication of other novirhabdoviruses, IHNV and HIRRV, was also inhibited by co-infection with high titers of rVHSV-ΔG. There were no big differences in mortalities between groups infected with wild-type VHSV plus rVHSV-ΔG and groups infected with wild-type VHSV alone, when the challenged wild-type VHSV was more than 10^2 PFU/fish. However, a group of fish infected with 10 PFU/fish of wild-type VHSV plus rVHSV-ΔG showed significantly lower and slowly progressing cumulative mortality than a group of fish infected with 10 PFU/fish of wild-type VHSV alone. This result suggests that rVHSV-ΔG has an ability to attenuate the disease progression caused by wild-type VHSV when co-infected with relatively low titers of wild-type VHSV. These results indicate that the propagation-incompetent rVHSV-ΔG would not worsen but attenuate the progression of a disease caused by wild-type VHSV infection. Therefore, rVHSV-ΔG-based vaccines can provide a safe and effective way to control VHSV.

1. Introduction

Various rhabdoviral diseases have been responsible for lots of economic damage in aquaculture industry worldwide [1–4]. Among them, viral hemorrhagic septicemia virus (VHSV) is the most notorious culprit in both freshwater and marine fish cultures, and many efforts have been made in the past decades to control VHSV disease through prophylactic vaccines or immunostimulants [5,6].

In adaptive immune responses against viral diseases, not only humoral but also cellular responses play an important role in protection. Attenuated live viruses can effectively induce both humoral and cellular adaptive memories, and, recently, the availability of attenuated VHSVs (that were generated by reverse genetics technology) as a vaccine candidate has been reported [7,8]. However, as the attenuated viruses still possess the ability to replicate in the host cells, concerns over the possibility of virulence restoration and the possible harmful effects on weakened individuals or non-target organisms are the main limiting factors for the practical use of the attenuated VHSVs in cultured fish. To overcome these problems, previously, we generated a replicon VHSV particle that was lacking the G gene in the genome (rVHSV-ΔG), and the potential of it as a protective vaccine was proved through an in vivo immunization experiment [9].

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As the rVHSV-ΔG has no G gene in the genome, viral particles with G proteins on their envelope cannot be produced. Only when the G protein is trans-supplied, a virus that can infect only a single round is generated. Although the inability of rVHSV-ΔG to form plaques without trans-supply of G protein was verified in the previous study [9], the fate of rVHSV-ΔG in the presence of wild-type VHSV, where the G proteins are available also for rVHSV-ΔG replication, is not known. Furthermore, the effect of rVHSV-ΔG on the replication of wild-type VHSV when both are in the same cell or in the same fish has not been investigated. As the effect of rVHSV-ΔG on the disease progression of fish infected with wild-type VHSV can be an important issue for the safety of the rVHSV-ΔG-based vaccines and for the further application of rVHSV-ΔG, in the present study, we analyzed the replication of rVHSV-ΔG in the presence of wild-type VHSV and the effect of rVHSV-ΔG on the replication of wild-type VHSV in *Epithelioma papulosum cyprini* (EPC) cells and in olive flounder fingerlings.

2. Materials and methods

2.1. Cells and viruses

Epithelioma papulosum cyprini cells purchased from the American Type Culture Collection (ATCC no. CRL-2872) were maintained in Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml, Gibco), streptomycin (100 μg/ml, Gibco) and 10% fetal bovine serum (FBS, Sigma). The wild-type VHSV (VHSV KJ2008) [8], wild-type HIRRV (HIRRV CA-9703) [10], wild-type IHNV and G gene-lacking recombinant VHSV (rVHSV-ΔG) [9] were propagated in monolayer of EPC cells at 15 °C in the presence of 2% FBS.

2.2. Effect of rVHSV-ΔG on the replication of wild-type VHSV, HIRRV, and IHNV in EPC cells

EPC cells (3×10^6 cells/35-mm dish) were infected with each wild-type rhabdovirus (VHSV, HIRRV, and IHNV) alone at a MOI of 0.00001 or co-infected with each wild-type virus (MOI 0.00001) plus rVHSV-ΔG (MOI 0.1, 0.01, and 0.001), and incubated at 15 °C. To know the effect of rVHSV-ΔG on the replication of wild-type viruses, each supernatant was collected at 1, 3, 5, 7, and 10 days post-infection, and plaque assay was performed [11]. Briefly, EPC cells (3×10^6 cells/35-mm dish) were inoculated with serially diluted each kind of viral supernatants, then, incubated at 15 °C for 1 h. Thereafter, the inoculum was removed and cells were overlaid with plquing medium (0.7% agarose in L-15 containing 2% FBS, penicillin 100 U/ml, and streptomycin 100 μg/ml). After 7 days of incubation to allow plaque formation, the cells were fixed by 10% formalin and stained with 5% crystal violet for 30 min at room temperature. After rinsing of the cells with distilled water, the plaque-forming units (PFU) were counted. To estimate the amount of rVHSV-ΔG by real-time RT-PCR, the gene region that is specific for rVHSV-ΔG was PCR-amplified using a primer pair (Table 1), and cloned into pGEM T-easy vector (Promega). The constructed vector was used for the absolute quantification. For real-time RT-PCR, total RNA was extracted from 1 ml of each supernatant using Hybrid-R Kit (Gene all, Korea), and all of the total RNA was used to synthesize complementary DNA (cDNA) using HyperScript First strand synthesis Kit according to the manufacturer's instruction (Gene all, Korea). Real-time RT-PCR was carried out using the Light Cycler 480 (Roche). PCR primers used for the detection of rVHSV-ΔG are in Table 1. The PCR reactions in a volume of 20 μl were run using 2 × SYBR Green Premix (Enzynomics, Korea) with 5 μl of cDNA or serially diluted plasmid vector for standard curve, and 5 pM of each primer. Thermal cycling condition was 1 cycle of 15 min at 95 °C (pre-incubation), followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C.

2.3. Effect of rVHSV-ΔG on the replication and virulence of wild-type VHSV in olive flounder

Olive flounder fingerlings (approximate 3 g in body weight, confirmed free-from VHSV and showing no signs of illness) were infected by an intra-muscular (i.m.) injection with the wild-type VHSV (10^1, 10^2, 10^3, 10^4 PFU/fish) or co-infection with both the wild-type VHSV and rVHSV-ΔG (10^4 PFU/fish). Control fish were injected with an equal volume of L-15 alone. Experimental fish in each group (45 fish) were kept in nine 30 L tanks at 13–14 °C and mortality was recorded daily for 14 days post-injection. At 1, 3, 14 days post-injection, the kidney was sampled from 3 survived fish in each group, and total RNA was extracted for real-time RT-PCR. The primers used for the estimation of wild-type VHSV titer by absolute quantification are in Table 1.

2.4. Statistical analysis

Statistical analysis was performed using SPSS for Windows (Chicago, IL, USA). Differences in viral titers among groups were analyzed using ANOVA followed by Tukey HSD post-hoc test. Kaplan-Meier method was used to analyze significance of the cumulative mortality. A probability (P) value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of rVHSV-ΔG on the replication of wild-type VHSV, HIRRV, and IHNV in EPC cells

The titer of wild-type VHSV in EPC cells was significantly lowered by coinfection with 0.01–0.1 MOI of rVHSV-ΔG (Fig. 1a). CPE was also evidently decreased by coinfection with rVHSV-ΔG.

Table 1
Summary of primers used in this study.

Name of primer		Sequence (5′ to 3′)
For detection of wild-type VHSV by real-time PCR		
wtVHSV (G-NV)	G1415F	TGGGAGGGGCCTTCCTTCTG
	NV24R	GCTGTGTGCCGACTGGGTCGTC
For detection of rVHSV-ΔG by real-time PCR		
rVHSV-ΔG (ΔG-NV)	ΔGF	CAAGTCACCGGTCCGCGGCC
	NV24R	GCTGTGTGCCGACTGGGTCGTC
For standard curve of real-time PCR		
wtVHSV (G-NV)	F	ATGGAATGGAATACTTTTTTCTTG
	R	TCATGGGGGAGATTCGGAGCC
rVHSV-ΔG (ΔG-NV)	F	ATGGCTCTATTCAAAAGAAAGC
	R	TCATGGGGGAGATTCGGAGCC