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Short communication

Therapeutic potential of CpG-ODN 1668 against scuticociliatosis in olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

Scuticociliatosis caused by *Miamiensis avidus* is one of the major diseases in cultured olive flounder (*Paralichthys olivaceus*) in Korea. However, until recently, no effective chemotherapy or vaccine has been developed. Oligodeoxynucleotides containing unmethylated deoxycytidyl-deoxyguanosine motifs (CpG-ODNs) have been shown to be effective in enhancing innate immune responses and preventing infectious diseases in fish. However, it has not been experimented whether CpG-ODNs can be used as a therapeutic tool against infectious diseases in fish. In the present study, we evaluated the therapeutic potential of CpG-ODN 1668 against scuticociliatosis in olive flounder for the first time. The group of fish administered CpG-ODN 1668 at 3 days after the infection showed clearly higher survival rates than the groups of fish administered GpC-ODN 1720 or PBS, indicating a high therapeutic potential of CpG-ODN 1668 against scuticociliatosis. However, the treatment efficacy of CpG-ODN 1668 was diminished when administered at 6 days post-infection, and no treatment effect was found when administered at 9 days after the infection. These results suggest that early diagnosis through continuous monitoring and early treatment with CpG-ODN 1668 are essential for increase of treatment efficacy. In conclusion, the present study demonstrated for the first time the possible use of CpG-ODN 1668 in treatment of scuticociliatosis in olive flounder.

1. Introduction

Oligodeoxynucleotides containing unmethylated deoxycytidyl-deoxyguanosine motifs (CpG-ODNs) are artificially synthesized DNA fragments that mimic bacterial genomic DNA that contains a higher frequency of unmethylated CpG motifs than vertebrate genome (Ahmad-Nejad et al., 2002; Akira and Takeda, 2004; Hemmi et al., 2000). This difference makes CpG-ODNs be recognized as a pathogen-associated molecular pattern (PAMP) by the Toll-like receptor 9 (TLR9) of vertebrates, and activate innate immune responses which are different according to classes of CpG-ODNs (Hartmann and Krieg, 2000; Krieg, 2002; Krieg et al. 1995; Verthelyi et al., 2001; Vollmer et al., 2004).

Scuticociliatosis caused by histophagous ciliates, *Miamiensis avidus*, has been a major cause of economical loss in olive flounder (*Paralichthys olivaceus*) farms in Korea (Jung et al., 2007; Kim et al., 2004). Although substantial efforts have been made to develop effective therapeutics and vaccines against scuticociliatosis, there is still no acceptable therapeutic or vaccine for farm fish. Recently, we have reported the high effectiveness of doxycycline in treatment of scuticociliatosis in olive flounder (Kang et al., 2013), but more studies are still needed for its application into commercial farm fish. Furthermore, considering induction of drug resistance in parasites, efforts on the development of other chemotherapeutic and immunotherapeutic measures to control scuticociliatosis should be devoted.

Generally, immunostimulants are used for prevention of infectious diseases by enhancing innate immunity. However, as the stimulatory effects are not long-lasting, several or continuous administration of the stimulants is needed before outbreaks of diseases. Furthermore, administration of certain immunostimulants for a long time can not only increase economical burden but also deplete immunity rather than enhance immunity (Bricknell and Dalmo, 2005). Previously, we had demonstrated that CpG-ODN 1668 had a strong preventive ability against scuticociliatosis in olive flounder (Kang and Kim, 2012; Lee and Kim, 2009). However, as the outbreaks of scuticociliatosis in olive flounder are all year round and prediction of the infection time is not possible, determination of CpG-ODN administration schedule for prevention of scuticociliatosis is difficult. Thus, therapeutic approach using CpG-ODNs can be a way to control scuticociliatosis in a practical aspect.

CpG-ODNs have been shown to be effective in enhancing innate immune responses and preventing infectious diseases in fish (Carrington and Secombes, 2007; Jørgensen et al., 2001, 2003; Lee et al., 2003; Tassakka and Sakai, 2003). However, there are no reports on the therapeutic use of CpG-ODNs against fish diseases. In the present study, we analyzed the treatment effectiveness of CpG-ODN 1668 against scuticociliatosis in olive flounder, and showed for the first time that

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CpG-ODN 1668 could be used as an immunotherapeutic in fish infected with scuticociliates.

2. Materials and methods

2.1. Ciliates

Scuticociliates, *M. avidus*, isolated from the brain of diseased olive flounder were cultured using *Epithelioma papulosum cyprini* (EPC) cells in Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS, Gibco) at 20 °C.

2.2. CpG-ODNs

CpG-ODNs were synthesized from Bioneer Corporation (Korea). The sequence of CpG-ODN 1668 was 5′-TCCATGACGTTCCTGATGCT-3′. and GpC-ODN 1720 (5′-TCCATGAGCTTCCTGATGCT-3′) was synthesized to use as a control of CpG-ODN 1668. The underlined nucleotides indicate phosphorothioate linkage.

2.3. Fish and experimental regime

2.3.1. Experiment 1

Olive flounder fingerlings weighing approximately 1.5 g were obtained from a local fish hatchery in Korea, and were acclimated for 1 week at 20–21 °C. During the acclimation period, 10 fish were randomly sampled and were confirmed free from scuticociliates by microscopic observation of skin and internal organs. A total of 90 fish were immersed in seawater containing 4×10^7 ciliates/30 l for 3 days, and randomly divided into 3 groups with 2 replicates (15 fish in a tank). Fish were intraperitoneally (i.p.) injected with 10 μg of CpG-ODN 1668, or GpC-ODN 1720. Fish in the control group were i.p. injected with 20 μl of phosphate buffered saline (PBS). Mortality was recorded daily for 3 weeks post-challenge.

2.3.2. Experiment 2

Olive flounder fingerlings weighing approximately 10 g were purchased from a local fish farm. After acclimation for 1 week at 20–21 °C, 270 fish were immersed in seawater containing 1×10^8 ciliates/100 l. At 3, 6, and 9 days after the infection initiation, 90 fish were randomly collected from the infection tank, divided into 3 groups with 2 replicates (15 fish in a tank), and i.p. injected with 10 μg of CpG-ODN 1668, or GpC-ODN 1720, or 50 μl of PBS. Mortality was recorded daily for 4 weeks from the infection initiation, and dead fish were necropsied to confirm the presence of ciliates. Relative percent survival (RPS) was calculated as follows (Amend, 1981):

$$\text{RPS} = [1-(\text{percent of experimental group}/\text{percent of control group})] \times 100\%.$$

3. Results

3.1. Experiment 1

To know whether CpG-ODN 1668 has a therapeutic potential against scuticociliatosis, fish infected with *M. avidus* by immersion for 3 days were treated with CpG-ODN 1668, GpC-ODN 1720, or PBS. The groups of fish administered GpC-ODN 1720 or PBS showed 90–100% mortalities, however, the group of fish administered CpG-ODN 1668 showed clearly higher survival rates (60% in both replicates) than the control groups (Fig. 1).

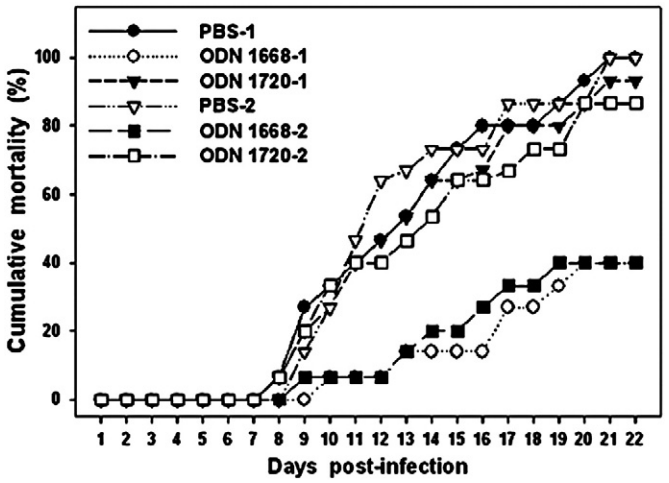

Fig. 1. Cumulative mortality rates of olive flounder (*Paralichthys olivaceus*) fingerlings that were infected by being immersed into seawater containing 4×10^7 *Miamiensis avidus*/30 l for 3 days and then, intraperitoneally (i.p.) injected with phosphate buffered saline (PBS), 10 μg of CpG-ODN 1668 (ODN 1668), or GpC-ODN 1720 (ODN 1720). The experiment was conducted with two replicates (-1 and -2).

3.2. Experiment 2

To evaluate the therapeutic potential of CpG-ODN 1668 according to the progression of scuticociliatosis, fish were treated with CpG-ODN 1668 at 3, 6, and 9 days after the infection with *M. avidus*. The group of fish administered CpG-ODN 1668 at 3 days post-infection initiation showed 54.6% and 63.2% of RPS (Fig. 2a), which was similar to the results of Experiment 1 above. Although fish administered GpC-ODN 1720 showed slightly higher survival rates than PBS injected fish, the RPSs were below 20%. The group of fish administered CpG-ODN 1668 at 6 days after the infection initiation still showed higher survival rates than control groups, and the RPSs of the replicates were 46.5% and 30.7% (Fig. 2b). However, no treatment effect of CpG-ODN 1668 was found when administered at 9 days after the infection initiation (Fig. 2c).

4. Discussion

CpG-ODNs are artificially synthesized single-stranded DNA fragments, and their immunostimulatory ability and safety in fish have been well demonstrated (reviewed in Carrington and Secombes, 2006). The application of CpG-ODNs in fish has been focused on the preventive aspect. However, it has not been experimented whether CpG-ODNs can be used as a therapeutic tool against infectious diseases in fish. In the present study, we evaluated the therapeutic potential of CpG-ODN 1668 against scuticociliatosis in olive flounder for the first time.

Recently, we have reported that doxycycline was effective in treatment of scuticociliatosis in olive flounder by oral administration or by bath. In the present results, the treatment efficacy of CpG-ODN 1668 in the fish administered at 3 days post-infection was similar to that of doxycycline, indicating a high therapeutic potential of CpG-ODN 1668 against scuticociliatosis. The ability of CpG-ODN 1668 in enhancing complement-mediated serum scuticocidal activity has been demonstrated in olive flounder (Kang and Kim, 2012; Lee and Kim, 2009). As CpG-ODN itself has no direct scuticocidal activity, increased innate immune responses by CpG-ODN 1668 administration would be the major factor of the present therapeutic effect. It has been reported that scuticociliates can invade host tissues and evade host immune responses by their immunomodulatory ability (Paramá et al., 2004, 2007a,b). The high treatment efficacy of CpG-ODN 1668 in the present results suggests that enhancement of immune responses by CpG-ODN