Developmental and Comparative Immunology 60 (2016) 218–227

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

A specific CpG oligodeoxynucleotide induces protective antiviral responses against grass carp reovirus in grass carp *Ctenopharyngodon idella*

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ARTICLE INFO

Article history:
Received 20 January 2016
Received in revised form
8 March 2016
Accepted 8 March 2016
Available online 10 March 2016

Keywords:
Grass carp (*Ctenopharyngodon idella*)
Grass carp reovirus
CpG oligodeoxynucleotides
Toll-like signaling pathway
RIG-I-like signaling pathway
Interferon

ABSTRACT

CpG oligodeoxynucleotides (ODNs) show strong immune stimulatory activity in vertebrate, however, they possess specific sequence feature among species. In this study, we screened out an optimal CpG ODN sequence for grass carp (*Ctenopharyngodon idella*), 1670A 5′-TCGAACGTTTTAACGTTTTAACGTT-3′, from six published sequences and three sequences designed by authors based on grass carp head kidney mononuclear cells and CIK (*C. idella* kidney) cells proliferation. VP4 mRNA expression was strongly inhibited by CpG ODN 1670A in CIK cells with GCRV infection, showing its strong antiviral activity. The mechanism via toll-like receptor 9 (TLR9)-mediated signaling pathway was measured by real-time quantitative RT-PCR, and TLR21 did not play a role in the immune response to CpG ODN. The late up-regulation of CiRIG-I mRNA expression indicated that RIG-I-like receptors (RLRs) signaling pathway participated in the immune response to CpG ODN which is the first report on the interaction between CpG and RLRs. We also found that the efficient CpG ODN can activates interferon system. Infected with GCRV, type I interferon expression was reduced and type II interferon was induced by the efficient CpG ODN in CIK cells, especially IFNγ2, suggesting that IFNγ2 played an important role in response to the efficient CpG ODN. These results provide a theoretical basis and new development trend for further research on CpG and the application of CpG vaccine adjuvant in grass carp disease control.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is a fish species of the largest production in the world and is an important economical species farmed extensively in China and other Asian countries. However, development of grass carp cultivation industries has been threatened by increased disease outbreaks associated with grass carp reovirus (GCRV) (Rao and Su, 2015). GCRV is the most toxic species in genus *Aquareovirus* in the family Reoviridae. Nowadays, grass carp hemorrhagic disease vaccine mainly focuses on inactivated vaccine and attenuated vaccine but their immune protective activity remain to be further studied. Immune adjuvant, a component that potentiates the immune responses to an antigen and/or modulates it towards the desired immune responses, coordinate applying with vaccines is of increasingly importance in grass carp disease control and prevention.

New type immune adjuvant like CpG oligodeoxynucleotide (ODN) has the advantage comparing with traditional ones which have uncertain toxic and side effects (Harandi et al., 2010). CpG ODNs are a type of pathogen-associated molecular patterns (PAMPs) containing dinucleotides with unmethylated CpG motifs. They have shown strong immune stimulatory activity due to their appearance commonly in the genomes of microbial pathogens while a much less frequency in vertebrate genomes (Bird, 1987; Cardon et al., 1994; Krieg, 2002). The ODN sequences containing an unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines were found to activate the mouse immune system efficiently (Krieg et al., 1995). Owing to their immune stimulatory activity, artificial synthetic CpG ODNs with a length of 25 bp around containing one to three motifs have been used as immune protective agents and immune adjuvants to mediate protective immune response against various cancers, allergies, and infectious diseases, especially those caused by viruses, such as respiratory syncytial virus (Yamaguchi et al., 2012), swine-origin influenza virus (Gong et al., 2012),

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http://dx.doi.org/10.1016/j.dci.2016.03.007
0145-305X/

hepatitis C virus (Naarding et al., 2011), and human immunodeficiency virus (Donhauser et al., 2010). CpG ODNs are classified into four types according to their activation on peripheral blood mononuclear cells (PBMCs), class-A/type D, class-B/type K, class-C and class-P (Vollmer and Krieg, 2009). Class-A CpG ODNs mainly activate plasmacytoid dendritic cells (PDC) secreting IFN-I and natural killer (NK) cells excitation; Class-B CpG ODNs promote B cells proliferation, differentiation and secretion of immune globulin (Ig), interleukin 6 (IL-6) and IL-12. They also accelerate PDC maturation, but have little stimulation on NK cells; Class-C CpG ODNs have the function of both Class-A and Class-B; Class-P CpG ODNs have the similar function to Class-C, but their stimulation on dendritic cells (DC cells) is slightly stronger than on B cells (Donhauser et al., 2010). The optimal CpG ODN sequence for different species varies considerably. Human PBMCs are potentially activated by ODN containing 'GTCGTTT', 'AACGTTT' or 'TTCGTTT' motifs (Krieg et al., 2000). CpG motifs that most effectively activate mice immune cells are poorly stimulatory in human, due to evolutionary divergence in CpG recognition between species (Bauer et al., 1999; Hartmann and Krieg, 2000; Verthelyi et al., 2001). The number of CpG motif in the ODN sequence also influences the immune stimulatory activity while ODN containing two motifs have a better immune stimulatory effect than one, but four motifs have no more effect, and eight motifs even have an inhibition on immune stimulatory (Shi et al., 2001). Synthetic CpG ODN have the same immune stimulatory activity as natural bacterial CpG DNA. Phosphorothioate backbone can protect Synthetic CpG ODN from nuclease degradation, but the effect on IFN-I in PDC may not as strong as CpG ODN with a partly phosphorothioate backbone (Krieg, 2006).

The majority of studies into the immune effects of CpG ODN to date have been carried out on mammals where they are proving very successful at stimulating innate and adaptive immune responses in a variety of species as well as protecting them from bacterial, viral and protozoan pathogens (Krieg, 2002; Mutwiri et al., 2004). Fish also possess the ability to raise both innate and adaptive immune responses to invading pathogens and interest in the effect of CpG ODN on the piscine immune system is growing with various studies having now been carried out to elicit their effects on different fish species including salmonids, cyprinids and pleuronectiformes (Carrington and Secombes, 2006). However, CpG ODN motif possesses specific sequence feature among species, the optimum CpG ODN for many species are not explicit and working mechanisms remain largely unknown.

As a PAMP, being recognized by the pattern recognition receptors (PRRs) in innate immune system, CpG ODN is recognized by toll-like receptor (TLR) 9 on B cells, dendritic cells, macrophages and other immune cells (Bauer et al., 2001; Hemmi et al., 2000; Krieg, 2002). In mammalian cells, TLR9 is localized to endoplasmic reticulum and translocates to endocytic structures upon exposure to CpG ODNs (Latz et al., 2007). It has been shown that TLR9-deficient mice expressed a nonresponsive phenotype toward CpG ODN, implying that TLR9 is the only component of the CpG ODNs receptor (Hemmi et al., 2000). Supporting this concept, it was subsequently found that expression of TLR9 correlated with CpG ODN responsiveness in primary human cell (Bauer et al., 2001). Previous studies have shown that TLR9 knockdown significantly reduced CpG ODN-mediated up-regulation of immune genes in the TLR9 signaling pathway, suggesting that CpG ODN-mediated immune response is TLR9-dependent, indicating a vital contribution of the TLR9 signaling pathway to the antiviral activity of CpG ODN (Zhou et al., 2014). However, CpG ODNs may also work via a TLR9-indepednent mechanism (Barrier et al., 2006). In fish, it has been reported that CpG ODN interacted with Atlantic salmon TLR9 and enhanced TLR9 expression in Atlantic salmon, Japanese flounder, and sea bream (Cuesta et al., 2008; Iliev et al., 2013; Skjaeveland et al., 2008; Takano et al., 2007). Grass carp TLR9 gene has been cloned and characterized (Yang et al., 2011). Synthetic CpG ODNs could up-regulate many immune genes related to TLR9 signaling pathway, TLR9, IL-1β, IFN-I, myxovirus resistance (Mx), and interferon regulatory factors (IRFs) (Jorgensen et al., 2003; Liu et al., 2010a; Martinez-Alonso et al., 2011; Strandskog et al., 2008). Previous studies showed that the chicken TLR21 plays similar roles to the mammalian TLR9, which works as a receptor to recognize CpG ODN (Brownlie et al., 2009; Keestra et al., 2010). TLR21 has also been molecular cloned and its expression patterns were analyzed in various tissues in grass carp (Wang et al., 2013). However, the immune response of TLR21 to CpG ODN have not been studied.

In this study, to screen out an optimal CpG ODN sequence in grass carp, six published sequences and three sequences designed by authors were used to stimulate grass carp mononuclear cells from head kidney tissue and CIK (*C. idella* kidney) cells for proliferation activity examination. The mRNA expression of the possible receptor gene (CiTLR9 and CiTLR21), VP4 (grass carp reovirus strain GCRV-097 segment 6 outer capsid protein) which can reflect the antiviral activity of CpG ODNs, and other immune related genes in grass carp, including RIG-I (retinoic acid inducible gene I), MDA5 (melanoma differentiation-associated gene 5), CCL20 (chemokine ligand 20), IFN1 (interferon 1), IFN2 (interferon 2), IFNγ1 (interferon γ 1) and IFNγ2 (interferon γ 2), was measured after CpG ODN stimulation or subsequently GCRV infection. These immune related genes report the mechanism of immune response to CpG ODN.

This work provides a better understanding of the function of CpG ODN in antiviral immune response to GCRV in grass carp, and can facilitate the development of immune adjuvant for grass carp disease control and long term sustainability of grass carp farming.

2. Materials and methods

2.1. Fish and cells

Grass carps were obtained from Hubei Bairong Improved Aquatic Seed CO., LTD. (Huanggang, 438800, China) and kept in 1000-L tanks at 25 °C with a constant flow of filtered water. Fish (50–100 g) were fed with pellet food twice a day at 8:00 am and 5:00 pm at a daily ration of 0.7% of their body weight to be acclimated to feeding conditions for 2 weeks.

Three grass carp were randomly sampled and deeply anesthetized with MS-222 (200 mg L^{-1}) and moved into clean bench after sterilized with 75% ethanol. Head kidney tissues were collected from grass carp, washed three times with phosphate buffer solution (PBS) and passed through a 100 mesh stainless steel screen. Grass carp head kidney cells were re-suspended in L-15 medium (Thermo Scientific HyClone, Beijing, China) containing 10% fetal bovine serum (FBS; Biosourse, USA), 100U/ml of penicillin (Sigma, USA) and 100 U/ml of streptomycin (Sigma, USA). The cells were incubated in 96-well culture at 28 °C in a 5% CO_2 humidified atmosphere.

CIK cell line is provided by China Center for Type Culture Collection and grown in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Biosource, USA), 100 U/ml of penicillin (Sigma, USA) and 100 U/ml of streptomycin (Sigma, USA) according to a previous report (Chen et al., 2012). CIK cells consist of many kinds of cells including fibre cells, polygon cells and giant cells in grass carp kidney (Zuo et al., 1986). The cells were incubated at 28 °C in a 5% CO_2 humidified atmosphere.

2.2. CpG ODNs designing and synthesis

The synthetic ODNs are short single stranded unmethylated DNA sequences. In this study, we chose six different CpG ODNs from