Vaccine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Involvement of two microRNAs in the early immune response to DNA vaccination against a fish rhabdovirus

Q1 Dennis Berbulla Bela-ong[a,b,*], Brian Dall Schyth[b], Jun Zou[c], Christopher J. Secombes[c], Niels Lorenzen[a,*]

Q2 [a] Fish Health Section, Department of Animal Science, University of Aarhus, Hangøvej 2, DK-8200 Århus N, Denmark
[b] Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Bulowsvej 27, DK-1870 Frederiksberg C, Denmark
[c] Scottish Fish Immunology Research Centre, University of Aberdeen, Zoology Building, Tillydrone Avenue, Aberdeen AB24 2TZ, Scotland, United Kingdom

ARTICLE INFO

Article history:
Received 13 January 2015
Received in revised form 22 March 2015
Accepted 27 April 2015
Available online xxx

Keywords:
microRNA
Viral hemorrhagic septicemia virus (VHSV)
Rhabdovirus glycoprotein
DNA vaccination
Interferons
Rainbow trout

ABSTRACT

Mechanisms that account for the high protective efficacy in teleost fish of a DNA vaccine expressing the glycoprotein (*G*) of *Viral hemorrhagic septicemia virus* (VHSV) are thought to involve early innate immune responses mediated by interferons (IFNs). Microribonucleic acids (miRNAs) are a diverse class of small (18–22 nucleotides) endogenous RNAs that potently mediate post-transcriptional silencing of a wide range of genes and are emerging as critical regulators of cellular processes, including immune responses. We have recently reported that miR-462 and miR-731 were strongly induced in rainbow trout infected with VHSV. In this study, we analyzed the expression of these miRNAs in fish following administration of the DNA vaccine and their potential functions. Quantitative RT-PCR analysis revealed the increased levels of miR-462, and miR-731 in the skeletal muscle tissue at the site of vaccine administration and in the liver of vaccinated fish relative to empty plasmid backbone-injected controls. The increased expression of these miRNAs in the skeletal muscle correlated with the increased levels of the type I interferon (IFN)-inducible gene *Mx*, type I IFN and IFN-γ genes at the vaccination site. Intramuscular injection of fish with either type I IFN or IFN-γ plasmid construct resulted in the upregulation of miR-462 and miR-731 at the site of injection, suggesting that the induction of these miRNAs is elicited by IFNs. To analyze the function of miR-462 and miR-731, specific silencing of these miRNAs using anti-miRNA oligonucleotides was conducted in poly I:C-treated rainbow trout fingerlings. Following VHSV challenge, anti-miRNA-injected fish had faster development of disease and higher mortalities than control fish, indicating that miR-462/731 may be involved in IFN-mediated protection conferred by poly I:C.

1. Introduction

Q4 DNA vaccines encoding the glycoprotein (G) of fish rhabdoviruses like *viral hemorrhagic septicemia virus* (VHSV) have been shown to confer efficient protection against lethal virus challenge under laboratory conditions [1,2]. The immune response elicited by these vaccines involves early, short-term non-specific immunity, followed by specific, long-lasting protection [1,3]. Neutralizing antibodies and cytotoxic cells are believed to be involved in the specific protection [1,4]. The early protection correlates with upregulation of the interferon (IFN)-induced antiviral myxovirus resistance Mx protein. Initial studies suggested that expression of the G protein on the surface of transfected cells induces expression of IFN in the neighbouring cells [5], but the exact protective mechanism remains to be elucidated [3,6–8]. Type I IFN responses implicate activation of an array of innate antiviral defense mechanisms. Recent reports suggest that these include small regulatory RNAs called microRNAs (miRNAs) [9–13].

MicroRNAs are short (19–25 nucleotides) endogenous RNAs that regulate gene expression by repressing translation of and/or degrading target mRNAs, resulting in altered mRNA and protein expression profiles [14]. Each miRNA may regulate multiple genes [15,16] and can control a broad spectrum of cellular processes, including function and development of immune cells [17] and host-pathogen interactions [18–20].

Recently we reported that two miRNAs in teleost fishes, miR-462 and miR-731, were the most highly induced miRNAs in the liver of rainbow trout (*Oncorhynchus mykiss*) following VHSV infection

Q3 * Corresponding authors at: Animal Science-Fish Health Section, Aarhus University, Hangøvej 2, 8200 Aarhus N, Denmark. Tel.: +45 25520730.
E-mail addresses: dbelaong@yahoo.com (D.B. Bela-ong), niels.lorenzen@anis.au.dk (N. Lorenzen).

http://dx.doi.org/10.1016/j.vaccine.2015.04.092
0264-410X/

Table 1
Anti-miRNAs and mismatched controls used in the experiments.

Name	Sequence (5′ → 3′)[a]
dre-anti-miR-462 probe	mA mU IT mA mU IG mG mG IT mU mC IC mG mU IT mA
dre-anti-miR-731 probe	mG mA IG mA mA IA mA mC IG mU mG IT mC mA IT mU
dre-anti-miR-462 mismatch probe	mA mU IT mA mU IG mG mG IA mU mC IA mG mU IT mA
dre-anti-miR-731 mismatch probe	5′mG mA IG mA mA IA mA mC IC mU mG IA mC mA IT mU

[a] Positions in which the control anti-miRNAs differed from the anti-miRNAs are underlined and are located in positions complementary to that of the seed sequence in the mature miRNAs. 2′-*O*-methylated positions are indicated/preceded by the letter m, while the LNA coupled positions are indicated by the letter I.

(Schyth et al., submitted). Here, we report the upregulation of these miRNAs in fish immunized with a VHSV G-expressing DNA vaccine and determine the kinetics of expression following vaccination. We further show that the expression of these miRNAs is elicited by IFN such that inhibition of the two miRNAs in fish treated with the IFN inducer poly I:C reduces the ability of poly I:C to protect fish against lethal VHSV infection.

2. Materials and methods

2.1. Fish and virus

Outbred *O. mykiss* were hatched from disinfected eggs obtained from a commercial fish farm certified as disease-free. The fish were kept in pathogen-free aquarium facilities and acclimatized to laboratory conditions several weeks prior to experiments. Throughout the experiments, fish were maintained in 8 L aquaria at 10 ± 2 °C supplied with flow through partly deionized water. VHSV isolate DK-3592B [21] was used in infection trials. All fish experiments were conducted according to European and Danish guidelines for the use of experimental animals and permitted by the Danish Committee for Animal Experiments (license no. 2007/561-1312).

2.2. DNA vaccine

The DNA vaccine (herein referred to as pcDNA3-vhsG) included the VHSV G gene under the control of the cytomegalovirus (CMV) promoter at a multiple cloning site in the plasmid pcDNA3 (Invitrogen) as described previously [2]. Control fish were injected with either pcDNA3 (empty vector) diluted in 0.9% NaCl (physiological saline, PS) or PS only.

2.3. IFN-expressing plasmids

The IFN constructs consisted of the full-length coding sequence of IFNa1 (GenBank accession no. AJ580911; [22,23] or IFN-γ (GenBank accession no. AJ616215; [24]) inserted at a multiple cloning site in the plasmids, pcDNA3.1/3.3 (Invitrogen) driven by the CMV promoter. The constructs are herein referred to as pcDNA3.3-IFNa1 and pcDNA3.1-IFN-G. Control fish were injected with pcDNA3.1 (empty vector) dissolved in PS or with PS only.

2.4. Poly I:C and anti-miRNAs

Polyinosinic: polycytidylate sodium salt (Poly I:C; CAS # 42424-50-0) was purchased from Sigma-Aldrich GmBH (Steinheim, Germany). The anti-miR-462 and anti-miR-731 and their corresponding mismatch controls were unconjugated 2′-O-methylated Locked Nucleic Acid (LNA)-based oligonucleotide probes (RiboTask Langeskov, Denmark). The anti-miRNAs were designed as complete anti-sense sequences of the mature zebrafish miRNA sequences, whereas the corresponding mismatch anti-miRNA controls differed from the anti-miRNAs by two nucleotides (underlined bases) within the anti-seed region (Table 1).

2.5. Vaccination and injection with IFN constructs

Fish (approx. 1 g each) were anaesthetized with 0.01% benzocaine were injected with 10 μg of pcDNA3-vhsG or 10 μg of pcDNA3 empty plasmid backbone in 20 μL phosphate buffered saline (PBS) or PBS only in the right epaxial muscle in front of the dorsal fin as described [25]. For injection with IFN-expressing plasmids, benzocaine-anaesthetized fish were injected with 10 μg of either pcDNA3.3-IFNa1 or pcDNA3.1-IFN-G in 20 μL PBS. Positive controls were injected with 5 μg poly I:C (Sigma) in 20 μL PBS. Negative controls were injected with 10 μg of the empty vector in 20 μL PBS or 20 μL PBS alone.

At 1, 4, 7, and 21 days post-vaccination (dpv), muscle tissue from the injection site and liver from 6 fish were sampled for each group. Muscle tissue was immediately processed for RNA isolation while liver samples were stored at −80 C in RNALater until examined.

2.6. RNA isolation, cDNA synthesis, and quantitative RT-PCR for miRNAs and mRNAs

Total RNA was isolated and purified from tissue samples using the miRNeasy Kit (cat.# 217004, Quiagen SA Biosciences, Hilden, Germany), with DNase treatment (Qiagen RNase-free DNase Set, cat. # 79254). For miRNA expression studies, total RNA (1 μg) was reverse transcribed with the QuantiMiR RT Kit Small RNA Quantitation System (cat. # RA420A-1, System Biosciences, Mountain View, CA, USA) to convert small non-coding RNAs into cDNAs. For mRNA (*Mx, IFN-1, IFN-G*) expression analyses, 1 μg RNA was used to make cDNA using iScript cDNA synthesis kit from BioRad (cat. # 170-8891, Hercules, CA, USA).

Levels of mature miR-462 and miR-731 and those of *Mx, IFN-1*, and *IFN-G* mRNAs were detected by qPCR. Primer sequences for both miRNA and mRNA expression analysis are given in Table 2. See Supplementary information for a complete description of the qPCR protocol.

2.7. Anti-miRNA injection and virus challenge experiments

All groups were in duplicate aquaria (18–27 fish/aquarium). A flow diagram of the experiment is presented in Fig 1. Fish fingerlings (approx. 0.5 g each) anaesthetized in benzocaine were injected intraperitoneally (IP) with poly I:C in PS or PS only (control). 24 h post-poly I:C treatment or PS injection, fish received IP anti-miRNA treatments or controls according to group designations (Fig. 1). On the day of virus challenge, water flow was discontinued and VHSV isolate DK-3592B [21] was added to each experimental aquarium to a final titer of approximately 10^4 tissue culture infectious dose$_{50}$ (TCID$_{50}$)/mL per aquarium. Water flow was subsequently restarted after virus challenge of 2 h. Unchallenged controls consisted of a mix of fish from each of the treatment groups. Disease development in each aquarium was monitored daily for 21 days post-challenge (dpc). Moribund fish were terminated, and along with dead fish removed, registered and examined for external manifestations of disease (skin darkening, bleeding, ascitis, exopthalmia) and frozen (for virological examination). Initially, the effect of anti-miRNA on susceptibility to VHSV without prior poly I:C was analyzed (Supplementary information).

Please cite this article in press as: Bela-ong DB, et al. Involvement of two microRNAs in the early immune response to DNA vaccination against a fish rhabdovirus. Vaccine (2015), http://dx.doi.org/10.1016/j.vaccine.2015.04.092