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Single-walled carbon nanotubes as candidate recombinant subunit vaccine carrier for immunization of grass carp against grass carp reovirus

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ABSTRACT

Grass carp reovirus (GCRV), the most pathogenic aquareovirus, can cause fatal hemorrhagic disease in fingerling and yearling grass carp. Vaccination by injection is by far the most effective method of combating disease. However it is labor intensive, costly and not feasible to vaccinate large numbers of the fish. Thus, an efficient and economic strategy for the prevention of GCRV infection becomes urgent. Here, functionalized single-walled carbon nanotubes (SWCNTs) as carrier were used to manufacture SWCNTs-VP7 subunit vaccine with chemical modification. Different developmental stages of grass carps were immunized by VP7/SWCNTs-VP7 subunit vaccine against GCRV by intramuscular injection and bath immunization. The results indicate that better immune responses of grass carp immunized with the SWCNTs-VP7 subunit vaccine were induced in comparison with VP7 subunit vaccine alone. Immunization doses/concentrations are significantly reduced (about 5–8 times) to prevent GCRV infection in different developmental stages of grass carp with injection or bath treatment when SWCNTs carrier was used. A good immune protective effect (relative percentage survival greater than 95%) is observed in smaller size fish (0.2 g) with SWCNTs-VP7 bath immunization. In addition, serum respiratory burst activity, complement activity, lysozyme activity, superoxide dismutase activity, alkaline phosphatase activity, immune-related genes and antibody levels were significantly enhanced in fish immunized with vaccine. This study suggested that functionalized SWCNTs was the promising carrier for recombinant subunit vaccine and might be used to vaccinate fish by bath approach.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is an economically important freshwater fish, and cultured extensively in more than 40 countries [1]. The production of grass carp is more than 4.3 million tonnes globally in 2012, where of China produces 4.2 million tonnes [2]. However, the development of grass carp aquaculture has been hampered by outbreaks of various infectious diseases, such as hemorrhagic disease, bacterial enteritis and bacterial septicemia, which have resulted in serious economic losses [2]. Grass carp reovirus (GCRV), as a prototype member of aquareovirus, can provoke severe hemorrhagic disease in fingerling and yearling populations of grass carp, and cause a mortality rate of up to 85% during the outbreak [3]. It has been recognized that GCRV is the most pathogenic among all aquareovirus species reported to date [4].

In an attempt to control the spread of the GCRV, antibiotics have been widely used over the years and partially solved the problem, but have also raised concerns regarding antibiotic's residues in fish and environment [1]. Another approach to disease prevention is to culture fish with enhanced resistance to GCRV through vaccination. An inactivated vaccine is applied as the main method to prevent GCRV, but this kind of vaccine has subtype specificity, which limits its application [5]. To better mitigate the disease, it is necessary to characterize the antigenicity of GCRV in grass carp to develop a novel vaccine against it. Among the structural proteins of GCRV,

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VP7 is the outer capsid protein and encoded by the S10 gene fragment [6]. GCRV VP7 had been expressed in *Escherichia coli* and suggested that recombinant VP7 could be used as a potential subunit vaccine against GCRV infection [7]. The potential advantages of subunit vaccines over inactivated vaccine include: (i) the high stability of recombinant protein, (ii) low manufacturing cost, (iii) no infection risk [8].

Until recently, intramuscular injection was the primary route for subunit vaccine administration. However, there are some limitations in this method. Not only is the process of handling, anesthetizing and injecting stressful for the fish, but it is also labor intensive and costly for the farmer [9]. Meanwhile, it is not practical to inject fish under 20 g in large numbers quickly [10], and fish smaller than this are often the most susceptible to disease. As an alternative to intramuscular injection, bath immunization is probably the simplest method of vaccination and easy to be administered without any stress to the fish. However, the efficacy is still not satisfactory on controlling the infectious disease with the traditional immersion methods [11]. In many cases they didn't work because of several formidable barriers, such as gastrointestinal tract, skin and cell [9,11].

To improve the production of grass carp and reduce the economic losses, more economic and efficient preventative strategy of GCRV infection is urgently desired in China [12]. The rapid development of nanomaterials and nanotechnology shows potential applications in many fields, such as electronic devices, biological and chemical sensors, and biomaterials [13–18]. Nanomaterials have special thermodynamic, mechanical, magnetic, optical, and quantum properties, and their use in the preparation of tumor vaccines has enabled controlled release, stability, targeting, magnetic properties, greater penetration, and antigenic features to be incorporated [13,14]. Nanomaterials such as carbon nanotubes (CNTs), particularly the functionalized water-soluble CNTs, have a wide range of promising applications in drug delivery [15,16] and therapy [17,18]. Functionalized CNTs can interact with mammalian cells and enter cells via cytoplasmic translocation [19,20]; and they therefore can deliver a range of therapeutic reagents into the cell. For example, *in vitro* study showed CNTs encapsulation permitted the drugs to be used at a 100-fold lower concentration compared to exogenous treatment yet achieve a comparable 70% cancer kill rate [18]. Thus, functionalized CNTs as vaccine carrier can be used to break through these biological barriers (gastrointestinal tract, skin and cell) and enhance the immune effect of vaccine. In addition, if functionalized CNTs as vaccine carrier can play a role in bath immunization of smaller size fish, it will provide broad prospects for application to aquatic vaccine.

In this study, functionalized single-walled CNTs (SWCNTs) as carrier were used to manufacture SWCNTs-VP7 subunit vaccine by chemical modification method. Different developmental stages of grass carps were immunized by VP7/SWCNTs-VP7 subunit vaccine against GCRV. The immune response on grass carp was evaluated in various ways. Blood leukocyte respiratory burst activity, complement activity, lysozyme activity, superoxide dismutase activity, alkaline phosphatase activity, immune-related genes and antibody levels were measured. After challenge with GCRV, the relative percentage survival was also recorded. To our knowledge, there is no report on the effect of CNTs-vaccine on immune system in fish. This work has laid a foundation for future work on a wide range of CNTs-vaccine delivery systems on fish.

2. Materials and methods

2.1. Experimental fish and virus

Grass carps were kindly provided by a fish farm in Heyang (Shannxi, China) and acclimatized in the laboratory for two weeks before experimental manipulation. Fish were maintained at 28 °C in aerated water and fed daily with commercial dry feed pellets (Hello Fish Dry Pellets; CVM Products, Beijing, China). Possible virus contamination in fish and feed was evaluated by reverse transcription quantitative real-time PCR (RT-qPCR) to confirm they were free from GCRV [21]. The grass carp in two different developmental stages (Type I: 0.21–0.24 g in body weight and 2.5–2.8 cm in body length; Type II: 25.5–28.5 g in body weight and 12.4–13.3 cm in body length) without any physical deformities, swimming behavior abnormities or clinical signs were used in the present study.

The GCRV strain used as a challenge pathogen in this study was isolated from the infected grass carp in fish farm located in Rougu (Shaanxi, China) and stored in our laboratory. The virus was cultured in *Ctenopharyngodon idellus* kidney cell (CIK). The CIK cell culture methods and 50% tissue culture infective doses ($TCID_{50}$) of the virus were performed according to established protocols [22]. Care of animals was in compliance with the guidelines of the Animal Experiment Committee, Northwest A&F University.

2.2. Cloning of vp7 gene

Viral genomic RNA was extracted with QIAGEN Viral RNA Mini Kit (Qiagen, Hilden, Germany) and converted to cDNA with RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) according to the manufacturer's instructions. The incipient primers used to explore *vp7* gene were designed from the GCRV cDNA sequence (GenBank accession NO., AF403396), using Primer Premier 5.0 software. A primer pair consisting of GCRV-1F (5′-GAATTCATGCCACTTCAC ATGATTCC-3′, the underline indicates *EcoR* I site) and GCRV-1R (5′-AAGCTTAATCGGATGGCTCCACATG-3′, the underline shows *Hind* III site) was utilized. The PCR protocol was 5 min at 95 °C, 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min. The product was purified by using the DNA Fragment Purification Kit (Beijing CoWin Biotech Corp., Beijing, China), ligated with pMD19T vector (Takara, Dalian, China) and then transformed into competent *E. coli* DH5α cells (Beijing CoWin Biotech Corp., Beijing, China), and plated on

Table 1
Primers used for the analysis of mRNA expression by RT-qPCR.

Genes	Accession no.		Primer sequences (from 5′ to 3′)	Product size (bp)
18S	EU047719	Forward	ATTTCCGACACGGAGAGG	90
		Reverse	CATGGGTTTAGGATACGCTC	
CRP	FJ547474	Forward	CTGCCTCCGCTCTCCATCTT	300
		Reverse	TCCCTTTGGCACATACGGTTCCTGA	
Mx2	JF699168	Forward	ACATTGACATCGCCACCACT	129
		Reverse	TTCTGACCACCGTCTCCTCC	
TNF-α	EU047718	Forward	TGTGCCGCCGCTGTCTGCTTCACGCT	291
		Reverse	GATGAGGAAAGACACCTGGCTGTAGA	
IL-1β	EU047716	Forward	GGAGAATGTGATCGAAGAGCGT	448
		Reverse	GCTGATAAACCATCCGGGA	
IL-8	EU047717	Forward	AGGTCTGGGTGTAGATCCACGCTG	137
		Reverse	TTAGTGTGAAAACTAACATGATCTCT	
IFN-I	AB196166	Forward	GGTGAAGTTTCTTGCCCTGACCTTAG	173
		Reverse	CCTTATGTGATGGCTGGTATCGGG	
C3	AY374472	Forward	GTCCTTCCGCTTCGTGGCTTATT	346
		Reverse	TCCTGCGGTGTGCGACTCAAAAC	
IgM	DQ417927	Forward	GCTGAGGCATCGGAGGCACAT	170
		Reverse	TTGGGTCTCGCACCATTTTCTC	
MHC-I	AY391782	Forward	CCTGGCAGAAAAATGGACAAG	271
		Reverse	CCAACAACACCAATGACAATC	
MHC-IIB	JF436931	Forward	TACTACCAGATTCACTCGG	111
		Reverse	CGGGTTCCAGTCAAAGAT	
IgD	GQ429174	Forward	CTGGCGCAGCTCTGAATTTG	287
		Reverse	TCGGAGGATGCTCACAATGG	
CD8α	GQ355586	Forward	GAGTCTCTGCACGGATCTAT	172
		Reverse	GTGTAGTGTTCCGAATTTAAGT	