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In addition to its antiviral and immunomodulatory properties, the zebrafish β-defensin 2 (zfBD2) is a potent viral DNA vaccine molecular adjuvant

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ABSTRACT

It is well known that β-defensins are key components of the host innate immune response against pathogens and potentially provide a link between innate and adaptive immunity. In zebrafish (*Danio rerio*), a vertebrate model species in numerous biomedical fields, three β-defensin isoforms were recently identified. To our knowledge, however, studies describing antimicrobial or immunomodulatory properties of any of the zebrafish β-defensins isoforms are absent today. Since it is indubitable that deepening the study of zebrafish β-defensins would be of interest in this work we investigated whether or not the zebrafish β-defensin 2 (zfBD2) has the antiviral properties described for their vertebrate counterparts. Our *in vitro* and *in vivo* studies showed that zfBD2 has antiviral activity, immunomodulatory properties and, most importantly, is a potent viral DNA vaccine molecular adjuvant. In addition, a potential relationship between zfBD2 activity and the NF-κB signaling pathway is suggested. Altogether these results show that the zebrafish could be a suitable *in vivo* animal model to study the roles played by β-defensin 2 in viral diseases, vaccinology and even in clinical dermatology. To note that psoriasis can be induced in zebrafish and the over-expression of β-defensin 2 is implicated in the inflammatory response associated with this human skin disorder.

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1. Introduction

Pathogen resistance to conventional anti-infective drugs (antibiotics and antivirals) has created a major global health issue. Therefore, there is urgent need to find other non-mainstream therapeutic options (Peters et al., 2010). In this regard, natural anti-infective agents or natural host-defense peptides (HDPs) represent one of the most promising future strategies for combating/preventing infections and microbial drug resistance.

HDPs are gene-encoded components of the immune system that have been selected by evolution as crucial tools of the first line of defense against invading microbes in all living organisms (Hancock and Sahl, 2006; Zasloff, 2002). Overall, these peptides display a potent antimicrobial activity and are rapidly mobilized in order to neutralize pathogens, including viruses, bacteria, protozoa, and fungi (Peters et al., 2010). In addition, the role of HDPs in modulating the innate immune response and boosting infection-resolving immunity, while dampening potentially harmful pro-inflammatory (septic) responses, gives these peptides the potential to become an entirely new therapeutic approach against pathogens (Hancock and Sahl, 2006).

Defensins constitute a family of HDPs with broad range of antimicrobial, antiviral and immunomodulatory activities (Ulm et al., 2012) that have a characteristic β-sheet-rich fold and a framework of six disulfide-linked cysteines (Falco et al., 2008; Ganz, 2005; Lehrer and Ganz, 2002). Among the three subfamilies of defensins (α-, β- and θ-defensins), β-defensins are probably the most interesting because, in contrast to α- and θ-defensins (Owen et al., 2004; Selsted, 2004), they are present in most of the plant and animal species explored so far (Ganz, 2004; Pazgier et al., 2006; Thomma et al., 2002).

The broad spectrum of activities of β-defensins is well-illustrated in studies carried out in both higher and lower vertebrates. Overall, these studies show that in addition to their role as potent antiviral/bacterial agents (Chattopadhyay et al., 2006; Falco et al., 2008, 2009; Garcia et al., 2001; Guo et al., 2012; Harder et al., 1997; Howell et al., 2007; Jiang et al., 2009; Jin et al., 2010; Klotman and Chang, 2006; Liu et al., 2002; Quinones-Mateu et al., 2003; Schroeder et al., 2011; Selsted and Ouellette, 2005; Sun et al., 2005; Valore et al., 1998), β-defensins are key components of the innate response and potentially provide a link between

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innate and adaptive immunity (Bowdish et al., 2006; Funderburg et al., 2007; Ganz, 2002; Oppenheim et al., 2003; Pazgier et al., 2006; Pingel et al., 2008; Yang et al., 2002, 1999, 2010). Moreover, these immunomodulatory properties are consistent with several studies that use β-defensins as vaccine adjuvants (Biragyn et al., 2002; Kohlgraf et al., 2010; Mei et al., 2012; Mutwiri et al., 2007; Tani et al., 2000; Zhang et al., 2010). On the other hand, β-defensins have also relevance in other biomedical fields as dermatology since they are implicated in human skin disorders including psoriasis and atopic dermatitis (Jansen et al., 2009).

In the zebrafish (*Danio rerio*), a vertebrate model species in numerous biomedical fields (Rakers et al., 2013; Rauta et al., 2012), three β-defensin isoforms were recently identified (Zou et al., 2007). To our knowledge, however, studies describing antimicrobial or immunomodulatory properties of any of the zebrafish β-defensins are absent. Taking into account that (i) the successful zebrafish developmental model has expanded and become also a model for the analysis of host-pathogen interactions during infectious disease (Phelps and Neely, 2005), (ii) numerous pathogens have been demonstrated to infect zebrafish, such as rhabdoviruses (Boltana et al., 2013; Encinas et al., 2010; Sanders et al., 2003), and new mechanisms of virulence and host defense have been revealed using this new model (Phelps and Neely, 2005) and (iii) β-defensins are crucial players of the first line of defense against invading microbes, it would be of interest to deepen the study of zebrafish β-defensins.

To that end, in this work we investigated both *in vitro* and *in vivo*, whether or not zebrafish β-defensins have some of the properties described for their vertebrate counterparts. Out of the three isoforms, we chose the zebrafish β-defensin 2 (zfBD2) to carry out this study because a transcriptomic study (Encinas et al., 2010) suggests that, *in vivo*, the induction of zfBD2 may be involved in the early immune response of zebrafish skin and secondary lymphoid-organs to the viral haemorrhagic septicaemia virus (VHSV), a fish rhabdovirus (Gomez-Casado et al., 2011). Our *in vitro* and *in vivo* studies showed that zfBD2 has antiviral activity, immunomodulatory properties and is a potent DNA vaccine adjuvant. In addition, a potential relationship between zfBD2 activity and NF-κB signaling pathway is suggested.

Altogether these results show that the zebrafish could be a suitable *in vivo* animal model to study the roles played by β-defensin 2 in viral infectious diseases, vaccinology and even in clinical dermatology. Psoriasis, for instance, can be induced in zebrafish (Webb et al., 2008) and the overexpression β-defensin 2 is implicated in the inflammatory response associated with this skin disorder.

2. Materials and methods

2.1. DNA constructs

The cDNA encoding the sequences of the zebrafish β-defensin 2 (zfBD2) (GenBank acc. number NM_001081554) and the spring viraemia of carp virus (SVCV) glycoprotein G (gpG_{SVCV}) (Genebank acc. number Z37505) were synthesized by GenScript (CA, USA) and then sub-cloned into the plasmid constructs pAE6 (containing the 5′-regulatory sequences of the carp β-actin gene, (Brocal et al., 2006; Ortega-Villaizan et al., 2009) and pMCV1.4 (containing the CMV promoter, (Chico et al., 2009), respectively, following standard procedures to generate the plasmid constructs pAE6-gpG_{SVCV} and pMCV1.4-zfBD2.

2.2. Cell cultures and virus

The fish cell line ZF4 (zebrafish embryonic fibroblast) (Driever and Rangini, 1993) purchased from the American Type Culture Collection (ATCC number CRL-2050) was used in this work. ZF4 cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen corporation, UK) cell culture medium containing 10% fetal calf serum (FCS) (Sigma, St. Louis, USA), 1 mM Pyruvate (Gibco, Invitrogen Corporation, UK), 2 mM Glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone.

The isolate 56/70 of SVCV isolated from carp (Stone et al., 2003) was propagated in ZF4 cells at 22 °C. Supernatants from SVCV-infected cell monolayers were clarified by centrifugation at 4000*g* for 30 min and kept in aliquots at −70 °C. Clarified supernatants were used for both *in vitro* and *in vivo* infection assays.

2.3. Cell transfection assays

Cell transfection assays were performed as previously described (Ortega-Villaizan et al., 2011). Briefly, ZF4 cell monolayers, grown in culture flasks of 75 cm^2, were detached using trypsin (Sigma), washed, resuspended in cell culture medium supplemented with 10% of FCS and dispensed into 96-well cell culture plates. The following day, plasmid DNA incubated with 0.3 μl of FuGene 6 (Roche, Barcelona, Spain) for 30 min in RPMI-1640 was added (1/5 of the total volume of the culture medium in each well) to the wells containing ZF4 cells in culture medium with 10% of FCS. The plates were further incubated at 24 or 28 °C for the times indicated in each experiment. The expression of zfBD2 and gpG_{SVCV} in ZF4-transfected cells was evaluated by quantitative PCR (qPCR) in real time as described below.

2.4. In vitro cell infection assays

ZF4 cells transfected with pMCV1.4.zfBD2 or pMCV1.4 were infected with SVCV (multiplicity of infection, m.o.i., of 3×10^{-1}) in a final volume of 100 μl/well of culture medium supplemented with 2% FCS at 22 °C for 90 min. Infected cell monolayers were then washed, fresh medium added, and plates further incubated until the end of each experiment. SVCV replication in ZF4 cells was evaluated by qPCR using specific primers and probe sequences for the gene encoding the protein N of SVCV (Table 1). Non-transfected ZF4 cells infected with SVCV were included as control.

2.5. In vitro zfmx gene expression

ZF4 cells were transfected with pMCV1.4-zfBD2 or pMCV1.4 as indicated above and then incubated at 28 °C for 72 h. After the incubation period, cell total RNA was extracted and cDNA synthesis carried out as indicated below. The *zfmx* gene transcript levels (both isoforms A and B) were evaluated by RT-qPCR as described below.

2.6. Fish

Adult zebrafish (*D. rerio*) of 2–3 g (∼4 cm in length) were obtained from a local fish pet shop and maintained at 28 °C in 30 l tanks equipped with a re-circulating dechlorinated water system. Fish were fed daily with a commercial feed diet. Prior to experiments, fish were acclimatised to laboratory conditions for 2 weeks. All experiments with live animals (zebrafish) were performed using protocols approved by the European Union Council Guidelines (86/609/EU).

2.7. Intramuscular injection of zebrafish with pMCV1.4-zfBD2

To evaluate the expression, protection against SVCV challenge or effect of zfBD2 on the immune system, zebrafish were anaesthetized by immersion in 50 μg/ml buffered tricaine

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