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Tilapia hepcidin (TH)2-3 as a transgene in transgenic fish enhances resistance to *Vibrio vulnificus* infection and causes variations in immune-related genes after infection by different bacterial species

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ABSTRACT

Hepcidin is an antimicrobial peptide (AMP) secreted by the liver during inflammation that plays a central role in mammalian iron homeostasis. But the function of hepcidin in fish is still not completely understood. We recently described three different hepcidins (named tilapia hepcidin (TH)1-5, TH2-2, and TH2-3) from tilapia *Oreochromis mossambicus*, the cDNA sequences were determined, the predicted peptides were synthesized, and the TH2-3 peptide showed antimicrobial activity against several bacteria. We hypothesized that TH2-3 may have a biological function like an AMP in fishes and can be used as a transgene to boost resistance against bacterial infection. To examine the antimicrobial effects of TH2-3, we produced and engineered the overexpression of TH2-3 in zebrafish (*Danio rerio*) and the convict cichlid (*Archocentrus nigrofasciatus*). The microinjected plasmid also contained a green fluorescent protein (GFP) which was used as an indicator to trace germline transmission. In vivo, transgenic TH2-3 fish (of the F3 generation) were challenged with *Vibrio vulnificus* (204) and *Streptococcus agalactiae* (SA). Results showed significant clearance of bacterial numbers of *V. vulnificus* (204) but not of *S. agalactiae* in transgenic TH2-3 fish. A gene expression study using a real-time RT-PCR revealed that transgenic TH2-3 zebrafish showed increased endogenous expressions of Myd88, tumor necrosis factor-α, and TRAM1 in vivo. After transgenic TH2-3 zebrafish were infected with *V. vulnificus* (204), interleukin (IL)-10, IL-26, lysozyme, toll-like receptor (TLR)-4a, and Myd88 were upregulated, but IL-1β (at 12–24 h) and IL-15 (at 1–12 h) were downregulated post-infection. After transgenic TH2-3 zebrafish were infected with *S. agalactiae*, IL-1β (at 1–24 h), IL-15 (at 6 h), IL-22 (at 1–6 h), and TLR3 (at 1–24 h) were downregulated, but TLR4a (at 6–12 h) and c3b (at 12 h) were upregulated post-infection. Our findings identify the TH2-3 transgene in transgenic fish as an active component of the host response to bacterial pathogens. These results suggest that using TH2-3 as a transgene in zebrafish can effectively inhibit bacterial growth, specifically the *V. vulnificus* (204) strain for up to 24 h.

1. Introduction

Hepcidin, a small peptide hormone made in the liver that is also called liver-expressed antimicrobial peptide (AMP) (LEAP)-1, was originally identified by two research groups [1,2]. It is a conserved 25-amino acid peptide produced in the liver and detectable in blood and urine in humans. In addition to its direct antimicrobial activity in vitro, human hepcidin exerts antibacterial and antifungal activities at concentrations of 10–30 μM [1,2]. Synthetic fish hepcidin peptides were also shown to be bactericidal and fungicidal [3,4]. Those above-described results suggest that hepcidin may play an important role in host defense against infections, and it may be possible to develop it as an anti-infective drug. Recently, hepcidin was demonstrated to regulate iron homeostasis and likely acts on iron metabolism by limiting intestinal iron absorption and release from macrophages [5], which suggests that hepcidin is instrumental in regulating normal homeostasis of iron levels in humans. Overexpression of hepcidin from hepatic adenomas was found in a patient with refractory anemia, and mutations in the hepcidin gene were present in severe cases of juvenile hemochromatosis (JH) [6,7].

Hepcidin genes and peptides were identified in a number of mammalian, amphibian, and fish species [4,8–13]. The structures and sequences of hepcidin genes are conserved between mammals and fish. Our previous work demonstrated that three hepcidin-like AMPs (named tilapia hepcidin (TH)1-5, TH2-2, and TH2-3) exist in

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tilapia (*Oreochromis mossambicus*). The minimal inhibitory concentration (MIC) results suggested that tilapia-synthesized TH1-5 and TH2-3 peptides possess antimicrobial activity at 50–100 μg/ml against gram-negative bacteria [4]. In another report, synthetic bass hepcidin was found to inhibit gram-negative bacteria and fungi at concentrations of 10–100 μM [3]. Weak antimicrobial activities may be due to the disulfide bond not forming when the TH peptides are synthesized. Most AMPs are cationic peptides, and the disulfide bond plays an important role in the antimicrobial activity by supporting the tertiary structure [14].

In fact, it has long been known that multiple mechanisms contribute to the development of gene functions in the setting of transgenic animal technology. The technology for producing transgenic animals exists for a variety of vertebrate and invertebrate species [15]. In a transgenic model, overexpression of hepcidin induced iron-restricted anemia similar to anemia associated with inflammation [16]. Another report indicated severe iron-deficient anemia in transgenic mice expressing liver hepcidin [17]. Thus, embryonic hepcidin transgene expression decreasing transferring receptor 1 messenger (m)RNA levels in mouse placenta suggests that hepcidin's action on the placenta is mostly through transcriptional downregulation of the iron uptake machinery [18]. However, in contrast to the severe iron-deficient anemia characterized in hepcidin 1 transgenic mice, hepcidin 2 transgenic mice develop normally similarly to non-transgenic mice [19]. Taken together, these data from a transgenic mouse model provide important insights into anemia associated with inflammation. But these animal models still do not elucidate the specific role of hepcidin in fish, and an open question about whether fish hepcidin has any antimicrobial activity in vivo remains. The relation of hepcidin to the immune response comes from a study of the regulation of hepcidin gene expression following inflammatory stimuli in transgenic fish.

The molecular and biological functions of fish hepcidin mostly remain unknown. The antimicrobial activity of fish hepcidin may lend itself to potential applications in aquaculture species against bacterial and viral infections [20]. Herein, we describe the generation of a muscle-specific mylz2 promoter-driven TH2-3 gene in transgenic zebrafish (*Danio rerio*) and convict cichlid (*Archocentrus nigrofasciatus*) that conferred resistance to bacterial infections. To assess whether this transgenic TH2-3 zebrafish model faithfully reproduces all of the features of the immune responses after a bacterial infection, we investigated the impact of TH2-3 overexpression on muscle after a bacterial infection compared to wild-type (WT) groups after a bacterial infection by qualitative reverse-transcription polymerase chain reaction (qRT-PCR). We inferred that TH2-3 may induce immune-related gene expressions, resulting in significant clearance of bacterial numbers of *Vibrio vulnificus* but not *Streptococcus agalactiae* in transgenic TH2-3 fish. Our results attempt to shed light on fish hepcidin's specific functions for future applications.

2. Materials and methods

2.1. Generation of muscle-specific mylz2 promoter-driven TH2-3 transgenic fish

A 273-base pair (bp) fragment of TH2-3 was amplified from a tilapia complementary (c)DNA library using the forward (5′-cta cgaattccgccaccatgaagacgttcagtgttgcagttgc-3′) and reverse primers (5′-ccccccgggtcaatgatgatgatgatgatggaacctgcagcagaagccgcag-3′). The product was gel-purified and subcloned into a pTLR cloning vector using EcoRI and XmaI restriction sites [21]. A fragment of the BGH polyadenylation sequence (pA) was amplified from the pcDNA3.1 vector (Invitrogen, Madison, WI, USA) using the primers, 5′-tgacccgggctgtgccttctagttgccagccat-3′ and 5′-gtggcggccgcccatagagcccaccgcatccccagc-3′. The product was gel-purified and subcloned into the pTLR cloning vector after TH2-3 using NotI and XmaI restriction sites. The mylz2 promoter fragment was amplified from the pm2.5K-DsRed vector [21] using the primers 5′-cttctcgagatgctgtgaagtattctctacttatc-3′, 5′-ggcggctagcgtagtgtcctgtacttgaggggct-3′, 5′-gtataagcttatgctgtgaagtattctctacttatc-3′, and 5′-cgaattcgtagtgtcctgtacttgaggggct-3′. The two fragments were gel-purified and subcloned into the pTLR cloning vector (which already possessed the TH2-3 and BGH pA sequences) by XhoI and NheI or HindIII and EcoRI restriction sites, respectively. A 702-bp fragment of enhanced green fluorescent protein (EGFP) was amplified from the pEGFP-N1 vector (Clontech Laboratories, Mountain View, CA, USA) using the forward (5′-ctacgctagccgccaccatggtgagcaagggcg-3′) and reverse primers (5′-agtggatccagagtgatcccggcggcggtcacg-3′). The product was gel-purified and subcloned into the NheI and BamHI restriction sites in the pTLR cloning vector (which already possessed the mylz2 promoter, TH2-3, and BGH pA sequences). A 213-bp fragment of SV40 early mRNA polyadenylation signal (SV40 pA) was amplified from the pEGFP-N1 vector using the forward (5′-tctggatccactctagatcataatcagccatac-3′) and reverse primers (5′-gcataagcttatacattgatgagtttggacaaacc-3′). The product was gel-purified and subcloned into the HindIII and BamHI restriction sites in the pTLR cloning vector (which already possessed the mylz2 promoter, TH2-3, EGFP, and BGH pA sequences), and the final constructed plasmid was named the pTLR-Mylz-EGFP-Mylz-TH2-3 vector. DNA sequence analysis confirmed that every step of the construct was the correct sequence. The L200- and R150-flanking region was a transposon arm for transposon excision events (Fig. 1). We introduced two polyA signals (BGH pA and SV40 pA) into the expression vector because the pEGFP-N1 vector contained the enhanced green fluorescent protein (EGFP) gene ligated with an SV40 early mRNA polyadenylation signal. The TH2-3 ligated with BGH pA followed sequences constructed with the pcDNA3.1 vector.

Plasmid DNA of pTLR-Mylz-EGFP-Mylz-TH2-3 (Fig. 1) and transposase mRNA were injected into the one-cell stage of ~1000 zebrafish or convict cichlid eggs following our previous report [21]; after injection, the eggs were placed in a 28 °C incubator. The whole body of zebrafish or convict cichlid was examined for the presence of green fluorescent color expression by fluorescence microscopy using an FITC filter every 120 min (IX71; Olympus, Tokyo, Japan). We obtained several transgenic zebrafish and convict cichlids, and chose only one transgenic zebrafish or convict cichlid to mate with a wild-type (WT) zebrafish or convict cichlid, respectively. To obtain sufficient numbers of transgenic zebrafish and convict cichlid for bacterial challenge and subsequent studies, we followed the mating, breeding, and propagation procedures in our previous report [21].

2.2. Bacterial challenge analysis in transgenic fish

The F3 transgenic zebrafish (about 4 cm in body length) and convict cichlid (about 6 cm in body length) were placed communally in 500-L aquaria for the bacterial challenge. *V. vulnificus* (204; from Dr. Chun-Yao Chen, Department of Life Science, Tzu Chi University, Hualien, Taiwan) was cultured in trypticase soy broth and agar at 28 °C. *S. agalactiae* (SA; from Dr. Stone S.-C. Chen, Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan) was cultivated in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) overnight at 28 °C with rotary shaking (150 rev/min). Stock cultures were kept at −70 °C in 25% glycerol. Ten microliters of *V. vulnificus* (strain 204; at 1×10^6 colony-forming units (CFU)/ml) or *S. agalactiae* (strain SA; at 1×10^7 CFU/ml) was injected into the caudal peduncle of transgenic zebrafish and WT zebrafish. The same bacterial number