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Identification and characterization of matrix metalloproteinase-13 sequence structure and expression during embryogenesis and infection in channel catfish (*Ictalurus punctatus*)

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ABSTRACT

Matrix metalloproteinase-13 (MMP-13), referred to as collagenase-3, is a proteolytic enzyme that plays a key role in degradation and remodelling of host extracellular matrix proteins. The objective of this study was to characterize the MMP-13 gene in channel catfish, and to determine its pattern of expression in various healthy tissues and during embryogenesis. Since MMP-13 has been shown to have importance in tissue remodelling and some pathological processes, we further studied its involvement in the defense responses of catfish after bacterial infection. The channel catfish MMP-13 cDNA contains an open reading frame of 1416 bp encoding 471 amino acids. Using RT-PCR analysis, MMP-13 was widely expressed in various health tissues. Using quantitative real-time PCR analysis, expression of MMP-13 gene was up-regulated by bacterial infection. During normal embryological development, MMP-13 expression was slightly increased in the first day post-fertilization and sharply up-regulated from 1-day post-fertilization through hatching.

1. Introduction

Matrix metalloproteinases (MMPs), also called matrixins, are a large family of zinc-dependent endoproteinases. The main function of MMPs is to degrade and turnover extracellular matrix, the important cellular environment that supports and directs tissue remodelling, development, and morphogenesis [1]. MMPs not only act upon extracellular matrix, but also on non-matrix proteins such as cytokines, chemokines, and antimicrobial peptides [2]. MMPs are also extracellular enzymes that play a role in regulating cell–matrix and cell-to-cell signalling events [3,4].

MMPs are involved in numerous physiological and pathological processes, such as development, wound healing, inflammation, cell invasion, angiogenesis, and immune surveillance [5–9]. MMPs have been demonstrated to be involved in various inflammatory processes, such as regulating physical barriers, cytokines and chemokines, and further downstream leukocyte migration [10]. Specifically, MMPs participate in re-epithelialization, resolution of scar formation, and inflammation by loosening the cell–cell and cell–extracellular matrix contacts at the wound margin, activating chemokines, establishing the chemotactic gradient, extravasating the leukocytes out of the blood to the injury, degrading existing collagen fibrils, and synthesizing new fibrils [6].

MMPs, therefore, are further involved in regulation of the host immune system. A first-line of host defense against the environment is the epithelium. The epithelium acts as a barrier to the external environment, regulates inflammation, and secretes antimicrobial peptides [11]. A critical process in innate immunity includes the repair of epithelium. Many MMPs are associated with the repair of epithelium [12–14]. A few members have been demonstrated to have an indirect role in removal of bacteria. For instance, MMP-7 deficient mice have an impaired ability to clear enteric pathogens, such as *Escherichia coli* and *Salmonella typhimurium* [15].

The MMPs also play an important role in development. Zebrafish lacking specific MMPs such as MMP-2, MT1-MMP, or

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MMP-13, through embryogenesis developed morphological abnormalities during somitogenesis, organ development, and tissue architecture [16–18]. In mice, a few MMPs were found to be critical during bone and vascular remodelling as well as mammary development [19–22]. A recent report on *Tribolium* demonstrated that knock-down of certain MMPs resulted in larvae with tracheal defects and abnormal intestines [23].

Currently, 25 members of the MMP family have been reported in vertebrates [2]. All MMPs share three common domains: the predomain, which is necessary for secretion; the prodomain, which regulates the molecule's function; and the catalytic domain containing the zinc-binding site, which is responsible for substrate processing [7]. MMPs are synthesized as zymogens. After the removal of the propeptide to reveal the zinc-binding site, MMPs are activated. Based on their substrate specificity and domain organization, MMPs are classified into collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and a few other ungrouped members [1].

MMP-13 is the third member in the collagenase subfamily of proteases. While MMP-13 preferentially hydrolyzes collagen type II, it has wide substrate specificity, allowing it to degrade several other forms of collagen and other extracellular matrix components.

MMP-13 is also referred to as collagenase-3, where the numbering was based on the order in which it was identified. MMP-13 was first recognized and cloned from human breast carcinoma [24]. Subsequent work showed that MMP-13 was expressed in chondrocytes of human cartilage [25]. As a collagenase-type MMP, MMP-13 hydrolyzes collagen into N-terminal and C-terminal polypeptide fragments [25,26]. MMP-13 was also found to be expressed in osteoblasts and periosteal cells during human fetal ossification [27]. Hence, MMP-13 plays an important role in the process of bone formation and remodelling during development [28], and normal remodelling of bone and cartilage during skeletal repair [29]. MMP-13 has been further shown to be expressed in various pathological conditions [26,30], during wound healing [31], and bacterial infections [32,33].

In teleosts, only limited information on the biological function of MMP-13 is available. The gene structure was identified and found capable of degrading type I collagen in rainbow trout [34]. In zebrafish, MMP-13 is required for normal embryogenesis [15]. MMP-13 expression varied during zebrafish development, with peak expression at 48 h post-fertilization. In Japanese flounder, MMP-13 was up-regulated during the course of *Edwardsiella tarda* infection [35]. No information is available on the MMP-13 gene and its function for channel catfish *Ictalurus punctatus*, the predominant aquaculture species in the United States (USDA-NASS, 2008). In this study, we identified and characterized a complete cDNA transcript and the MMP-13 gene in channel catfish, determined its pattern of expression in various healthy tissues and during embryogenesis and evaluated its involvement in the defense responses of catfish against the Gram-negative bacteria *Edwardsiella ictaluri*, the causative agent in enteric septicemia of catfish (ESC).

2. Materials and methods

2.1. Identification and sequencing of the catfish MMP-13 cDNA

A partial cDNA sequence of channel catfish MMP-13 was initially identified from an EST sequence (GenBank: CK411123) using BLAST similarity comparison. MMP-13 cDNA partial sequence was obtained by sequencing each end of the existing EST clone (AUF_IpHdk_44_p11) corresponding to this accession number. The clone was completely sequenced using a primer-walking strategy. Using BLAST analysis, the completely sequenced clone was putatively missing the 5′-end of the transcript. Therefore, 5′-rapid amplification of cDNA ends (5′-RACE) was conducted to complete the sequence of the MMP-13 transcript. RACE was conducted using the SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA) following the manufacturer's instructions. The 5′-RACE amplicon was gel purified and cloned using the pGEM-T Easy cloning kit (Promega, Madison, WI). Plasmid DNA was purified and sequenced. Sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3130*XL* automated sequencer (Applied Biosystems). Primers used for RACE and sequencing reactions are listed in Supplemental Table 1. Vector NTI 10 software (Invitrogen, Carlsbad, CA) was used to analyze and cluster sequences.

2.2. BAC library screening and genomic DNA sequencing

The MMP-13 gene was screened from the CHORI-212 BAC library [36] purchased from the Children's Hospital of the Oakland Research Institute. A gene-specific probe was designed from the cDNA sequence and generated by PCR using primers listed in Supplemental Table 1. The probe was labeled with ^{32}P-dCTP (Amersham Biosciences, Piscataway, NJ) by the Random Primed DNA Labeling kit (Roche Applied Science, Indianapolis, IN). After removing the unincorporated nucleotides with Sephadex® G-50 spin columns (Amersham Biosciences), the labeled probe was denatured at 95 °C for 5 min and added to hybridization tubes, which had been pre-hybridized for 2 h with 30 ml of hybridization buffer (20× SSPE, 20% SDS, 100× Denhardt's solution and 3 mg salmon sperm DNA). The hybridization was performed at 63 °C for 16 h. The filters were washed twice and exposed to X-ray film at −80 °C overnight. Positive BAC clones were identified and cultured in 2× YT medium for 20 h. BAC DNA was isolated using the R.E.A.L. Prep 96 BAC DNA isolation kit (Qiagen, Valencia, CA) and sequenced using a primer-walking method. Primers used for BAC sequencing are listed in Supplemental Table 1. Vector NTI 10 software (Invitrogen) was used to analyze and cluster sequences. BLAST searches were conducted to identify the gene. The MMP-13 cDNA and genomic DNA sequences were aligned using the Spidey program at the NCBI.

2.3. Bacterial challenge and sample preparation

The ESC challenge was conducted following established protocols, with modification [37,38]. Fish were randomly divided into different time points: 4 h control (3 pools), 4 h treatment (3 pools), 24 h control (3 pools), 24 h treatment (3 pools), 3-day control (3 pools), 3-day treatment (3 pools), 7-day control (3 pools) and 7-day treatment (3 pools). Treated group were injected with *E. ictaluri*, while control group with PBS. At each time point, 15 fish from each pool were collected, euthanized with MS-222 (300 mg/ml), and liver, spleen, intestine, and skin were collected, pooled, and immediately submerged in RNAlater solution (Invitrogen) per the manufacturer's protocol. Tissues were stored at −80 °C until RNA extraction. Samples were homogenized using a mortar and pestle under liquid nitrogen, and total RNA extracted using the RNeasy Plus Mini kit (Qiagen, Valencia, CA) according to the supplied protocol for tissue extraction.

To determine catfish MMP-13 gene expression in healthy channel catfish, 13 tissues were collected including brain, skin, muscle, blood, head kidney, trunk kidney, liver, spleen, intestine, stomach, gill, heart, and ovary. To determine the expression of MMP-13 occurring during the normal catfish embryogenesis, channel catfish embryos were collected at different time points: 2 h post-fertilization (hpf), 6, 12, 24, 48, 72, 96 hpf, and newly hatched. Samples from the healthy catfish and embryos were flash