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Functional roles and gene regulation of tumor necrosis factor receptor 1 in freshwater striped murrel

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ABSTRACT

In this study, a complete molecular characterization of tumor necrosis factor receptor 1 (TNFR1) which was identified from the constructed cDNA library of striped murrel *Channa striatus* (*Cs*) is reported. The *Cs*TNFR1 encoded a type I membrane receptor protein that contains 399 amino acids including three cysteine-rich domains (CRDs) at CRD1$^{41–46}$, CRD2$^{79–118}$ and CRD3$^{120–159}$ in the extracellular region and a putative TNF receptor-associated factor (TRAF) site at 245–253 and a death domain between 297 and 388 in the cytoplasmic region which is essential for induction of apoptosis. The predicted molecular mass of *Cs*TNFR1 is 45 kDa and its corresponding theoretical isoelectric point (pI) is 6.3. *Cs*TNFR1 shared maximum identity with TNFR1 from olive flounder *Paralichthys olivaceus* (82%). Real-time PCR showed that *Cs*TNFR1 gene was expressed most abundantly ($P < 0.05$) in the head kidney. Further, *Cs*TNFR1 mRNA transcription was studied after challenge with fungus *Apanomyces invadans* and bacteria *Aeromonas hydrophila.* The fungus injected murrels showed a highest expression at 48 h, whereas bacteria injected murrels showed at 24 h. The gene expression studies revealed that *Cs*TNFR1 may be involved in innate immune process of murrels against pathogenic infections. The over-expressed and purified recombinant *Cs*TNFR1 protein (r*Cs*TNFR1) was subjected to TNF-α inhibition assay to confirm their specificity and activity against TNF-α which confirmed that the r*Cs*TNFR1 inhibits the activity of TNF-α in a dose dependent manner where maximum inhibition (97%) was observed at 10,000 fold concentration of r*Cs*TNFR1. In addition, the direct cytotoxic effect of r*Cs*TNFR1 was analyzed against head kidney phagocyte. The results showed that the recombinant *Cs*TNFR1 protein does not exhibit any significant cytotoxicity against head kidney phagocyte cells even at higher concentration (8 μg/ml). Moreover, the recombinant protein was analyzed for respiratory burst activity in the presence of two different ROS inducers, opsonized zymosan (fungal cell wall component) and phorbol 12-myristate 13-acetate (PMA). The findings showed that the *C. striatus* head kidney phagocyte exposed to purified recombinant *Cs*TNFR1 protein alone do not produced any ROS. However, opsonized zymosan induced recombinant *Cs*TNFR1 protein significantly ($P < 0.05$) enhanced the ROS production on concentration basis which is revealed that the ROS production depends on the concentration of the recombinant *Cs*TNFR1 protein. Overall, the study showed that the *Cs*TNFR1 plays an important role in the pathogen-induced inflammatory process of striped murrel.

1. Introduction

Tumor necrosis factor (TNF) is a multifunctional potent cytokine belonging to ligand family that is secreted mainly by activated macrophages/monocytes. It is involved in pleiotropic actions including cell growth, cell differentiation, inflammatory reaction, apoptotic process, necrotic cell death mechanism, immune function and activation of various elements of immune system (Ashkenazi and Dixit, 1998; Wallach et al., 1999; Locksley et al., 2001; Aggarwal, 2003; Goetz et al., 2004; Xiao et al., 2007). TNF cytokine mediates its effects through interacting with two receptors, designated as tumor necrosis factor receptor 1 (TNFR1) and TNFR2 (Smith et al., 1994; Old, 1998). These two receptors are belongs to family of surface proteins including CD40, Fas, OX40,

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low affinity nerve growth factor receptor (NGFR) and several other receptors (Brockhaus et al., 1990; Tartaglia et al., 1991; Smith et al., 1994; Rusten and Jacobsen, 1995; Grell, 1995; Sato et al., 1997).

Based on the cytoplasmic region and signaling properties, TNFRSF members have been classified into three major groups including death domain-containing receptors, TNF receptor-associated factor (TRAF) binding receptors and decoy receptors (Locksley et al., 2001; Hehlgans and Pfeffer, 2005; Yuan et al., 2007; Li et al., 2009). TNFR1 is categorized under the group death domain containing receptors (DDCRs) which contain death domain in its cytoplasmic region. TNFR1 mediates many biological functions especially TNFR1 driven cytotoxic pathway through TNF-α (Chen and Goeddel, 2002; Dranoff, 2004; Wallach et al., 1999). Upon binding TNFR1 with ligand TNF-α, it activates various downstream pathways of adapter proteins such as FADD, RIP and TRADD, nuclear factor *kappa* B (NF*k*B) and caspase cascade to initiate the apoptosis. This resulted in the activation of caspase cascade, activation of nuclear factor *kappa* B (NF*k*B), induction of apoptosis and also sometimes acts as anti-apoptosis (Chen and Goeddel, 2002, Dranoff, 2004; Wallach et al., 1999). Locksley et al. (2001), Wiegmann et al. (1992), Hsu et al. (1995) and Tartalia et al. (1993) also reported a few other death domain containing receptors which include DR3, TRAIL-R1, TRAIL-R2, and DR6.

Locksley et al. (2001), Hehlgans and Pfeffer (2005) and Li et al. (2009) stated that some receptors namely TNFR2, CD40 and CD27 lacked death domain in the cytoplasmic region; instead they contain TRAF-interacting motifs (TIMs). Upon activation of these TIMs, it recruits TRAF family members which results in the activation of intracellular signal transduction pathways such as NF*k*B, Jun N-terminal kinase (JNK), P38, extracellular signal-related kinase (ERK) and phosphoinositide 3-kinase (PI3K) (Rothe et al., 1994; Cheng et al., 1995; Rothe et al., 1995; Cheng and Baltimore, 1996; Kanda et al., 2002; Dempsey et al., 2003). Thus, TNFR2 entails in the anti-apoptotic effect of TNF, whereas TNFR1 is involved in both apoptotic and anti-apoptotic signaling pathways.

The characteristic features of all TNFRSF members are well identified by presence of cysteine-rich repeats in their extracellular domains. TNFR1 and 2 have been identified and are found to bind both TNF-α and TNF-β in mammals. The extracellular domain of TNFR1 and 2 share significant similarity in the primary protein structure, but not in their cytoplasmic region (Dembic et al., 1990). It has been shown that each receptor has a different signal transduction pathway thus; it has unique downstream intracellular processes (Vilcek and Lee, 1991). Tartaglia et al. (1991), Rothe et al. (1994) and Hsu et al. (1996) observed that the cell responding to TNF is completely dependent on the type of TNF receptor which is predominant on the cell surface. Hohmann et al. (1989) and Ware et al. (1991) stated that most of the cells express both TNFR1 and 2 on their surface, however; in general, one of these two receptors is predominantly expressed. For example, epithelial cells predominantly express TNFR1 but, myeloid and lymphoid cells predominantly express TNFR2 (Hohmann et al., 1989; Ware et al., 1991).

In vertebrates, TNFR1 has been cloned and gene expression analyzed from human (Dembic et al., 1990), bovine (Newton et al., 1994; Mwangi et al., 2000), porcine (Boury et al., 1998) and fish (Park et al., 2003). In invertebrates, *Drosophila* genes Eiger and Wengen were found to be the first members of the TNFSF and TNFRSF proteins, respectively (Kanda et al., 2002; Igaki et al., 2002; Li et al., 2009). Igaki et al. (2011), Kauppila et al. (2003), Mabery and Schneider (2010), Mekata et al. (2010) and Moreno et al. (2002) reported that Eiger (TNFSF) binds to Wengen (TNFRSF); it resulted in the activation of caspase-independent JNK pathway which stimulate the apoptosis. A few recent studies from mollusks (Yu et al., 2007; Park et al., 2008; Li et al., 2009) have also confirmed the presence of TNFR in the TNF system as well as their binding nature.

The striped murrel *Channa striatus* is an air breathing favorite freshwater food fish in many parts of India due to its taste, market value and high medicinal qualities. It is also widely cultured in China and Southeast Asian countries. With fast development of fish farming activities, a diverse group of biotic agents such as fungus, bacteria, and cutaneous ectoparasites may initiate skin lesions in murrel, which are consequently colonized by fungus *Aphanomyces invadans* and bacteria *Aeromonas hydrophila* and eventually lead to epizootic ulcerative syndrome (EUS). Outbreaks of EUS had a drastic impact on *C. striatus* culture industry and caused significant economic losses to fish farmers in recent years (Jayaram, 1981; Haniffa and Marimuthu, 2004; Das, 1994; Bondad-Reantaso et al., 1992; Lilley et al., 1992). The widespread use of antibiotics as an additive in fish feeds for preventing infectious diseases has raised concerns about the development of many antibiotic-resistant microorganisms. Residues of antibiotics deposited in the aquaculture pond which leads to environmental pollution. Thus, enhancing the disease resistance population of this species using molecular techniques would be a perfect tool.

The aim of present study is to focus on the roles and mechanisms of the TNFs-TNFR complexes in the immune system of *C. striatus*. In order to accomplish those, we have characterized TNFR1 cDNA clones from *C. striatus* (*Cs*). In addition, tissue-specific expression of *Cs*TNFR1 and its modulation upon pathogenic biotic agents were investigated. Furthermore, recombinant TNFR1 (r*Cs*TNFR1) proteins were expressed in *E. coli* and purified. The purified recombinant protein was subjected to TNF-α inhibition assay to determine their specificity and inhibitory activity against TNF-α. Moreover, cytotoxic assay was performed to study its direct cytotoxicity against head kidney phagocytes. In addition, to confirm the role of recombinant *Cs*TNFR1 in pathogen induced inflammatory pathway, the ROS production capacity of recombinant *Cs*TNFR1 along with ROS inducers were assessed.

2. Materials and methods

2.1. Establishment of C. striatus cDNA library and CsTNFR1 identification

C. striatus cDNA library was established by utilizing the total RNA extracted from the tissue pool of spleen, liver, kidney, muscle and gills. Briefly, the total RNA from the tissue pools was isolated using Tri Reagent™ (Life Technologies) method as per the manufacturer's instruction. Then, the mRNA was purified using an mRNA isolation kit (Miltenyi Biotech) according to the manufacturer's protocol. Further, the first strand cDNA conversion and normalization were carried out using CloneMiner™ cDNA library construction kit (Invitrogen) and Trimmer Direct Kit: cDNA Normalization Kit (BioCat GmbH) as per the manufacturers' protocol. Thereafter, the genome sequencing FLX™ (GS-FLX™) technology was applied according to the manufacturer's protocol (Roche) to obtain the *C. striatus* cDNA sequences. Then, the process of raw data including screening, removal of normalization adaptor sequences and assembly were carried out as reported in our earlier studies (Arockiaraj et al., 2013; Arasu et al., 2013). A *Cs*TNFR1 cDNA sequence was identified through BLAST annotation program (http://www.blast2go.com/b2ghome) from the established *C. striatus* cDNA library.

2.2. In silico analyses of CsTNFR1

Homologues analysis of *Cs*TNFR1 was conducted using BLAST server in NCBI (http://blast.ncbi.nlm.nih.gov/Blast).