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# Constitutive expression of tumor necrosis factor-alpha in cytotoxic cells of teleosts and its role in regulation of cell-mediated cytotoxicity

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**Abstract**

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are the main killer cell populations of the immune system. The mechanisms by which these cells recognize target cells vary considerably, while the effector molecules used to facilitate target cell death are highly conserved. The main pathways utilized by killer cells consist of granule exocytosis and those mediated by members of the TNF superfamily. Nonspecific cytotoxic cells (NCC) are the first identified cytotoxic cell population in teleosts. We have previously demonstrated the expression of granzymes and Fas ligand in these cells. This is the first report of the expression of tumor necrosis factor-alpha in these killer cells. A cDNA coding for TNF was cloned and sequenced from NCC purified from Nile tilapia (*Oreochromis niloticus*). Factors regulating the transcriptional modulation of TNF in these cells were identified by RT-PCR analysis. The mature form of tilapia TNF was expressed as a recombinant protein and biological activities were analyzed. Using a cross-reacting anti-TNF polyclonal antibody, analysis of TNF expression suggested that tilapia NCC constitutively express the membrane-bound as well as secreted forms of TNF. Recombinant tilapia TNF effectively induced cytotoxicity in the mammalian cell line WEHI, although to a lesser extent compared to the murine TNF. Treatment with recombinant TNF protected NCC from activation-induced cell death. Recombinant tilapia TNF was also effective in upregulation of granzyme transcription in tilapia NCC. These data suggest that teleost TNF may play a role in diverse effector functions of cytotoxic cells from ectotherms, similar to the biological functions described for mammalian TNF.
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*Keywords:* Tumor necrosis factor-alpha; Cytotoxicity; Nonspecific cytotoxic cells; Activation-induced cell death; Stress activated serum factors

## 1. Introduction

Apoptotic cellular death is initiated by two main signaling mechanisms called the intrinsic and extrinsic pathways. The intrinsic pathway triggers apoptosis as a result of DNA damage, cell cycle checkpoint defects, loss of survival factors and other types of severe cellular stress conditions. In all of those circumstances death occurs as a consequence of the activation of the pro-apoptotic arm of the Bcl-2 gene superfamily. This engages mitochondria to cause the cytosolic release of apoptogenic factors such as cytochrome C and SMAC/DIABLO (Adams and Cory, 1998; Green, 2000; Hunt and Evan, 2001). The extrinsic pathways of cellular death are initiated by binding of members of the TNF superfamily ligands to their death receptors (Ashkenazi, 2002).

The TNF ligand super-family is composed of members such as TNF, Fas ligand and TRAIL that are grouped by their structural and functional similarities. These ligands recognize different specific receptors, which together constitute the TNF receptor super family (Kwon et al., 1999; Locksley et al., 2001; Wallach et al., 1999). TNF binds to two membrane receptors of 55–60 kDa (TNF-R1) and 75–80 kDa (TNF-R2). TNF-R1 is widely expressed on a variety of cell types and mediates most of the TNF functions. TNF-R2 expression is tightly regulated and restricted primarily to cells of lymphoid tissue (Grell et al., 1995). Like other members of the TNF super-family, TNF is synthesized as type II transmembrane protein that may be expressed in either of two forms: the membrane embedded “pro” molecule or the cleaved “ma-

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ture" form (Idriss and Naismith, 2000). Both forms are active and self assemble into non-covalent trimers, whose individual chains fold into compact jelly roll β-sandwiches and interact at hydrophobic interfaces (Fesik, 2000).

The physiological significance and difference between the membrane and soluble forms of TNF super-family members has been the focus of many investigations. Recombinant soluble TNF-alpha and lymphotoxin are capable of inducing apoptotic cell death in mouse as well as human T lymphocyte blasts in vitro (Sarin et al., 1995). It has also been demonstrated that the membrane-bound TNF and FasL are more potent inducers of cytotoxicity than their soluble trimeric forms (Eissner et al., 1995; Grell et al., 1995; Schneider et al., 1998; Tanaka et al., 1998). Membrane-bound FasL was cytotoxic for human peripheral blood lymphocytes, while the soluble form blocked the killing (Suda et al., 1997). Although these studies have shed light into the possible mechanisms of actions of the different forms of TNF, the role of TNF alpha in immune functions is still unclear. In mammalian natural killer (NK) cells, expression of TNF alpha alone was not sufficient to induce tumor cell killing while simultaneous interactions of at least three TNF family ligands (TNF, LT-α1β2 and FasL) with their corresponding receptors effectively generated pro-apoptotic signals in tumor cells (Kashii et al., 1999).

Teleost TNFs have been sequenced and expressed from a variety of species (Garcia-Castillo et al., 2002; Hirono et al., 2000; Laing et al., 2001; Saeij et al., 2003; Zou et al., 2003b). Comparisons of the gene structure and phylogenetic analysis of the amino acid sequences appear to indicate that the teleost TNFs are more closely related to mammalian TNF alpha than beta (Goetz et al., 2004). It has been suggested that fish do not have a TNF beta lymphotoxin-like gene. Although this could suggest a more prevalent role for TNF alpha in fish species, there are many unanswered questions about its functional role. While it is clear that fish TNF is produced by macrophages in response to bacterial stimulus, other cell sources for this cytokine have not been determined (Goetz et al., 2004). Most of the studies on teleost TNF have utilized mixed populations of myeloid cells, which make the functional characterization difficult to achieve (Goetz et al., 2004). Using long-term hematopoietic cell-lines, expression of TNF in channel catfish was shown to be mostly in T cells and macrophages, but not in B cells and fibroblasts (Zou et al., 2003b). In the present study, we have cloned and expressed the TNF-alpha gene from freshly purified nonspecific cytotoxic cells (NCC) of tilapia. NCC have been well characterized in a number of fish species and they appear to be functionally related to mammalian NK cells (Carlson et al., 1985; Evans et al., 1984; Faisal et al., 1989; Graves et al., 1985; Greenlee et al., 1991; Jaso-Friedmann et al., 2002; Jaso-Friedmann and Evans, 1999; McKinney and Schmale, 1994). We have previously shown that tilapia NCC expressed a molecule functionally characterized as Fas ligand and we have molecular information about teleost granzymes. (Bishop et al., 2002; Jaso-Friedmann et al., 2000a; Praveen et al., 2004). However, this is the first report on the molecular identity of a death ligand in tilapia. Our results show that, much like its mammalian counterpart, TNF alpha from tilapia NCC appears to have pleiotropic immunological functions.

## 2. Materials and methods

### 2.1. Experimental animals and isolation of NCC

Outbred tilapia (*Oreochromis niloticus*) weighing 60–100 g were obtained from Americulture, Inc. Animas, NM. Fish were maintained in fiberglass aquaria equipped with a constant flow through system at 23–25 °C. Fish were fed a commercial diet of pelleted fish food (Southern States Co-operative Inc., VA). Fish were acclimatized for a minimum of 3 months prior to the study and were free from any clinical infections. NCC were purified from peripheral blood, anterior kidney or spleen of tilapia as previously described (Bishop et al., 2000). Purity of cell preparation was verified by flowcytometric analysis using 5C6 (a monoclonal antibody detecting NCCRP-1, which is an activation marker found exclusively on NCC (Evans et al., 1998; Jaso-Friedmann et al., 2002).

### 2.2. Construction and screening of tilapia cDNA library

Construction of cDNA libraries from NCC of different fish species has been previously described (Praveen et al., 2004). Conserved regions in various teleost TNF-alpha sequences were identified by multiple sequence alignments using Clustal W provided with Vector NTI package, version 6 (InforMax Inc). Two degenerate primers (TNFDGN1F: 5′-GNGCHAARGCHGCHATYCA-3′ and TNFDGN3R: 5′-CARRTARATDGCRTTRTACCA-3′) were used to amplify a portion of tilapia TNF, using tilapia NCC cDNA library as template. Amplicons were TA cloned in to pDrive cloning vector using a PCR cloning kit (Qiagen, Valencia, CA). Inserts were sequenced in two directions and compared with the known sequences in DDBJ/EMBL/GenBank databases using BLAST version 2.2.5 (Altschul et al., 1997). Sequence from one of the amplicons which had closest similarity to the known TNF sequences was used to design primers to amplify the entire 5′ and 3′ ends of the tilapia TNF as described previously (Praveen et al., 2004).

### 2.3. Phylogenetic analysis and protein modeling

Similar analyses as those previously done with catfish granzyme and NCCRP-1 of zebrafish were performed (Jaso-Friedmann et al., 2002; Praveen et al., 2004). The three dimensional structure of tilapia TNF was modeled using SWISS-MODEL in the first approach mode accessible via the internet (http://www.expasy.org/swissmod). The co-ordinate files were imported to RasWin software version 2.6 for ana-

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