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Molecular cloning, functional identification and expressional analyses of FasL in Tilapia, *Oreochromis niloticus*

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ABSTRACT

FasL is the most extensively studied apoptosis ligand. In 2000, tilapia FasL was identified using anti-human FasL monoclonal antibody by Evans's research group. Recently, a tilapia FasL-like protein of smaller molecule weight was predicted in Genbank (XM_003445156.2). Based on several clues drawn from previous studies, we cast doubt on the authenticity of the formerly identified tilapia FasL. Conversely, using reverse transcription polymerase chain reaction (RT-PCR), the existence of the predicted FasL-like was verified at the mRNA level (The Genbank accession number of the FasL mRNA sequence we cloned is KM008610). Through multiple alignments, this FasL-like protein was found to be highly similar to the FasL of the Japanese flounder. Moreover, we artificially expressed the functional region of the predicted protein and later confirmed its apoptosis-inducing activity using a methyl thiazolyl tetrazolium (MTT) assay, Annexin-V/Propidium iodide (PI) double staining, and DNA fragment detection. Supported by these evidences, we suggest that the predicted protein is the authentic tilapia FasL. To advance this research further, tilapia FasL mRNA and its protein across different tissues were quantified. High expression levels were identified in the tilapia immune system and sites where active cell turnover conservatively occurs. In this regard, FasL may assume an active role in the immune system and cell homeostasis maintenance in tilapia, similar to that shown in other species. In addition, because the distribution pattern of FasL mRNA did not synchronize with that of the protein, post-transcriptional expression regulation is suggested. Such regulation may be dominated by potential adenylate- and uridylate-rich elements (AREs) featuring AUUUA repeats found in the 3′ untranslated region (UTR) of tilapia FasL mRNA.

1. Introduction

FasL (CD95L), along with TNF-alpha, LT (TNF-beta), TRAIL, VEGI (TL1A), TWEAK, and LIGHT, is a death ligand in the extrinsic apoptosis pathway (Aggarwal, 2003). The binding of these ligands to the corresponding receptors can induce programmed cell death within the targeted cell.

FasL functions as a major death inducer in the immune system. It eliminates infected cells and tumor cells, downregulates immune response by killing T cells (Nagata, 1999), and maintains B cell tolerance (Lee et al., 2006) and the homeostasis of cell population and quality (Andreu-Vieyra et al., 2005; Prisco et al., 2003). The expression of FasL is limited to only certain cell types. T cells, NK cells, monocytes and mast cells express FasL (Blott et al., 2001; Kiener et al., 1997; Wagelie-Steffen et al., 1998). Usually the protein is stored intracellularly as a transmembrane protein or as a soluble truncated form in the secretory vesicles of those hematopoietic lineages. Given appropriate stimulation, FasL could be infused into the cell surface (Lettau et al., 2006) or released into the extracellular milieu as a membrane component of exosomes (Andreola et al., 2002) or as soluble FasL (Kiener et al., 1997). The FasL expression of tumor cells, which is believed to be an immune-escape mechanism, remains controversial (Lettau et al., 2009). Some non-lymphoid cells also constitutively express FasL on the membrane, including certain cells in the eye; neurons, astrocytes, oligodendrocytes, microglia and the vascular endothelium in the central nervous system; and the fetal cytotrophoblasts and maternal decidual cells of the placenta. FasL helps to establish

Abbreviations: RT-PCT, reverse transcription polymerase chain reaction; MTT, methyl thiazolyl tetrazolium; PI, Propidium iodide; RACE, rapid amplification of cDNA ends; AREs, adenylate and uridylate rich elements; UTR, untranslated region; DISC, death-inducing signaling complex; MMP, matrix metalloproteinases; NCC, non-specific cytotoxic cell; ORF, open reading frame; rtFasL, recombinant tilapia FasL.

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so-called immune privilege at those sites (Niederkorn, 2006). High expression was also identified on mature spermatozoa, but its role remains to be clarified (Li et al., 2012).

FasL is a type II transmembrane protein belonging to the TNF family. Its detailed structure was shown in Fig. 1.

FasL exists in a membrane-bound or truncated soluble form. The soluble FasL originates from membrane FasL that is cleaved by the matrix metalloproteinases (MMP) in extracellular space (Kayagaki et al., 1995) or produced by the alternative splicing of the FasL mRNA (Ayroldi et al., 1999). The apoptosis-inducing ability of FasL processed by MMP is largely compromised compared with membrane-bound FasL (Schneider et al., 1998). It has also been reported that naturally cleaved FasL may dampen apoptotic activity by competing with mFasL or blocking Fas receptors (Gregory et al., 2011; Knox et al., 2003; O' Reilly et al., 2009; Suda et al., 1996; Tanaka et al., 1998). When binding to the matrix proteins or cross-linking by antibodies, the cytotoxicity of naturally cleaved soluble FasL was potentiated in vitro and in vivo (Aoki et al., 2001; Schneider et al., 1998). Recombinant soluble FasL corresponding to the entire extracellular domain of FasL shows considerable cytotoxicity compared with naturally cleaved sFasL, although lower than membrane-bound FasL (Hohlbaum et al., 2000; Suda et al., 1996). Recombinant soluble FasL aggregation by fusing a self-assembling motif (Daburon et al., 2013; Shiraishi et al., 2004) showed extensively enhanced apoptosis-inducing activity, both in vitro and in vivo.

Using anti-human FasL monoclonal antibody clone 33, Evans' group identified tilapia FasL of similar molecular weight as the human counterpart of 37 k (moderately glycosylated (Diestre et al., 2006)) in both the tilapia non-specific cytotoxic cell (NCC) lysates and the supernatants from NCC incubated media (Evans et al., 2000; Jaso-Friedmann et al., 2000). However, the authenticity of this protein begs considerable doubt.

Correspondently, clone 33 antibody completely blocked the lysis activity of tilapia NCC towards HL-60 cell and the apoptosis-inducing activity of the supernatant containing the tilapia FasL towards the pathogenic protozoans (Bishop et al., 2002; Jaso-Friedmann et al., 2000). Because only one Western blot band was identified in the NCC lysate and supernatant of the culture media, only one form of FasL was expressed in tilapia NCC, either intact membrane-bound FasL or truncated soluble form.

For the FasL identified in the supernatant, there are only two circumstances for it to be free of the cell: as a transmembrane protein on the surface of an exosome (Andreola et al., 2002) or as a truncated soluble molecule that is devoid of the transmembrane domain, at least (Kiener et al., 1997), as produced by the alternative splicing of the FasL mRNA (Ayroldi et al., 1999).

If the FasL exists as a surface protein on an exosome, the result that the apoptosis-inducing activity of the supernatant containing this form of FasL was blocked by clone 33 antibody (Jaso-Friedmann et al., 2000) contradicts the fact that surface FasL cannot be recognized by this antibody (Herr et al., 2000; Kiener et al., 1997). If the molecule in question is the truncated soluble form of FasL, its molecule weight was uncommonly large, compared with the results shown in other studies (Ayroldi et al., 1999; Cuesta et al., 2003; Kiener et al., 1997; Long et al., 2004).

Another grounds for doubt is the questionable specificity of the clone 33 antibody against the FasL protein propogated by several researchers. In particular, this antibody detected a different protein than FasL, as revealed by 2D electrophoresis (Maher et al., 2002). The antibody may recognize other death-inducing molecules, such as TRAIL, TNF, perforin and granzymes in the tilapia NCC immune synapse (de Saint Basile et al., 2010) other than FasL, and caused similar blockage of the cytotoxicity towards HL-60 cell and protozoans. In this regard, more direct evidence should be provided, for instance, to enrich the identified tilapia NCC FasL by immune precipitation (IP) and to analyze its sequence; alternately, Western blots could be performed using a more convincing antibody.

Recently, a predicted tilapia FasL-like protein sequence with a smaller molecule weight of 26.3 k (calculated by Compute pI/Mw tool http://web.expasy.org/cgi-bin/compute_pi/pi_tool) than its human counterpart of 31.5 k was present in Genbank. This clue again challenged the authenticity of the identified tilapia FasL by Evans' group.

To confirm the existence of the predicted tilapia FasL-like molecule in Genbank and to identify its functions and tissue distribution patterns, we performed the following experiments.

2. Materials and methods

2.1. Fish

Tilapia weighing 130 ± 30 g were acclimated to the lab environment for one week before being sacrificed for experiments. They were kept in 0.3 m^3 glass tanks at 28 °C (water temperature) on a 12-h light/12-h dark cycle. Water quality was maintained by filtration, aeration, and a 1 L min^{-1} circulating water supply.

2.2. Gene cloning of tilapia FasL

After the fish were anesthetized with MS-222, the tissues were sampled, frozen immediately in liquid nitrogen and stored at −80 °C. The total RNA was isolated from the head kidney of the tilapia using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Then, the first-strand cDNAs were synthesized with a TOYOBO Reverse Transcription Kit (TOYOBO, Japan, Catalog No. FSK-100). A partial

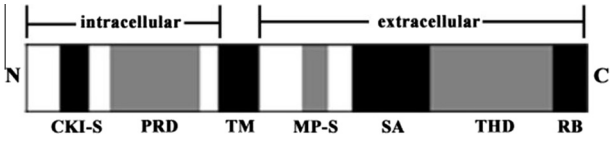

Fig. 1. Structural composition of FasL. CKI-S, casein kinase substrate motive; PRD, proline-rich domain; TM, transmembrane region; MP-S, metalloprotease cleavage site; SA, self-assembly region; THD, TNF homology domain; RB, receptor binding site (Adapted from (Lettau et al., 2009)).

Table 1
Primers used in this study.

Primer	Sequence(5′–3′)
pF1	GTGGGACACGACGCATTCTG
PR1	ATCTCCCTGAGTGGCTGTGC
pF2	TGGTTGGCGTAGTGGTGCTG
PR2	ACCTTAGAATCGCCCTTGGA
5′-R1	CCACTGAAGACAACCCGATA
5′-R2	AATAAATGCAGCACCACTACGC
3′-F1	TCCAAGGGCGATTCTAAGGT
3′-F2	ACTAGCCAGCAAGGTCCAGC
5′-CDS	AAGCAGTGGTATCAACGCAGAGTACGCGGGGGGGGHN[a]
NUPA	AAGCAGTGGTATCAACGCAGAGT
UPMA long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UPMA short	CTAATACGACTCACTATAGGGC
ORF-F	TGTCACCGCTCCGCTCTAT
ORF-R	TCCACGAAGATGTTTGAGCC
rF	CGCGGATCCGAAAAACATTTCTCAGCAGAAGATA
rR	CCCAAGCTTTTATAGTTTGTATATCCCAAAGTTTGT
Fa-Q-F	CCAAGGGCGATTCTAAGGTC
Fa-Q-R	ATCTCCCTGAGTGGCTGTGC
Beta-Q-F	ATGGTGGGTATGGGTCAGAAAGAC
Beta-Q-R	TCTCCATGTCATCCCAGTTGGTC

[a] N: A/T C/G.