Fish & Shellfish Immunology 55 (2016) 559–567

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

TNF-α is involved in apoptosis triggered by grass carp reovirus infection *in vitro*

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ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form
13 June 2016
Accepted 22 June 2016
Available online 23 June 2016

Keywords:
Grass carp reovirus
TNF-α
Apoptosis
Capase-8
TRADD

ABSTRACT

Grass carp reovirus (GCRV) infection causes apoptosis in *Ctenopharyngodon idella* kidney cells (CIK). However, the cause of GCRV-induced apoptosis and its signaling pathways remain unknown. This study investigated the role of TNF-α-induced capase-8 pathways in mediating GCRV-induced apoptosis in the grass carp (*Ctenopharyngodon idella*). Recombinant TNF-α was expressed and purified from *Escherichia. coli*. The western blot assay indicated that TNF-α expression level in kidney and spleen was higher than that in liver. In apoptosis assay, recombinant TNF-α triggered significant apoptosis in CIK cells, which was characterized by increased mRNA levels of TNF-α, TRADD or caspase-8, and enhanced caspase-8 activity in CIK cells. To confirm the biological activity of TNF-α during GCRV infection, significant apoptosis in CIK cells was induced by GCRV correlating with enhanced caspase-8 activity, increased mRNA level of TNF-α, TRADD or caspase-8, increased protein level of TNF-α in CIK cells and cell supernatant, suggesting that TNF-α-induced capase-8 pathways might be involved in GCRV-triggered apoptosis. Furthermore, treatment with an anti-TNF-α polyclonal antibody significantly decreased the degree of apoptosis in infected CIK cells compared with cells treated with a control antibody, which confirmed that TNF-α was a key mediator involved in GCRV-induced apoptosis. Taken together, these results indicated that GCRV might trigger apoptosis via TNF-α induced capase-8 pathways in CIK cells.

1. Introduction

Grass carp (*Ctenopharyngon idellus*) is a major freshwater aquaculture fish species in Asia [1]. Grass carp reovirus (GCRV) leads to grass carp hemorrhagic disease, which is a major problem for grass carp aquaculture [2,3]. GCRV belongs to the genus Aquareovirus in the family of reoviridae [4], and the virion comprises 11 dsRNA genomic segments that encode seven structural proteins (VP1 – VP7) and five non-structural proteins (NS16, 26, 31, 38 and 80) [5]. Although cell death induced by GCRV has been reported, including typical apoptosis and non-apoptotic cell death, the signaling pathways involved in these processes remain unknown [6,7].

Apoptosis is a highly regulated cell death process in the normal development and homeostasis of multicellular organisms [8]. Apoptosis is also used by viruses to evade the host immune system to ensure viral replication and persistent infection [9,10]. Apoptosis occurs via two fundamental pathways: extrinsic and intrinsic [11]. The common event in the end point of both the intrinsic and extrinsic pathways is the activation of a set of cysteine proteases (caspases) [12]. The extrinsic pathway is processed by the combination of death ligands and receptors, such as tumor necrosis factor receptor-1 (TNF-R1) and their cognate ligands (TNF-α). Ligand/receptor binding induces the recruitment of several adaptor proteins and proenzymes, which activates the caspases, ultimately resulting in apoptotic cell death [13]. The intrinsic pathway is controlled by the mitochondria and is triggered by stimuli-like reactive oxygen species (ROS), endoplasmic reticulum stress, lysosomal stress or calcium overloading. When the mitochondria are stimulated, cytochrome c is released into the cytoplasm to activate caspases (e.g. caspase-9 and caspase-3), causing caspase-dependent intrinsic apoptosis [14,15].

Tumor necrosis factor alpha (TNF-α) is a cytokine that regulates pleiotropic biological activities, including the immune response, maintenance of homeostasis of the immune system, induction of programmed cell death (apoptosis), cell proliferation and differentiation [16–18]. Binding of TNF-α to TNFR1 results in the sequential formation of two signaling complexes [19,20]. The quickly formed plasma membrane-bound complex I is assembled

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http://dx.doi.org/10.1016/j.fsi.2016.06.033
1050-4648/

on the receptor's cytoplasmic tail and comprises the adaptor TRADD, the protein kinase RIP1, and the signal transducer TRAF2. This complex signals inflammation and cell survival through IkB kinase (IKK)-dependent activation of transcription factor NF-κB [21]. Subsequently, complex I dissociates from the receptor and forms cytoplasmic complex II, in which TRADD, together with RIP1, associate with the adaptor protein FADD and pro-caspase-8 (also called FLICE). In cells that lack substantial NF-κB activity, the formatted complex II results in the induction of apoptotic cell death and caspase-8 activation [20]. Many reports have demonstrated analogous functions of the grass carp TNF-α with its mammalian counterparts [22–25], and modulation of its expression has been detected in response to GCRV, lipopolysaccharide, polyinosinic-polycytidylic acid, bacterial or recombinant TNF-α protein [26], TNF-α has also been demonstrated to regulate the fish NF-κB pathway [27]. TNF-α is upregulated during several virus infections in several cells [28–31]. However, data regarding how TNF-α is involved in apoptosis triggered by GCRV in grass carp kidney cells remains unknown.

In this study, recombinant TNF-α was expressed and purified from *Escherichia. coli*, and we also detected TNF-α in different tissues form healthy organs of the grass carp. Furthermore, we investigated the role of TNF-α-induced capase-8 pathways in mediating GCRV-induced apoptosis in the grass carp. We aimed to investigate TNF-α′ s involvement in the host's immune response to GCRV infection by studying the relationship between TNF-α and GCRV-induced apoptosis.

2. Materials and methods

2.1. Animals, cell lines and virus

Healthy grass carps (100–200 g) were obtained from the SHOU experimental fish breeding farm. CIK cells were grown in M199 medium with 10% fetal bovine serum (Gibco, USA) [32]. Monolayers of CIK cells were infected by GCRV (type I, strain JX-01) at a multiplicity of infection (MOI) of 1.0 and then the cells were incubated for 2–3 days at 28 °C. Infected cells or supernatants were harvested when the CPE (Cytopathic effect) was greater than 90% [33]. A standard end-point dilution assay was used to determine the titer of GCRV, and the titers were expressed as median tissue culture infection dose (TCID50) per mL, using the method of Reed–Muench method, as previously described [3].

2.2. Expression and purification of pET-32a-TNF-α from Escherichia coli

The expression plasmid pET-32a-TNF-α was constructed previously [34]. Briefly, the TNF-α ORF was amplified from the *Ctenopharyngodon idella* mRNA by RT-PCR using the primer pairs sTNF-P32-F (CGCGGATCCATGAGAGATCATTTTTCAAAAGC) and sTNF-P32-R (CCGCTCGAGGCAGAGCAAACACCCCAAAAAAG), and than cloned into pET-32a vector. To express the fusion protein in *Escherichia coli* BL-21 (DE3) cells, 200 ml of Luria-Bertani medium containing 100 μg ampicillin/ml was inoculated with 1/100 of an overnight culture and grown to an optical density of 0.6 at 600 nm. Protein expression was induced using 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 8 h. The bacteria were collected by centrifugation at 8000 rpm for 5 min, and the pellet was resuspended in 10 ml of PBS. The protein expression level was assessed by SDS-PAGE. Recombinant proteins were purified on Hislink™ Protein Purification Resin (Promega, America), according to the manufacturer's protocol. The specificity of the expressed protein was further analyzed by immunoblotting analysis using a specific monoclonal antibody (1:5000 dilution) specific for the his-tag (Abmart, China). Proteins were resolved by SDS-PAGE and transferred to PVDF (GE Healthcare) as described previously [33]. After blocking with 5% skimmed milk and binding of primary antibodies in PBS with 0.1% Tween 20 (PBST), the membranes were incubated with the secondary antibody conjugated with alkaline phosphatase (Abmart, China). Reactive bands were developed with an alkaline phosphatase substrate solution (NBT/BCIP) (Boster, China).

2.3. Extraction of whole-tissue proteins

To monitor the expression level of TNF-α in the kidney, spleen and liver, whole-tissue proteins were extracted using a Total Protein Extraction Kit (Beyotime, China), according to the manufacturer's instructions. Briefly, tissues were washed in PBS and homogenized in ice-cold cell lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.4% IGEPAL, 1 mM PMSF). The samples were then centrifuged at 13000 rpm for 5 min at 4 °C; at this stage, the whole-tissue proteins were in the supernatants. The proteins were quantified using a BCA Protein Assay kit (Sangong, China). The expression level of TNF-α was determined by western blotting using the homemade anti-TNF-α polyclonal antibody [34]; GAPDH served as the internal reference protein, using an anti-GAPDH monoclonal antibody (Abmart, China).

2.4. Immune stimulation of CIK cells and quantification of gene expression

For the viral challenge, CIK cells were infected with GCRV at an MOI of 1, and PBS-treated cells were used as the control. Cells were sampled at 0, 6, 12, 24 and 48 h post-infection. For the recombinant TNF-α stimulation, CIK cells were exposed to 150 ng/ml of recombinant TNF-α for 0, 6, 12, 24 and 48 h. Total RNA was extracted from the cells using TRIzol (Invitrogen, USA), according to the manufacturer's protocol. The extracted RNA was assayed using a Nanodrop 2000 spectrophotometer. Reverse transcription was performed using a PrimerScript First Strand cDNA Synthesis Kit (Takara, Japan). The first-strand cDNA was subsequently used as the template for the RT-PCR. The grass carp elongation factor 1α gene (*EF1α*) was used as the internal control gene for cDNA normalization [35]. Real-time PCR (RT-PCR) was carried out in a 20 μL reaction volume containing 5 μL of cDNA temple, 10 μL of 2 × SYBR Green Master Mix (Bio-Rad, USA), 1 μL of each specific primer (10 mM), and 7 μL of PCR grade water using the CFX96 Real-time PCR Detection System (Bio-Rad, USA). After the PCR program was completed, the threshold cycle (CT) value was determined using the manual setting on the CFX Manager 2.1 software (Bio-Rad, USA). The $2^{-\Delta\Delta CT}$ method was used to analyze the expression of the different genes [36]. The $2C^{Treference}/2CT^{target}$ ratio of the target gene to the reference gene (*EF1α*) was calculated [37]. All of the expression data were subjected to a one-way analysis of variance ANOVA, followed by a paired-samples *t*-test, and P values less than 0.05 ($P < 0.05$) were considered statistically significant. The mRNA expression levels of TNF-α, TRADD and caspase-8 were analyzed by real-time-PCR using the primers in Table 1.

Table 1
Primers used in the present study (all 5′–3′).

qTNFa-F	TAGATTGGAGAGTGAACC
qTNFa-R	GCTGTAGACGAAGTAAATG
qCaspase8-F	ATGTCTCACGCTATTACG
qCaspase8-R	GTTCACTTGTCCATCTGA
qEF-1a-F	CGCCAGTGTTGCCTTCGT
qEF-1a-R	CGCTCAATCTTCCATCCCTT
qTRADD-F	CAGTCCAGAACACAACCT
qTRADD-R	CCAGTCCACCAACAGAAT