Available online at www.sciencedirect.com
SCIENCE DIRECT
Developmental and Comparative Immunology 30 (2006) 639–647
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

TNF induces the growth of thymocytes in rainbow trout

Kazuyoshi Hino [a,*], Osamu Nakamura [a], Yasutoshi Yoshiura [b], Hiroaki Suetake [c], Yuzuru Suzuki [c], Tasuku Watanabe [a]

[a] *Laboratory of Fish Pathology, School of Fisheries Sciences, Kitasato University, Ofunato, Iwate 022-0101, Japan*
[b] *Inland Station, National Research Institute of Aquaculture, Tamaki, Mie 519-0423, Japan*
[c] *Fisheries Laboratory, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Maisaka, Shizuoka 431-0211, Japan*

Received 24 June 2005
Available online 5 October 2005

Abstract

In order to investigate the effects of TNFα upon the growth of fish thymocytes, rainbow trout thymocytes were cultured in the conditioned medium (CM): the supernatants of the macrophage cultures stimulated with chitin derivative and LPS. Synthesis of TNFα by macrophages and subsequent secretion into CM were ascertained by RT-PCR and western blotting. While most of the thymocytes cultured in normal medium died within 7 days, the thymocytes cultured in CM exhibited markedly better growth as monitored by alamarBlue™ assay and BrdU assay. The proliferating cells appeared to be small lymphocytes. Since such activity in CM was significantly inhibited by an anti-trout TNF antibody, it was clearly evident that TNFα in the CM induced the proliferation of the thymocytes. Production of TNFα in the thymus of healthy fish was also demonstrated by RT-PCR. Collectively, this data suggest that TNFα is involved in T cell development in the trout thymus.

Keywords: TNFα; Thymocyte; Macrophage; Rainbow trout; Cytokine; Recombinant TNFα; Conditioned medium; Proliferation

1. Introduction

Tumor necrosis factor α (TNFα) is a key molecule in immune and inflammatory responses. A diverse array of biological activities has been demonstrated for TNFα in mammals: cytotoxicity against tumor cells, acceleration of tissue remodeling and activation of immunocytes during infection [1,2]. Furthermore, TNFα is also known to be involved in the differentiation, proliferation and apoptosis of thymocytes [3–13]. Mouse [5] and human [4] TNFα can each induce the proliferation of thymocytes without the involvement of any other molecule. This activity is further enhanced with interleukin (IL)-1 [9], IL-2 [3,7] and IL-7 [7] on the adult murine thymocytes, and with IL-2, IL-4 and IL-7 on the fetal thymocytes [6]. TNFα can also induce the differentiation of $CD3^-CD4^-CD8^-$ thymocytes [8,12]. Expression of TNFα mRNA has been demonstrated in the normal murine thymus [14,15]. Furthermore, Giroir et al. [16] showed that the thymus is the only organ where TNFα is secreted constitutively in mouse. These findings indicate that TNFα plays a crucial role in T cell production in the mammalian thymus.

The complementary DNA encoding for TNFα has been cloned in several teleost species including flounder [17], channel catfish [18], and fugu [19]. Two TNFα-like genes have been sequenced in rainbow trout (TNF1 and TNF2) [20,21]; these genes share 94% amino acid identity. While human recombinant TNFα enhances O_2^- producing activity [22] and expression of class II major histocompatibility complex (MHC)

* Corresponding author. Tel.: +81 192 44 1907; fax: +81 192 44 2125.
E-mail address: f034z@st.kitasato-u.ac.jp (K. Hino).

0145-305X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2005.09.005

products [23] in rainbow trout macrophages, there have been obtained only a few information on biological activities of fish own TNFα. Qin et al. demonstrated that supernatants obtained from trout macrophage cultures were cytotoxic to murine L929 cells, a cell line sensitive to mammalian TNFα. Since this observed activity was inhibited by rabbit anti-mouse TNFα, the authors suggested that cell lysis was attributable to trout TNFα [24]. A more recent study documented that recombinant trout TNF (rTNF) molecules induced the expression of an array of proinflammatory factors including IL-1β, TNF1, TNF2, IL-8 and cyclo-oxygenase 2 in leucocytes isolated from the head kidney and a macrophage cell line [25]. Our understanding of the precise biological role of TNFα in fish, however, is still far from sufficient. Of particular note is that there is currently no evidence to support the contention that TNFα might participate in the production of T cells in fish.

In the present study, therefore, we aimed to ascertain whether trout TNF has a proliferative effect upon thymocytes. In order to achieve our aims, we incubated trout thymocytes in the supernatant of cultured media containing macrophages that had been stimulated by LPS and S-chitosan [26], a water soluble hydrolysate of chitin prepared using hydrochloric acid (Approximate molecular weight 11,000 Da). Our study revealed that conditioned media could promote the proliferation of thymocytes. This activity was markedly inhibited by anti-TNF. This is the first study to report that TNF can exert an influence upon thymocyte growth in fish.

2. Materials and methods

2.1. Fish

Rainbow trout were purchased from Iwate Prefectural Inland-Water Fisheries Station, Matsuo, Iwate. Fish were subsequently maintained in running fresh water and fed a commercial trout diet (Nihon Nosan Kogyou, Japan).

2.2. Production of recombinant trout TNF and anti-rTNF rabbit serum

Trout rTNF was produced as described by Zou et al. [25]. Briefly, the cDNA fragments encoding the putative mature TNF1 and TNF2 were ligated into a bacterial expression vector (pQE30, Qiagen, UK) containing a 6× His tag at the N-terminus. In order to produce rTNF protein in bacteria, the expression plasmids were transformed into *E.coli* M15 cell (Qiagen). The resultant recombinant proteins formed insoluble inclusion bodies which were purified under denaturing condition using an Ni-NTA column according to the manufacturer's instructions (Qiagen). Purified rTNF1 and rTNF2 were dialyzed against phosphate buffered saline (PBS, 20 mM phosphate, 100 mM NaCl, pH 7.4). About 90% of both the rTNF molecules were found as insoluble aggregates. To produce polyclonal antisera against rTNF1 and rTNF2, rabbits were subcutaneously injected with 300 μg of either protein together with Freund's complete adjuvant. Rabbits were given two subsequent booster injections and then 2 weeks later, blood samples taken and sera isolated. The antisera raised against rTNF1 or rTNF2 cross-reacted with both molecules. For further experiments, antiserum against rTNF1 was used. For cell proliferation assays, the IgG fraction was purified from anti-rTNF rabbit serum using an ImmunoPure® *Plus* Immunobilized Protein A IgG Purification Kit (Pierce, USA).

2.3. Establishing cultures of rainbow trout macrophages

RPMI1640 medium (Nissui Pharmaceutical, Japan) supplemented with 10% of fetal bovine serum (FBS), 100 international units (IU)/ml of penicillin sulphate, and 100 μg/ml of streptomycin (RPMI-10) was used for cell culture. Six fish (160–876 g in body weight) were anesthetized with 200 ppm of 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, USA). After laparotomy, RPMI-10 was introduced into the abdominal cavity and peritoneal cells were harvested using a pipette. The cells were counted and transferred to flasks (Corning No. 430639 USA). After incubation for 1 h at room temperature, cells attached to the flasks were separated from non-adherent cells by washing three times with RPMI-10. Adherent cells were cultured at 20 °C, until confluent cell sheets were formed. Cells were then treated with 0.1% trypsin (Invitrogen, USA)/0.02% EDTA in 0.01 M PBS (pH 7.4), and then transferred to new 25 or 75 cm^2 tissue culture flasks (Greiner Nos 690160, 658170, Germany) as appropriate. In three of the six fish used in this study, cells proliferated slowly and reached confluence within 50 days. A culture derived from one of these three fish, designated as RTM5, grew more rapidly than the primary cultures, and was chosen as the source of TNF for use in further experiments (Fig. 1). A high incidence of phagocytic activity against latex beads (about 90%) [26] indicated that these cells are mainly composed of macrophages. In this study, RTM5 cells passaged within 15 and 30