Cytokine 60 (2012) 498–504

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

TNF-inducible expression of lymphotoxin-β in hepatic cells: An essential role for NF-κB and Ets1 transcription factors

Lily S. Subrata [a], Dominic C. Voon [a], George C.T. Yeoh [a,b], Daniela Ulgiati [a], Elizabeth A. Quail [a], Lawrence J. Abraham [a,*]

[a] Biochemistry and Molecular Biology, School of Chemistry and Biochemistry, The University of Western Australia, Crawley 6009, Australia
[b] The Western Australian Institute for Medical Research & Centre for Medical Research, The University of Western Australia, Crawley 6009, Australia

ARTICLE INFO

Article history:
Received 23 March 2012
Received in revised form 30 May 2012
Accepted 31 May 2012
Available online 26 June 2012

Keywords:
Oval cells
Transcription factor
Ets1
TNF
Lymphotoxin

ABSTRACT

As TNF is one of the earliest signals that can be detected in the leukocyte-derived inflammatory cascade which drives subsequent cytokine production, we are interested in determining whether TNF is one of the initiating factors controlling liver remodeling and regeneration following chronic liver damage. One of the early responses is the expression of lymphotoxin-β by hepatic progenitor oval cells. The aim of this study was to determine whether hepatic expression of LT-β was controlled by TNF and to understand the basis of this regulation. We previously showed that LT-β expression is transcriptionally controlled via the TNF-induced, inflammatory NF-κB pathway in T lymphocytes. Here we show that TNF is able to upregulate LT-β expression in hepatic cells at the transcriptional level by the binding of NF-κB *p*50/*p*65 heterodimers and Ets1 to their respective sites in the LT-β promoter.

1. Introduction

Lymphotoxin-beta (LT-β) is a membrane-bound member of the TNF family and is closely related to TNF itself. LT-β exists as part of a heterotrimer, comprising, in its most abundant form, two LT-β subunits and one lymphotoxin-α (LT-α) subunit (i.e., LTα1β2) [1,2]. The LTαβ ligand is expressed on the surface of activated lymphocytes [3] and signals via the lymphotoxin-β receptor (LTβR) [4], a protein ubiquitously expressed by nonlymphoid cells [5,6]. We have shown previously that LT-β expression is transcriptionally controlled via the TNF-induced, inflammatory NF-κB pathway in T lymphocytes [7,8]. Recent work has shown that LT-α and LT-β are also expressed in hepatic progenitor (oval) cells [9] and virally infected hepatocytes [10]. Further, in both LT-β and LTβR knockout mice, oval cell mediated liver regeneration was impaired indicating an important role for LT-β signaling in this process [11]. Current evidence suggests that the appearance of oval cells following chronic liver injury is preceded by the infiltration of lymphocytes in an initial inflammatory burst [12,13]. We have shown previously that signaling via the major inflammatory cytokine TNF is necessary for an optimal oval cell-mediated regenerative response [14]. Recent evidence suggests that macrophages may also play a major role in the response [15]. Accordingly, the aim of this study was to determine whether hepatic cell expression of LT-β was controlled at the transcriptional level by TNF and to identify the transcription factors that regulate the response.

2. Materials and methods

2.1. RT-PCR analysis of LT-β mRNA

Total RNA was isolated from cells using RNAzol™B (Tel-Test Inc., TX, USA) and reverse transcribed using the Reverse Transcription System (Promega Corp.) according to the manufacturer's instructions. The mRNA levels of LT-β and GAPDH were analyzed by quantitative real-time PCR using the iQ SYBR green supermix (Biorad Laboratories, Inc.). All the samples were assayed in triplicate and analyzed using the iQ5 real-time PCR Detection System (Biorad). To calculate relative expression, all results were analyzed according to the ΔC_t method using GAPDH as a reference gene. The PCR primer sequences were LT-F AAGCTGCCAGAGGAGGAGCC, LT-R TCCCGCTCGTCAGAAACGCC, GAPDH-F TGCCCCCTCTGCTGATGCC,

Abbreviations: TNF, tumor necrosis factor; TNFRI, TNF receptor type I; CDE, choline-deficient ethionine-supplemented; LT-β, lymphotoxin-β; LTβR, lymphotoxin-β receptor; NF-κB, nuclear factor κB; EMSA, electrophoretic mobility shift assays; PIL, $p53^{-/-}$ immortalized liver oval cell line; PMA, phorbol myristate acetate.

* Corresponding author. Address: Biochemistry and Molecular Biology, School of Chemistry & Biochemistry, The University of Western Australia, 35 Stirling Highway, Crawley 6009, Western Australia, Australia. Tel.: +61 8 6488 3041; fax: +61 8 6488 1148.
E-mail address: labraham@cyllene.uwa.edu.au (L.J. Abraham).

1043-4666/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cyto.2012.05.029

GAPDH-R CCTCCGACGCCTGCTTCACCAC. PCR reactions conditions were as described [16].

2.2. Cell culture

The murine $p53^{-/-}$ immortalized liver cell line, PIL-4, was cultured in Williams' Medium E plus supplements [17]. HepG2 (ATCC HB-8065) and HepB3 (ATCC HB-8064) human cell lines were obtained from the American Type Culture Collection (ATCC, VA, USA), and maintained in MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, CA, USA). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Transfections and luciferase reporter assays

The full-length pLTBL-1828 reporter [7] and derivatives carrying mutations in putative transcription factor binding sites were described previously [8]. Cells were transfected by lipid-mediated transfection as described [16] and cultured for 24 h prior to induction with recombinant human TNF (5 ng/ml; Roche Diagnostics Australia, NSW, Australia) or PMA (20 ng/ml; Calbiochem-Novabiochem, NSW, USA) for 18 h. For cotransfections using pcDNA3.1 derivatives expressing human Ets1 and Ets2 cDNA [8] the expression constructs were used in 2 molar-fold excess over the LT-β reporter construct. Relative LT-β promoter activity was expressed as luciferase activity normalized to the respective *Renilla* luciferase activity. Transfection data obtained from at least three independent experiments were analyzed for statistical significance using analysis of variance (ANOVA).

2.4. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared and EMSA reactions carried out as previously [16,18]. Oligonucleotides used in this study are as follows (mutant nucleotides in lower case): Region D (region containing NF-κB binding site; −90 to −71 of the LT-β promoter), TGTGCGGAAAGTCCCAGCCA; mutant RegD-M, TGTGCGGgtacCCCAGCC; NF-κB-consensus, AGTTGAGGGGACTTTCCCAGGC; Region C2 (Ets binding site; −130 to −91), AGCTCTGGGTAAACAGGAAGC TGGGTGAGGGGAGCAGGGG; mutant RegC2-M2, AGCTGTGGGTAA ACggtAccCTGGGTGAGGGGAGCAGGGG; Ets-consensus, CGAGCAG GAAGTTCGACG [19]. Supershift antibodies used were anti-NF-κB *p*65 (A-X, sc-109X rabbit polyclonal IgG), anti-NF-κB *p*50 (NLS-X, sc-114X rabbit polyclonal IgG) and anti-Ets1/Ets2 (C-275X, sc-112X rabbit polyclonal IgG) (Santa Cruz Biotechnology).

2.5. Chromatin immunoprecipitation (ChIP)

ChIPs were performed using the Upstate Biotechnology (Lake Placid, NY) ChIP assay kit as previously described [20] with minor modifications. Briefly, 1×10^7 cells were fixed with formaldehyde (Sigma-Aldrich, St Louis, MO) and sonicated 10 times for 5 sec (pulses separated by 3 min on ice) using a Sonifier 250 (Branson Ultrasonics Corporation, Danbury, CT) set at duty cycle 100% and output 4.5. Soluble chromatin was precleared with Salmon Sperm DNA/Protein A/G-agarose beads (Upstate Biotechnology) and incubated overnight with either 2 μg of Ets1 antibody (C-20, Santa Cruz Biotechnology) or 2 μg of normal rabbit IgG (Santa Cruz Biotechnology). Immune complexes were collected and DNA isolated using the QIAmp DNA blood mini-kit (Qiagen). Quantitative PCR was carried out using SYBR Green PCR mastermix (Qiagen) and the LT-β promoter specific primers, TCAGAGAACCCAGGCATC-CAGCTG and TGCACACCTGGCTGGGACTTTCC. Cycling conditions were 96 °C for 5 min, followed by 28 cycles of 96 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec. Off-target control primers, TCCTGCATCCTGTCTGGAA and GTCTTCTGGGCCACTGACTG amplified a region of the TNF promoter that does not contain any Ets-consensus motifs or known to bind any Ets proteins. Cycling conditions were 96 °C for 5 min, followed by 28 cycles of 96 °C for 30 sec, 59 °C for 30 sec, 72 °C for 30 sec. Samples were run on a Rotor Gene 2000 real-time PCR Thermalcycler and the results analyzed using Rotor Gene 6 software (Corbett Research Pty Ltd., Sydney, Australia).

3. Results

3.1. Response of LT-β in oval cells and hepatocellular carcinoma cell lines to TNF

We have previously shown that LT-β mRNA and protein is expressed in the human hepatocellular carcinoma cell lines Hep3B and HepG2 and the murine oval cell line PIL4 [16]. To determine whether these cell lines were appropriate models to study the

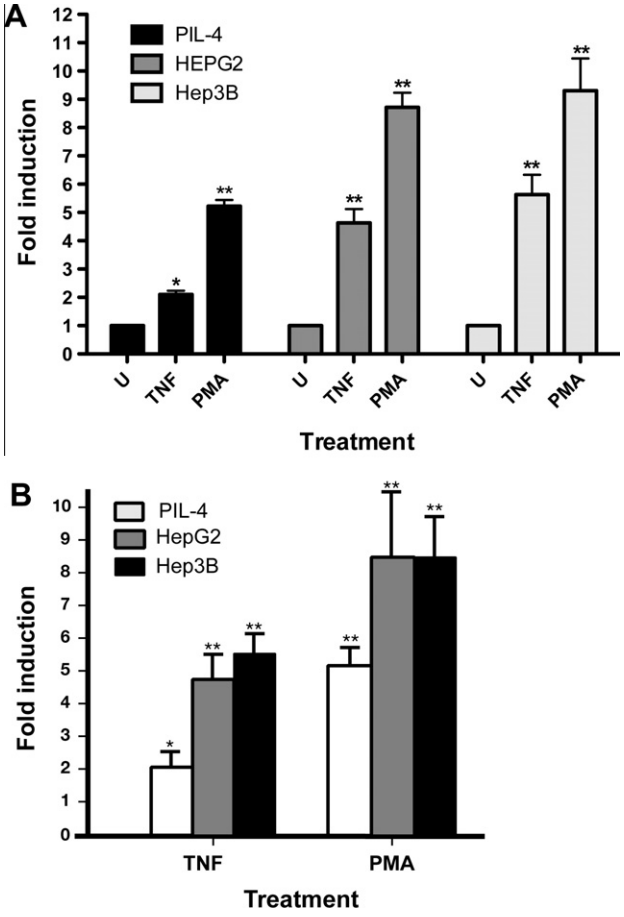

Fig. 1. Hepatic expression of LT-β is inducible by TNF and PMA. Inducible expression of LT-β steady-state mRNA levels (A) and relative LT-β promoter activity (B) was investigated in PIL-4 oval cell and HepG2 and Hep3B hepatoma cell lines. Following transfection with the full-length LT-β promoter-luciferase reporter and a 24 h recovery period, transfected cells were either left untreated or treated with recombinant human (h) TNF (5 ng/ml) or PMA (20 ng/ml) for 18 h. Cells were then harvested for both total protein and RNA. Steady state mRNA levels were determined by quantitative PCR normalized against GAPDH mRNA levels and expressed as -fold induction over untreated cells. Relative LT-β promoter activity is expressed as firefly luciferase activity normalized to the respective *Renilla* luciferase activity. The data presented for each cell type are mean values (±SEM) of at least three independent transfections, expressed relative to the respective untreated values. Treatments resulting in statistically significant induction of LT-β compared to untreated activity, are indicated $^{*}(p < 0.05)$ or $^{**}(p < 0.01)$.