Cellular Signalling 21 (2009) 1495–1503

Contents lists available at ScienceDirect

Cellular Signalling

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

Down-regulation of FoxO-dependent c-FLIP expression mediates TRAIL-induced apoptosis in activated hepatic stellate cells

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ARTICLE INFO

Article history:
Received 27 March 2009
Received in revised form 15 May 2009
Accepted 19 May 2009
Available online 24 May 2009

Keywords:
TRAIL
FoxO
Hepatic stellate cells
FLIP
Apoptosis

ABSTRACT

Activated hepatic stellate cells which contribute to liver fibrosis have represented an important target for antifibrotic therapy. In this study, we found that TRAIL inhibited PI3K/Akt-dependent FoxO phosphorylation and relocated FoxO proteins into the nucleus from the cytosol in activated human hepatic stellate LX-2 cells. The accumulated FoxO proteins in the nucleus led to down-regulation of c-FLIP$_{L/S}$ expression, resulting in the activation of apoptosis-related signaling molecules including the activation of caspase-8, -3, and Bid, as well as mitochondrial cytochrome *c* release. These results were supported by showing that siRNA-mediated knockdown of FoxO led to restoration of c-FLIP$_{L/S}$ expression and resistance to TRAIL-induced apoptosis after treatment of LX-2 cells with TRAIL. Furthermore, c-FLIP$_{L/S}$-transfected LX-2 cells showed the decreased sensitivity to TRAIL-induced apoptosis. Collectively, our data suggest that sequential activation of FoxO proteins under conditions of suppressed PI3K/Akt signaling by TRAIL can down-regulate c-FLIP$_{L/S}$, consequently promoting TRAIL-induced apoptosis in LX-2 cells. Therefore, the present study suggests TRAIL may be an effective strategy for antifibrotic therapy in liver fibrosis.

1. Introduction

Liver fibrosis is the excessive accumulation of extracellular matrix proteins that occurs in most types of chronic liver diseases due to viral, metabolic, and genetic liver injury [1,2]. Prolonged liver injury results in hepatocyte damage, which triggers activation of hepatic stellate cells (HSCs) [3,4]. HSCs are recognized as the primary cellular source of matrix components in chronic liver disease, and play a critical role in the development and maintenance of liver fibrosis [5]. Therefore, HSCs represent an appealing target for antifibrotic therapy to prevent the deposition of extracellular matrix proteins [6]. Although biologically plausible pathways involved in liver fibrosis have led to the identification of numerous compounds that have received great interest as potential future therapeutic agents for liver fibrosis [7–9], their efficacy and safety in humans still remains unknown.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is a novel anticancer agent, capable of inducing apoptosis preferentially in a wide variety of cancer cell lines but not in most normal cells including hepatocytes [10,11]. TRAIL binds to two transmembrane receptors TRAIL-R1/DR4 and TRAIL-R2/DR5, resulting in the recruitment of the adaptor molecule FADD, which recruits caspase-8 into the death-inducing signaling complex (DISC) [12]. Once recruited to FADD, caspase-8 drives its autoactivation through oligomerization and subsequently activates other caspases including caspase-3, -6, and -7 [13]. Activated caspase-8 also cleaves and activates the BH3 domain-containing proapoptotic molecule Bid, a truncated form of which then translocates to mitochondria triggering the proapoptotic mitochondrial events including the cytosolic release of cytochrome *c* [10]. A previous report has suggested a role of TRAIL for the apoptosis induction of activated HSCs [14]. In this study, we revaluated this issue in more detail and showed that TRAIL-induced apoptosis in HSCs is regulated through FoxO-dependent FLIP expression.

FoxO proteins are a subgroup of the Forkhead family of transcription factors [15,16]. These proteins regulate the expression of a wide variety of genes involved in the control of cell survival and apoptosis, cell cycle progression, protection against oxidative stress, and DNA repair [17]. In the mammals, four different FoxO members have been identified: FoxO1, FoxO3, FoxO4 and FoxO6 [18]. Akt directly phosphorylates the FoxO1, FoxO3 and FoxO4 at three regulatory serine/threonine sites and their phosphorylation causes redistribution of FoxO from the nucleus to the cytoplasm, and the resulting decrease in nuclear FoxO has been proposed as the possible mechanism for the regulation of FoxO-mediated transcription [19]. Conversely, the decreased activity of the PI3K/Akt pathway also leads to nuclear translocation of FoxO and resulting in activation or inhibition in gene transcription. One of FoxO targets, cellular-FLICE inhibitory protein (c-FLIP), has been identified as the most important regulator of TRAIL-induced apoptosis [19–21]. c-FLIP exists as

Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; HSC, hepatic stellate cell; FoxO, forkhead box O; PARP, poly(ADP-ribose) polymerase; DR4, death receptor 4; DR5, death receptor 5; c-FLIP, cellular-FLICE inhibitory protein; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA; PBS, phosphate-buffered saline.

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doi:10.1016/j.cellsig.2009.05.008

two alternatively spliced isoforms: c-FLIP$_L$ is homologous to caspase-8 that lacks amino acids for proteolytic caspase activity, and c-FLIP$_S$ consists of only two death effector domains [22]. c-FLIP is recruited to the activated death receptor via FADD, thereby either preventing the recruitment of caspase-8 to the DISC or the proximity-induced activation of caspase-8 [22]. Hence, the regulation of c-FLIP expression by FoxO is a potentially important mechanism for controlling the TRAIL signaling pathway.

In this study, we demonstrated that TRAIL-induced apoptosis in HSCs is mediated by down-regulation of FoxO-dependent c-FLIP expression followed by activation of a caspase-dependent mitochondrial apoptotic pathway. Therefore, our results suggest that TRAIL may be an effective strategy in anti-liver fibrosis therapy.

2. Materials and methods

2.1. Cell culture, materials, and antibodies

The immortalized human hepatic stellate LX-2 cells were kindly obtained from Dr. Friedman SL (Mount Sinai School of Medicine, NY) and maintained in DMEM supplemented with 2% fetal calf serum (FCS), 2 g/ml glucose, and 100 U/ml penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. In this study the following inhibitors were used: caspase inhibitor, z-VAD-fmk (R&D Systems, Minneapolis, MN) and PI3K/Akt inhibitor, LY294002 (Sigma, St. Louis, MO). These inhibitors were dissolved in dimethyl-sulfoxide (DMSO; Sigma, St. Louis, MO) and the final concentration of DMSO was 0.1%. TRAIL was purchased from R&D Systems (Minneapolis, MN). The following primary antibodies were used: Bcl-x$_L$ (S-18), Bcl-2 (N-19), Bax (N-20), PARP (H-250), Caspase-3 (H-277), cytochrome *c* (H-104), β-tubulin (D-10), and Lamin A/C (N-18) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Caspase-8 (1C12), p-AKT (D9E), FoxO1 (C29H4), p-FoxO1 (Ser256), p-FoxO1 (Thr24)/FoxO3a (Thr32), and p-FoxO3a (Ser318/321) were purchased from Cell Signaling Technology (Danvers, MA). FoxO3a was from Upstate (Billerica, MA). c-FLIP (NF-6) was purchased from Alexis Biochemicals (San Diego, CA). Anti-Bid (AF860), anti-DR4 (AF347), and anti-DR5 (AF631) antibodies were from R&D systems (Minneapolis, MN). Anti-β-actin (AC-74) antibody was purchased from Sigma (St. Louis, MO).

2.2. Annexin V analysis for apoptosis measurement

Cells were seeded into 6-well plates at a concentration of 1×10^6 cells/well and treated with increasing concentrations of TRAIL for the indicated times. The cells were then resuspended in 100 µl of staining solution containing FITC-conjugated annexin V and propidium iodide (PI) (BioBud, Seoul, Korea) in a HEPES buffer. After incubation at room temperature for 20 min, annexin V-positive cells were analyzed using a FACSVantage flow cytometer (Becton Dickinson, San Jose, CA). To determine whether caspases were involved in TRAIL-induced apoptosis, caspase inhibitor, z-VAD-fmk was used. Cells (5×10^5) were pre-incubated in the absence or presence of 20 µM z-VAD-fmk for 3 h at 37 , treated with or without TRAIL for 24 h, and then processed for annexin V binding assay as described above. To evaluate whether inhibition of PI3K/Akt pathway was involved in TRAIL sensitivity in LX-2 cells, 10 µM LY294002 was used. Cells (5×10^5) were pre-incubated in the absence or presence of LY294002 for 3 h at 37 °C, treated with or without TRAIL for the indicated times, and then processed for annexin V binding assay.

2.3. Methylene blue assay for cell survival

The methylene blue assay was performed as previously described [23]. Cells were seeded into 24-well plates at a concentration of 1×10^4 cells/well and treated with increasing concentrations of TRAIL for 24–48 h. After treatment, the cells were fixed with 100% ethanol and then stained with methylene blue. The absorbance of dye eluted from the fixed cells in each well was measured on an automated scanning photometer at a wavelength of 630 nm. Results are presented as percentage of survival, with the control set at 100%.

2.4. Flow cytometric analysis of TRAIL receptors

Cells (5×10^5 cells/well) from the culture media were spun down at 500×g, washed with phosphate-buffered saline (PBS), and resuspended in 500 µl PBS. The cells were then incubated with 5 µl goat IgG2a, or anti-DR4 and anti-DR5 polyclonal goat antibody (1:100) for 1 h, respectively. After washing with PBS, FITC-conjugated rabbit anti-goat polyclonal antibody (1:200, Sigma Chemical Co., St. Louis, MO) was added to the cell suspension and incubated for 1 h on ice, followed by washing with PBS. After additional washes in PBS, the samples were analyzed by flow cytometry using a FACSVantage flow cytometer (Becton Dickinson, San Jose, CA).

2.5. RT-PCR analysis

RNA extraction and relative RT-PCR analysis were performed as previously described [23]. Briefly, total cellular RNA was extracted from cells harvested at the indicated times using Trizol reagent (Life Technologies, Carlsbad, CA). One µg of total cellular RNA from cells was reverse transcribed using maloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) with each dNTP and 1 µg oligo dT. Amplification of 2.5 µl of these cDNA by PCR was performed using the following gene-specific primers: DR4 (forward), 5′-CTGAG CAACGCAGACTCGCTGTCCAC-3′ and (reverse), 5′-AAGGACACGGCAG AGCCTGTGCCAT-3′; DR5 (forward), 5′-CTGAAAGGCATCTGCTCAG GTG-3′ and (reverse), 5′-CAGAGTCTGCATTACCTTCTAG-3′; c-FLIP$_L$ (forward), 5′-GCTGAAGTCATCCATCAGGT-3′ and (reverse), 5′-CAT-ACTGAGATGCAAGAATT-3′; c-FLIP$_S$ (forward), 5′-GCTGAAGTCATCCAT-CAGGT-3′ and (reverse), 5′-GATCAGGACAATGGGCATAG-3′; GAPDH (forward), 5′-ACCACAGTCCATGCCATCAC-3′ and (reverse), 5′-TCCAC-CACCCTGTTGCTGTA-3′. PCR was performed in 30 µl reaction volume using the TaKaRa Taq™ Kit (Takara, Japan) with the following conditions: 30 cycles of 94 °C for 60 s (denaturation), 56 °C for 60 s (annealing), and 72 °C for 90 s (amplification), followed by a final extension at 72 °C for 12 min. The amplified fragments were separated on 1% agarose gel and visualized by ethidium bromide staining.

2.6. DNA and siRNA transfection

For over-expression of cellular Akt with point mutations at the kinase domain (Akt-KD), LX-2 cells were transfected with pcDNA3.1 as mock control or pcDNA3.1/Akt-KD using Lipofectamine (Invitrogen Life Technologies, Carlsbad, CA). For over-expression of c-FLIPs or c-FLIP$_L$, LX-2 cells were transfected with pCA as mock control, pCA/c-FLIPs, or pCA/c-FLIP$_L$ using Lipofectamine. Transfection solutions consisted of 0.2 ml/well serum-free OPTI-MEM medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 3 µl of Lipofectamine and 2 µg of plasmid DNA were prepared according to manufacturer's instructions. After transfection, the cells were incubated in antibiotic-free medium for 4 h, followed by replacement with fresh normal growth medium for a further incubation of 24–48 h before determination of the Akt, c-FLIPs, or c-FLIP$_L$ levels.

To silence FoxO gene expression, transfection of a small interfering RNA (siRNA) duplex was performed using oligofectamine reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's protocol. The FoxO siRNA which can suppress both FoxO1 and FoxO3a corresponding to nucleotides 961 to 979 of the human FoxO1 coding region (GAGCGTGCCCTACTTCAAG) was synthesized by Bioneer (South Korea). A scrambled siRNA, which targets the green fluorescent protein DNA sequence (CCACTACCTGAGCACCCAG), was used as a control.