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TWEAK/Fn14 promotes pro-inflammatory cytokine secretion in hepatic stellate cells via NF-κB/STAT3 pathways

Aixiu Wang[a,1], Feng Zhang[b,1], Hui Xu[a], Mingcui Xu[b], Yu Cao[a], Chen Wang[a], Yuanyuan Xu[a], Min Su[a], Ming Zhang[a], Yuzheng Zhuge[a,*]

[a] Department of Gastroenterology, Drum Tower Hospital, Medical School of Nanjing University, Nanjing, Jiangsu, China
[b] Department of Gastroenterology, Affiliated Drum Tower Clinical Medical School of Nanjing Medical University, Nanjing, Jiangsu, China

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ABSTRACT

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor fibroblast growth factor-inducible 14 (Fn14) have been associated with liver disease. Hepatic stellate cells (HSCs) play a critical role in the hepatic wound-healing response after liver injury, but there is little information available on the role of the TWEAK/Fn14 pathway in human HSCs. In this study, we explored the role of TWEAK/Fn14 in activated human HSCs. The LX-2 cells were treated with TWEAK, and the expression of pro-inflammatory cytokines was assayed by enzyme-linked immunosorbent assay (ELISA) and real-time PCR (RT-PCR). Western blotting and RT-PCR were performed to evaluate the expression of Fn14 after TWEAK stimulation. Total and phosphorylated of inhibitor-κB (I-κB), nuclear factor kappa B (NF-κB), Janus kinase 2 (JAK2), and signal transducers and activators of transcription 3 (STAT3) were examined by western blotting after TWEAK stimulation and small interfering RNA (siRNA) transfection. The result showed that TWEAK upregulated the expression of Fn14 and pro-inflammatory factors interleukin-8 (IL-8), interleukin-6 (IL-6), regulated upon activation normal T cell expressed and secreted (RANTES), and monocyte chemotactic protein-1 (MCP-1). In LX-2 cells, the pro-inflammatory cytokine secretion was closely related to the activation of the NF-κB and STAT3 pathways. Furthermore, our research showed that STAT3 and NF-κB could interact with each other, which resulted in a significant increase of pro-inflammatory cytokine secretion. The activation of NF-κB and STAT3 signalling-dependent pro-inflammatory cytokine expression may be responsible for such a novel principle and new therapeutic targets in chronic liver disease.

1. Introduction

Hepatic stellate cells (HSCs) play an important role in the occurrence and development of hepatic fibrosis. In the normal liver, HSCs reside in the subendothelial space of Disse and are the major cell type responsible for vitamin A storage (Bataller and Brenner, 2005). Following liver injury, quiescent HSCs change to an activated state, losing vitamin A droplets, producing extracellular matrix proteins (Seki and Brenner, 2015; Asahina, 2012). Inflammation is a key component and contributor to hepatic wound healing and fibrogenic response. Activated HSCs can secrete many pro-inflammatory and fibrogenic cytokines, such as transforming growth factor-β1 (TGF-β1), regulated upon activation normal T cell expressed and secreted (RANTES), interleukin-10 (IL-10), interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), which further accelerate the recruitment of inflammatory cells into the injured liver and exacerbate liver injury (Kisseleva and Brenner, 2007; Kisseleva and Brenner, 2006; Ruddell et al., 2009). Activated HSCs also contribute to the regulation of the liver's acute injury response by secreting interleukin-6 (IL-6) (Friedman, 2008). IL-6 and IL-8 can be induced by TNFα and lead to liver inflammation and injury (Wehbe et al., 2006; Liu et al., 2015). Besides, upon liver injury, the pro-inflammatory cytokines participate in regulating HSCs activation and promoting the survival of activated HSCs (Seki and Schwabe, 2015).

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is expressed as a type 2 transmembrane protein and functions primarily as a soluble cytokine, which have many biological functions, such as pro-inflammatory activity, anti-apoptosis and the increased of cell survival and proliferation (Bertin et al., 2013; Fick et al., 2012; Jain et al., 2009). TWEAK is widely expressed in neurons and astrocytes and acts through its signalling receptor Fn14 to trigger Fn14-associated signalling pathways (Mustafa et al., 2016). Fn14 is upregulated in tissue

* Corresponding author at: Department of Gastroenterology, Drum Tower Hospital, Medical School of Nanjing University, 321#, Zhongshan Road, Nanjing, 210008, Jiangsu, China.
E-mail address: yuzheng9111963@aliyun.com (Y. Zhuge).
[1] The authors equally contributed to this work.

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injury, including in hepatic, vascular and neuronal cells, and in tumors (Burkly et al., 2011). Meanwhile, TWEAK/Fn14 participates in different types of reactions primarily via the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (Dohi and Burkly, 2012; Tran et al., 2005). Additionally, the TWEAK/Fn14 pathway promotes glioblastoma cell survival though increasing the activation of the Akt2 serine/threonine protein kinase (Fortin et al., 2009). The pro-inflammatory effect of TWEAK has been studied in detail in many cells. For example, TWEAK promotes a variety of cells to produce pro-inflammatory cytokines involved in tissue damage response. After folic acid–induced acute kidney injury, TWEAK/Fn14 plays a pro-inflammatory role on tubular epithelial cells by activation of the NF-κB pathway (Sanz et al., 2008). TWEAK can induce an inflammatory profile on human cerebral micro-vascular endothelial cells (HCMECs) and contribute to neuroinflammation (Stephan et al., 2013).

TWEAK plays an important role in the development of liver disease. After liver injury, the TWEAK/Fn14 signalling is mainly involved in regulating liver regeneration in vivo (Wilhelm et al., 2016). In chronic liver injury and recovery, the principal function of TWEAK appears to initiate ductular expansion proliferation and hepatic progenitor cells (HPCs) proliferation via activation of NF-κB signalling, extracellular signal-regulated kinase (ERK), and c-Jun N- terminal kinase signalling (Bird et al., 2013; Kitade et al., 2016). TWEAK/Fn14 signalling is critical for mice livers to regenerate normally after partial hepatectomy (Kitade et al., 2016). Recent research found that TWEAK modulated HSCs proliferation, and TWEAK is required for liver fibrogenesis following acute hepatic injury in vivo (Wilhelm et al., 2016). However, we do not know much about the relationship between TWEAK and HSCs. In this study, we aim to focus on the effects of TWEAK on the expression of pro-inflammatory cytokines in activated human HSCs and explore the possible mechanism.

2. Material and methods

2.1. Reagents, antibodies, and siRNAs

RhTWEAK (1090-TW) was purchased from R & D Systems (Minneapolis, MN, USA). Anti-phospho-NF-κB p65 (Ser536) (3033), anti-NF-κB p65 (8242), anti-I-κBα (Ser32/36) (9246), anti-STAT3 (9132), anti-phospho-(Tyr705)-STAT3 (9145), anti-Jak2 (3230), and anti-phospho-Jak2 (Tyr1007/1008)(3771) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-I-κB (ab32518) and anti-TWEAKR (ab109365) were from Abcam (Cambridge, UK). Anti-β-actin (A5441) antibody was from Sigma-Aldrich (St. Louis, MO, USA). Anti-Laminin β-1 was from Santa Cruz Biotechnology (Dallas, TX, USA) (sc-33709). SiRNAs targeting STAT3, Fn14, and negative control siRNA were purchased from Invitrogen (Carlsbad, CA, USA), and siRNAs targeting p65 were purchased from RiboBio (Guangzhou, China).

2.2. Cell culture

The human hepatic stellate cell line LX-2 was purchased from Merck Millipore Corporation (Billerica, MA, USA). The LX-2 cells were cultured in DMEM medium supplemented with 10% or 2% foetal bovine serum (FBS) in a humidified incubator at 37 ˚C with 5% CO2. Recombinant human TWEAK was reconstituted with sterile phosphate-buffered saline (PBS) containing at least 0.1% FBS to 25 ug/ml and stored at − 20 ˚C. Immediately prior to treating the cells, TWEAK stock solution was diluted to 250 ng/ml with DMEM containing 2% FBS. When treating the LX-2 cells with TWEAK, the medium was changed to DMEM that contained 2% foetal bovine serum.

2.3. Western blotting

Total protein was extracted from LX-2 cells using standard protocols in a RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM PMSF) and a protease inhibitor cocktail (Roche). Nuclear and cytoplasmic protein was extracted from LX-2 cells using NE-PER nuclear and cytoplasmic extraction reagents (thermos 7883) according to the manufacturer's specifications. Protein concentration was determined with a BCA protein kit (Beyotime Institute of Biotechnology, China). The protein lysates were resolved by 8–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membrane blockade was accomplished with 5% nonfat milk in TBST for 2 h at room temperature. Then the membranes were incubated overnight at 4 ˚C with specific antibodies for target molecules and then incubated with appropriate secondary HRP-conjugated antibodies for 1 h at room temperature. Signals generated by enhanced chemiluminescence (Millipore) were recorded with a CCD camera (Clinx, Shanghai, China).

2.4. Quantitative real-time PCR

Total RNA was isolated from LX-2 cells by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized by using PrimeScript RT Master Mix kit (Takara Bio, Inc., Japan) according to the manufacturer's instructions. Real-time quantitative PCR assays were performed using the SYBR Premix Ex Taq II kit (Takara) by the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All data were normalised to the human β-actin. Primers used in real-time PCR experiments are shown as follows:β-actin, 5′-CTGGCACCACACCT TCTACAATG-3′(forward) and 5′-AATGTCACGCACGATTTCCCGC-3′(reverse); MCP-1, 5′-GGCTGAGACTAACCCAGAAAC-3′ (forward) and 5′-GAATGAAGGTGGCTGCTATGA-3′ (reverse); IL-8, 5′- AGCTCTGTGTG AAGGTGCAGT-3′ (forward) and 5′- AATTTCTGTGTTGGCGCAGT-3′ (reverse); IL-6 5′-ATGAA CTCCTTCTCCACAAGCGC-3′ (forward) and 5′-ATGAACTCCTTCTCCACAAGCGC-3′ (reverse); RANTES, 5′-TGCCCACATCAAGGAGTATTT-3′ (forward) and 5′-GAT GTACTCCCG AACCCATTT-3′ (reverse); Fn14 5′-AACAGAAAGGGAGCCTCACG-3′ (forward) and 5′-GTGGGGCCTAGTGTCAAGTC-3′ (reverse).

2.5. Cytokine measurement by ELISA

The ELISA kits used were as follows: IL-8(R & D Systems, Minneapolis, MN, USA D8000c), IL-6 (D6050, R & D Systems, Minneapolis, MN, USA), MCP-1 (DCP00, R & D Systems, Minneapolis, MN, USA), and RANTES (DRN00B, R & D Systems, Minneapolis, MN, USA). Cells were seeded into 6-well plates at 15 × 103 cells/well and allowed to adhere overnight. The following day, LX-2 cells were treated with TWEAK or transfected with siRNA for an indicated time, and the supernatants of the culture medium were measured using ELISA kits following the manufacturers' protocols.

2.6. siRNA transfection

Transfection was carried out using Lipofectamine RNAiMax Reagent (Invitrogen, Carlsbad, CA, USA). SiRNA sequences were as-follows: STAT3 (5′-GCCAATTGTGATGCTTCCCTGATTG-3′), Fn-14 (5′-GACAAGTGCATGGACTGCGCGTCTT-3′), p65 (5′-GGACAT ATGAGACCT-3′) and negative control (5′-TTCTCCGAACGTGTC ACGTTT'-3′) was used as the control. Briefly, 50 pmol siRNA, 7.5 μl Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA), and 0.5 ml Opti-MEM I Medium (Invitrogen, Carlsbad, CA, USA) without serum was mixed and incubation at room temperature for 20 min; afterward, 1.5 ml of cell suspension including 20 × 104 cells in complete growth medium was added to each well. This gives a final siRNA concentration of 20 nM.