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Adiponectin inhibits hepatic stellate cell activation by targeting the PTEN/AKT pathway

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ABSTRACT

Adiponectin inhibits hepatic stellate cell (HSC) activation and subsequent development of liver fibrosis via multiple mechanisms. Phosphatase and tensin homolog deletion 10 (PTEN) plays a crucial role in suppression of HSC activation, but its regulation by adiponectin is not fully understood. Here, we investigated the effect of adiponectin on PTEN in LX-2 cells, a human cell line and examined the underlying molecular mechanisms involved in adiponectin-mediated upregulation of PTEN activity during fibrosis. PTEN expression was found to be significantly reduced in the livers of mice treated with CCl_4, whereas its expression was rescued by adiponectin treatment. The DNA methylation proteins DNMT1, DNMT3A, and DNMT3B are all highly expressed in activated primary HSCs compared to quiescent HSCs, and thus represent additional regulatory targets during liver fibrogenesis. Expression of DNMT proteins was significantly induced in the presence of fibrotic stimuli; however, only DNMT3B expression was reduced in the presence of adiponectin. Adiponectin-induced suppression of DNMT3B was found to be mediated by enhanced miR-29b expression. Furthermore, PTEN expression was significantly increased by overexpression of miR-29b, whereas its expression was markedly reduced by a miR-29b inhibitor in LX-2 cells. These findings suggest that adiponectin-induced upregulation of miR-29b can suppress DNMT3B transcription in LX-2 cells, thus resulting in reduced methylation of PTEN CpG islands and ultimately suppressing the PI3K/AKT pathway. Together, these data suggest a possible new explanation for the inhibitory effect of adiponectin on HSC activation and liver fibrogenesis.

1. Introduction

Hepatic fibrosis is a reversible wound-healing process characterized by excessive deposition of extracellular matrix (ECM) proteins, especially fibrillar collagen [1,2]. Fibrosis is a consequence of chronic injury associated with alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD) as well as chronic viral diseases such as hepatitis C viral infection [3,4]. Hepatic stellate cells (HSCs) are the primary players in hepatic fibrosis development and progression [5]. When activated, HSCs transdifferentiate into α-smooth muscle actin (α-SMA) expressing myofibroblast-like cells, which are the major matrix-producing cells involved in hepatic fibrosis [1]. We and others have recently demonstrated that adiponectin, a 30 kDa adipocytokine primarily secreted by white adipose tissue (WAT), has anti-fibrotic properties both in vivo and in vitro [6–10]. Adiponectin signaling occurs via its two cognate receptors: adiponectin receptor 1 and 2 [11,12]. However, the molecular mechanism responsible for anti-fibrotic effects induced by adiponectin remained unexplored.

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual phosphatase, and its major function is to dephosphorylate phosphatidylinositol 3, 4, 5-triphosphate (PIP3) to phosphatidylinositol 4, 5-bisphosphate [13]. Decreased PTEN expression has been reported in fibrotic diseases of the lungs, heart, skin as well as liver [14–18]. Specifically, deletion of the *Pten* gene in mice results in

Abbreviations: HSCs, hepatic stellate cell; TGFβ1, Transforming growth factor β1; PTEN, Phosphatase and tensin homolog deletion 10; DNMT, DNA methyltransferase; ECM, extracellular matrix; α-SMA, α-smooth muscle actin; CCl_4, carbon tetrachloride; PTP1B, protein tyrosine phosphatase 1B; AMPK, adenosine monophosphate-activated protein kinase; SOCS-3, suppressor of cytokine signaling-3; PPARα, peroxisome proliferator-activated receptor-α; FAK, focal adhesion kinase; SIRT, Sirtuin

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excess deposition of type I collagen, while PTEN overexpression can reverse chemical-induced liver fibrosis [19]. PTEN activity and expression is controlled by several mechanisms including phosphorylation, acetylation, oxidation, ubiquitination, non-coding RNAs, and DNA methylation [20–22]. For instance, aberrant PTEN promoter methylation is demonstrated in CCl_4-induced liver fibrosis [23]. DNA methylation is mainly carried out by three highly conserved enzymes, DNMT1, DNMT3A, and DNMT3B [24].

Recent studies indicate that aberrant microRNA (miRNA) expression is correlated with liver fibrosis [25,26]. miRNAs are a class of endogenous small non-coding RNAs that are typically 18–22 nucleotides in length [25]. These miRNAs typically work as posttranscriptional regulators of gene expression by binding with a portion of the 3′-untranslated-region (3′-UTR) of target mRNAs resulting in degradation or inhibition of the target mRNAs and thus initiation of translation [27]. miRNAs play critical roles in developmental and cellular processes such as growth, differentiation, apoptosis, and oncogenesis [28].

In the setting of liver fibrosis, miR-19, miR-29, and let-7 overexpression reduce α-SMA and collagen type I expression [29–31]. Additionally, miR-33a and miR-181b inhibitors reduce collagen type I and α-SMA expression in HSCs [21,32]. Moreover, published reports demonstrate that PTEN expression is tightly regulated by miR-29b in addition to DNMT3B [33]. However, how adiponectin modulates PTEN promoter methylation and miR-29b expression has not yet been fully described. Here, we hypothesized that adiponectin may play a role as an upstream regulator of PTEN expression via either DNMTs, miR-29b, or both. Hence this study aimed to investigate the molecular mechanisms underlying adiponectin-mediated increased PTEN expression as a novel pathway in targeting liver fibrosis.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, and penicillin-streptomycin were all purchased from Invitrogen® (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, GA. Recombinant TGFβ1 was purchased from R&D systems (Minneapolis, MN). Recombinant high molecular weight (HMW) human adiponectin was purchased from Biovendor (Candler, NC). Antibodies p-AKT, AKT, PTEN, DNMT1, DNMT3A and DNMT3B were purchased from Cell Signaling (Danvers, MA). CCl_4 and antibodies against α-SMA and anti-β-actin were obtained from Sigma-Aldrich (St. Louis, MO). Collagen type I antibody was purchased from Abcam (Cambridge, MA).

2.2. Animals and CCl_4-induced liver fibrosis in mice

Eight-week -old male C57BL/6 J mice were purchased from Jackson Laboratories for animal studies (Bar Harbor Maine; Stock no. 000664). Animals were cared for in accordance to protocols approved by the Animal Care and Use Committee of Emory University. All animals were housed in a temperature-controlled environment with a 12:12 h light/dark cycle. Animals were fed ad libitum with Purina Laboratory Chow (Ralston Purina, St. Louis, MO) and water. The study included three groups of mice: (I) Control mice that received olive oil by gavage and saline injections; (II) mice gavaged with CCl_4 and injected with a recombinant deficient adenovirus vector carrying *E. coli* β-galactosidase gene (Ad-LacZ); and (III) mice gavaged with CCl_4 and administered the recombinant deficient adenoviral vector carrying the human full-length adiponectin cDNA under the regulation of the CMV promoter (Ad-Adipo). Mice weighing 22–25 g were gavaged with olive oil as control or CCl_4 (1:1 ratio CCl_4 to oil; 2 ml/kg for both groups) thrice weekly for 6 weeks. Mice were given viral particle via tail vein injection (Ad-LacZ or Ad-Adipo (1×10^9 viral particles)) every third day for two weeks following 4 weeks of CCl_4 gavage. Saline was injected via tail vein in the control group. We measured serum adiponectin concentration following Ad-Adipo injection as previously described [7] (mouse adiponectin ELISA kit; Millipore, Billerica, MA, USA). Human adiponectin (NM_004797) containing adenovirus (Ad-Adipo) and Ad-LacZ were propagated in AD293 cells (Stratagene, La Jolla, CA). Adenoviruses were concentrated and purified with an Adeno-X virus purification kit (Clonetech Laboratories, Mountain View, CA), and viral titers were determined with Adeno-XMT rapid titer kit (Clonetech Laboratories).

2.3. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Serum ALT and AST levels were determined with an ALT and AST activity assay kit as per manufacturer's instruction (Sigma-Aldrich, St. Louis, MO).

2.4. Picrosirius red staining and quantification

Formalin-fixed, paraffin-embedded liver sections (5 μm) were deparaffinized and washed with double distilled water. Deparaffinized sections were incubated for 60 min with Sirius red solution (Abcam, Cambridge, MA) followed by brief rinses with acetic acid (0.05%). Sections were dehydrated by washing with absolute alcohol. Sections were observed under a light microscope (Axioplan2; Carl Zeiss, Hallbergmoos, Germany). The collagen staining was quantified by using Image J software (NIH, Bethesda).

2.5. RNA extraction and qRT-PCR analysis

Total RNA was extracted from liver tissue or LX-2 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), and one microgram of total RNA was reverse transcribed to cDNA using the Bio-Rad's iScript™ cDNA synthesis kit according to the manufacturer's instructions. Gene expression was measured with real-time PCR using IQ™ SYBR® Green Supermix (Bio-Rad) according to standard protocol. All human and mouse primers were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following primers were used in this study: mouse PTEN (NM_008960): forward 5′-TGGATTCGACTTAGACTTGACCT-3′ and reverse 5′-GCGGTGTCATAATGTCTCTCAG-3′; mouse DNMT1 (NM_001199433) forward 5′-CCGTGGCTACGAGGAGAAC-3′ and reverse 5′-CCGTGGCTACGAGGAGAAC-3′; mouse DNMT3A (NM_007872) forward 5′-GATGAGCCTGAGTATGAGGATGG-3′ and reverse 5′-CAAG ACACAATTCGGCCTGG-3′; mouse DNMT3B (NM_001122997) forward 5′-CGTTAATGGGAACTTCAGTGACC-3′ and reverse 5′-CTGCGTGTAAT TCAGAAGGCT-3′; mouse Acta2 (NM_007392) forward 5′-CCCAGACA TCAGGGAGTAATGG-3′ and reverse 5′-TCTATCGGATACTTCAGCG TCA-3′; human PTEN (NM_000314) Forward 5′-TGGATTCGACTTAGA CTTGACCT-3′ and reverse 5′-GGTGGGTTATGGTCTTCAAAAGG-3′. To detect the expression of miR-29b (Accession MIMAT0000100) 5′-CGC TAGCACCATTTGAAATCAG-3′, RT PCR was performed using the Quanta bio microRNA assay (Beverly, MA) according to user manual. The assays were performed in triplicate using the Mastercycler® eprealplex (Eppendorf®), with internal controls (18 s) for the expression of mRNA and U6 for miR-29b. The cycle threshold (Ct) values were normalized to reference gene 18 s/U6 and fold changes in expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5.1. Isolation of rat hepatic stellate cells

Primary rat HSCs were isolated from Male Sprague-Dawley® rats as previously described [7].

2.6. Cell lines

Human LX-2 cells, an immortalized human-derived cell line was a kind gift from Dr. Scott Friedman (Mount Sinai Hospital, New York).