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Involvement of STAT3-regulated hepatic soluble factors in attenuation of stellate cell activity and liver fibrogenesis in mice

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ABSTRACT

Glycoprotein 130 (gp130)/signal transducer and activator of transcription 3 (STAT3) signaling in hepatocytes controls a variety of physiological and pathological processes including liver regeneration, apoptosis resistance and metabolism. Recent research has shed light on the importance of acute phase proteins (APPs) regulated by hepatic gp130/STAT3 in host defense through suppression of innate immune responses during systemic inflammation. To examine whether these STAT3-regulated soluble factors directly affect liver fibrogenic responses during liver injury, hepatocyte-specific STAT3 knockout (L-STAT3 KO) mice and control littermates were subjected to bile duct ligation (BDL) and examined 10 days later. In contrast to controls, L-STAT3 KO mice failed to produce APPs, such as serum amyloid A and haptoglobin, after BDL. Whereas L-STAT3 KO mice displayed similar levels of cholestasis, inflammatory cell infiltration and regeneration in the liver, they developed exacerbated liver injury and fibrosis with significant increases in expression of alpha-smooth muscle actin and type I collagen genes. *In vitro* experiments revealed that attenuated expression of APPs in primary hepatocytes isolated from L-STAT3 KO mice with IL-6 exposure, compared to wild-type hepatocytes. The cultured supernatant from IL-6-treated wild-type hepatocytes inhibited expression of alpha-smooth muscle actin and type I collagen genes in activated hepatic stellate cells (HSCs), whereas this did not occur with the supernatant from IL-6-treated knockout hepatocytes or with control medium. In conclusion, the absence of STAT3 in hepatocytes leads to exacerbation of liver fibrosis during cholestasis. Soluble factors released from hepatocytes, dependent on STAT3, collectively play a protective role in liver fibrogenesis through an inhibitory effect on activated HSCs.

1. Introduction

Cholestatic liver injury is characterized by bile flow impairment of different parts of the biliary tree, which can be caused by gallstones, autoimmunity or unknown etiology. Persistent cholestasis eventually progresses toward biliary fibrosis and cirrhosis because of bile acid-induced cholangiocyte and hepatocyte damage, leading to failure of cellular repopulation and excessive deposition of extracellular matrix (ECM) proteins. Hepatic stellate cells (HSCs) are the main ECM-producing cells in the injured liver [1]. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cells, acquiring contractile, proinflammatory and fibrogenic properties. Activated HSCs produce and deposit ECM proteins in the pericentral and periportal regions.

The signal transducer and activator of transcription 3 (STAT3) is known to be ubiquitously expressed in a wide range of tissues where it is activated by tyrosine phosphorylation in response to a variety of cytokines and growth factors (e.g. interleukin (IL)-6 family, IL-10, leptin, IL-17, IL-23, interferons and EGF). STAT3, formerly known as acute phase response factor, regulates the expression of genes involved in the acute phase response, a series of inflammatory reactions induced in response to infection and tissue

Abbreviations: ECM, extracellular matrix; HSCs, hepatic stellate cells; STAT, signal transducer and activator of transcription; IL, interleukin; gp, glycoprotein; APPs, acute phase proteins; L-STAT3 KO, hepatocyte-specific STAT3 knockout; WT, wild-type; BDL, bile duct ligation; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; BrdU, 5-bromo-2-deoxyuridine; rtPCR, reverse-transcription polymerase chain reaction; SAA, serum amyloid A; αSMA, alpha-smooth muscle actin; TGFβ, transforming growth factor beta; PDGF, platelet derived growth factor; ALT, alanine aminotransferase.

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injury [2]. The IL-6 family is one of the major cytokines involved in triggering the acute phase response and all members of the IL-6 family use glycoprotein 130 (gp130) as a receptor to induce nuclear translocation of STAT3 [3] as well as to activate the Ras/mitogen-activated protein (MAP) pathway. Since systemic deletion of STAT3 leads to embryonic lethality in mice, the significance of STAT3 in adult organs has been investigated using conditional knockout animals generated by the Cre/loxP recombination system [4]. Previous reports suggested that STAT3 signaling in hepatocytes controls a variety of physiological and pathological processes, including hepatocyte proliferation after partial hepatectomy [5], apoptosis resistance of hepatocytes during Fas-mediated liver injury [6] and regulation of hepatic gluconeogenic genes [7]. Further study showed that the soluble factors dependent on gp130/STAT3 signaling such as acute phase proteins (APPs) suppress innate immune cell overactivation and hypercytokinemia, leading to host-defense during systemic inflammation [8,9]. Very recently, research has shown that gp130/STAT3 signaling is protective against liver fibrogenesis by regulating inflammation and injury in the liver during chronic cholestasis [10,11]. However, it is not clear whether STAT3-dependent soluble factors from hepatocyte, such as APPs, affect the activation of HSCs and their collagen synthesis.

In the present study, we used conditional knockout mice, carrying hepatocyte-specific deletion of STAT3, and determined the effects dependent on the hepatocyte-specific STAT3 signaling pathway during cholestasis. We found that its signaling pathway offered protection from liver injury and fibrogenesis in a murine model of cholestatic liver injury. Moreover, STAT3-dependent soluble factors released from hepatocytes directly suppressed the activated HSCs and their collagen synthesis *in vitro*. Hepatocyte STAT3 signaling plays an important role in attenuation of liver disease by modulating liver damage and fibrogenesis through their collective effect on HSCs.

2. Materials and methods

2.1. Animals

Mice carrying a STAT3 gene with 2 loxP sequences flanking exon 22 have been described previously [12]. Hepatocyte-specific STAT3 knockout (L-STAT3 KO) mice were generated by crossing $STAT3^{fl/fl}$ mice with albumin-promoter Cre (Alb-Cre) transgenic mice [13]. Sex-matched $STAT3^{fl/fl}$ mice obtained from the same litter were used as wild-type (WT) controls. All mice were used at the age of 7–10 weeks. All animals were housed with 12-h light/dark cycles with free access to food and water under specific pathogen-free conditions and were treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

2.2. Bile duct ligation

Bile duct ligation (BDL) is a well-established murine model of cholestasis. L-STAT3 KO mice and WT littermates were subjected to BDL as previously reported [14]. Briefly, the common bile duct was ligated 3 times with 5–0 silk sutures and then cut between the ligatures. After 10 days, the animals were sacrificed for the following analyses.

2.3. Histologic analyses

The liver sections were stained with H&E or picrosirius red. The percentage of oncotic necrosis or fibrotic area was calculated using image analysis software (win-ROOF visual system; Mitani Co., Tokyo, Japan). To assess intrahepatic macrophage accumulation, liver sections were stained with F4/80 using an anti-F4/80 rat monoclonal antibody (Abcam, Cambridge, MA). To detect apoptotic cells, the liver sections were also subjected to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining as previously reported [15]. To assess regenerative status, nuclear 5-bromo-2-deoxyuridine (BrdU) incorporation was evaluated as previously described [16].

2.4. Isolation and culture of murine hepatic stellate cells

HSCs were isolated from C57BL/6J mice by 2-step collagenase-pronase perfusion of mouse liver as previously described [16]. Activated HSCs after a few passages were cultured with the supernatant from primary hapatocyte or recombinant Apo-SAA (PEPROTECH, Rocky Hill, NJ).

2.5. Primary culture of hepatocytes

Hepatocytes were isolated from the liver of L-STAT3 KO mice and WT mice by 2-step collagenase-pronase perfusion of mouse liver as previously described [8]. Isolated hepatocytes were stimulated with 20 ng/ml recombinant mouse IL-6 (R&D Systems, Minneapolis, MN). The cells or the supernatant were harvested after 24 h.

2.6. Western blot analysis

Western blotting was performed as previously described [16].For immunodetection, the following antibodies were used: phospho-STAT3 (Tyr705) antibody, anti-STAT3 antibody (Cell Signaling Technology, Danvers, MA) and anti-β-actin antibody (Sigma–Aldrich, St. Louis, MO).

2.7. Real-time reverse-transcription polymerase chain reaction

Total RNA extracted from the liver tissue and HSCs were reverse transcribed and subjected to real-time reverse-transcription polymerase chain reaction (rtPCR) as previously described [15]. mRNA expression of the specific genes was quantified using TaqMan Gene Expression Assays (Applied Biosystems Inc., Foster City, CA). Assay IDs of the specific genes are provided in Supplementary Table 1. Transcript levels are presented as fold induction.

2.8. Enzyme-linked immunosorbent assay

The levels of serum amyloid A (SAA) and haptoglobin in serum and cultured supernatant were measured using SAA ELISA kit (Invitrogen, Camarillo, CA) and Mouse Haptoglobin ELISA kit (Immunology Consultants Laboratory, Newberg, OR), according to the manufacturer's protocol.

2.9. Statistical analysis

Data are presented as median and interquartile range or mean ± standard deviation, compared using the Mann–Whitney U test and unpaired *t*-test, respectively. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Lack of acute phase response in L-STAT3 KO mice after BDL

L-STAT3 KO mice were produced by crossing floxed STAT3 mice and Alb-Cre transgenic mice which express Cre recombinase gene under regulation of the albumin gene promoter. To determine the