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Demonstration of cooperative contribution of MET- and EGFR-mediated STAT3 phosphorylation to liver regeneration by exogenous suppressor of cytokine signalings ☆,☆☆

Ekihiro Seki[1,2,3], Yuichi Kondo[1,2], Yuji Iimuro[1], Tetsuji Naka[4], Gakuhei Son[1], Tadamitsu Kishimoto[5], Jiro Fujimoto[1], Hiroko Tsutsui[6], Kenji Nakanishi[2,*]

[1]*Department of Surgery, Hyogo College of Medicine, Nishinomiya, Japan*
[2]*Department of Immunology and Medical Zoology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, Japan*
[3]*Department of Medicine, University of California, San Diego, School of Medicine, CA 92093-0702, USA*
[4]*Department of Molecular Medicine, Osaka University Graduate School, Osaka, Japan*
[5]*Osaka University Graduate School, Osaka, Japan*
[6]*Department of Microbiology, Hyogo College of Medicine, Nishinomiya, Japan*

***Background/Aims*: As conditional knockout mice for *stat3* are impaired in liver regeneration after partial hepatectomy while those for *gp130* have defects in early STAT3 phosphorylation but have normal DNA synthesis, late STAT3 phosphorylation induced independently of gp130 seems to be essential for liver regeneration. Since HGF and EGF can activate STAT3 via gp130-independent MET and EGFR, respectively, we assumed that these factors account for STAT3-dependent liver regeneration. Here, we investigated this hypothesis by introducing suppressor of cytokine signaling (SOCS)-1 and SOCS3, potent negative regulators of STAT3 signaling, selectively in hepatocytes.**

***Methods*: We generated recombinant adenoviruses expressing *socs1* and *socs3*.**

***Results*: Hepatocytes infected with *socs1*-virus lacked STAT3 phosphorylation in response to IL-6 and HGF, while cells infected with *socs3*-virus lacked the response to all of IL-6, HGF and EGF, indicating that those SOCS proteins differently regulate EGFR signaling. Mice infected with *socs3*-virus exhibited severe and persistent impairment while those with *socs1*-virus showed only delayed regeneration, indicating requirement of both MET and EGFR signalings.**

***Conclusions*: These results clearly demonstrated that MET- and EGFR-mediated STAT3 signalings cooperatively contribute to liver regeneration and could provide new insights into tissue homeostasis.**

***Keywords*: SOCS1; SOCS3; Adenovirus vector; ERK1/2; Hepatocyte**

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* Corresponding author. Tel.: +81 798 45 6574; fax: +81 798 40 5423.
E-mail address: nakaken@hyo-med.ac.jp (K. Nakanishi).

Abbreviations: gp, glycoprotein; PHx, partial hepatectomy; SOCS, suppressor of cytokine signaling; JAK, Janus-activated kinase; moi, multiplicity of infection; Ab, antibody.

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1. Introduction

Orchestrated activation of a signal network connecting cytokines, cell cycle transition, and growth factors is essential for homeostatic liver regeneration after liver resection or injury [1–3]. Immediately after liver resection or massive liver damage, nonparenchymal cells produce IL-6-related cytokines [1–6], and quiescent liver parenchymal cells start to replicate in response to various growth factors, such as HGF and EGF, via activating MET and EGFR, respectively [1–7]. These events converge into homeostatic liver regeneration. Conditional knockout mice for gene encoding glycoprotein (gp) 130, the common signaling component of receptors for IL-6-related cytokines, have severe defects in the early activation of STAT3 but are intact for DNA synthesis after partial hepatectomy (PHx) [8], indicating that gp130-mediated early STAT3 phosphorylation is dispensable for DNA synthesis. In contrast, conditional knockout mice for *stat3* have severe impairment in DNA synthesis [9], demonstrating the importance of STAT3 in the replication of hepatocytes. These observations allow us to assume that the early and late STAT3 phosphorylations in hepatocytes are induced by the gp130-dependent and -independent signalings, respectively, and that factors other than IL-6-related cytokines are essential for liver regeneration by inducing late STAT3 phosphorylation independently of gp130.

Suppressor of cytokine signaling (SOCS) is a family of intracellular molecules that negatively regulates various signal pathways [10–12]. SOCS1 and SOCS3 play their own roles in different biological situations by negatively regulating partially overlapped signal pathways [13–15]. SOCS1 binds to Janus-activated kinase (JAK) family members to negatively regulate various cytokine signalings, such as IL-6-induced STAT3 phosphorylation [13–16], while SOCS3 strongly interacts with activated cytokine receptors, such as gp130, to negatively regulate STAT3 phosphorylation [14–18]. Because HGF and EGF induced post PHx activate STAT3 via gp130-independent receptors, MET and EGFR, respectively [19–21], we assumed that MET- and EGFR-mediated signalings might account for the gp130-independent STAT3 phosphorylation. Here, we assessed this assumption by hepatocyte-selective introduction of SOCS1 and SOCS3. Infection of hepatocytes with recombinant adenoviruses expressing *socs1* only inhibited HGF-induced STAT3 phosphorylation, while those infected with recombinant virus expressing *socs3* could inhibit both EGF- and HGF-induced STAT3 phosphorylation. After PHx mice with exogenous SOCS3 exhibited persistently abolished DNA synthesis and liver mass restoration, those with ectopic SOCS1 expression showed only delay of these responses. These results indicated that absence of both MET- and EGFR-mediated STAT3 signalings almost completely abrogated liver regeneration and that absence of the MET-mediated STAT3 phosphorylation alone induces only slow-onset liver regeneration. These results demonstrated that the MET- and EGFR-mediated signalings cooperatively evoke liver regeneration process and shed light on the molecular mechanism for tissue homeostasis.

2. Experimental procedures

2.1. Mice

C57BL/6 mice were purchased from Clea Japan (Osaka, Japan). Six- to eight-week-old male mice were used. All mice were maintained under specific pathogen-free conditions, and received humane care as outlined in the Guide for the Care and Use of Experimental Animals in Hyogo College of Medicine.

2.2. Construction of recombinant adenovirus

Recombinant adenoviruses expressing genes encoding GFP (AxCA-GFP), murine SOCS1 (AxCA-SOCS1) and murine SOCS3 (AxCA-SOCS3), which contain a CAG promoter (chicken β-actin promoter with cytomegalovirus enhancer), were grown in 293 cells and prepared as shown previously [22]. AxCA-GFP was gifted from Dr. K. Ikeda at Osaka City University (Osaka, Japan). Viral titers were determined by optical densitometry (particles/ml) and by plaque-forming assay on HEK293 cells [22].

2.3. Adenovirus infection and operation procedure

Recombinant virus solution was injected (5×10^8 pfu in 0.2 ml) into mice via a tail vein (Supplementary Fig. 1A). At 48 h after infection, mice underwent 70% hepatectomy [4]. At the indicated time points after operation, serum and liver specimens were sampled. In some experiments, we measured the liver weight and calculated liver/body weight ratio [4]. Five to seven mice were used for each experimental group.

2.4. Hepatocyte preparation

Cells (5×10^5/ml) of hepatocytes were incubated with various adenoviruses at 50 of multiplicity of infection (moi) for 3 h [2]. The hepatocytes were incubated with recombinant murine IL-6 (20 ng/ml), EGF (20 ng/ml) or HGF (20 ng/ml) (R&D systems, Minneapolis, MN) for 15 min for detection of STAT3 phosphorylation. The culture medium generally used in this study is William E medium containing 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine [23].

2.5. [^{3}H]-TdR incorporation assay

The hepatocytes were incubated with EGF (20 ng/ml) and HGF (20 ng/ml) for 48 h, during last 16 h with [^{3}H]-TdR, and incorporated [^{3}H]-TdR was counted [24]. Stimulation index was calculated as follows: Stimulation index = [^{3}H]-TdR incorporated in sample cells (cpm)/[^{3}H]-TdR incorporated in control cells (cpm).

2.6. Western blot analysis

Protein electrophoresis, protein transfer, and detection by Western blot were performed [4]. The primary Abs used were: anti-phosphotyrosine STAT3 Ab, anti-STAT3 Ab, anti-phospho ERK and anti-ERK from Cell Signaling (Beverly, MA), anti-cyclin D1 Ab (sc-717) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-SOCS1 Ab and anti-