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Bone marrow-derived c-jun N-terminal kinase-1 (JNK1) mediates liver regeneration

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ABSTRACT

Liver regeneration is controlled by a complex network of signaling molecules, and a prominent role for c-jun N-terminal kinase has been suggested during this process. In the present study, we aimed to characterize and define the cell-type-specific contribution of JNK1 activation during liver regeneration. We used hepatocyte-specific JNK1 knockout mice ($JNK1^{\Delta hepa}$) using the *cre/lox-P* system. We performed partial hepatectomy (PH) in WT, $JNK1^{\Delta hepa}$ and $JNK1^{-/-}$ animals and investigated time-points up to 72 h after PH. Additionally, bone marrow transplantation experiments were conducted in order to identify the contribution of hematopoietic cell-derived JNK1 activation for liver regeneration. Our results show that liver regeneration was significantly impaired in $JNK1^{-/-}$ compared to $JNK1^{\Delta hepa}$ and WT animals. These data were evidenced by lower BrdU incorporation and decreased cell cycle markers such as Cyclin A, Cyclin D, E2F1 and PCNA 48 h after PH in $JNK1^{-/-}$ compared with $JNK1^{\Delta hepa}$ and WT livers. In $JNK1^{-/-}$ mice, our findings were associated with a reduced acute phase response as evidenced by a lower activation of the IL-6/STAT3/SAA-1 cascade. Additionally, $CD11b^{+}Ly6G^{+}$-cells were decreased in $JNK1^{-/-}$ compared with $JNK1^{\Delta hepa}$ and WT animals after PH. The transplantation of bone marrow-derived $JNK1^{-/-}$ into WT recipients caused significant reduction in liver regeneration. Interestingly, the transplantation of $JNK1^{-/-}$ into mice lacking JNK1 in hepatocytes only partially delayed liver regeneration. In summary, we provide evidence that (1) JNK1 in hematopoietic cells is crucial for liver regeneration, and (2) a synergistic function between JNK1 in hepatocytes and hematopoietic-derived cells is involved in the hepatic regenerative response.

1. Introduction

The regenerative capacity is dormant in a healthy liver. However, surgical resection, drug intoxication or viral infections trigger a complex process with the ultimate aim to restore liver mass and cell function. In rodents, the preferred experimental model to study liver regeneration is two-thirds partial hepatectomy (PH). This model is highly reproducible, consists of a controlled sequence of events and has direct clinical implications [1]. In fact, in humans, liver resection is frequently performed during surgery of liver metastases or repair after liver trauma [2].

Partial resection of the liver triggers activation of the c-Jun N-terminal kinases (JNK), members of the mitogen-activated protein kinase (MAPK) family [3,4]. The TNF-mediated activation of JNK within 1 h after PH leads to the activation of AP-1, which, in turn, promotes the expression of Cyclin D, initiating G0-G1 transition and, thus hepatocyte proliferation [3–7]. In contrast to the central role of JNK in promoting liver regeneration, persistent JNK activation attenuates liver regeneration, clearly suggesting that the magnitude and duration of JNK activation is a critical regulator of liver homeostasis.[3]

Cell-type-specific ablation using the *cre/lox-P* system has helped to improve the understanding of the specific functions of JNK in distinct tissues and the interaction between the liver and other organs [7,8]. This is of clinical relevance since targeted pharmacological modulation of JNK function might be beneficial for patients with malignant liver tumors.

Abbreviations: ANOVA, analysis of variance; ALT, alanine aminotransferase; AP-1, activator protein 1; APR, acute phase response; AST, aspartate aminotransferase; BM, bone marrow; BMT, bone marrow transplantation; BrdU, 5-bromo-2′-deoxyuridine; KC, Kupffer cells; FXR, farnesoid X receptor; GR, glucocorticoid receptor; H&E, hematoxylin and eosin; HRP, horseradish peroxidase; IL-6, interleukin-6; $JNK1^{\Delta hepa}$, hepatocyte-specific deletion of JNK1; LXR, liver X receptor; MAPK, members of the mitogen-activated protein kinase family; PH, partial hepatectomy; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; PPARγ1, peroxisome proliferator-activated receptor γ1; qPCR, quantitative real-time PCR analysis; RXR and RARγ, retinoic acid receptors; SAA-1, serum amyloid-1; STAT3, signal transducer and activator of transcription-3; TNFα, tumor necrosis factor-α; WT, wild type

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Therefore, in the present study, we aimed to characterize and define the cell-type-specific contribution of JNK1 activation during liver regeneration using the two-thirds PH model. We addressed this task by combining global and hepatocyte-specific JNK1 knockout animals. Additionally, we performed bone marrow transplantation (BMT) to finally establish that whereas JNK1 in hematopoietic cells is crucial for liver regeneration, JNK1 in hepatocytes alone is irrelevant. However, a synergistic function between JNK1 in hematopoietic cells and hepatocytes is necessary for restoring liver mass.

2. Methods

2.1. Housing, generation of knockout mice and bone marrow transplantation

Animals were maintained in the animal facility of the University Hospital RWTH Aachen according to the German legal requirements. Wild-type (WT), constitutive JNK1$^{-/-}$ animals as well as hepatocyte-specific knockout mice JNK1$^{\Delta hepa}$ were bred in a defined C57BL/6 background. Genotypes were confirmed via PCR for the respective genes. Genotypes were confirmed by PCR analysis of genomic DNA. We transferred bone marrow from JNK$^{\Delta hepa}$ and JNK1$^{-/-}$ mice into 6- to 7-week-old JNK1$^{-/-}$ and WT isogeneic recipients ($n = 6$–7 mice per group) after ablative γ-irradiation, as described previously [7,9].

2.2. Partial hepatectomy

Two thirds partial hepatectomy (PH) as described previously[1] was performed in male mice in the age of 7 to 8 weeks. Animals were euthanized after varying periods of time. Control samples were taken from explanted livers of the respective strains at the time of PH.

2.3. Immunoblot analysis

Protein extracts were electrophoresed and then blotted following standard procedures. Blots were incubated with primary antibodies anti-Ccn D1, anti-Ccn A2 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-pSTAT3 from Cell Signaling (Beverly, MA). As secondary antibodies, anti-rabbit-HRP (Cell Signaling) and anti-mouse-HRP (Santa Cruz) were used. GAPDH from AbD SeroTec (Düsseldorf, Germany) was used as loading control.

2.4. Quantitative real-time PCR

Total RNA was purified from liver tissue using Trizol reagent (Invitrogen, Karlsruhe, Germany). Total RNA (1 μl) was used to synthesize cDNA using SuperScript first-Stand Synthesis System (Invitrogen, Karlsruhe, Germany) and was resuspended in 100 μl of H_2O. Quantitative real-time PCR was performed using SYBR Green Reagent (Invitrogen, Karlsruhe, Germany) in 7300 real-time PCR system (Applied Biosystem, Darmstadt, Germany). GAPDH expression was used to normalize gene expression in a given sample which is represented as fold induction *versus* WT basal expression in control samples. Primers can be provided upon request.

2.5. Histological, immunofluorescence analysis

Livers from mice were harvested and, following fixation with 4% PFA, were embedded in paraffin for further histological evaluation. H&E staining was performed on paraffin-embedded liver sections. Samples were reviewed by a blinded pathologist who analyzed the degree of liver injury. For immunofluorescence staining, liver cryosections of 5 μm were stained with CD11b (Santa Cruz, Heidelberg, Germany), Oil Red O or anti-BrdU antibody as previously described [1]. Examination was performed using a Leica automatic stainer (Wetzlar, Germany). Mounting solution containing 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was used to counterstain the nuclei of hepatocytes and incubated with fluorescence labeled secondary antibodies (AlexaFluor 488 and 564, Invitrogen, Carlsbad, CA, USA).

2.6. Flow cytometry analysis

Immune cells from whole liver were isolated and stained with fluorochrome-conjugated antibodies (CD4-PE, CD3-APC, NK1.1/PE/Cy7, CD8-FITC, CD45-APC-Cy7, CD11b-PE, CD11c-APC, Ly6G-FITC, CD19-PERCP/Cy5.5, Gr1.1 PERCP/Cy5.5 and F4/80-Bio/Streptomycin/Cy7) (BD Biosciences, Heidelberg, Germany). All samples were acquired by flow cytometry (FACS Canto II; BD Biosciences) and analyzed using the Flowjo® software.

2.7. General procedures

Serum ALT, AST, cholesterol, glucose and triglycerides were processed by the Central Laboratory Facility at University Hospital RWTH Aachen. Blood counts were performed by the Central Laboratory Facility for laboratory animals at University Hospital RWTH Aachen.

2.8. Statistical analysis

Data are expressed as the mean ± SD. Statistical significance was determined via one- and two-way analysis of variance (ANOVA) followed by a Bonferroni test. Statistics regarding the BrdU stainings were determined via unpaired *T*-test.

3. Results

3.1. Liver regeneration after partial hepatectomy is independent of hepatocyte-derived JNK1

Following liver resection, hepatocyte proliferation starts in areas of the lobules surrounding the portal triads and then proceeds to the pericentral areas 36 to 48 after PH [10]. Thus, we studied DNA synthesis 48 h after surgery and first examined bromodeoxyuridine incorporation (BrdU). As expected, BrdU incorporation was significantly decreased in JNK1$^{-/-}$ compared with WT and JNK1$^{\Delta hepa}$ mice, 48 h after PH (Fig. 1a). This reduction was consistent with the lower number of mitotic figures we observed at this time point in the H&E staining (Supplementary Fig. 1). Moreover, we analyzed cyclin A protein expression (Fig. 1b) and also included other cell cycle markers such as Cyclin D, E2F1 and PCNA, which showed that hepatic regeneration is strongly reduced in JNK1$^{-/-}$ compared with JNK1$^{\Delta hepa}$ and WT mice (Fig. 1c–e).

To better characterize the differences between JNK1$^{\Delta hepa}$ and JNK1$^{-/-}$ livers, we examined in more detail the course of liver regeneration up to 72 h after PH (Suppl. Fig. 1). We observed that JNK1$^{-/-}$ and JNK1$^{\Delta hepa}$ knockout mice displayed lower BrdU incorporation compared with WT mice 72 h after PH (Fig. 2a + b). Furthermore, liver injury as evidenced by serum transaminases was less prominent in JNK1$^{-/-}$ compared with JNK1$^{\Delta hepa}$ and WT mice (Fig. 2c). However, we found no differences in liver *versus* body weight (LW/BW) ratio at this time point (Fig. 2d).

3.2. IL-6/SAA-1/STAT-3 activation is decreased in JNK1$^{-/-}$ mice after partial hepatectomy

Partial hepatectomy (PH) causes JNK activation and a robust regenerative response to restore liver mass [5]. First, Hui [11] and later Das [8] have clearly demonstrated that JNK1$^{-/-}$ knockout mice show impaired liver regeneration. IL-6-dependent STAT-3 activation plays a physiological role in promoting hepatic survival by stimulating proliferation of hepatocytes in a paracrine manner, specifically via inducing the acute phase response [12–14]. Thus, we examined whether the defect in liver regeneration in JNK1$^{-/-}$ mice was associated with changes in STAT-3/IL-6 activation (Fig. 3).