Research Article

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Haematopoietic cell-derived Jnk1 is crucial for chronic inflammation and carcinogenesis in an experimental model of liver injury

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Background & Aims: Chronic liver injury triggers complications such as liver fibrosis and hepatocellular carcinoma (HCC), which are associated with alterations in distinct signalling pathways. Of particular interest is the interaction between mechanisms controlled by IKKγ/NEMO, the regulatory IKK subunit, and Jnk activation for directing cell death and survival. In the present study, we aimed to define the relevance of Jnk in hepatocyte-specific NEMO knockout mice ($NEMO^{\Delta hepa}$), a genetic model of chronic inflammatory liver injury.

Methods: We generated $Jnk1^{-/-}/NEMO^{\Delta hepa}$ and $Jnk2^{-/-}/NEMO^{\Delta hepa}$ mice by crossing $NEMO^{\Delta hepa}$ mice with Jnk1 and Jnk2 global deficient animals, respectively, and examined the progression of chronic liver disease. Moreover, we investigated the expression of Jnk during acute liver injury, evaluated the role of Jnk1 in bone marrow-derived cells, and analysed the expression of NEMO and p-JNK in human diseased-livers.

Results: Deletion of Jnk1 significantly aggravated the progression of liver disease, exacerbating apoptosis, compensatory proliferation and carcinogenesis in $NEMO^{\Delta hepa}$ mice. Conversely, $Jnk2^{-/-}/NEMO^{\Delta hepa}$ displayed hepatic inflammation. By using bone marrow transfer, we observed that Jnk1 in haematopoietic cells had an impact on the progression of chronic liver disease in $NEMO^{\Delta hepa}$ livers. These findings are of clinical relevance since NEMO expression was downregulated in hepatocytes of patients with HCC whereas NEMO and p-JNK were expressed in a large amount of infiltrating cells.

Conclusions: A synergistic function of Jnk1 in haematopoietic cells and hepatocytes might be relevant for the development of chronic liver injury. These results elucidate the complex function of Jnk in chronic inflammatory liver disease.

Keywords: Jnk1; Jnk2; Necrosis; Apoptosis; IKKγ/NEMO; HCC.
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Abbreviations: HCC, hepatocellular carcinoma; $NEMO^{\Delta hepa}$, NEMO knockout mice; ConA, concanavalin A; AP, alkaline phosphatase; GS, glutamine synthetase; ALT, alanine aminotransferase; DEN, diethylnitrosamine.

Introduction

The consequences of chronic liver disease are liver fibrosis and hepatocellular carcinoma (HCC), which lead to organ failure and death. HCC is the fifth most common solid cancer, which affects one million people per year representing the third cause of mortality by cancer worldwide [1,2]. This high death rate occurs mainly because HCC frequently develops in a fibrotic liver, limiting treatment options. However, the pathogenesis of HCC remains associated with alterations in multiple cellular signalling pathways, limiting the clinical benefits of new treatment options. Therefore, the focus of future targeted therapy is to define novel pathways especially those involved in the promotion of the survival and/or death of transformed hepatocytes and the control of their progression into HCC.

In the last decade, NF-κB has emerged as an essential transcription factor for hepatocyte physiology. NF-κB activation is controlled by the IKK complex since it regulates IκB phosphorylation, which consequently gets degraded and releases NF-κB into the nucleus for the control of gene transcription. Knockout mice for the IKK complex members IKKβ or NEMO die *in utero* as a result of TNF-mediated hepatocyte cell death [3–8]. Additionally, mice with hepatocyte-specific ablation of IKKα

($IKK\alpha^{\Delta hepa}$), IKKβ ($IKK\beta^{\Delta hepa}$) or IKKγ/NEMO ($NEMO^{\Delta hepa}$) show increased sensitivity to TNF-induced liver injury [9,10]. Interestingly, $NEMO^{\Delta hepa}$ mice develop a spontaneous phenotype, characterized by increased hepatocyte apoptosis and compensatory proliferation, leading to chronic hepatitis, which triggers fibrosis and finally HCC, mimicking NASH and HCC development in humans. Moreover, chronic hepatitis in $NEMO^{\Delta hepa}$ mice is associated with steatosis and liver progenitor cell activation and these mice are more sensitive to Concanavalin A (ConA) or LPS-mediated hepatotoxicity [10].

Because loss of IKKγ/NEMO ($NEMO^{\Delta hepa}$) results in prolonged Jnk activation and the current discrepancies concerning the distinct roles of Jnk1 and Jnk2 in hepatic injury remain unsolved, we hypothesized that Jnk deletion in $NEMO^{\Delta hepa}$ mice would be beneficial for the progression of liver disease. Surprisingly, we found that $Jnk1^{-/-}/NEMO^{\Delta hepa}$ livers displayed exacerbated apoptosis, compensatory proliferation and carcinogenesis, whereas loss of Jnk2 led to a chronic inflammatory phenotype. Specifically, we found that Jnk1 in haematopoietic cells is protective against the progression of liver injury in $NEMO^{\Delta hepa}$ mice. Thus, the understanding of the cell-specific role of Jnk during chronic liver inflammation will be important in establishing more effective therapies.

Materials and methods

Housing and establishment of the knockout mice and human liver samples

Animals were maintained in the animal facility of the University Hospital RWTH Aachen according to the German legal requirements. We generated mice carrying the loxP-site-flanked NEMO gene under the control of the Alb-*cre* promoter as previously described [11]. From $NEMO^{\Delta hepa}$ mice, we generated double knockout animals by crossing $NEMO^{\Delta hepa}$ with either constitutive Jnk1- or Jnk2-deficient mice defined in a C57BL/6 background and purchased from the Jackson Laboratory (Bar Harbor, ME). Progression of liver disease was monitored in male mice ranging from 8 to 52 weeks of age (n = 20–34).

Liver paraffin sections from a total of six patients with confirmed diagnosis of mild fibrosis (n = 2; Patient # 1 + 2) and advanced liver cirrhosis (n = 2; Patient # 3 + 4), and hepatocellular carcinoma (HCC) (n = 2; Patient # 5 + 6) were obtained from the Department of Pathology of the University Hospital RWTH Aachen, Germany. Patients' clinico-pathologic characteristics were analysed, summarized and represented in Table 1.

Bone marrow transplantation

Bone marrow from WT, $Jnk1^{-/-}$ and $Jnk2^{-/-}$ donors (n = 4–5 mice per group) was transplanted into 4–6 week-old WT, $NEMO^{\Delta hepa}$, $Jnk1^{-/-}/NEMO^{\Delta hepa}$ and $Jnk2^{-/-}/NEMO^{\Delta hepa}$ recipients (isogenic on a C57BL/6J background) after ablative γ-irradiation, as described previously [12]. Mice were sacrificed 1 year later.

Liver microarray

RNA was isolated from 8 weeks-old male WT, $NEMO^{\Delta hepa}$, $Jnk1^{-/-}/NEMO^{\Delta hepa}$ and $Jnk2^{-/-}/NEMO^{\Delta hepa}$ mice using TRIzol reagent and purified with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions Samples were analysed by the Division of Nutrition, Metabolism and Genomics at Wageningen University as previously described [13]. We calculated average gene expression of the control mice and divided the log2 values of the individual mice by the mean of the controls. Log ratios were saved in a txt file and analysed with the Multiple Experiment Viewer. First we selected the top up- and downregulated genes in the dataset based on interquartile range and then selected genes associated with parameters related to hepatic fibrosis, liver metabolism, hepatic infiltration and inflammation and carcinogenesis. These genes were then hierarchically clustered and statistically analysed by Pearson's correlation (n = 3 livers per group).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was determined via a one-way analysis of variance (ANOVA), followed by a Newman-Keuls test.

Transcript profiling

Affymetrix microarray data have been deposited with the NCBI Gene Expression Omnibus under accession number GSE59601 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59601).

Results

NEMO expression in human and mouse chronic liver injury

To better define the relevance of both IKKγ/NEMO and Jnk during liver inflammation and carcinogenesis, we examined their expression in paraffin samples of patients with different stages of chronic liver disease ranging from mild or advanced liver cirrhosis to HCC (Fig. 1A, Table 1). Although reduced expression of NEMO was evident in advanced cirrhosis, we observed loss of IKKγ/NEMO expression in human HCC samples (Fig. 1A). Interestingly, NEMO expression was also found in infiltrating immune cells, co-localizing with p-JNK (Fig. 1A). JNK activation was typically detected in fibrotic areas and infiltrating cells (Fig. 1A). The combination of high p-JNK activity and lack of IKKγ/NEMO expression nicely reflected the situation normally found in

Table 1. Origin and clinical features of liver samples.

Parameter/sex	P1 fibrosis	P2 fibrosis	P3 cirrhosis	P4 cirrhosis	P5 HCC	P6 HCC	Reference values
	F	M	M	M	F	M	
Age	60	73	54	55	61	62	-
Leukocytes (g/L)	8.2	3.2	3.2	9.4	7.4	8.7	4.3-10.0
Hematocrit (L/L)	0.40	0.43	0.31	0.28	0.36	0.51	0.40-0.54
Platelets (g/L)	91	92	43	28	227	285	150-350
INR (ratio)	1.09	1.04	1.38	1.70	1.10	1.06	0.9-1.1
Bilirubin (mg/dl)	2.9	0.7	3.7	40.1	0.4	0.5	0.2-1.0
AST (37 C) (U/L)	45	46	37	62	23	103	10-50
ALT (37 C) (U/L)	31	35	39	47	11	63	10-50
γ-GT (U/L)	76	51	172	24	12	614	10-71
AP (U/L)	134	104	89	92	56	197	40-129

P, patient; F, female; M, male; INR, international normalized ratio; AST, aspartate transaminase; ALT, alanine transaminase; γ-GT, gamma-glutamyl transferase; AP, alkaline phosphatase.