NF-κB Essential Modifier Is Required for Hepatocyte Proliferation and the Oval Cell Reaction After Partial Hepatectomy in Mice

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BACKGROUND & AIMS: The transcription factor nuclear factor κB (NF-κB) is activated by the IκB kinase complex. The regulatory subunit of this complex, NF-κB essential modifier (NEMO or IKBKG), is a tumor suppressor. Hepatocyte-specific deletion of NEMO induces chronic liver inflammation that leads to apoptosis, oxidative stress, development of nonalcoholic steatohepatitis, and hepatocarcinogenesis. **METHODS:** We performed partial hepatectomies in mice with hepatocyte-specific disruption of NEMO ($Nemo^{\Delta hepa}$). Some mice were fed a diet that contained the antioxidant butylated hydroxyanisole (BHA), and others were given daily intraperitoneal injections of the oxidant phenetyl isothiocyanate (PEITC). **RESULTS:** $Nemo^{\Delta hepa}$ mice had impaired liver regeneration after partial hepatectomy and 50% mortality, indicating that NEMO is required for the regenerative response. Liver cells of the mice had a strong oxidative stress response; these cells down-regulated the NF-κB–dependent antioxidant response and reduced levels of proteins that repair DNA double-strand breaks. However, the impairments to hepatocyte proliferation were compensated by a response of oval cells in $Nemo^{\Delta hepa}$ mice. Oval cells expressed low levels of albumin and thereby expressed normal levels of NEMO. Repopulation of the liver with oval cells that expressed NEMO reversed liver damage in $Nemo^{\Delta hepa}$ mice. Interestingly, these mice still developed hepatocellular carcinomas 6 months after partial hepatectomy, whereas $Nemo^{\Delta hepa}$ mice fed the BHA diet were protected from carcinogenesis. **CONCLUSIONS: In livers of mice, expression of NEMO and activation of NF-κB are required for hepatocyte proliferation and liver regeneration. These mechanisms require control of oxidative stress and DNA integrity.**

Keywords: PEITC; Liver Disease; Mouse Model; Tissue Regeneration.

Nuclear factor κB (NF-κB) is essential to preserve liver homeostasis and is a main regulator of immune and inflammatory responses. NF-κB controls the fine-tuning between life and death because it has both death-promoting and antiapoptotic functions, depending on the cellular source, damaging context, and stimulus. NF-κB activation relies on the IκB kinase (IKK) complex, which consists of the 2 kinases IKK1/IKKα and IKK2/IKKβ and the regulatory subunit IKKγ/NEMO. NEMO in hepatocytes acts as a tumor repressor molecule that protects the liver against chronic inflammation, progression of nonalcoholic steatohepatitis, and hepatocarcinogenesis.[1,2]

Oxidative stress is involved in several cellular responses, such as proliferation, cell growth, inflammation, apoptosis, necrosis, and autophagy.[3,4] Mitochondria are the main source of reactive oxygen species (ROS), which promote oxidative stress when their presence exceeds the cellular antioxidant defense capacity. NF-κB is essential to control cell death because it tightly regulates ROS production by promoting transcription of antioxidant and antiapoptotic molecules such as Mcl-1, Bcl-Xl, A-20, c-Flip, SOD2, and ferritin heavy chain (FHC).[5–7] In addition to cell death, ROS promote DNA double-strand breaks (DSBs) when oxidative stress exceeds the DNA repairing capacity. Phosphorylation of ATM and histone H2AX are well-accepted markers of oxidative DNA damage.[8] ATM and H2AX are phosphorylated during tumorigenesis, because DSBs contribute to genome instability and thus to development of hepatocellular carcinoma (HCC).[9] Moreover, ROS is directly linked to cell proliferation because hepatocyte proliferation is associated with inducible nitric oxide synthase (iNOS) expression during liver regeneration[10] and iNOS-deficient mice show impaired liver regeneration.[11] In this regard, excessive oxidative stress due to lack of antioxidants UCP-2 or Nrf-2 delays and impairs liver regeneration in mice.[12,13] Together, these studies show that the oxidative stress and antioxidant responses must be fine-tuned to preserve hepatocyte homeostasis.

Liver damage triggers compensatory cell proliferation in an attempt to restore tissue loss. We previously described that $Nemo^{\Delta hepa}$ mice show spontaneous liver damage and hepatocyte apoptosis accompanied by compensa-

Abbreviations used in this paper: BHA, butylated hydroxyanisole; BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-collidin; DSB, double-strand break; FHC, ferritin heavy chain; IHC, immunohistochemistry; IKK, IκB kinase; IL, interleukin; IL6R, interleukin-6 receptor; iNOS, inducible nitric oxide synthase; MnSOD, manganese superoxide dismutase; NCF-1, neutrophil cytosolic factor 1; NF-κB, nuclear factor κB; PEITC, phenethyl isothiocyanate; PH, partial hepatectomy; ROS, reactive oxygen species; TEM, transmission electron microscopy; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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tory hepatocyte proliferation and profuse presence of oval cells throughout the parenchyma.[1] Interestingly, disruption of death receptor–mediated oxidative stress significantly improved liver parenchyma and attenuated the compensatory proliferative response in hepatocytes. However, the regenerating capacity of NEMO-deficient hepatocytes and its impact on overall liver regeneration after tissue damage have not been studied. Moreover, the role of NF-κB during liver regeneration, although widely studied, remains controversial.[14–17]

Herein, we investigated the relevance of NEMO/NF-κB in hepatocytes for the regenerative capacity of the liver. We show that NEMO in hepatocytes is essential to promote liver regeneration because deletion of NEMO significantly increases oxidative stress that triggers DNA damage and hepatocyte apoptosis, which promote proliferation of the liver stem cell compartment. Furthermore, we show that liver regeneration mediated by oval cells reverses the damaging phenotype observed in NEMO-deficient mice, although this effect is temporary because development of HCC was still observed in oval cell–mediated regenerating livers from Nemo$^{\Delta hepa}$ mice. However, long-term antioxidant treatment completely abrogated tumor development in Nemo$^{\Delta hepa}$ mice, suggesting an essential role of oxidative stress in carcinogenesis.

Materials and Methods

Animal Procedures

We generated Nemo$^{\Delta hepa}$ mice as described.[2] Mice were treated in accordance with the guidelines of the National Academy of Sciences (NIH publication 86-23 revised 1985). Pathogen-free 8-week-old male mice underwent partial hepatectomy (PH) of 70% of the liver by resection of the left lateral and median lobes.[18]

Antioxidant Treatment (Butylated Hydroxyanisole Diet) and Induction of Oxidative Stress (Phenethyl Isothiocyanate Injection)

Four-week-old male mice were fed ad libitum with a standard chow containing 0.7% of butylated hydroxyanisole (BHA; Sigma St. Louis, MO) and normal drinking water. For 1 week, 7-week-old mice received daily intraperitoneal injections of 50 mg/kg phenethyl isothiocyanate (PEITC; Sigma) in a vehicle containing ethanol/Cremophor (Sigma, St. Louis, MO) EL/phosphate-buffered saline in a ratio of 1/1/8.

Oval Cell Assay

Liver progenitor cells/oval cells were detected by immunofluorescence with CK19 (goat, Santa Cruz Biotechnologies, Santa Cruz, CA; rabbit, AbboMax, Abbomax Inc, San Jose, CA), A6 (kindly provided by Valentina Factor, National Institutes of Health), and MIC1-1C3 (kindly provided by Craig Dorrell, Grompe Lab, OHSU) antibodies.

Determination of Proliferation

Hepatocyte proliferation was assessed by immunofluorescence with 5-bromo-2′-deoxyuridine (BrdU; GE Healthcare, Freiburg, Germany) or Ki67 (Dianova, Dianova, Hamburg, Germany) antibodies following Cy-3 and fluorescein isothiocyanate, respectively (Jackson Research, Suffolk, United Kingdom). Proliferating hepatocytes were quantified by counting the number of nuclei positive for BrdU or Ki67, relative to the total nuclei per power field (200×) stained with 4′,6-diamidino-2-phenylindole (DAPI).

Histologic Evaluation, Determination of Apoptosis, and DNA Damage

Paraffin-embedded liver sections were stained with H&E. Apoptosis was evaluated by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay performed on frozen liver sections using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). DNA damage was detected by using pH2A.X antibody (Cell Signaling Technology, Beverly, MA). Apoptotic cells or cells with DNA damage were quantified by counting TUNEL- or pH2A.X-positive nuclei relative to the total nuclei per power field (200×) stained with DAPI.

Statistical Analysis

Data are expressed as mean ± SD. Statistical significance was determined by 2-way analysis of variance followed by Student *t* test.

Results

High Mortality, Liver Apoptosis, and Attenuated Proliferative Response in Nemo$^{\Delta hepa}$ Mice After PH

All wild-type animals (Nemo$^{f/f}$) survived PH (Figure 1*A*), whereas 50% of the Nemo$^{\Delta hepa}$ mice died 5 days after PH, mainly within the first 72 hours. Accordingly with the poor prognosis, aspartate aminotransferase (AST) levels were consistently higher in Nemo$^{\Delta hepa}$ mice at all time points after PH compared with wild-type animals (Supplementary Figure 1*A*). We found massive hepatocyte apoptosis in Nemo$^{\Delta hepa}$ livers 32 and 48 hours after PH but detected no cell death in Nemo$^{f/f}$ livers (Figure 1*B* and Supplementary Figure 1*B*). H&E staining confirmed the presence of apoptotic bodies and a certain degree of parenchymal degeneration (Supplementary Figure 1*C*). Finally, strong caspase-3 activity confirmed the impact of NEMO deletion on hepatocyte apoptosis after PH (Figure 1*C*). We found no regulation of Mcl-1 (data not shown) and increased messenger RNA (mRNA) expression of Bcl-xL and Bak (Supplementary Figure 1*D*), which may be considered as a compensatory response of the liver due to the massive apoptosis found after PH.

We next asked if lack of NEMO affects hepatocyte proliferation. In Nemo$^{f/f}$ livers, the number of BrdU-positive cells increased transiently and peaked 48 hours after PH (Figure 1*D* and Supplementary Figure 1*E*). In contrast, a higher number of BrdU-positive cells was already evident before PH in Nemo$^{\Delta hepa}$ livers and proliferation significantly increased by 10% after 32 hours. However, the number of BrdU-positive cells remained unchanged at 48 and 72 hours after PH. Fifteen days after PH, the number of BrdU-positive cells was still slightly higher in PH/Nemo$^{\Delta hepa}$ than in untreated Nemo$^{\Delta hepa}$ livers (Supple-