Research Article

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Hepatocyte specific deletion of *c-Met* leads to the development of severe non-alcoholic steatohepatitis in mice

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Background & Aims: Non-alcoholic-fatty-liver disease (NAFLD) is part of the metabolic syndrome. The spectrum of NAFLD includes NASH (non-alcoholic steatohepatitis), which is characterised by progressive inflammation associated with oxidative stress and apoptosis, finally triggering liver cirrhosis and hepatocellular carcinoma. HGF (hepatocyte growth factor)/mesenchymal-epithelial transition factor (c-Met) receptor signalling is known to activate distinct intracellular pathways mediating among others anti-apoptotic properties to hepatocytes. Therefore, the aim was to characterise the role of c-Met during NASH development.

Methods: Hepatocyte specific *c-Met* knockout mice (*c-Met*Δ^{hepa}) using the cre-loxP system and wild type controls (*c-Met*$^{loxP/loxP}$) were fed a methionine-choline deficient (MCD) diet.

Results: MCD feeding triggered massive steatosis, decreased survival and higher transaminases in *c-Met*Δ^{hepa} livers compared to *c-Met*$^{loxP/loxP}$. Gene array analysis demonstrated that genes involved in fatty acid metabolism were strongly upregulated in *c-Met*Δ^{hepa} livers correlating with higher amounts of hepatic free fatty acids. Consequently, *c-Met*Δ^{hepa} mice showed significantly more TUNEL positive cells and more superoxide anion production than *c-Met*^{loxPloxP} animals. Additionally, *c-Met*Δ^{hepa} livers showed significantly larger fractions of infiltrating neutrophils, macrophages, and cytotoxic T cells. These changes correlated with an enhanced progression of liver fibrosis as evidenced by higher collagen deposition in *c-Met*Δ^{hepa} livers. As increased apoptosis was a prominent feature in *c-Met*Δ^{hepa} livers, we generated *c-Met*/Casp8Δ^{hepa} double knockout mice. In these animals compared to *c-Met*Δ^{hepa} animals the increase in apoptosis could be reverted.

Conclusions: *c-Met* deletion in hepatocytes triggers NASH progression. A prominent mechanism is higher fatty acid accumulation and increased apoptosis, which in part can be reverted by blocking caspase 8.

Introduction

Non-alcoholic steatohepatitis (NASH) is a chronic progressive liver disease, representing the cause of chronic liver injury with the strongest rising incidence [1] in western civilizations. NASH is also seen as the hepatic equivalent of the metabolic syndrome, which is characterized by fat accumulation in visceral organs, insulin resistance, dyslipidemia, and high blood pressure [2,3]. The fact that around 20% of the population in industrialized nations displays characteristics of the metabolic syndrome illustrates the tremendously rising socio-economical importance of this disease entity [4,5]. Approximately 5% of patients with NASH develop complications of end stage liver disease [6–10]. In a prospective study of patients with NASH cirrhosis, the cumulative incidence of hepatocellular carcinoma (HCC) was 2.6% per year [11].

During NASH pathogenesis, several disease stages can be differentiated. First, metabolic changes become evident and hepatic steatosis develops. During the course of NASH development, hepatocytes become sensitized for damage induced by oxidative stress, reactive oxygen species (ROS), and lipid peroxidation [12]. Subsequently, inflammation triggers the development of steatohepatitis and finally fibrotic remodelling of the liver [13]. This is associated with high human morbidity and mortality, as so far no causal therapeutics that could stop or revert fibrosis are

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Abbreviations: NAFLD, non-alcoholic-fatty-liver disease; NASH, non-alcoholic steatohepatitis; HGF, hepatocyte growth factor; c-Met, mesenchymal-epithelial transition factor; MCD, methionine-choline deficient; FATP5, fatty acid transport protein 5; FABP1, fatty acid binding protein 1; Mttp, microsomal triglycerides transfer protein; ApoB, Apolipoprotein B; CPT1a, carnitine palmitoyltransferase 1a; Acox1, acyl CoA oxidase 1; ROS, reactive oxygen species; FFA, free fatty acids; DHE, Dihydroethidium; HCC, hepatocellular carcinoma; α-SMA, alpha-smooth-muscle-actin; BDL, bile duct ligation; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3 kinase.

available. Additionally, NASH has a high probability to develop into hepatocellular carcinoma with and without underlying cirrhosis.

Mesenchymal-epithelial transition factor (c-Met) acts as the cellular receptor for the hepatocyte growth factor (HGF), thereby regulating cell growth, motility, and morphogenesis. HGF is mainly produced by cells of mesenchymal origin and acts in a pleiotropic manner on cells of epithelial and endothelial origin. Initially, *c-Met* was identified as a proto-oncogene. It serves as an important regulator for stem cell growth during embryogenesis and, in particular, also for the growth of hepatocytes. In this regard, it is of interest that HGF knockout mice as well as mice deleted for its receptor c-Met display severe developmental defects and both die between day 13 and 16 during embryogenesis. The lack of both genes manifests with impaired placental and liver development [14,15].

Binding of HGF to its receptor c-Met induces its dimerization and phosphorylation. Thereafter, intracellular adapter proteins bind to c-Met and lead to the activation of further specific intracellular cascades that activate PI3K, Ras, and ERK-dependent pathways thus, controlling HGF-induced pro-mitogenic and anti-apoptotic events. The latter are of particular importance, as interference with apoptosis-regulating pathways can have deleterious consequences for the entire organism. Usually, the apoptosis-inducing death-receptor Fas is sequestered by c-Met. This prevents uncontrolled Fas-ligand-induced death-receptor activation and inhibits apoptosis [16]. This control mechanism is disturbed during NASH pathogenesis, because Fas ligand is produced in excess and the physiological inhibition through c-Met is hampered [17]. As a consequence, apoptosis is induced and liver damage occurs [18,19]. Thus, HGF/c-Met has direct implications for the pathogenesis of NASH and seems to be hepato-protective.

Further light was shed on the manifold of c-Met-controlled activities that regulate liver physiology through the introduction of conditional *c-Met* knockout mice by Borowiak *et al.* [20]. Here, mice carrying the Mx-Cre-induced *c-Met* deletion displayed reduced liver regeneration. Analysis of hepatocellular cell cycle progression in conditional *c-Met* knockout mice indicated a defective exit from quiescence and diminished entry into the S-phase. This was accompanied by reduced activation of Erk1/2 kinase, while Akt phosphorylation was still intact–highlighting the importance of cytokine-induced intracellular crosstalks.

As the role of c-Met during the development from steatosis towards steatohepatitis is still not clarified, we here aimed to determine the role of hepatocellular c-Met during this process.

Materials and methods

Animals

We used wild type (*c-Met*^{loxPloxP}) and hepatocyte specific conditional *c-Met*-knockout (*c-Met*$^{\Delta hepa}$) mice under control of a postnatal activated albumin promoter (C57BL/6). Successful deletion of *c-Met* was verified by genotyping with the following primers: Met (forward): AGC CTA GTG GAA TTC TGT AAG; Met (reverse): CCA AGT GTC TGA CGG CTG TG; Cre (forward): CCG GTG AAC GTG CAA AAC AGG CTC TA; Cre (reverse): CTT GCA TGA TCT CCG GTA TTG AAA CTC CAG. Representative PCR analysis of DNA isolated from hepatocytes is shown in Fig. 1.

Mice were housed in 12-hour light/dark cycles, with free access to food and water and treated in accordance with the criteria of the German administrative panel on laboratory animal care and approved by the local Animal Care Committee. At least 5 animals per time point were analysed. All experiments were repeated at least three times.

For dietary treatment 8-12 weeks old male mice weighing 25 g were either fed a methionine- and choline-deficient diet (MCD) or chow diet (MP Biomedicals, LLC, Ohio, USA). Both, knockout and wild type animals, showed a food intake of 5-6 grams per day without differences between chow or dietary treatment.

Flow cytometry analysis

Immune cells from whole liver extracts were isolated. After red blood cell lysis (PharmLyse, BD Biosciences, Heidelberg, Germany) cells were stained in two different panels. (1) CD45-APC-Cy7 (BD Pharmingen, Heidelberg, Germany, 557659), Hoechst 33258 dye (Sigma Aldrich, St Louis, MO), CD3-APC (eBiosciences, Frankfurt, Germany, 17-0031-82), CD4-PE (eBiosciences 12-0041-83), CD8-FITC (eBiosciences 11-0081-85), CD19-PerCpCy5.5 (BD Pharmingen 551001), NK1.1-PE-Cy7 (BD Pharmingen 552878), or (2) CD45-APC-Cy7 (BD Pharmingen 557659), Hoechst 33258 dye (Sigma Aldrich, St Louis, MO), Ly6G-FITC (BD Pharmingen 551460), F4/80-PE-Cy7 (eBiosciences 25-4801-82), Gr1-PerCpCy5.5 (BD Pharmingen 552093). Then, cells were subjected to flow cytometry using a BD Canto II (BD Biosciences, Heidelberg, Germany). Data were analysed using FlowJo software (TreeStar, Ashland, USA).

Microarray

100 ng of RNA for individual samples was used for whole transcript cDNA synthesis (Affymetrix, Santa Clara, CA). Hybridization, washing, and scanning of Affymetrix GeneChip Mouse Gene 1.0 ST arrays were carried out according to standard Affymetrix protocols. All arrays of the liver were hybridized in one experiment. Arrays were normalized using the Robust Multi-array Average method (PMID: 12538238, PMID: 12582260). Probe sets were defined according to Dai *et al.* (PMID: 16284200). In this method probes are assigned to unique gene identifiers, in this case Entrez IDs. The probes on the Gene 1.0 ST arrays represent 21,187 Entrez IDs (CDF 17). For the analysis only genes were taken into account that had an intensity value of >20 on at least two arrays. This threshold was met by 13,280 genes. Gene set enrichment analysis was performed to identify differentially expressed pathways (PMID: 16199517). Pathways that had a false discovery rate q value of <0.01 were considered to be significantly affected.

Further applied methods are described in the Supplementary materials and methods.

Results

Conditional hepatocyte specific c-Met deletion triggers severe steatohepatitis

To better define the impact of c-Met for non-alcoholic steatohepatitis (NASH), we used hepatocyte specific conditional *c-Met* knockout mice. The efficiency of *c-Met* deletion and Cre expression in wild type (*c-Met*$^{loxP/loxP}$, littermate controls) and knockout (*c-Met*$^{\Delta hepa}$) livers is shown in Fig. 1A (Cre PCR and c-Met PCR).

Eight weeks old wild type and knockout littermates of the same weight were fed with the NASH inducing MCD (methionine- and-choline-deficient) diet *ad libitum*. Kaplan Meyer survival analysis showed an earlier and more severe mortality of *c-Met*$^{\Delta hepa}$ mice under diet as all mice died within 20 weeks, while wild type animals survived up to 25 weeks, although the exact cause of the earlier lethality remains unknown so far (Fig. 1B).

Already after 4 weeks of MCD feeding the phenotype in *c-Met*$^{\Delta hepa}$ mice was associated with stronger liver injury as reflected by significantly higher transaminase levels compared to wild type littermates (Fig. 1C and Supplementary Fig. 1A). At this time point the histological analysis showed only slight signs of fatty liver disease in wild type livers, whereas *c-Met*$^{\Delta hepa}$ mice displayed massive fatty liver degeneration (Fig. 1D and E). Both groups showed a significant increase of hepatic triglycerides after 4 weeks of treatment although no significant difference between *c-Met*$^{\Delta hepa}$ and wild type animals could be detected (Fig. 1F).