Neuron-Derived Orphan Receptor 1 Promotes Proliferation of Quiescent Hepatocytes

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BACKGROUND & AIMS: Studies of the transcriptional networks that regulate nuclear receptor-mediated proliferation of quiescent hepatocytes could lead to new information about liver growth and hepatoprotective strategies. **METHODS:** We used quantitative real-time PCR to analyze expression of neuron-derived orphan receptor 1 (Nor-1) and its target genes during liver regeneration after hepatectomy in mice, and in hepatocellular carcinoma (HCC) samples from patients. We used adenoviral vectors to express Nor-1 in normal liver (Ad/CMV/V5-Nor-1), or reduce its level with small hairpin RNAs (Ad/BLOCK-iT/Nor-1$^{\text{small hairpin RNA}}$) after partial hepatectomy. **RESULTS:** Levels of *Nor-1* messenger RNA and protein, and transcription of Nor-1 target genes (*Ccnd1* and *Vcam-1*), increased during the late priming and proliferative phases of liver regeneration after partial hepatectomy. Levels of *NOR-1* messenger RNA and transcription of its target gene *CCND1* and of the NOR-1 subfamily member *NUR-77* also increased in human HCC samples compared with paired HCC-free tissue. Ad-Nor-1$^{\text{small hairpin RNA}}$ reduced the hepatocyte proliferation after hepatectomy. Overexpression of Nor-1 in normal livers of mice induced proliferation of quiescent hepatocytes independently of interleukin-6 and tumor necrosis factor−α signaling. In gene expression profile analysis, Nor-1 altered expression of genes involved in the cell cycle, proliferation, and tumorigenesis. **CONCLUSIONS: In mice, the orphan nuclear receptor Nor-1 activates proliferation of quiescent hepatocytes and is required for hepatocyte proliferation after partial hepatectomy. Nor-1 and its gene targets are also up-regulated in human HCC samples. Nor-1 activates a transcriptional program that induces hepatocyte proliferation independently of inflammatory signaling pathways.**

Keywords: Nuclear Receptors; Liver Growth; Surgery; Metabolism.

BASIC AND TRANSLATIONAL LIVER

Among adult organs, the liver is unique because normal turnover results from self-duplication of fully differentiated cells and, in minor part, of liver progenitors.[1] The liver is able to rapidly increase its mass upon injury (compensatory regeneration), due to a loss of liver cells, or without cell loss or damage (direct hyperplasia).[2] After partial hepatectomy (PH), quiescent hepatocytes rapidly activate DNA synthesis and become able to replicate (priming phase); as a consequence, powerful mitotic waves of liver cells restore the original liver mass (proliferative phase) and reconstitute the liver lobule within 7 days, when various mechanisms limit further regeneration (growth termination phase).[3] Liver regeneration (LR) after PH is mediated mainly by cytokines, growth factors, and metabolic signals.[3] LR after PH is considered a valuable model for studying the complex mechanisms that allow quiescent hepatocytes to proliferate.[3] Unraveling the transcriptional networks governing the proliferative switch in quiescent hepatocytes is a key for developing therapeutic strategies for liver disease and hepatocellular carcinoma (HCC).

Nuclear receptors (NRs) are transcription factors acting as sensors of hormones and nutrients, able to program the genetic control of development, metabolism, proliferation, apoptosis, circadian rhythms, and behavioral responses.[4] Several NRs are direct modulators of liver functions and have been involved in the pathophysiology of hepatic diseases, including carcinogenesis. Many pathways involved in LR can be modulated by NRs,[5–7] and some NRs are able to induce direct hyperplasia, because they induce hepatocyte proliferation in the absence of liver injury,[5,8–11] promoting the transcription of cyclin D1 (Ccnd1),[5,10] which is able to induce a spontaneous and robust hepatocyte proliferation in the intact liver.[12,13]

Together with the nerve growth factor IB (NGFIB/Nur-77/NR4A1) and nuclear receptor related 1 (Nurr-1/NR4A2), neuron-derived orphan receptor 1 (Nor-1, NR4A3) is a member of a subfamily of "true orphan" NRs, not requiring ligand binding to induce transcription and controlled at the level of protein expression and by post-translational modification.[14–16] The 3 members of the

Abbreviations used in this paper: Ccnd1, cyclin D1; Ccne1, cyclin E1; HCC, hepatocellular carcinoma; IL, interleukin; LR, liver regeneration; mRNA, messenger RNA; Nor-1, neuron-derived orphan receptor 1; NR, nuclear receptor; Nurr-1, nuclear receptor related 1; PCNA, proliferating cell nuclear antigen; PH, partial hepatectomy; shRNA, small hairpin RNA; Stat 3, signal transducer and activator of transcription 3; Vcam-1, vascular cell adhesion molecule 1.

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NR4A subfamily are rapidly and transiently induced by various stimuli (growth factors, inflammatory cytokines, glucagon, prostaglandins).[16,17] Nor-1 is predominantly expressed in the brain, adrenal glands, inflammatory system, stomach, heart, kidney, and pancreas; and upon specific conditions can be found in skeletal muscle and fat.[17,18] Dietary restriction, glucagon stimulation,[16,19] and liver resection-reperfusion injury[20] also induce *Nor-1* in the liver. Nor-1 has also been described as a downstream target of the hypoxia-inducible factor 1α and of the vascular-endothelial growth factor.[21,22] Nor-1 has been involved in development and differentiation,[23] apoptosis, and metabolic homeostasis in different tissues.[14,16] Recently, Ccnd1 has been shown to be a direct target of Nor-1 in vascular smooth muscle cells,[24] thus highlighting a central role of Nor-1 in modulating cell growth and proliferation.[21,22,24]

The aim of the present study is to unravel the role of Nor-1 in hepatocyte proliferation. Using gain- and loss-of-function approaches, we show that Nor-1 is necessary for hepatocyte proliferation after PH, and sufficient to induce a proliferative switch in quiescent hepatocytes in wild-type mice and in models of defective hepatocyte proliferation.

Methods

Animals

C57BL/6 wild-type, tumor necrosis factor receptor 1 knockout mice (Tnfrsf1$^{-/-}$; Jackson Laboratory strain 003243), and interleukin (IL)-6 knockout mice (IL-6$^{-/-}$; Jackson Laboratory strain 002650) were housed under a standard 12-hour light/12-hour dark cycle and fed standard rodent chow and water ad libitum. The 10- to 12-week-old male mice were used in the experiments. Liver weight and body weight were estimated the day of sacrifice after 4 hours of fasting for all the experiments. All the animal protocols were approved by the Ethical Committee of the Consorzio Mario Negri Sud.

PH was performed according to the method of Higgins and Anderson under ketamine/xylazine anesthesia.[6,25] The left lateral and median lobes were completely excised. For the sham-operated controls, an excision was made into the peritoneal cavity, and the liver was exteriorized and put back into the peritoneal cavity, followed by closure of the incision. Mice (4–6 per group) were sacrificed at different time points after hepatectomy (days 0, 0.5, 1, 2, 3, and 7 after PH). Data were normalized to sham-operated controls at each time point after PH. To measure the fraction of hepatectomy, the livers were excised from each group of mice; their weights were compared to the initial total liver mass calculated from the total body weight of each animal.[6,7] For the experiments of partial hepatectomy with Nor-1 loss of function, we treated mice with PH 2 days after adenoviral infection, and we collected samples at different time points.

Adenovirus Injection

In order to evaluate the effects of Nor-1 induction and/or inhibition, we performed a series of experiments injecting adenoviruses for gain and loss of function of Nor-1 via the jugular vein. To obtain efficient Nor-1 overexpression, we used adenoviruses (Ad/CMV/V5-GW/LacZ vs Ad/CMV/V5-Nor-1) at the concentration of 5×10^{E9} pfu/mL. For partial hepatectomy experiments with Nor-1 loss of function, we used adenoviruses (Ad-GW/U6-LacZ$^{\text{small hairpin RNA [shRNA]}}$ vs Ad/BLOCK-iT™/Nor-1$^{\text{shRNA}}$) at the concentration of 6×10^{E9} pfu/mL. In all of the gain-of-function experiments, mice were studied for gene and protein expression, and for immunohistochemistry 3 days after virus injection. The gain-of-function experiments were performed in normal liver (no mitogens, no partial hepatectomy). In the loss-of-function experiments, mice were studied for gene and protein expression, and for immunohistochemistry at 2, 3, 4, and 9 days after virus injection according to the time points of LR (sham-operated controls, day 1, day 2, and day 7 after PH).

Results

Nuclear Receptor Nor-1 Is Induced During Liver Regeneration After Partial Hepatectomy and in HCC

We performed PH and we measured gene transcripts via quantitative real-time polymerase chain reaction at different time points, before PH and during the proliferating stages up to the restoration of normal liver (Figure 1*A*–*C*). One and 3 days after PH, *Nor-1* messenger RNA (mRNA) levels are induced up to 6-fold compared to normal liver, where *Nor-1* expression is almost undetectable; Nor-1 subfamily member *Nurr-1* mRNA levels are also induced during the proliferating stages, while no differences are detectable in *Nur-77* mRNA expression (Figure 1*D*). Changes in Nor-1 mRNA expression are coupled to increased Nor-1 immunostaining, with peak 1 day after PH (Figure 1*E*), and to increased mRNA levels of the Nor-1 target genes cyclin D1 (*Ccnd1*) and vascular cell adhesion molecule 1 (*Vcam-1*) (Figure 1*F*). These data point to a strong induction of Nor-1 and its target genes *Ccnd1* and *Vcam-1* during the late priming and proliferative phases after PH, when transcriptional regulatory processes support the proliferative networks.[1,3]

Because Nor-1 has been reported to play a putative role in different tumors,[26,27] we then verified whether Nor-1 could also be increased in a model of transformed proliferating hepatocytes. When comparing HCC to chronic hepatitis C at a cirrhotic stage, we document increased mRNA expression of *NOR-1,* its target *CCND1*, and Nor-1 subfamily member *NUR-77*, while no differences are observed for *NURR-1* mRNA (Figure 2*A*, *B*, and *C*). In a different set of HCC samples, we also show increased NOR-1 immunostaining when compared to paired reference tissues (Figure 2*D*).

Ablation of Nor 1 in Regenerating Liver Blunts Hepatocyte Proliferation Capacity After PH

The parallel increase of Nor-1 in mice during the proliferative stages of LR and of human NOR-1 in HCC suggests a putative role in the modulation of hepatocyte proliferation. To verify if Nor-1 expression is a causative event, we studied LR capacity after hepatic Nor-1 silencing. Mice were pretreated with Ad-Nor-1$^{\text{shRNA}}$ 2 days before PH and sacrificed at different time points after PH (days 0, 1, 2, and 7). We document a significant decrease of Nor-1 in terms of mRNA expression and protein expression (day 2, Figure 3*A*; day 0 and day 2, Figure 3*B*).