Osteopontin is Induced by Hedgehog Pathway Activation and Promotes Fibrosis Progression in Nonalcoholic Steatohepatitis

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Nonalcoholic steatohepatitis (NASH) is a leading cause of cirrhosis. Recently, we showed that NASH-related cirrhosis is associated with Hedgehog (Hh) pathway activation. The gene encoding osteopontin (OPN), a profibrogenic extracellular matrix protein and cytokine, is a direct transcriptional target of the Hh pathway. Thus, we hypothesize that Hh signaling induces OPN to promote liver fibrosis in NASH. Hepatic OPN expression and liver fibrosis were analyzed in wild-type (WT) mice, Patched-deficient (Ptc+/−) (overly active Hh signaling) mice, and OPN-deficient mice before and after feeding methionine and choline–deficient (MCD) diets to induce NASH-related fibrosis. Hepatic OPN was also quantified in human NASH and nondiseased livers. Hh signaling was manipulated in cultured liver cells to assess direct effects on OPN expression, and hepatic stellate cells (HSCs) were cultured in medium with different OPN activities to determine effects on HSC phenotype. When fed MCD diets, Ptc+/− mice expressed more OPN and developed worse liver fibrosis (P < 0.05) than WT mice, whereas OPN-deficient mice exhibited reduced fibrosis (P < 0.05). In NASH patients, OPN was significantly up-regulated and correlated with Hh pathway activity and fibrosis stage. During NASH, ductular cells strongly expressed OPN. In cultured HSCs, SAG (an Hh agonist) up-regulated, whereas cyclopamine (an Hh antagonist) repressed OPN expression (P < 0.005). Cholangiocyte-derived OPN and recombinant OPN promoted fibrogenic responses in HSCs (P < 0.05); neutralizing OPN with RNA aptamers attenuated this (P < 0.05).

Conclusion: OPN is Hh-regulated and directly promotes profibrogenic responses. OPN induction correlates with Hh pathway activity and fibrosis stage. Therefore, OPN inhibition may be beneficial in NASH (HEPATOLOGY 2011;53:106-115)

Nonalcoholic steatohepatitis is a potentially serious form of chronic liver injury because it increases the risk of developing cirrhosis and primary liver cancer. The mechanisms that lead to these outcomes have not been fully elucidated, but they appear to involve responses triggered...
by hepatocyte apoptosis and myofibroblast accumulation. Certain apoptotic stimuli have been reported to induce hepatocyte production of Hedgehog (Hh) ligands. Hh ligands, in turn, elicit several fibrogenic actions by engaging their receptors on Hh-responsive liver cells, such as ductular type cells, hepatic stellate cells (HSCs), and natural killer T (NKT) cells. In HSCs, for example, Hh pathway activation functions in a cell-autonomous fashion to promote transition of quiescent HSCs (Q-HSCs) to myofibroblastic HSCs (MF-HSCs), enhance MF-HSC proliferation, and inhibit MF-HSC apoptosis. Activating Hh signaling in other types of liver cells (such as ductular cells and NKT cells) also causes these cells to generate factors that promote MF-HSC accumulation through paracrine mechanisms.

Osteopontin (OPN), a proinflammatory cytokine and integrin-binding ligand, is highly expressed in many inflamed tissues and plays a critical role in wound healing. Recently, glioblastoma (Gli) binding sites were demonstrated in the OPN promoter, prompting speculation that Hh signaling might regulate OPN transcription. This concept is potentially relevant to NASH-related liver fibrosis, because Hh pathway activity increases in parallel with fibrosis stage in NASH. Moreover, in other tissues, OPN is secreted by cells that mediate fibrogenic repair in NASH (such as NKT cells and fibroblasts). Evidence that OPN messenger RNAs (mRNAs) increase during culture-related activation of Q-HSCs to MF-HSCs and correlate with fibrosis severity in biliary atresia further support a potential role for OPN in the pathogenesis of cirrhosis. Therefore, we manipulated Hh pathway activity in mice and cultured cells to determine effects on OPN production, and examined whether reduction of OPN affected Hh signaling or fibrogenesis. The results support and advance the concept that OPN is an Hh target gene and reveal a previously unsuspected role for OPN as a proximal mediator of the fibrogenic actions of Hh in NASH.

Materials and Methods

Animals. C57BL/6 Patched-deficient (Ptc+/−) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Ptc+/− mice have only one copy of Ptc, an Hh pathway repressor. Therefore, these mice are unable to silence Hh signaling and exhibit excessive Hh pathway activity. WT and Ptc+/− mice were fed a methionine and choline–deficient (MCD) diet to induce nonalcoholic steatohepatitis (NASH) and liver fibrosis, or control chow (n = 8/group) for 8 weeks. Additionally, 129/SvJ Black-Swiss OPN-deficient (OPN−/−) mice and littermate controls were fed the MCD diet or control chow, respectively (n = 6/group). Because 129/SvJ mice were reported to be more sensitive to an MCD diet than C57Bl/6 mice, OPN−/− mice and littermate controls were fed the diets for 4 weeks rather than 8 weeks.

Animal care and procedures were approved by the Duke University and Northwestern University Institutional Animal Care and Use Committees as set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Histopathologic Analysis. Serial sections were stained with hematoxylin and eosin. Nonalcoholic fatty liver disease (NAFLD) severity was assessed using criteria described by Brunt et al. (Supporting Information Materials and Methods). To quantify liver fibrosis, 5-μm sections were stained with Picrosirius red (Sigma, St. Louis, MO) and collagen stained with Sirius red (Sigma, St. Louis, MO) and counterstained with fast green (Sigma, St. Louis, MO). Collagen stained with Sirius red was quantitated in the sections that were randomly chosen (under ×20 magnification, 10 fields each from sample) as described.

Immunohistochemical Analysis. To localize and characterize cells that produce and/or respond to Hh ligands and OPN, formalin-fixed, paraffin-embedded livers were prepared for immunohistochemical analysis as described. Protocols and antibodies used are listed in Supporting Information Materials and Methods and Supporting Information Table 1.

Molecular Techniques. Real-time quantitative reverse-transcription polymerase chain reaction (QRT-PCR) and western immunoblot analysis were performed using established protocols. Details are provided in the Supporting Information Materials and Methods and Supporting Information Table 2.

Isolation and Culture of Primary Rodent Hepatic Stellate Cells. Hepatic stellate cells (HSCs) were isolated from normal Sprague-Dawley rats as described.
(Supporting Information Materials and Methods). A similar isolation/culture protocol was used for studies involving mouse primary HSCs. Day 4 HSCs were used in all experiments.

**Human HSCs.** The human HSC line LX-2 was cultured in serum-supplemented Dulbecco's modified Eagle's medium. Primary human HSCs were isolated as described.21

**Studies of Hh and OPN in Cultured HSCs.** To evaluate the effects of Hh signaling on HSCs, day 4 HSC cultures were grown for an additional 24 hours in medium containing either exogenous Hh agonist (SAG) at a concentration of 0.3 μM, 5 μM cyclopamine (Toronto Research Chemicals, Inc., Toronto, ON, Canada), an inhibitor of Hh signaling, or 5 μM tomatidine (Calbiochem, San Diego, CA), a catalytically inactive analog of cyclopamine (tomatidine serves as a control for cyclopamine).

In separate experiments, recombinant OPN (rOPN) or vehicle was added to cultures to assess their effects on HSC activation. A 100-ng/mL dose was used in this study because it stimulated the greatest effects *in vitro.*22 The effects of inactivating OPN were subsequently assessed by treating HSCs with the human OPN RNA aptamer OPN-R3 or its biologically inactive mutant, OPN-R3-2 (both synthesized by Dharmacon, Lafayette, CO).23 Aptamers (100 nmol/L) were added to medium for 48 hours before harvest. This concentration of OPN aptamer has been shown to inhibit adhesion, migration, and invasion in the breast cancer cell line MDA-MB-231 (which highly expresses OPN and is a standard tool for evaluating OPN actions).23 All cell experiments were performed at least in duplicate. Total RNA and protein were harvested before treatment and at the end of treatment and were analyzed by way of QRT-PCR and immunoblotting, respectively.

**Effect of Cholangiocyte-Conditioned Media on Primary HSC Activation.** The immortalized but non-transformed murine immature cholangiocyte cell line 603B was maintained in six-well plates (Costar 3516, Corning Incorporated) in standard culture media as described.24,25 At 90% confluence, cholangiocyte-conditioned media were harvested and added to primary HSC cultures together with OPN-targeted RNA aptamers or null aptamers. HSCs were harvested 2 days later, and mRNA expression was analyzed by way of QRT-PCR. Experiments were performed in duplicate and were repeated twice.

**Human Liver Studies.** Formalin-fixed, paraffin-embedded liver sections from deidentified controls and subjects with biopsy-proven NASH and explanted liver tissues from individuals undergoing liver transplantation for NASH or ASH-related cirrhosis (n = 6/group) from the Liver and Hepatobiliary Unit, Birmingham, UK, and Department of Pathology at Duke University were used. Normal tissues were obtained from nondiseased livers removed during resection for colorectal hepatic metastases or from split-liver grafts.

Freshly explanted livers with cirrhosis related to autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), and primary biliary cirrhosis (PBC) were also snap-frozen and used for total liver RNA analyses.

All studies using material from Duke University Hospital were conducted in accordance with National Institutes of Health and institutional guidelines for human subject research. Studies of samples acquired from the Hepatobiliary Unit in Birmingham were performed in accordance with local ethical approval 04/Q2708/41 and REC 2003/242 from the South-Birmingham Research Ethics Committee, UK (Supporting Information Materials and Methods and Supporting Information Table 1).

**Statistical Analysis.** The results are expressed as the mean ± SEM. Statistical significance was determined using Student *t* test and was set at *P* < 0.05.

**Results**

**Up-regulation of OPN Parallels Hh Pathway Activation During MCD Diet–Induced NASH.** WT mice (n = 8/group) develop hepatic necro-inflammation, accumulate markers of MF-HSCs, and exhibit liver fibrosis after 8 weeks of an MCD diet. Ptc+/− mice (with haplo-insufficiency of Ptc, a factor that constrains Hh signaling) exhibit worse liver fibrosis than WT mice after MCD diet exposure (Fig. 1A; Supporting Information Fig. 1A-D).6,7 These findings suggest that Hh pathway activation promotes fibrosis progression in this model of NASH, but the exact mechanisms involved have not yet been determined.

Hh pathway activation results in nuclear accumulation of Gli proteins (downstream targets of Hh signaling),26 and Gli proteins may regulate transcription of OPN, a potential profibrogenic factor.27-29 Therefore, we compared OPN expression in WT mice that were fed either control or MCD diets for 8 weeks. In WT mice, MCD diets caused a significant induction of OPN mRNA (>10-fold) and protein (approximately two-fold) expression (Fig. 1B,C; Supporting Information Fig. 1E). Ptc+/− mice exhibited even greater up-regulation of OPN expression when fed an MCD diet (Fig. 1B,C; Supporting Information Fig. 2A,B). The latter finding supports the concept that Hh signaling increases OPN expression.
In both strains, the OPN-immunoreactive cells were mostly ductular in appearance (Fig. 1D). Double immunostaining for Gli2 and OPN in liver samples from NASH patients demonstrated that Gli2 (+) ductular cells that coexpressed OPN localized within fibrous septae (Fig. 1E).

**MCD Diet–Fed OPN-Deficient Mice Develop Less Fibrosis.** To evaluate whether OPN directly contributed to the fibrogenic response evoked by MCD diets and gain further insight into the relationship between OPN and the Hh pathway, OPN $$^{-/-}$$ mice (n = 12) and littermate controls (n = 12) were fed MCD or control diets for 4 weeks. OPN deficiency had no obvious effect on expression of the Hh target gene Gli2, because both OPN $$^{-/-}$$ mice and littermate controls showed similar induction of Gli2 mRNA (data not shown) and protein (Fig. 2A) after 4 weeks of an MCD diet. Despite apparent similarities in Hh pathway activity, however, the fibrogenic responses of OPN $$^{-/-}$$ mice were markedly attenuated when compared with their littermate controls. After MCD diet feeding, for example, OPN $$^{-/-}$$ mice accumulated 50% fewer $$\alpha$$-smooth muscle actin ($$\alpha$$SMA)–positive cells (Fig. 2B) and significantly fewer Sirius red–stained fibrils (Fig. 2C) than comparably treated littermates. These results are consistent with an earlier report of reduced collagen gene expression in OPN $$^{-/-}$$ mice and suggest that the Hh pathway mediates its fibrogenic effects, at least in part, by inducing expression of OPN.
**Paracrine/Autocrine OPN Stimulates HSC Expression of Fibrogenic Phenotype.** Hh-responsive bile ductular cells are major sources of OPN (Fig. 1E). Therefore, we treated primary cultures of rodent HSCs with conditioned medium from monocultures of a cholangiocyte cell line, and assessed effects on HSC gene expression. To determine whether HSC responses were mediated by OPN, studies were repeated using cholangiocyte-conditioned medium plus OPN-targeted aptamers. Cholangiocyte-conditioned media augmented HSC expression of αSMA (Fig. 3A) and collagen (Fig. 3B); RNA aptamer treatment repressed αSMA induction by 50% and returned collagen expression to basal values, proving that paracrine signaling involving OPN promoted fibrogenic gene expression in HSCs. In separate studies, other primary HSC cultures were treated with rOPN (100 ng/mL) or vehicle for 24 hours, and RNA was analyzed by way of QRT-PCR (Fig. 3C,D). rOPN also augmented expression of αSMA (Fig. 3C) and collagen Iα1 expression (Fig. 3D). These findings support the concept that exogenous OPN can function as a paracrine factor to promote fibrogenic gene expression in HSCs.

Because it has been reported that MF-HSCs themselves also express OPN, 16 we next investigated changes in endogenous OPN gene expression during

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**Fig. 2.** OPN-deficient mice develop less fibrosis after MCD diet treatment. 129/SvJ OPN-deficient mice and littermate controls were fed an MCD diet or control chow (n = 6 mice/group/dietary treatment) for 4 weeks. At the end of treatment, mice were sacrificed. (A) Accumulation of Gli2 (+) cells in OPN−/− and littermates. Sections from six animals were used, and eight randomly selected, 400× fields chosen for cell counting. (B) αSMA morphometry. Sections from three animals were used at each time point, and 10 randomly selected, 400× fields were chosen for analysis with Metaview software. (C) Sirius red quantification by way of morphometric analysis. Sections from six animals were used, and 10 randomly selected 400× fields were chosen for analysis. Results are expressed as fold change relative to chow-fed littermates and are presented as the mean ± SEM. *P < 0.05 versus control mice.

**Fig. 3.** Paracrine OPN stimulates HSC activation and collagen expression. (A,B) Isolated mouse primary HSCs were cultured for 4 days and treated with conditioned medium (CM) from cholangiocytes for 48 hours, with or without OPN-targeted RNA aptamers. (C,D) In separate experiments, primary HSCs were directly treated with rOPN (0 or 100 ng/mL) for 24 hours and harvested for QRT-PCR analysis. (A,C) αSMA. (B,D) Collagen mRNA. Results of duplicate experiments are presented as the mean ± SEM. *P < 0.05 versus vehicle-treated stellate cells.
spontaneous culture-related activation of Q-HSCs to MF-HSCs. We confirmed that HSC expression of OPN mRNA and protein increased significantly as Q-HSCs transitioned to becoming MF-HSCs (Fig. 4A; Supporting Information Fig. 3A). Addition of OPN aptamers to day 4 cultures significantly repressed αSMA and collagen gene expression, providing novel evidence that HSC-derived OPN may help to maintain the myofibroblastic phenotype of cultured HSCs. As HSCs become MF-HSCs, they repress expression of the Hh inhibitor Hhip, induce expression of Hh ligands, and up-regulate various myofibroblastic genes while down-regulating markers of quiescence. To clarify the relationship between Hh pathway activation and OPN expression, day 4 culture-activated MF-HSCs were treated with cyclopamine to selectively inhibit Hh signaling. Cultures were harvested 24 hours later, RNA was isolated, gene expression was assessed by way of QRT-PCR, and results were compared with parallel cultures that had been treated with tomatidine, an inactive cyclopamine analog. Inhibiting Hh signaling with cyclopamine attenuated induction of OPN gene expression (Fig. 4B; Supporting Information Fig. 4B).

Conversely, addition of the Hh agonist SAG augmented OPN gene expression significantly (Fig. 4C). Therefore, endogenous OPN gene expression in MF-HSCs is regulated, at least in part, by the Hh pathway.

To determine the relative importance of OPN as a downstream target of the Hh pathway in HSCs, day 4 primary MF-HSCs from Ptc+/−/C0 mice were incubated with OPN aptamers for 48 hours. At baseline, HSCs from Ptc+/−/C0 mice expressed three-fold more gli2 mRNA than WT HSCs, confirming that Ptc deficiency enhances Hh signaling (Fig. 5A). Consistent with our in vivo findings (Fig. 1), Ptc+/−/C0 HSCs also expressed more OPN mRNA than WT HSCs (Fig. 5B). Neutralizing OPN significantly reduced collagen and αSMA mRNA levels in Ptc-deficient HSCs (Fig. 5C,D) but had little effect on gli2 mRNA expression (Fig. 5E). These findings suggest that the Hh pathway mediates induction of certain fibrogenic genes indirectly through up-regulation of the Hh-responsive gene OPN.

Evidence of OPN Overexpression in Humans with Progressive NAFLD. Because the mouse model of MCD diet–induced NASH differs from human NASH
in several aspects, it was important to determine whether OPN overexpression occurred in human patients with NAFLD. Coded liver sections from 11 patients with well-characterized NAFL (n = 3), NASH (n = 3), and NASH-related cirrhosis (n = 5) were stained to demonstrate OPN and analyzed using computer-assisted morphometry. Expression of OPN was lowest in patients with NAFL and highest in patients with NASH-related cirrhosis (Fig. 6A,B). To further validate the association between NAFLD-related fibrosis stage and OPN expression, total liver RNA was isolated from a separate cohort of 36 patients with early (stage F0-F1) or advanced (stage F3-F4) NASH-related fibrosis (n = 18/group) and analyzed by way of QRT-PCR. In livers with advanced fibrosis, OPN expression was double that of livers with early fibrosis (Fig. 6C). Additional analysis was conducted using RNA and protein harvested from explanted livers with NASH-related cirrhosis and residual tissue from nondiseased donor livers (n = 6/disease). Livers with NASH-related cirrhosis contained over 10 times more OPN protein (Fig. 6D; Supporting Information Fig. 4) and 5 times more OPN mRNA compared with nondiseased control livers (Fig. 7A). Interestingly, OPN was also significantly increased in livers from individuals with alcoholic liver disease (ALD)-related cirrhosis (Fig. 7A,B; Supporting Information Fig. 4), PBC (Fig. 7A,C), AIH, and PSC (Fig. 7A), suggesting that OPN induction is a conserved response to chronic liver injury.

Discussion

Cirrhosis rarely occurs unless NAFLD progresses to NASH, a condition that is characterized by inflammation and hepatocyte death. However, only a minority of individuals with NASH actually develop cirrhosis. Fibrosis stage in NASH has been shown to correlate with the level of hepatic apoptotic activity. Index liver biopsy specimens of NASH patients who eventually progress to cirrhosis also harbor more myofibroblasts (MFs) than specimens of patients who do not progress to cirrhosis. These observations link hepatocyte apoptosis with myofibroblast accumulation and fibrosis progression in NASH.

There is further evidence that phagocytosis of apoptotic debris stimulates HSCs to become MFs. Recently, we identified a potentially related mechanism by which hepatocyte apoptosis promotes MF accumulation and liver fibrosis by demonstrating that dying hepatocytes produce Hh ligands. Biologically active Hh ligands are detected in apoptotic fragments released by ligand-producing cells. Hh ligands, in turn, engage various types of Hh-responsive cells, including HSCs, ductular-type cells, and NKT cells, to trigger fibrogenic responses. Consistent with these findings, we observed that Hh activity correlated with MF accumulation and fibrosis stage in NAFLD patients and in rodent models of NAFLD, suggesting that interindividual differences in Hh signaling...
influenced intensity of fibrogenic responses during NASH. The present study provides further support for this concept, because it identifies OPN as a proximal effector of Hh-mediated fibrogenesis, and demonstrate that livers of OPN-deficient mice are significantly protected from NASH-related fibrosis.

A recent analysis of the OPN gene revealed binding sites for Gli transcription factors, suggesting that OPN transcription is likely to be regulated by Hh signaling. By demonstrating colocalization of Gli and OPN in liver cells, and proving that expression of OPN mRNA is increased by a Smoothened agonist but decreased by a Smoothened antagonist, our results support and advance this concept. In addition, our data demonstrate that changes in OPN gene expression are paralleled by changes in OPN protein content and biological activity, because OPN aptamers reverse the profibrogenic actions of OPN. The latter findings also verify that OPN is a significant downstream target of Hh signaling (rather than vice versa), because neutralizing OPN had no effect on cellular expression of the Hh target gene Gli2 but significantly diminished fibrogenic gene expression, even in Ptc-deficient cells with supranormal Hh pathway activity. Coupled with the evidence that OPN−/− mice are significantly protected from NASH-related fibrosis, the data explain why we noted that hepatic content of Gli2+ cells and OPN+ cells increased in parallel as liver fibrosis advanced in patients with NAFLD.

Although the present studies focused on the roles of OPN as a paracrine factor for cholangiocyte-stellate cell fibrogenic interactions, and an autocrine mediator of fibrogenic gene expression in MF-HSCs, other cell types might also contribute to the fibrogenic actions of OPN.
OPN in NASH. NKT cells are particularly noteworthy in this regard. These liver-enriched immune cells are capable of producing and responding to Hh ligands and are also known to secrete OPN. To our knowledge, the possibility that Hh signaling might regulate OPN expression in NKT cells has not been evaluated. However, we have demonstrated that Hh pathway activation enhances hepatic accumulation of NKT cells. We and others have also shown that hepatic NKT cell content is significantly increased in patients with NASH-related cirrhosis. Moreover, activated liver NKT cells generate soluble factors that evoke expression of fibrogenic genes in cultured HSCs, and mice that are genetically deficient in NKT cells are relatively protected from NASH-related fibrosis, similar to OPN-deficient mice.

Therefore, OPN induction may represent a conserved profibrogenic mechanism among several distinct types of Hh-responsive liver cells, including ductular cells, MF-HSCs, and NKT cells. Such reasoning suggests that interindividual differences in OPN production may contribute to differences in the outcomes of NASH. Indeed, OPN may also dictate the fibrogenic response in other chronic liver diseases, because it is significantly upregulated in livers with cirrhosis related to ALD, AIH, PBC, and PSC, and a recent study reported that plasma OPN levels correlate with hepatic inflammation and fibrosis in chronic hepatitis C.

Although more work is needed to delineate the interactions between OPN and other putative profibrogenic factors, this concept suggests that OPN levels may provide a useful biomarker for liver fibrosis in NASH, and that OPN neutralization might be useful for preventing progressive hepatic fibrosis in NASH patients.

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**References**


