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Viral Factors Induce Hedgehog Pathway Activation in Humans with Viral Hepatitis, Cirrhosis, and Hepatocellular Carcinoma

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Abstract

Hh pathway activation promotes many processes that occur during fibrogenic liver repair. Whether the Hh pathway modulates the outcomes of virally-mediated liver injury has never been examined. Gene-profiling studies of human hepatocellular carcinomas (HCC) demonstrate Hh pathway activation in HCCs related to chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). Because most HCC develop in cirrhotic livers, we hypothesized that Hh pathway activation occurs during fibrogenic repair of liver damage due to chronic viral hepatitis, and that Hh-responsive cells mediate disease progression and hepatocarciongenesis in chronic viral hepatitis. Immunohistochemistry and qRTPCR analysis were used to analyze Hh pathway activation and identify Hh-responsive cell types in liver biopsies from 45 patients with chronic HBV or HCV. Hh signaling was then manipulated in cultured liver cells to directly assess the impact of Hh activity in relevant cell types. We found increased hepatic expression of Hh ligands in all patients with chronic viral hepatitis, and demonstrated that infection with HCV stimulated cultured hepatocytes to produce Hh ligands. The major cell populations that expanded during cirrhosis and HCC (i.e., liver myofibroblasts, activated endothelial cells, and progenitors expressing markers of tumor stem/initiating cells) were Hh-responsive, and higher levels of Hh pathway activity associated with cirrhosis and HCC. Inhibiting pathway activity in Hh-responsive target cells reduced fibrogenesis, angiogenesis, and growth.

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Conclusions—HBV/HCV infection increases hepatocyte production of Hh ligands and expands types of Hh-responsive cells that promote liver fibrosis and cancer.

Keywords

fibrosis; hedgehog pathway; hepatitis B; hepatitis C; liver progenitors; morphogens

Cirrhosis and liver cancer are major causes of morbidity and mortality. Worldwide, most cases of cirrhosis and liver cancer are caused by chronic infection with hepatitis B (HBV) or C viruses (HCV) (1, 2). Unfortunately, despite effective vaccination for hepatitis B and improvements in anti-viral therapies for both hepatitis B and hepatitis C, the global burden of chronic hepatitis B and/or hepatitis C infection and virally-mediated liver disease remains enormous (2, 3).

Progressive liver damage from chronic HBV/HCV occurs because chronic viral infection increases the death rate of hepatocytes (4). This triggers repair responses to replace the cells that died (4). In some individuals, however, repair is fibrogenic and results in progressive accumulation of collagen matrix (5). Fibrogenic repair is also generally accompanied by vascular remodeling and hepatic accumulation of liver progenitor cells (5). Expansion of liver progenitor populations, in turn, provides a source of immature cells with survival advantages, some of which may eventually become tumor-initiating cells for primary liver cancers (6). Primary hepatocellular carcinoma (HCC) is a major cause of liver-related death in HBV/HCV-infected individuals with cirrhosis (3, 4). Therefore, fibrogenic repair contributes to the pathogenesis of the potentially fatal outcomes of chronic viral hepatitis.

Recent studies of cultured cells, animal models of metabolic and biliary types of liver disease, and small numbers of liver biopsy samples from patients with similar conditions have identified a mechanism that modulates fibrogenic repair, namely, activation of the Hedgehog (Hh) signaling pathway (7-9). Hhligands are morphogens that orchestrate tissue construction and remodeling by initiating autocrine and paracrine signaling in Hh-responsive cells (10). In such cells, interaction of Hh-ligands with Patched (Ptc) receptors on the cell surface releases the signaling-competent co-receptor, Smoothened (Smo), from Ptc-repression. This permits intracellular signal transduction that culminates in the nuclear localization of Glioma-associated oncogene family transcription factors (Gli1, 2, 3) that regulate the transcription of Hh-responsive genes (10). Hepatic stellate cells (HSCs), some cholangiocytes, liver progenitors, and lymphocytes, are capable of producing Hh-ligands (8, 11-13). Hh-ligands function as viability factors for multipotent progenitors that are capable of differentiating into mature hepatocytes or cholangiocytes (11). They also stimulate quiescent HSCs to acquire a more myofibroblastic phenotype, and promote the proliferation and survival of myofibroblastic cells (13). In addition, Hh-ligands act in a paracrine fashion to induce cultured ductular cells to undergo epithelial-to-mesenchymal transitions (EMT), providing another mechanism that might increase myofibroblastic cell numbers in livers (8, 14, 15). In vitro, myofibroblast-derived Hh ligands up-regulate expression of various chemokines and chemokine receptors in neighboring immature liver epithelial cells, facilitating hepatic recruitment and retention of certain types of immune cells (16). Some of these immune cells, in turn, produce and respond to Hh-ligands (12, 16), further enriching

the microenvironment with morphogens. Finally, Hh ligands may influence vascular remodeling during liver damage because they exert proangiogenic effects during development and adult wound healing responses (17, 18). Indeed, liver sinusoidal endothelial cells (SECs) were recently shown to be Hh-responsive cells that up-regulate their expression of adhesion molecules and other markers of activated sinusoidal endothelium when exposed to Hh-ligands (19). Hence, there is growing experimental evidence that Hh-signaling increases during liver injury and that activation of the Hh pathway promotes many processes that are known to occur during fibrogenic liver repair, including growth of myofibroblastic populations, EMT, vascular remodeling, and hepatic accumulation of inflammatory cells and liver progenitors.

Whether or not the Hh pathway modulates the outcomes of virally-mediated liver injury has never been examined. Recent gene-profiling studies of human liver cancer repositories provide evidence for Hh pathway activation in a substantial proportion of HBV/HCV-related HCCs (20-22). Given that virally-induced cirrhosis is a strong risk factor for HCC, the latter data suggest that Hh pathway activation might occur during chronic viral hepatitis. If verified, this raises additional questions about the role(s) of Hh-responsive cells in regulating disease progression and hepatocarcinogenesis during virally-mediated liver disease. The goals of this study, therefore, were to determine if the Hh pathway becomes activated in the livers of patients with chronic viral hepatitis; to characterize the types of liver cells that produce and/or respond to Hh ligands in virus-infected livers; and to delineate potential mechanisms by which Hh-signaling might contribute to fibrotic repair and hepatocarcinogenesis in this context. We hypothesized that Hh-signaling would increase in cell populations that mediate fibrogenic repair during chronic viral hepatitis and induce such cells to acquire phenotypes that promote liver fibrosis, vascular remodeling, and hepatocarcinogenesis. Hence, fibrosis stage and HCC development were predicted to increase in parallel with the level of Hh pathway activity in patients with chronic viral hepatitis.

Material and Methods

Human Samples

45 formalin-fixed, paraffin-embedded (FFPE) liver biopsies from subjects with progressive hepatitis B (n=23) and C (n=22) were obtained from the Department of Pathology tissue repository at Universidade Federal do Espírito Santo, Brazil (Details in Suppl Material & Methods). Demographic data of selected subjects are described in Table 1. FFPE liver sections were also obtained from residual healthy liver tissues of two donor livers that were utilized for split liver transplantation at Duke University Hospital. The study was approved by the Human Ethics Committee of Centro de Ciências da Saúde, Universidade Federal do Espírito Santo (033-09) and by the Duke Institutional Review Board and fulfilled Institutional and NIH Guidelines for human subject research.

Histology and Immunohistochemistry (IHC)

FFPE livers were prepared for immunohistochemistry and serial sections were stained with H&E and Sirius red. For each immunostain, sections from control livers and sections from

livers with viral hepatitis were always processed identically and concurrently. Each assay also always included appropriate positive controls (other types of diseased human liver with known Hh pathway activation) and negative controls (diseased human liver incubated with secondary antibody but without primary antibody). Detailed protocols are in Supplemental Material and Methods and antibodies used are listed in Supplemental Table 1.

Cell lines and studies to assess effects of viral proteins on Hh ligand production

Huh-7 cells (gift from Eckard Wimmer, Stony Brook University, NY), were grown according to standard protocols (23). After pilot experiments were completed to establish optimal conditions for viral infection, Huh-7 cells were infected with HCV genotype 2a JFH-1 virus (MOI of 0.1) (24, 25) or control adenoviral vectors (gift from P. Goldschmidt-Clermont, University of Miami, FL; MOI of 50) (26) and harvested 72h later for RNA isolation/analysis.

Isolation and culture of Human Primary Hepatic Stellate (HSC) and Sinusoidal Endothelial Cells (SEM) to assess effects of hedgehog pathway activity

Approximately 25g of finely-cut liver obtained from a residual segment of healthy human liver was used for primary HSCs and SECs isolation as previously described (27, 28)

To assess the effects of Hh pathway activity, primary HSCs and SECs were treated with cyclopamine (5μM, Toronto Research Chemicals, Toronto, Canada), an inhibitor of Hh-signaling, or its catalytically-inactive analog, tomatidine (5μM) (29). RNA and protein were harvested for qRT-PCR and Western blot analysis 48h later.

Angiogenesis Assay

A total of 12,000 primary human SECs (from Sciencecell, San Diego, CA) were seeded in each well of chamber slide with 100μl Matrigel after 30min of reincubation at 37°C (30). Wells were photographed at different time points at random fields with the use of a confocal microscope (Zeiss LSM Pascal Axiovert; Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK). The length of the vascular tubes after 16h was digitally analyzed using AnalySISD software (Olympus Biosystems, Münster, Germany). Cultures were treated with vehicle, recombinant Shh (5μg/ml, Stem Cell Tech Inc, Vancouver, Canada), primary HSC (from Sciencecell, San Diego, CA) conditioned medium with or without cyclopamine, or pre-treated with short hairpin RNAs (shRNA) targeted against Smo to determine effects on tube formation, a well characterized surrogate for angiogenesis (30). shRNA was designed as previously described (31); Smo shRNA sequence is shown in Supplemental Table 2. For the shRNA delivery we used the BLOCK-iT Inducible H1 Lentiviral RNAi System (Invitrogen).

RNA and Protein Analysis

Real-time reverse-transcription polymerase chain reaction (RT-PCR) and Western immunoblot were performed using established protocols (12); details are in Supplemental Material & Methods and Supplemental Table 3.

RNA isolation from FFPE tissue and qRT-PCR

Total RNA was isolated from archival FFPE tissue using RNeasy FFPE kit (Qiagen, Valencia, CA) following the manufacturer's protocols. Since the concentration of obtained cDNA was very low, qRT-PCR cycles were increased to 50 (60 for Laser Capture samples), and all work involving mixing of reagents and loading of plates was performed in a sterile hood to prevent contamination.

For Laser Capture Microdissection (LCM) Palm MicroBeam Microlaser and Zeiss microscope was used. First, FFPE tissue was deparafinized in xylene for 5min and then was cut using Auto-LPC option that allows catapulting of selected tissue parts into collection vessel. Following tissue collection, RNA was isolated using RNeasy FFPE kit. Final cDNA was diluted to 50μl and 5μl was used per reaction.

Statistical Analysis

Results are expressed as means ± SEM. Comparisons between groups were performed using the non-parametric Wilcoxon-Rank-Sums test using SAS version 9.1 software (SAS Institute, Cary, NC). P-values are two-tailed; significance was accepted at the 5% level.

Results

Patient populations

22 patients with liver disease caused by chronic HBV infection and 23 patients with chronic HCV-induced liver disease underwent clinically-indicated liver biopsies and residual, de-identified formalin-fixed, paraffin-embedded tissues were used for the present study. Table 1 summarizes the clinical information that was submitted with each liver biopsy. Males predominated in the subgroups with HBV and HCC. Patients tended to be middle-aged. However, the group without cancer was generally about one decade younger than the group with coincident HCC. Serum aminotransferase values were less than twofold elevated in patients with chronic HBV, including the sub-groups with advanced fibrosis and/or HCC. HCV patients who had no fibrosis (F0) or only mild-moderate fibrosis (F1, F2) also had relatively normal serum aminotransferases, while HCV patients with F3-4 fibrosis and/or HCC had significant aminotransferase elevations.

Chronic viral hepatitis increases hepatic production of Hh ligands

Compared to liver biopsies from controls without chronic liver disease which rarely, if ever, demonstrated cells that expressed Hh-ligands (Fig 1A, Suppl Fig 1A, Suppl Fig 2A-B), biopsies from patients with viral hepatitis demonstrated significantly increased expression of Sonic hedgehog (Shh) and Indian hedgehog (Ihh) mRNAs, as well as accumulation of cells that produced these proteins (Fig 1B-C, Suppl Fig 1 B-C). Increased expression of Shh and Ihh mRNAs was noted consistently in virus-infected patients with relatively little liver fibrosis, but higher levels of Shh-Ihh mRNA were generally exhibited by patients with bridging fibrosis. In patients with HBV, mRNA levels of Shh and Ihh remained relatively flat across the spectrum of more advanced fibrosis, whereas levels of ligand mRNAs more closely paralleled fibrosis stage in HCV (Fig 1B, Suppl Fig 1B). Shh protein displayed a strong positive correlation with fibrosis stage in HBV and HCV (Suppl Fig 2C-D), but not

with inflammatory grade (data not shown). In both diseases, the types of cells that were producing Hh-ligands differed according to fibrosis stage. In livers with relatively little fibrosis (F0-1), production of Shh occurred predominately in hepatocytes, although the ductular cells that were present also expressed this ligand. In contrast, Ihh production tended to occur mainly in stromal and sinusoidal cells in livers with F0-1 fibrosis. As fibrosis advanced, the ductular reaction increased, Shh production progressively shifted to stromal and ductular-type cells, and these cell types were the predominant source of Shh in livers with advanced fibrosis (F3-4), although Shh production remained detectable in some hepatocytes (Fig 1C, Suppl Fig 3AD). During advanced fibrosis, however, Ihh expression remained largely confined to the stromal compartment. (Suppl Fig 1C, Suppl Fig 3E-F).

Evidence for substantially increased production of Hh-ligands in early stage viral hepatitis suggested that viral infection *per se* might induce expression of Shh and/or Ihh mRNA in liver cells. We infected cultured human hepatoma cells (Huh-7) with HCV (JFH-1 strain) to address this issue. Cell culture derived HCV JFH-1 virus was incubated with Huh-7 cells and Hh-ligand expression was assessed by qRT-PCR 72h later. Results were compared to Shh expression in Huh-7 cells that had been infected with various adenoviral vectors. Although none of the adenovirus-infected cells demonstrated increases in Shh mRNA levels **(data not shown)**, Shh mRNA expression was significantly induced in cells that were infected with HCV JFH-1 virus (Fig 1D). These findings complement the IHC data and suggest that infection of hepatocytes with the hepatitis virus directly stimulates such cells to produce Shh. Also consistent with the IHC evidence that stromal cells (rather than liver epithelial cells) were the major producers of Ihh during chronic viral infections, viral factors did not influence expression of Ihh in the cultured liver cells **(data not shown).**

Accumulation of Hh-responsive cells correlates with fibrosis stage in viral hepatitis

Next we determined if production of Hh-ligands resulted in expression of Hh-regulated genes, such as Ptc and Gli2. Healthy control livers harbored only rare Gli2(+) or Ptc(+) cells, and these localized within and near portal tracts (Figs 2A , Suppl Fig 4A). In contrast, livers that were infected with HBV or HCV demonstrated significantly increased mRNA levels of Ptc and Gli2, with greatest expression of both target gene mRNAs occurring in livers with bridging fibrosis (Fig 2B, Suppl Fig 4B). IHC verified that cells expressing Ptc and Gli2 proteins accompanied the accumulation of Ptc and Gli2 mRNAs, and revealed that the distribution of such Hh-responsive cells varied with fibrosis stage (Fig 2C, Suppl Fig 4C-D). In livers with relatively little fibrosis (F0-F1), Ptc(+) cells were scattered throughout the lobular parenchyma where they mainly localized along sinusoids. Gli2(+) cells were localized mainly in and around portal tracts in livers with F0-1 fibrosis. In livers with advanced (F3-4) fibrosis, cells that expressed Ptc and/or Gli2 appeared to localize mainly within/along fibrous septae, although the numbers of Hh-responsive sinusoidal cells within the lobular parenchyma also increased.

Hh-responsive cells localize in areas of fibrosis and liver myofibroblasts require Hh pathway activity to retain their fibroblastic phenotype

To better characterize populations of Hh-responsive cells, serial sections were stained with Sirius red to demonstrate fibrosis and αSMA to demonstrate myofibroblasts (Suppl Fig 5).

As expected, Sirius red staining increased with fibrosis stage in both HBV and HCV (Suppl Fig 5A). Consistent with evidence that myofibroblasts are major producers of collagen matrix during liver fibrogenesis, α-sma mRNA levels and accumulation of αSMA(+) cells increased with fibrosis stage in both diseases (Fig 3A, Suppl Fig 5B). Inspection of the various immuno-stained sections at higher magnification demonstrated that sinusoids harbored many αSMA(+) cells, even in livers with relatively little fibrosis (stage F0-1); cells that produce Ihh, and Hh-responsive (i.e., Ptc-positive) cells were similarly distributed (Fig 3B). These findings suggested that the myofibroblast population was enriched with Hh-responsive cells.

To more directly examine the potential significance of Hh-signaling in myofibroblatic liver cells, HSCs were isolated from residual healthy liver allograft tissue, and cultured to induce myofibroblastic trans-differentiation. Myofibroblastic human HSCs were then treated with cyclopamine, a highly specific Hh-signaling antagonist, or its biologically inactive analog, tomatidine. Primary myofibroblastic HSCs that were treated with tomatidine demonstrated Hh pathway activation, as evidenced by expression of Hh-target genes (Gli2), as well as the expected production of various myofibroblast markers, such as α-sma and collagen 1α1 (Col1α1) (Fig 3C-D). In contrast, treating myofibroblastic HSCs with cyclopamine inhibited Hh-signaling and significantly repressed expression of myofibroblast-related genes (Fig 3C-D). These *in vitro* data complement the findings in whole liver tissue, and suggest that the Hh pathway plays an important role in maintaining the myofibroblastic phenotype of stromal cells that accumulate during the progression of chronic viral hepatitis.

Hh-ligands increase expression of activation markers in human hepatic sinusoidal endothelial cells and promote angiogenesis

Further inspection of the fibrous septae in immunostained sections of HBV- or HCV-infected livers with advanced fibrosis demonstrated Ptc-expressing cells and cells with Gli2-positive nuclei lining apparent vascular spaces (Suppl Fig 6A-B). Strong expression of CD31, a marker of activated SECs, was also noted in these areas (Suppl Fig 6C). In order to determine if the activated endothelial cells were Hh-responsive, additional sections were double-stained for CD31 and Gli2. Many cells that lined vascular spaces within fibrous septae co-expressed CD31 and Gli2 in livers with chronic viral hepatitis, indicating that activated hepatic endothelial cells are Hh-responsive (Fig 4A).

To more directly assess the role of Hh signaling in hepatic vascular remodeling, primary endothelial cells were isolated from residual human liver allograft tissue from a healthy donor, placed in culture to induce activation, and then treated with cyclopamine or tomatidine. Culture-activated endothelial cells exhibited evidence of Hh pathway activation (increase in Gli2 mRNA level); cyclopamine treatment inhibited Hh-signaling and repressed endothelial cell gene expression of the activation marker CD31 (Fig 4B). In separate experiments, human sinusoidal endothelial cells were cultured in matrigel and Hh-signaling was manipulated to determine effects on vascular tube formation. In this angiogenesis model, treatment with recombinant Shh or myofibroblast-conditioned medium (a source of Hh-ligands) stimulated tube formation, while various strategies that inhibited Hh-signaling blocked tube formation (Fig 4C-E). These in vitro data support the concept that Hh-ligands

released from liver cells provide paracrine signals that lead to Hh pathway activation in neighboring endothelial cells, thereby regulating vascular remodeling responses that occur in livers with chronic viral hepatitis.

Hh-responsive cells in the ductular reaction co-express markers of liver epithelial progenitors and mesenchymal cells

Virally-infected livers with advanced fibrosis demonstrated increased numbers of ductular structures embedded within fibrous septae (Figs 1C, 2C & 5; Suppl Figs 4C-D). Many of the cells in such ductular structures stained positively for Shh (Figs 1C,5A; Suppl Fig 3C-D) and some had Gli2(+) nuclei (Fig 2C, Fig 5B, Suppl Fig 7), demonstrating that many epithelial cells in the ductular reaction were producing and responding to Hh-ligands. Autocrine Hh-signaling is a general property of progenitor cells (11, 32). Therefore, we used immunohistochemistry to assess progenitor markers in the ductular cells of the virally-infected livers. The results indicate that such cells generally express several well-accepted markers of liver epithelial progenitors, including Krt7 (Fig 5C) and CD133 (Fig 5D). Interestingly, many of these cells also stained positively for typical mesenchymal markers, such as αSMA (Fig 5E) and S100A4 (Fig 5F). Co-expression of epithelial and mesenchymal genes is an accepted characteristic of cells that are undergoing EMT (14, 33). Thus, the new results support earlier work which showed that Hh pathway activation induced EMT in immature ductular cells (8), and demonstrate that livers with chronic viral hepatitis become enriched with populations of Hh-responsive progenitors that are undergoing EMT.

Hh-responsive cells are particularly enriched in peri-tumoral stroma of hepatocellular carcinomas

Because chronic viral hepatitis is a major risk factor for HCC (2, 3), and Hh pathway activation has been demonstrated in certain HCC cell lines (21), we used immunohistochemistry to localize Hh-ligand producing cells and Hh-responsive cells in livers with chronic viral hepatitis and co-incident HCC. To our surprise, we were generally unable to demonstrate Shh, Ihh, Ptc, or Gli2 staining in malignant hepatocytes. However, the peri-tumoral stromal tissue was enriched with cells that expressed Ptc and S100A4. Laser capture micro-dissection and qRT-PCR analysis revealed robust expression of Ptc mRNA in the fibrous tissue that surrounded tumor nodules (Fig 6). Closer inspection revealed that this fibrous tissue was riddled with vascular spaces and enriched with poorly formed ductular structures. Immunohistochemistry demonstrated that fibrous tissue adjacent to tumors harbored numerous ductular structures that expressed Shh and Gli2, as well as stromal cells that expressed Ihh, Gli2 (Fig 7A,C) and/or Ptc (Suppl Fig 7B).

Further immunostaining demonstrated that these ductular structures tended to express Krt7, a marker of immature liver epithelial cells (Fig 7D **and** Suppl Fig 7C); most also stained positively for the tumor stem/progenitor markers CD133, EpCam (Fig7 E-F), and survivin (Suppl Fig 7D). Malignant hepatocytes along the edge of this peri-tumoral stroma also expressed EpCam and survivin (Fig 7F, Suppl Fig 7D), but not Krt7 (Fig 7D) or CD133 (Fig 7E). Hence, cells with features of tumor stem/initiating cells that accumulate in livers with chronic viral hepatitis are Hh-responsive, and many produce Shh. Such cells localize in

ductular structures that are embedded in tumor-associated fibrous stroma that is enriched with cells that are Hh-responsive (i.e., Ptc and/or Gli2-positive), and that produce Ihh ligand.

Discussion

Chronic infection with HBV and HCV, is a major cause of cirrhosis and liver cancer worldwide (2, 3). Nevertheless, many individuals do not develop significant liver fibrosis or neoplasia. (3, 4), and although there is little argument that viral infection increases hepatocyte death, viral levels do not reliably predict important outcomes of liver injury. At present, the mechanisms that account for the variable consequences of chronic viral hepatitis are not fully understood.

We performed a cross-sectional analysis of liver biopsy samples from patients with chronic HBV or chronic HCV and differing degrees of liver fibrosis. A sub-group of the patients had co-incident HCC. Our results suggest that inter-individual differences in the degree of Hh pathway activation that occur during chronic viral infection may mediate some of the variability in viral hepatitis outcomes. We found that chronic infection with hepatitis viruses significantly increased hepatic expression of Hh-ligands (Shh and Ihh) and target genes (Ptc and Gli2), and noted that individuals with more advanced stages of liver disease (i.e., bridging fibrosis to cirrhosis) expressed higher levels of Shh, Ptc and Gli2 than those with little or no fibrosis. In our study population, all of the HCCs occurred in livers with advanced fibrosis/cirrhosis; cirrhotic livers also tended to have the greatest net Hh pathway activity. Thus, higher levels of Hh pathway activity associated with both of the potentially fatal outcomes of chronic viral hepatitis (i.e., cirrhosis and primary liver cancer).

The design of our study (retrospective, cross-sectional analysis) and nature of our study population (humans) prevented us from manipulating Hh-signaling to test the potential cause-effect relationship between Hh pathway activity and outcomes of advanced virus-mediated liver disease in our study cohort. However, to overcome this limitation, we performed IHC to characterize the types of liver cells that produced and responded to Hh-ligands in our patients, and then used cultured human liver cells to determine if (and how) manipulating Hh-signaling altered the phenotypes of the relevant Hh-responsive cells.

Immunohistochemistry revealed that liver epithelial cells (i.e., hepatocytes and ductular cells) were the major source of Shh ligands in patients with viral hepatitis, whereas stromal cells were the predominant producers of Ihh ligands in these subjects. Introducing HCV proteins into cultured liver epithelial cells induced significant expression of Shh mRNA but had no appreciable effect on Ihh expression. To our knowledge, these data provide the first evidence that hepatitis-viral infection of liver epithelial cells stimulates such cells to produce Shh ligand, a potent morphogen and viability factor for various stem/progenitor cells.

Immunostaining also demonstrated that the major liver cell populations that typically expand during cirrhosis (i.e., myofibroblasts, activated SEC, and liver epithelial progenitors) are all enriched with cells that respond to Hh-ligands. Treating cultures of primary human myofibroblastic HSCs with cyclopamine, a highly-specific antagonist of Smoothened, inhibited Hh-signaling and repressed expression of fibroblast-associated genes. Similarly,

the Hh pathway was active in activated human liver SECs, and blocking Hh-signaling with cyclopamine repressed the activated phenotype of such cells. Recombinant Shh also induced vascular tube formation in cultured human endothelial cells, and various strategies that inhibited Hh-signaling prevented angiogenesis in that assay. We had previously demonstrated that Hh-ligands promoted viability, growth, and EMT in cultures of immature ductular cells (7-9). Together, these data indicate that Hh pathway activation is not merely an epiphenomenon that occurs during chronic viral hepatitis. Rather, it likely plays an important causal role for the accumulation of myofibroblasts, activated endothelial cells, and hepatic epithelial progenitors during this disease. Hence, virally-mediated increases in Hh-ligand production activate Hh-signaling in Hh-target cells that reconstruct virally-damaged livers, thereby influencing the levels of fibrosis, vascular remodeling, and progenitor accumulation that ensue.

Many types of adult cancers, including HCC, exhibit aberrant activation of the Hh pathway (20, 21, 34). We were unable to demonstrate Hh-ligand or target gene expression within nodules of HCC in livers with chronic viral hepatitis, but noted striking enrichment of peri-tumoral stromal tissue with a spectrum of cells that produced and/or responded to Hh-ligands, ranging from ductular-appearing cells to fibroblastic cells. Moreover, some of these peri-tumoral cells co-expressed markers of cancer stem/initiating cells and mesenchymal cells. Co-expression of epithelial and mesenchymal genes is a characteristic of cells that are undergoing EMT/MET (14, 33). Hence these findings suggest that “epithelioid” nodules of HCC were derived from sub-populations of malignantly-transformed, Hh-responsive liver progenitors. Such tumor stem/initiating cells have relatively mesenchymal phenotypes, and seem to reside in peri-tumoral stroma until Hh signaling is repressed sufficiently to permit them to undergo mesenchymal-to-epithelial transition, whereby they progressively acquire more hepatocytic features. Additional research is necessary to prove/disprove this concept. However, it is consistent with the known ability of Hh-ligands to promote EMT (8, 10), the fact that HCC cell lines display variable levels of Hh pathway activity and mesenchymal gene expression (35), evidence that viral factors induce EMT in liver cells (36, 37), and findings of EMT-related gene expression in HCV-related HCC (15, 38).Therefore, like cirrhosis, HCC may be a direct consequence of Hh-dependent remodeling of liver damage caused by chronic viral infection. If confirmed, such results would have important implications for patients with viral hepatitis because 1) inter-individual differences in Hh responsivness may underlie observed differences in the outcomes of chronic HBV/HCV and 2) Hh inhibitors may help to prevent cirrhosis and HCC in patients with these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

HCV	Hepatitis C virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HSC	Hepatic Stellate Cells
SEC	sinusoidal endothelial cells
shRNA	short hairpin RNA
Shh	Sonic hedgehog
Ihh	Indian hedgehog
Ptc	Patched
Smo	Smoothened
Gli	Glioma-associated oncogene homolog
α-sma	smooth muscle α-actin
Krt7	keratin 7
CD133	prominin 1
Epcam	tumor-associated calcium signal transducer 1
MOI	Multiplicity of infection
S100a4	S100 calcium binding protein A4
CD31	platelet/endothelial cell adhesion molecule
Hh	hedgehog
HBX	hepatitis B X protein
qRT-PCR	quantitative reverse transcription polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LCM	laser capture microdissection
Col1α1	type I α1 collagen
IHC	immunohistochemistry
FFPE	formalin-fixed, paraffin-embedded
JFH-1	HCV genotype 2a JFH-1 virus clone
EMT	epithelial-to-mesenchymal transition

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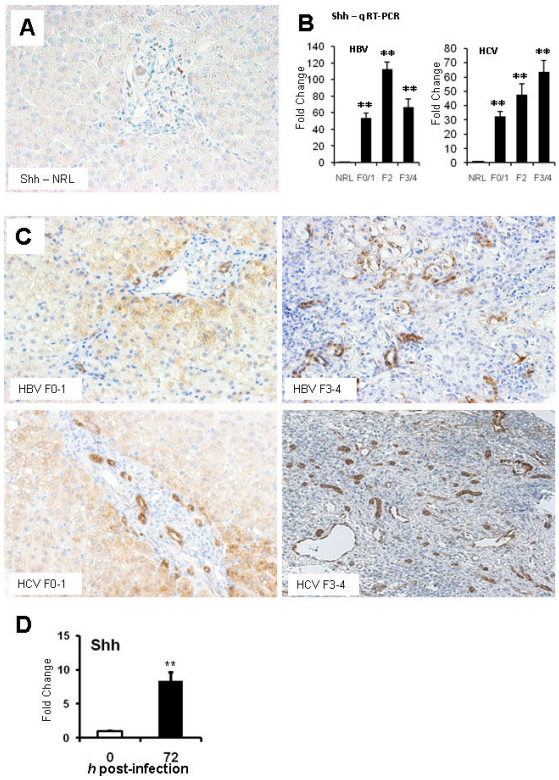

Figure 1. Liver cell production of Sonic Hh ligands (Shh) increases during chronic viral hepatitis

A) Liver section from a representative control liver without viral hepatitis (NRL). Final magnification ×200. B) qRT-PCR analysis of RNA isolated from normal liver, and HBV- or HCV-infected livers with F0/1, F2, or F3/4 fibrosis (n = 3/group). Results were normalized to Shh expression in normal control livers; means ± SEM are displayed. **P < 0.005 vs. controls. C) Immunohistochemical staining for Shh in representative sections from patients with HBV (top) and HCV (bottom). Left panels display findings in livers with F0 fibrosis; right panels display findings in livers with F3-4 fibrosis. Final magnification ×200. D-E) qRT-PCR analysis of Shh expression in Huh-7 cells infected with HCV JFH-1 (D). Results were normalized to the respective controls and mean ± SEM data are shown. **P < 0.005 vs. controls.

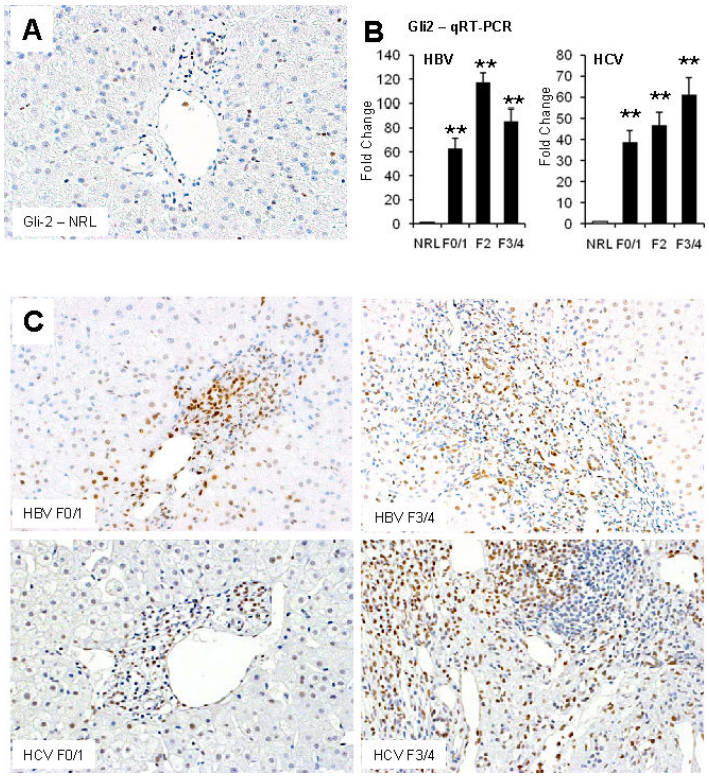

Figure 2. Liver cells that express the Hh-regulated transcription factor, Gli2, accumulate during chronic viral hepatitis

A) Liver section from a representative control liver without viral hepatitis (NRL). Final magnification ×200. B) qRT-PCR analysis of RNA isolated from normal liver, and HBV- or HCV-infected livers with F0/1, F2, or F3/4 fibrosis (n = 3/group). Results were normalized to Gli2 expression in normal control livers; mean ± SEM data are displayed. **P < 0.005 vs. controls. C) Immunohistochemical staining for Gli2 in representative sections from patients with HBV (top) and HCV (bottom). Left panels display findings in livers with F0 fibrosis; right panels display findings in livers with F3-4 fibrosis. Final magnification ×200.

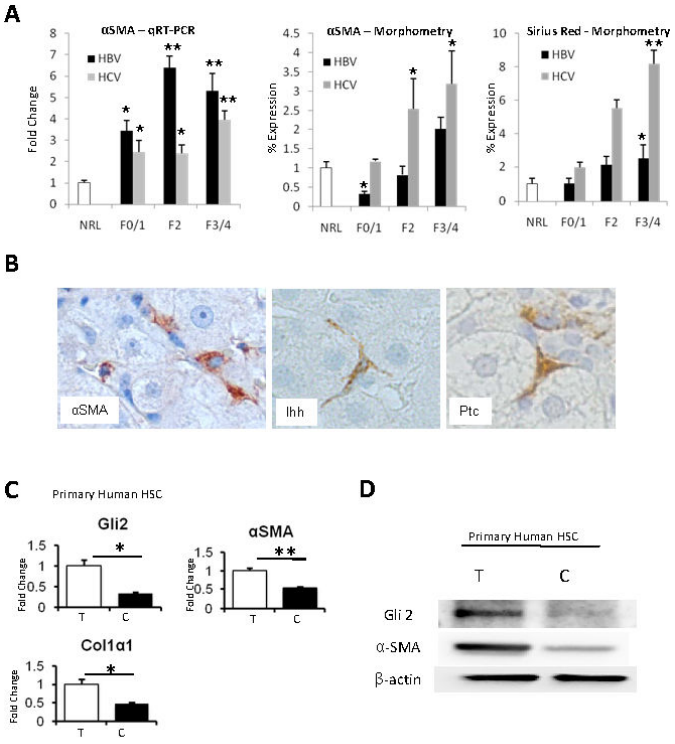

Figure 3. Myofibroblastic cells that accumulate during chronic viral hepatitis are Hh-responsive and Hh signaling is necessary for fibrogenic gene expression

A) RNA was isolated from normal liver, and HBV- or HCV-infected livers with F0/1, F2, or F3/4 fibrosis (n = 3/group); qRT-PCR was done to assess expression of α-sma (left panel); accumulation of αSMA-expressing cells (middle panel) and Sirius red stained fibrils (right panel) were quantified in serial liver sections using morphometric analysis. Means ± SEM results are graphed. *P < 0.05, **P < 0.005 vs controls. B) Immunohistochemistry demonstrating sinusoidal cell expression of αSMA (left panel), Ihh (middle panel), and Patched (Ptc) (right panel) in representative liver sections with F0-1 fibrosis. Final magnification ×600. C-D) Hepatic stellate cells were isolated from non-diseased human liver and cultured to induce transition to myofibroblastic cells (MF); then treated with tomatidine (T) or cyclopamine (Cy) for an additional 48h. RNA and protein were isolated from triplicate plates and qRT-PCR (C) and Western blot (D) were used to assess effects on gene expression. Experiments were replicated a total of three times; data were normalized to tomatidine-treated cultures. Means ± SEM are displayed. * P< 0.05, **P < 0.005 vs tomatidine-treated cultures.

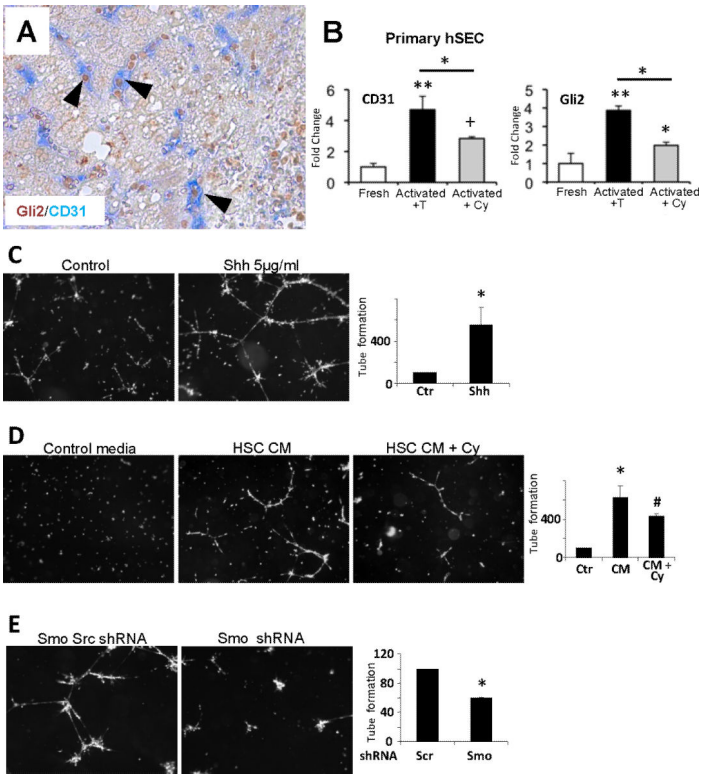

Figure 4. Activated endothelial cells are Hh responsive during chronic viral hepatitis and Hh pathway activation promotes vascular remodeling responses

A) Immunohistochemistry was used to characterize the types of cells that accumulated in fibrotic septae. Representative photomicrograph from patient with stage F3-4 chronic hepatitis C is shown. Double immunohistochemistry for Gli2 (brown) and CD31 (blue). Final magnification ×600. B) Primary liver endothelial cells were isolated from a non-diseased liver; RNA was harvested from freshly-isolated cells and from triplicate plates of cells that had been cultured to induce transition to an activated phenotype and then treated with either tomatidine (T) or cyclopamine (Cy) for an additional 48h. qRTPCR analysis was done to determine effects on expression of CD31 (left) and Gli2 (right). Data were normalized to gene expression in the freshly-isolated cells. Means ± SEM results are displayed. * $P < 0.05$, ** $P < 0.005$, + $P < 0.01$ vs fresh cells. C-D) SECs were seeded into matrigel and treated with vehicle or Shh ligand (5µg/ml) (C) and HSC condition media (CM) with or without Cy (D). E) SECs were pre-treated with lentivirus containing Smo scramble (scr) shRNA or Smo shRNA. Effects on vascular tube formation were assessed in triplicate experiments. Representative results are displayed as arbitrary units. Means ± SEM are graphed. *$P < 0.05$ vs control; # $P < 0.05$ vs condition medium alone.

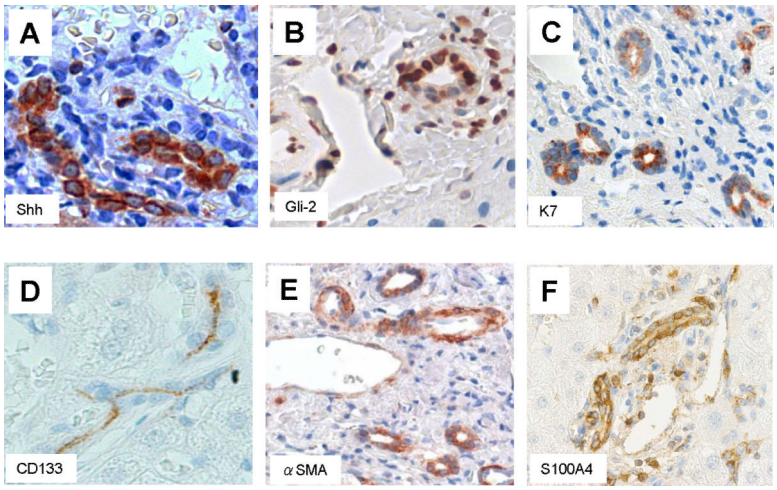

Figure 5. The ductular reaction that occurs during chronic viral hepatitis is enriched with Hh-responsive ductular cells that co-express epithelial progenitor cell and mesenchymal cell markers

Immunohistochemistry was used to characterize the types of cells that accumulated in fibrotic septae. Representative photomicrographs from patients with stage F3-4 chronic hepatitis C are shown. Mono-staining for A) Shh, B) Gli2, C) Krt 7, D) CD133, E) αSMA, F) S100A4. Final magnifications ×600.

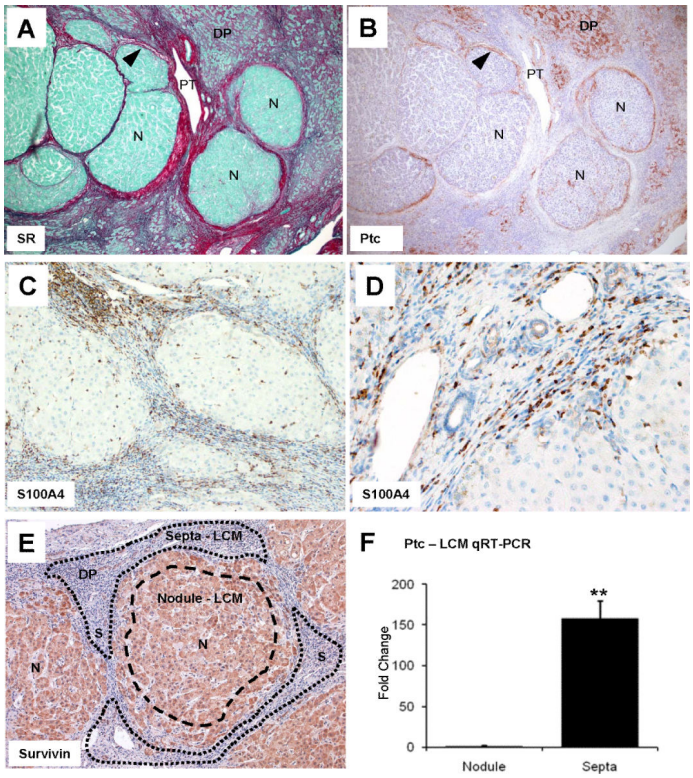

Figure 6. Peri-tumoral stromal tissue adjacent to HCC is enriched with Hh-responsive stromal cells and ductular cells that express markers of tumor stem/progenitor cells

A) Nodules of HCC (N) and adjacent peri-tumoral tissue in representative patient with chronic hepatitis C and HCC. Final magnification ×100. B-E) Immunohistochemistry for Ptc (B), S100A4 (C-D) and Survivin (E) in serial sections from this patient. Dotted lines delineate in (E) margins of samples that were procured using laser capture dissection microscopy (LCM). Final magnifications ×100 (B-C), ×400 (D) and ×200 (E). F) qRT-PCR analysis of peritumoral fibrotic septae and nodules of malignant hepatocytes from 3 patients with chronic viral hepatitis and HCC. Data were normalized to Ptc gene expression in the tumor nodules. Means ± SEM are displayed. ** $P < 0.005$ vs tumor nodules.

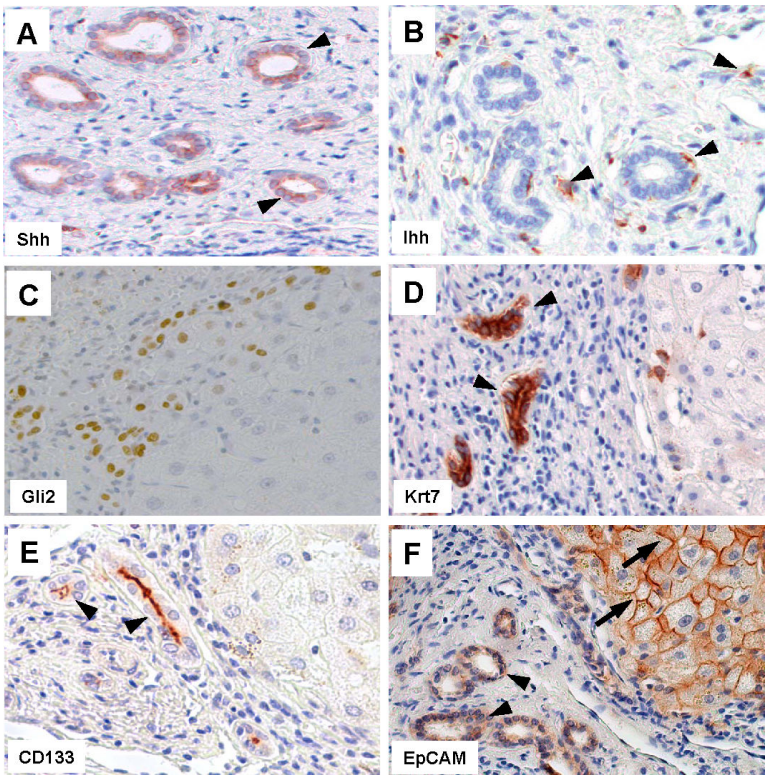

Figure 7. Peri-tumoral tissue adjacent to HCC is enriched with Hh-responsive cells

Immunohistochemistry was used to characterize the types of cells that accumulated in peri-tumoral fibrotic tissue. Representative photomicrographs are shown. Mono-staining (arrowheads) for A) Shh, B) Ihh, C) Gli2, D) Krt7, E) CD133, F) EpCam. Final magnifications ×600 (A, B, D, E and F) or ×400 (C).

Table 1

The demographic data of the selected patients from whom the tissue was used for the study.

HBV

	No. of Patients	Average Age	Gender	Inflammation	hbsag	ALT	AST
F0	5	40.4 (±16)	2M/3F	0	pos	41.5 (±5)	34.0 (±6)
F1	5	31.6 (±9)	4M/1F	0/1//2	pos	40.6 (±12)	36.7 (±10)
F2	4	41.8 (±10)	3M/1F	2/2//2	pos	56.7 (±3)	50.0 (±10)
F3/4	4	39.8 (±15)	3M/1F	2/3//2	pos	52.0 (±29)	41.5 (±15)

HCV

	No. of Patients	Average Age	Gender	Inflammation	Genotype	ALT	AST
F0	4	47.3 (±15)	3M/1F	0/1//1	1,3	55.0 (±24)	41.0 (±17)
F1	5	49.8 (±8)	3M/2F	1/2//1	1	47.4 (±11)	33.5 (±7)
F2	4	47.3 (±8)	1M/3F	2/2//1	1,2	52.5 (±9)	36.0 (±1)
F3/4	7	54.3 (±12)	3M/4F	2/3//1	1	204.3 (±88)	155.5 (±78)

HCC

	No. of Patients	Average Age	Gender	ALT	AST
HBV	4	66.2 (±12)	3M/1F	35.7 (±26)	28.6 (±14)
HCV	3	57.3 (±15)	3M	117.5 (±40)	90.5 (±46)