Detection of HCV in Bile Duct Epithelium by Laser Capture Microdissection (LCM)

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Abstract. Chronic hepatitis C affects 0.3 to 1.5% of the general population worldwide. The estimated total number of newly acquired hepatitis C virus (HCV) infections is 28,000 in the USA, with 10,000 deaths each year resulting from HCV-associated chronic liver disease. Histological examination of liver tissue from chronic HCV infection shows lymphoid aggregates or follicles in the portal triads, focal fatty change and lobular inflammation. Hepatitis-associated bile duct lesion (HBL) is seen in 5-91% of the cases. While the morphological spectrum of HBL has been well described, its pathogenesis in hepatitis C is not known. To this date, evidence supports both the direct injury and immune-mediated mechanisms, but to what extent these mechanisms are involved in the pathogenesis of HBL in chronic hepatitis C remains unclear. Our study showed the presence of HCV in the bile duct epithelium of patients with chronic hepatitis C infection, using the laser capture microdissection technique. These results will enhance our diagnostic capabilities and treatment of chronic hepatitis C infection.

There is a large reservoir of chronically infected Americans who can serve as a source of transmission to others, and who are at risk of the severe consequences of chronic liver disease such as cirrhosis and hepatocellular carcinoma.

Hepatitis C virus is a spherical, enveloped, positive sense, single-stranded RNA virus with a genome size of 9.6 kb and is classified in the Flaviviridae family. The genome consists of a 5'-noncoding region, a single large open reading frame and a 3'-noncoding region. The open reading frame encodes for a core protein (C), two envelope proteins (E1, E2) and a small putative protein of unknown function, and at least six nonstructural proteins: protease, helicase, RNA polymerase and regulatory proteins. The most important characteristic of the HCV genome is its sequence heterogeneity. A total of 6 major genotypes and more than 50 subtypes have been identified. They differ in their distribution worldwide. Infection with different genotypes results in different clinical outcomes, both in terms of disease severity and response to antiviral therapy. The consequence of the genomic diversity of HCV (quasispecies) and the tendency of the envelope gene of the virus to mutate rapidly, enables the virus to escape the immune surveillance, leading to a high rate (>80%) of chronic infections, and lack of immunity to re-infection in repeatedly exposed individuals (1).

Histological examination of liver tissue from chronic HCV infection shows lymphoid aggregates or follicles in the portal triads, focal fatty change and lobular activity. Bile duct damage is seen in 5-91% of the cases. However, there is no widespread loss of ducts and clinically cholestasis is not significant. Intrahepatic bile duct lesions are found in a large variety of liver diseases: primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), suppurrative cholangitis, parasitic infestation, graft-versus-host reaction, liver transplant rejection and drug toxicity. The bile duct lesions include proliferative, inflammatory and degenerative changes. A particular morphologic presentation was first described in 1969 by Poulsen and Christoffersen (2), as hepatitis-associated bile duct lesion (HBL). Despite the fact

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Key Words: Hepatitis C virus, bile duct epithelium, laser capture microdissection.
that virtually nothing is known about the pathogenesis of HBL, its morphological spectrum has been described in detail. The affected bile ducts are small or medium-sized (50-70 ìm in outer diameter) and the duct epithelium is swollen and rounded. The epithelial nuclei may show pyknosis or karyorrhexis; whereas, the cytoplasm is pale, mostly vacuolated or "empty looking". Often cells are partly confluent with more or less ill-defined cellular borders. The duct epithelium is almost always infiltrated with lymphocytes and occasionally with plasma cells and neutrophils, with focal cellular attenuation or loss. Duct ectasia is often seen. The basement membrane may be indistinct, but is usually described as being preserved (3). The bile duct lesions in chronic HCV infection are undoubtedly important, due to their association with more active portal and lobular inflammation. Some authors suggest that HBL may serve as a useful diagnostic marker for hepatitis C. Others have suggested a strong correlation between bile duct injury and poor prognosis and development of cirrhosis. Despite its significant role in progression of liver disease and response to treatment, the exact mechanism for the development of hepatic duct lesions in chronic hepatitis C infection remains a mystery. To this date, evidence supports both the direct injury and immune-mediated mechanisms, but to what extent these mechanisms are involved in the pathogenesis of HBL in chronic hepatitis C remains unclear. In addition, members of the Flaviviridae family are often directly cytopathic, such as yellow fever virus. Thus, HCV causing direct bile duct epithelium injury should be considered (4). In this study, bile ducts from North American patients were examined for the presence of HCV nucleic acids.

Materials and Methods

Preparation of samples. To determine the presence of HCV in bile duct epithelium of chronic hepatitis C infection, we studied liver samples obtained from 7 autopsy patients with diagnosis of chronic HCV infection, and one liver sample from a patient with no liver disease (7 males, 1 female; ages 28-59 y/o). We also obtained samples of the placenta from a patient sero-negative for HCV, as a negative control. As a control, a separate liver sample from the same patient was obtained and ethanol-fixed. Slides were stained with hematoxylin and eosin (H&E) and evaluated microscopically for HBL. For each patient, the samples were fixed in neutral buffered formaldehyde and embedded in paraffin. Liver tissue samples were snap-frozen in liquid nitrogen. Four to 8 micron serial frozen sections were cut using a cryostat. Three sections each were mounted on non-charged, non-coated glass slides. The unfixed sections were stored immediately at –80ÆC.

Laser capture microdissection. For separation of bile ducts, a LCM technique (PixCell laser capture microscope, Arcturus Engineering, Santa Clara, CA, USA) was used. Tissue sections were deparafinized with two changes of xylene dehydrated with two 100% ethanol washes and stained with hematoxylin and eosin for laser capture microdissection (LCM). The prepared (dehydrated tissue) section was overlaid with a thermoplastic membrane mounted on an optically transparent cap. The cap was placed above the selected area containing cells of interest. The cells were captured by focal melting of the membrane through laser activation. The efficacy of transfer in our institution is usually between 70-90%.

RNA isolation. RNA was extracted using Qiagen tissue extraction columns (Valencia, CA, USA). For detection of the HCV sequence, the extracted RNA was used for RT-PCR.

Reverse transcription. A portion (40 ìl) of the sample was digested with DNase I (Roche Applied Sciences, Indianapolis, IN, USA) to remove any contaminating DNA molecules. DNase I was inactivated per the manufacturer’s instructions. The sample was split into two separate tubes. The samples were heated to 65°C for 5 minutes and chilled immediately on ice. The reverse primer (100 pM) was added to both tubes (RT+ and RT-) and 10 ìl of master mix was added. The master mix consisted of 6 ìl of 10 mM dNTPs mixture; 6 ìl of 10x RT buffer; 5 ìl of 25mM MgCl2; 2 ìl of 0.1M DTT and 1 ìl of RNaseout (Ambion, Austin, TX, USA). The samples were placed at 42°C and RT was added (superscript II
RNase H enzyme) to the RT + tube only and incubated for 1 hour at 42°C. To inactivate the enzyme, the samples were heated at 70°C for 15 minutes.

PCR. A master mix was prepared for PCR of the final volume of 100 µl using the forward primer (100 pM for each) and reverse primer; 10x dNTPs; PCR buffer; 25mM MgCl and ampligold Taq polymerase.

Control b2M RT primer: CCT CCA TGA TGC TTA CAT G
Control b2m Forward: 5'fluorescein- CTT GTC TTT CAG CAA GGA CTG G
HCV primers to 5'noncoding region:
Forward: TCT GCG GAA CCG GTG AGT AC
Reverse: CCC AAA TCT CCN GGC ATW GAG

Three microliters of RT- and RT+ were added to two PCR tubes with the master mix, and 27 µl of RT- and RT+ cDNA into two other tubes with master mix. A reagent blank was also prepared with only the master mix. The samples were placed in a thermocycler and the cycling parameters were as follows: 10 minutes at 95°C; 30 seconds at 94°C; 15 seconds at 60°C; 15 seconds at 72°C; 10 minutes at 72°C for 35 cycles. cDNA was fractionated on 2% agarose gel, stained with Sybergold (Molecular Probes Inc, Eugene, OR, USA).

Results

Seven of the 8 specimens (1 sero-negative specimen and 6 sero-positive specimens) had detectable cellular mRNA. Our study showed the presence of HCV in the bile duct epithelium of six patients with chronic hepatitis C infection, using the laser capture microdissection technique. Figure 1 demonstrates amplification of the positive control beta 2 microglobin mRNA in paraffin-embedded sections. Figure 2 demonstrates amplification of HCV nucleic acids in laser captured paraffin-embedded sections. Figure 3 shows the dissection of the bile duct using the laser capture.

Conclusion

The bile duct lesions in chronic HCV infection are undoubtedly important. Although, our study shows the presence of HCV in the bile duct epithelium, it still does not prove that a direct cytopathic effect is responsible for HBL. Despite its significant role in progression of liver disease and response to treatment, the exact mechanism for the development of hepatic duct lesions in chronic hepatitis C infection remains a mystery.

Our study does indicate that, using LCM, one can detect HCV within bile duct epithelium. The strengths of this LCM are simple: only the cells of interest for our particular indication were microdissected and subjected to RT-PCR which generated cDNA in 5 hours compared to 3-4 days turn around using conventional techniques. Thus, our results may provide a method to enhance the diagnostic capabilities and treatment of chronic hepatitis C infection.

Acknowledgements

This study was funded by a grant from the College of American Pathology awarded to Ewa Fillipowicz.

References


Received March 28, 2005
Accepted April 12, 2005