



Review

Chronic Hepatitis C Virus Infection and the Pathogenesis of Hepatocellular Carcinoma

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Abstract. There is a strong epidemiologic relationship between chronic hepatitis C virus (HCV) infection and the development of hepatocellular carcinoma, although the cellular and molecular mechanisms of tumor formation remain to be firmly established. Clearly, HCV is associated with the development of chronic hepatitis and cirrhosis, so it may contribute to hepatocarcinogenesis as a consequence of its central role in the appearance and progression of necroinflammatory liver disease. There is also increasing evidence for a direct contribution of several HCV gene products to the development of the transformed phenotype, although none of the putative mechanisms involved in tumor formation have been strongly supported by *in vivo* evidence. Even if HCV is not shown to be a complete carcinogen, it may act as a co-carcinogen with underlying (serologically negative) hepatitis B virus infection, in the context of alcoholic cirrhosis, and in patients with long term exposure to chemical hepatocarcinogens such as aflatoxin B1.

Key words: hepatitis C virus; hepatocellular carcinoma; tumor pathogenesis.

Introduction

Hepatitis C virus (HCV) is a major etiologic agent of post-transfusion hepatitis²⁹. Although this was suspected during the 1970s and 1980s by transmission experiments of “non-A, non-B hepatitis” to chimps^{2, 114}, the partial cloning of the HCV genome in 1989¹⁸, followed by the rapid development of serological assays to detect infection in blood⁶³, have reduced the incidence of HCV transmission by more than 85%. In the

United States alone, HCV infections declined from an annual incidence of roughly 180 000 in 1985 to 28 000 a decade later⁴. The risk of acquiring HCV now is between 0.01–0.001% per unit transfused¹⁰⁸. Among the remaining major risk factors for the transmission of HCV, the sharing of needles among intravenous drug users, reuse of improperly sterilized glass syringes by physicians in poorer countries, and occupational or domestic exposure to contaminated blood or body fluids continue to spread HCV at significant rates⁴. Anti-HCV

Abbreviations used: ALT – alanine aminotransferase, CLD – chronic liver disease, CPE – cytopathic effect, CTL – cytotoxic T lymphocyte, eIF2 α – eukaryotic initiation factor 2 α , E2 – hepatitis C virus-encoded envelope protein 2, Grb2 – growth factor receptor bound protein 2, HBV – hepatitis B virus, HCV – hepatitis C virus, HCC – hepatocellular carcinoma, IFN- γ – interferon γ , PKR – interferon-induced protein kinase, NS5A – hepatitis C virus nonstructural protein 5A, PCNA – proliferating cell nuclear antigen, RT/PCR – reverse transcriptase/polymerase chain reaction, TNF- α – tumor necrosis factor α , SH3 – src homology domain 3.

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has also been detected in 10–20% of hemodialysis patients⁸². The increased frequency of anti-HCV with time in such patients suggests that HCV transmission occurs through shared supplies and dialysis machines⁸². As a consequence of these risk factors, an estimated 1.8% of the U. S. population has detectable anti-HCV, which translates into approximately 4 million infected individuals who are at risk for the development of chronic liver diseases (CLD), including cirrhosis and hepatocellular carcinoma (HCC)⁴. Hence, considerable HCV transmission occurs, and infection remains as an important public health problem.

Pathogenesis of HCV Infection

Acute exposure to HCV results in two major outcomes. In most patients, acute infection results in the appearance of HCV in the blood by 1–2 weeks postexposure^{5, 27}. In these cases, HCV is detected by reverse transcriptase/polymerase chain reaction (RT/PCR) of its RNA genome^{39, 50}. By 7 weeks postexposure, up to 30% of infected patients develop clinical symptoms, while the rest are subclinical, although nearly all have elevated alanine aminotransferase (ALT) levels, which is a biochemical marker in blood reflecting liver cell damage. Anti-HCV usually develops during the peak of ALT and persists for many years. Acute hepatitis usually resolves in 2–12 weeks and is signaled by clearance of HCV RNA from blood, the return of ALT to normal levels, and the disappearance of symptoms⁴⁸. Unfortunately, the resolution of acute infection occurs in only about 15% of cases.

In up to 85% of acutely infected patients, the ALT levels remain elevated and the HCV RNA persists in blood^{3, 27, 73}. In acutely symptomatic patients, symptoms usually resolve, but liver disease often progresses and becomes more severe with the passage of time. Most patients remain asymptomatic for 10–30 years, even though they have persistently elevated ALT levels and moderate or severe CLD^{48, 109}. For this reason, chronic HCV infection has been dubbed the “silent epidemic”⁶⁴. Interestingly, up to 1/3 of chronically infected patients with persistent viremia have consistently normal ALT levels, while ALT levels in others are intermittently elevated^{6, 23}. Among chronically infected patients with elevated ALT who become symptomatic, up to 35% develop cirrhosis by year 20 after infection, while almost a quarter of chronically infected patients develop HCC by year 30^{59, 109, 122}. These observations underscore the variability in the natural history of in-

fection and highlight the difficulties in assessing the risk that chronically infected patients will develop liver cancer.

Association of HCV with HCC

The association between HCV and HCC predates the discovery of the virus, since early on it had been shown that there was a close relationship between non-A, non-B hepatitis virus infection and the development of HCC^{57–59, 103}. However, once HCV was discovered¹⁸ and serological assays became available for its detection⁶³, a close association was established between anti-HCV and HCC all over the world^{12, 22, 43, 56, 102, 112, 125, 128}. In southern Europe and Japan, for example, more than half of the reported HCC has been associated with HCV^{12, 22}. On the other hand, the prevalence of anti-HCV in Alaskan patients with HCC is nearly zero. Intermediate frequencies of anti-HCV have been reported in patients from Greece, Austria, and Taiwan^{8, 40, 53}, indicating geographic variation in this association. Worldwide, there are an estimated 170 million people who are chronically infected with HCV, and the annual incidence rate of newly diagnosed HCC cases associated with HCV infection ranges from 1–7% in different populations²⁶. This high incidence, however, does not uniformly take into account the contribution of other important risk factors, such as age, gender, alcohol intake, long term aflatoxin consumption, and coinfection with HBV. In addition, most of these observations were made at about 20 years postinfection, suggesting that they do not represent the true incidence of HCV-associated HCC in chronically infected patients, nor the contribution of HCV to the development of this tumor.

Putative Indirect Mechanisms Whereby HCV Contributes to HCC

Most HCV-associated HCCs arise from a background of cirrhosis. This implies that chronic HCV infection may contribute importantly to HCC by stimulating a long-term necroinflammatory response that promotes the development of fibrosis and cirrhosis^{11, 43, 57, 59}. In one study, for example, between 5 and 28% of chronically infected patients with cirrhosis also had HCC²⁴. This suggests that HCV contributes indirectly to the development of HCC. While the mechanisms of

chronicity are not firmly established, HCV is highly immunogenic, since both humoral and cell-mediated immune responses have been detected against most of the virus antigens in chronically infected patients^{16, 62, 111}. However, the specificities and duration of cytotoxic T lymphocyte (CTL) responses are often modest and variable in chronic infections, while they are stronger and more consistently present in patients that resolve acute infections^{16, 89}. Further, there do not appear to be clear immunodominant epitopes in chronic infection⁶², suggesting that the virus fails to reproducibly present consistent targets and/or trigger immune recognition. In chronic infection, it is likely that HCV survives immune elimination by developing "escape" mutants⁸⁷. Such infections are characterized by the presence of virus particles in the blood that have slightly different sequences, known as quasispecies⁸⁷. Immunological responses generated against dominant quasispecies result in the clearance of virus particles encoded by these quasispecies from blood, as well as the removal of hepatocytes infected with the corresponding quasispecies. Following acute exposure to HCV, quasispecies diversity decreased among those that recovered, remained stable in those who developed mild CLD, and increased in those who developed severe CLD^{28, 47}, suggesting a correlation between quasispecies diversity and outcome. These quasispecies may arise, in part, due to the lack of proofreading function in the virus-encoded polymerase during replication⁷⁸. The dominant sequences observed at any time is likely to result from immunological selection. Hence, chronicity is promoted by the genetic heterogeneity of the virus, combined with the likelihood that this heterogeneity keeps the virus one or more steps ahead of the host immunological responses aimed at the elimination of HCV and resolution of CLD.

The persistence of HCV during chronic infection is not only important for the appearance and progression of CLD, but also for the development of HCC. Indeed, HCV RNA has been detected in both tumor and surrounding non-tumor liver tissue of patients with HCC^{25, 37, 42}. Although the levels of HCV RNA in tumor tissue is generally lower than surrounding non-tumor liver¹¹⁸, tumor cells appear to support virus replication, since both plus- and minus-strand RNA were observed in HCC⁴⁹. In addition, HCV gene expression has been detected in HCC cells¹²⁴, further suggesting an association between virus and tumor. However, the HCV genome does not seem to be integrated into the host DNA^{25, 31}, so that the mechanism(s) which support(s) persistence of the viral genome in cells during chronic infection remain(s) to be worked out.

Putative Direct Mechanisms Whereby HCV Contributes to HCC

HCV has a number of properties similar to that of flaviviruses^{99, 115}, and since flaviviruses cause cytopathic effects (CPE), it is possible that the pathogenesis of HCV infection involves the direct injury or destruction of the infected cells by the virus. In this context, some chronically infected patients with CLD who are immunosuppressed develop a rapid and severe exacerbation of CLD, followed by liver failure and death²¹, implying CPE as contributing significantly to this outcome. The development of hepatitis in some transgenic mice expressing HCV envelope and core antigens⁴⁶ suggests that the expression of these proteins can be directly cytopathic. The development of steatosis in HCV core transgenic mice⁸¹ is also consistent with a direct alteration of cellular metabolism by core protein. However, other studies have shown that HCV transgenic mice expressing virus structural proteins develop no liver pathology^{60, 66, 90}, suggesting that the core and envelope proteins are not directly cytopathic. The differences that distinguish these results remain to be sorted out.

In the context of pathogenesis, most evidence is consistent with the hypothesis that the development and progression of HCV-associated CLD and HCC is mediated by immune responses against virus-infected cells. For example, in chronic HCV, there is a correlation between intrahepatic lymphoid aggregates and the severity of CLD⁶⁵. CD8⁺ T cells dominate HCV-associated hepatitis and they are capable of lysing a variety of target cells *in vitro*^{62, 111}. In addition, CD4⁺ T cells have been shown to proliferate in response to HCV proteins *in vitro*^{67, 89}. HCV infection is also associated with increased levels of inflammatory cytokines (e.g. TNF- α and IFN- γ) that may contribute to hepatocellular injury^{10, 61, 89, 111}. All of these lines of evidence are consistent with an immune-mediated pathogenesis for HCC. The finding of no correlation between ALT and virus levels in the blood^{17, 85, 100}, and that some chronically infected HCV patients with virus in blood have reportedly normal transaminases²³, also suggest that HCV is not cytopathic. This is further supported by the finding that more than half of infectious volunteer blood donors have normal transaminases⁷⁵. In experimental systems, transgenic mice replicating HCV do not develop any pathology, suggesting that HCV is not directly cytopathic⁷². Conditional expression of HCV proteins in transgenic mice, followed by the development of T cell-dependent liver disease¹²⁶, is also consistent with the immune-mediated nature of the as-

sociated liver disease. The role of virus-specific CTL has also been demonstrated in HLA-A2.1 transgenic mice by immunization of these mice with the appropriate HCV epitopes^{107, 127}. Hence, there is both clinical and experimental evidence suggesting that the pathogenesis of chronic HCV-infection, which is important for the development of HCC, is immune mediated.

In addition to triggering anti-viral immune responses, the action of HCV-encoded gene products may also contribute directly to the pathogenesis of CLD and HCC. For example, the HCV core protein sensitizes the human hepatoblastoma cell line, HepG2, to anti-Fas- and TNF- α -mediated apoptosis^{101, 130}. In addition, HCV core protein has been shown to bind to and stimulate the *trans*-activation properties of the tumor suppressor p53, which also promotes apoptosis⁶⁹. If this occurs in the chronically infected liver, it would promote hepatocellular turnover and the accumulation of mutations in rapidly regenerating cells that have limited time for lesion recognition and repair. Expression of HCV core protein in human cervical epithelial cells, however, inhibited cisplatin-mediated apoptosis⁹⁴. Independent observations have also shown that HCV core protein has antiapoptotic properties^{71, 95}. If this occurs in infected liver, it would help to sustain the survival of infected hepatocytes replicating HCV, thereby promoting chronic infection. These observations also point out that the function of HCV core protein may depend upon the cell type or the state of the cell in which it is expressed, and perhaps whether core protein is expressed in the context of HCV replication or independent of it.

The finding that HCV core protein may alter the response of cells to apoptotic stimuli suggests a direct role for HCV in the development of HCC. This is further supported by the finding that HCV core protein activates the human *c-myc* promoter⁹³. Interestingly, *c-myc* gene amplification has also been detected during tumor progression¹, suggesting that the sustained elevation of *myc* expression may be an important component in the pathogenesis of HCC. This is further supported by the finding that overexpression of *c-myc* and transforming growth factor α in transgenic mice results in a major enhancement of tumor development^{105, 106}. HCV core protein also transforms the rat embryo fibroblast cell line, Rat-1¹⁵. HCV core protein also appears to cooperate with an activated *ras* oncogene in transformation of rat embryo fibroblasts⁹², although this observation has not been confirmed¹⁵. Strikingly, at least one strain of transgenic mice overexpressing the HCV core antigen develops HCC in the absence of inflammatory liver disease⁸⁰. This not only underscores the potentially direct contribution of high levels of HCV

core protein to the development of HCC, but also suggests that necroinflammatory liver disease and hepatocellular turnover are not absolute requirements for the pathogenesis of this tumor.

The integrity of the p53 gene and p53 protein function appear to be important in the pathogenesis of HCC, since p53 mutations occur in high frequency during tumor progression^{77, 83, 86}. As indicated above, the HCV core gene appears to bind and stimulate the *trans*-activating activity of p53⁶⁹, but contrasting evidence suggests that the HCV core protein transcriptionally represses the p53 promoter⁹⁶, suggesting decreased p53 expression. The levels of exogenous p53, combined with the use of different cell lines in these contrasting studies, may account for the differences. HCV core protein has also been shown to repress the *p21^{WAF1/CIP1/SDI1}* promoter in several cell types in a p53 independent manner⁹⁷. Down-regulation of *p21^{WAF1}* expression may perturb cell cycle checkpoint control at G1-S and contribute to the escape of HCV-infected cells from senescence. Interestingly, it has also been reported that the HCV-encoded nonstructural protein 3 (NS3), also binds to p53⁵². Although the consequences of NS3/p53 complex formation is uncertain, NS3 has been shown to transform NIH 3T3 cells¹⁰⁴ and inhibit actinomycin D-induced apoptosis (which is p53 dependent)³², suggesting that NS3 may inactivate p53. However, in the context of the virus life cycle, NS3 acts as a serine proteinase that is involved in the cleavage and maturation of the HCV nonstructural proteins⁷, and also acts as a helicase and ATPase, which is likely to play an important role in virus replication¹¹³. Since the transformation of NIH 3T3 cells mapped to the proteinase domain of NS3¹⁰⁴, it is possible that NS3 activates oncogene proteins or inactivates tumor suppressor proteins by cleavage. Alternatively, or in addition, the NS3 helicase activity may promote genetic instability in cellular DNA by stimulating DNA recombination¹¹⁵. However, the contribution of these mechanisms to the development and/or progression of HCC remain to be evaluated.

Based upon genome sequence heterogeneity, HCV clones have been divided into more than 11 genotypes (1, 2, 3, ...), and heterogeneity within virus genotypes further subdivided into a total of more than 50 subtypes (a, b, c, ...) ^{14, 110}. Among these, HCV subtype 1b appears to be more commonly associated with HCC than other subtypes^{13, 44, 129}. The enhanced oncogenicity of subtype 1b compared to other subtypes is in doubt, however, since 1b is the predominant subtype in many infected populations⁶⁸, and not all studies see a close association between subtype 1b and HCC^{9, 84, 98}. The

correlation between 1b infection and CLD may reflect the increased propensity of subtype 1b to establish chronic infections^{13, 54} and to be associated with CLD^{13, 30}. However, other work has shown no correlation between subtype of infection and CLD^{9, 41}, suggesting that cirrhosis is the dominant risk factor for tumor development¹²⁰. These results suggest that the pathogenic potential of HCV subtypes may be variable in different populations, and that other risk factors also contribute to the development of HCC to varying extents throughout the world.

Only about 20–25% of chronically infected patients with HCV respond to IFN treatment, as measured by clearance of viral RNA and normalization of transaminases, within one year after the start of therapy¹¹⁷. Prolonged IFN therapy in responders with CLD^{74, 88}, or successful retreatment of partial responders¹²³, has shown a statistically significant decrease in the incidence of HCC. Among non-responders, the most common HCV sequence found corresponds to genotype 1⁷⁰. Closer examination has shown that sequence variability within the nonstructural protein 5A (NS5A) of HCV subtype 1b correlates with IFN resistance^{13, 30}. Further work has shown that NS5A of genotype 1 can directly bind to and block the function of the IFN-induced protein kinase, PKR^{33, 35}, which is an immediate early effector of the cellular antiviral response induced by IFN^{33, 34}. PKR phosphorylates eukaryotic initiation factor 2 α (eIF2 α), thereby inhibiting viral mRNA translation and replication²⁰. PKR has also been identified as a tumor suppressor⁷⁶ that regulates stress-induced apoptosis in dividing cells²⁰. Independent observations have also shown that the HCV-encoded envelope 2 (E2) protein from most subtypes also inhibits PKR¹²¹, so that the combined action of NS5A and E2 may result in sustained virus replication and abrogation of negative growth regulation. Sustained virus replication promotes chronicity, while the abrogation of negative growth regulation may be an important step whereby HCV contributes to the development of HCC. The inhibition of IFN-mediated responses is also important in the context of virus replication, since without this inhibition the double-stranded viral RNA formed during HCV replication would otherwise strongly induce an IFN response, which would sharply decrease the frequency whereby acute HCV infection lapses into chronic. Hence, inhibition of the IFN response appears to link virus replication to chronicity and the development of HCC³⁶.

The role of NS5A in the biology of HCV is poorly understood, although its presence in complexes with other HCV nonstructural proteins suggest that NS5A

may be a cofactor in virus replication⁹¹. Removal of the amino terminal one third of NS5A yields a truncated polypeptide that has been shown to have *trans*-activation activity^{19, 55, 119}. Consistent with these roles is the finding of full-length NS5A in the perinuclear cytoplasm, which is the site of virus replication, and of amino-terminally truncated NS5A in the nucleus, where it can act as a *trans*-regulatory protein^{51, 79}. It is thought that in infected cells the amino-terminal region of NS5A blocks the *trans*-regulatory and nuclear localization domains located in the carboxy-terminal 2/3 of the molecule. A conformational change, promoted by binding of NS5A to other proteins or by the phosphorylation of NS5A, may result in the nuclear translocation of a protein with *trans*-regulatory activities⁹¹. The translocation of NS5A to the nucleus may repress the transcription of the cyclin kinase inhibitor, p21^{WAF1/CIP1/SDI1} and stimulate the expression of proliferating cell nuclear antigen (PCNA)³⁸, resulting in stimulated cellular growth. In addition, NS5A has recently been shown to interact with a cellular protein containing src homology (SH3) binding domains, which may be a tumor suppressor⁴⁵. Further, NS5A appears to bind to growth factor receptor-bound protein 2 (Grb2) adaptor protein, and may inhibit signal transduction which normally regulates cell growth¹¹⁶. While the functional consequences of these interactions remain to be studied, NS5A stimulates anchorage-independent growth of NIH3T3 cells in soft agar and promotes tumor formation of these cells in nude mice³⁸, suggesting that NS5A may contribute to increased cellular growth and to tumor formation. However, it is not clear whether the behavior of NS5A in NIH3T3 cells accurately reflects the effects of this protein upon hepatocytes, nor whether any of the molecular based mechanisms discussed above contribute to malignant transformation.

Conclusions

There appears to be a close correlation between chronic infection with HCV and the development of HCC. Most of the time, HCC develops in the context of cirrhosis, and it is likely that co-infection with HBV and/or chronic alcohol intake contribute importantly to HCV-associated HCC because they also promote the development of cirrhosis. Beyond these epidemiological associations, however, the mechanism(s) whereby HCV brings about the development of HCC are not known. It is likely that the ability of HCV to establish and maintain chronic infections, often characterized by

ongoing necroinflammatory liver disease, contributes importantly to the development of HCC. In addition to these indirect mechanism(s), there is increasing evidence that HCV may contribute directly to HCC by the action of one or more of its encoded gene products, which include NS3, NS5A, core and E2. The relative contributions of these proteins to the development of tumor remain speculative, as does the idea that tumor formation requires their combined action. The further development of cell culture systems that support virus gene expression and replication, as well as animal models of pathogenesis, will go far in dissecting the indirect and direct contributions of HCV to HCC. These systems will also permit evaluation of cofactors, such as ethanol and HBV, in the appearance of this tumor type.

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