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EDITORIAL

Occult persistence and lymphotropism of hepatitis C virus infection

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Abstract

Recent discovery of occult hepatitis C virus (HCV) infection persisting after spontaneous or antiviral therapy-induced resolution of hepatitis C was made possible by the introduction of nucleic acid amplification assays capable of detecting HCV RNA at sensitivities superseding those offered by clinical tests. Although individuals with this seemingly silent HCV infection are usually anti-HCV antibody reactive and have normal liver function tests, occult HCV infection has also been reported in anti-HCV-negative individuals with persistently elevated liver enzymes of unknown etiology. Studies have shown that HCV RNA can persist for years in serum, lymphomononuclear cells and liver in the absence of clinical symptoms, although histological evidence of a mild inflammatory liver injury can be occasionally encountered. Furthermore, while HCV RNA can be detected in circulating lymphoid cells in approximately 30% of cases, a short-term culture under stimulatory conditions augments HCV replication in these cells allowing detection of virus in otherwise HCV-negative cases. HCV infects different immune cell subsets, including $CD4^+$ and $CD8^+$ T lymphocytes, B cells and monocytes. Studies employing clonal sequencing and single-stranded conformational polymorphism analyses have revealed unique HCV variants residing in immune cells, further strengthening the notion of HCV lymphotropism. Overall, the data accumulated suggest that occult HCV infection is a common consequence of resolution of symptomatic hepatitis C and that examination of the cells of the immune system is an effective approach to diagnosis of HCV infection and its long-term persistence. Further work is required to fully realize pathogenic and epidemiological consequences of occult HCV persistence.

Key words: Hepatitis C virus; Chronic hepatitis C; Occult viral persistence; HCV lymphotropism; Consequences of occult HCV persistence

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INTRODUCTION

Hepatitis C virus (HCV) is an important human pathogen which infects over 170 million people world-wide and causes symptomatic chronic hepatitis in up to 85% of those inflicted. In a significant number of patients, chronic hepatitis C (CHC) eventually progresses to fibrosis, cirrhosis and hepatocellular carcinoma (HCC). In fact, it is estimated that cirrhosis can manifest in up to 35% of patients with CHC, of whom approximately 3% would develop HCC[1,2]. End-stage liver disease due to CHC is currently the number one reason for liver transplantation in many parts of the world. The global socioeconomic burden of HCV infection is further magnified by hundreds of thousands of infections identified each year.

HCV is a positive single-stranded RNA virus which belongs to the *Flaviviridae* family and replicates by making the so-called negative strand, which is also referred to as the anti-genomic strand. The virus genome of approximately 9600 base pairs in length contains an internal ribosomal entry site (IRES) located at the 5'-untranslated region (5'-UTR), which drives the translation of the viral RNA transcript. Subsequent processing of the polyprotein precursor gives rise to over ten proteins, including structural proteins which form the viral nucleocapsid and envelope, as well as several non-structural proteins which are essential to replication[3].

The current standard treatment for CHC is a combination of pegylated-interferon alpha and Ribavirin (P-IFNα/RBV), which is administered for 24 wk to patients infected with HCV genotype 2 or 3 and for 48 wk

to those with genotype 1 or 4[4,5]. At present, patients who are serum HCV RNA non-reactive for at least 6 mo after completion of treatment, as determined by clinical laboratory assays, of which the sensitivities range between 9.6 and 615 IU or 30 and 1000 virus genome copies (also referred to as virus genome equivalents, vge) per mL, are considered to have achieved a sustained virological response (SVR) and would clinically be deemed "cured" of HCV. Thus, by this definition, SVR is attainable in approximately 40%-45% of patients infected with genotype 1[6,7] and in up to 69% of those carrying genotype 4[5]. HCV genotypes 2 and 3 are generally easiest to treat with up to 80% of patients afflicted with these strains achieving a SVR, as defined above[6,7].

IDENTIFICATION OF OCCULT HCV INFECTION

In the past four years, the identification of a new entity of HCV infection termed as occult HCV infection was made possible by the introduction of research assays which are capable of detecting HCV RNA at lower quantities ($\leqslant$ 2 IU or $\leqslant$ 10 virus genome copies per mL) than those used in clinical laboratories. One such research assay sequentially involves: (1) a reverse transcription (RT) of high quality intact total RNA extracted from serum or plasma, peripheral blood mononuclear cells (PBMC) or, when feasible, hepatic tissues; (2) a two-round (i.e., direct and nested) amplification of the resulting cDNA by polymerase chain reaction (PCR) using primers spanning different regions of the HCV genome; and (3) validation of the amplified products by nucleic acid hybridization (NAH) to recombinant HCV DNA probe[8]. By employing this assay or those with comparable sensitivities, low levels of HCV RNA can be detected in individuals for many years after clinical and biochemical recovery from hepatitis C[9-12]. In our studies, HCV RNA loads, as determined by the aformentioned method, in individuals who were followed for up to 7 years after SVR, were usually below 100 virus copies per mL of plasma or serum and, in most cases, ranged 100-1000 virus copies per 10^7 circulating lymphoid cells[9,13]. Comparable levels of HCV genomes were also observed by others[10]. Furthermore, longitudinal analyses of serum or plasma and PBMC samples obtained from the same patients at different time points of SVR duration or after spontaneous recovery from hepatitis C revealed that HCV RNA typically would not fluctuate by more than ten-fold between collections and that screening sequential samples enhanced identification of occult HCV persistence[8,13,14]. In this regard, when serum samples collected 12 mo after the first one were analyzed, the overall HCV RNA positivity was increased by as much as 15% of the cases examined[13].

The discovery of occult HCV infection has, in essence, directly challenged the accepted paradigm that apparent complete resolution of hepatitis C, either spontaneously or therapeutically-induced, would be indicative of eradication of HCV[8]. It should be pointed out that although HCV persistence after resolution of CHC was first made evident from studies using the RT-PCR/NAH or equivalent research assays, data from more recent studies suggested that this form of clinically unapparent, but molecularly evident HCV infection could also be identified when clinical assays of enhanced sensitivity are employed. On this note, it was reported that using the Roche Cobas-Amplicor assay (sensitivity: 50 virus copies/mL), HCV RNA was detected in freshly isolated blood mononuclear cells of approximately 20% of individuals with clinical SVR[12]. Furthermore, in another study conducted by another group, over 11% of CHC patients who initially failed IFNα monotherapy, but achieved clinical SVR after successful completion of P-IFNα/RBV were also found to carry residual HCV RNA when their sera were tested by the Cobas-Amplicor assay[15].

In addition to the documentation of HCV RNA in serum or plasma, PBMC and hepatic tissue in patients with resolved hepatitis C in whom alanine aminotransferase (ALT) levels were deemed repeatedly normal, HCV genomes were also identified in the same three aforementioned compartments in patients with persistently elevated liver enzymes, who were consistently negative for serological markers typical of a *bona fide* HCV infection[11,16-18].

LYMPHOTROPISM OF HCV

Although hepatocytes are considered to be primary targets of HCV, clinical and experimental evidence strongly indicates that the virus also invades and replicates in cells of other organs, particularly the immune system[19-21]. In doing so, HCV may effectively equip itself with one of the most efficient mechanisms to establish long-term, if not life-long, persistence, as it has been documented for other viruses equally capable of inducing protracted infections[22-24]. The notion of HCV lymphotropism is further supported by a greater representation of disorders of the lymphatic system in patients with CHC than in those without, including type Ⅱ mixed cryoglobulinemia[25] and non-Hodgkin's lymphoma[26].

Recent findings from our studies with different cohorts of patients with either spontaneous or therapy-induced resolution of hepatitis C showed that HCV RNA, on average, is detectable in freshly isolated PBMC in about 30% of cases, at levels ranging 100-1000 virus copies per 10^7 cells. However, in approximately 10% of such individuals, HCV RNA can reach 10^4 virus copies per 10^7 cells or higher, a level which is typically observed in patients with CHC[13]. In individuals where PBMC were apparently negative for HCV RNA, the use of mitogen cocktails supplemented with interleukin-2 (IL-2) and IL-4 to stimulate T and B lymphocytes and monocytes in 72-h cultures, augments HCV replication in the respective cells leading to enhanced detection of the residing virus[9,13,27]. Using this approach, HCV RNA positive strand could be identified in approximately 75% of seemingly HCV-negative cases[9,13,14,27]. Of note, the presence of HCV RNA positive strand in mitogen-treated cells is nearly always accompanied by that of HCV RNA negative (replicative) strand, indicative of authentic viral replication. Moreover, non-structural HCV proteins, such as NS5A, are also

detectable in circulating immune cells, as our recent study clearly documented[14]. Interestingly, in many cases of occult infection, HCV RNA expression was found to be higher in cells treated with a cocktail of mitogens, which simultaneously stimulated different immune cell types, compared to those treated with single mitogens[13,27]. This implied that within a given individual, different immune cell subsets may be infected with HCV. Indeed, this observation was unequivocally confirmed in our most recent study[14] in which different affinity-purified immune cell types, e.g. $CD4^+$ and $CD8^+$ T lymphocytes, B cells and monocytes, were found to be infected to a varying extent in different individuals. We have also established that HCV infection can be confined to a specific immune cell subtype, as evidenced by the presence of replicating HCV in affinity-purified cell types but not in total PBMC. This finding highlights the need to screen individual immune cell populations, in addition to PBMC, for possible HCV presence before occult infection can be irrefutably excluded.

The use of clonal sequencing and highly sensitive single-stranded conformational analysis (SSCP), which allows for identification of even single nucleotide polymorphisms, has enabled the identification of HCV variants which appeared unique to immune cells. For example, sequence polymorphisms located at the IRES of the 5'-UTR and the hypervariable region (HVR) of the second envelope glycoprotein (E2) were observed in lymphoid cells of individuals with occult persistent HCV infection[10,12-14,28]. The fact that these variants were different from those found in the serum or liver obtained in parallel further strengthened the notion of HCV lymphotropism[10,12-14]. Additional support for the inherent propensity of HCV to infect and propagate in cells of the immune system came from *in vitro* studies documenting that certain substitutions found in individuals with occult HCV infection[10,12,14] were identical to those that emerged from wild-type HCV passaged through untransformed T cell and lymphoblastoid cell cultures[29,30].

POTENTIAL CLINICAL CONSEQUENCES OF OCCULT HCV INFECTION

As of now, clinical data pertinent to the clinical significance of occult HCV persistence are still in its infancy. However, it is hypothesized that persistent HCV replication in hepatocytes and lymphoid cells would likely lead to continuous antigenic stimulation of the immune system in immunocompetent patients, which in turn, allows the host to keep this silent infection under relative control. This concept has been supported by demonstration of sustained HCV-specific T cell cytotoxic and proliferative responses in patients years after recovery from hepatitis C[31-33]. Similarly, T cell responses to HCV antigens were also evident in patients with persistently elevated liver enzymes of unknown etiology who were HCV RNA reactive in both lymphoid cells and the liver[34]. On the other hand, such prolonged HCV replication associated with the continuous presentation of HCV antigens by infected B cells and monocytes may contribute to the immune tolerance of HCV, hence, favouring even further virus persistence. At present, it remains unknown whether and how infection of the immune cells by HCV may alter their functions, although impairment in the allostimulatory capacity of HCV-infected dendritic cells derived from patients with CHC has been reported[35].

In certain scenarios, including immunosuppression, immunomodulatory therapy or co-infection, instead of eliciting desirable T cell responses in the host, persistent replicating HCV could represent a potential source for virus reactivation, as it has been shown in other viruses, including hepatitis B virus[8] and human herpesvirus 6[36]. In this regard, corticosteroid-induced immunosuppression has been shown to affect HCV reactivation years after spontaneous resolution of acute hepatitis C[37]. Along this line, recurrent HCV infection has been reported in liver transplant recipients who were deemed free of HCV RNA at the time of transplantation, as evidenced by negativity of HCV RNA in serum and the explanted liver tissue assessed by Cobas Amplicor assay with a sensitivity of 50 copies/mL[38]. Furthermore, HCV replication was found to be frequent among patients positive for antibodies to HCV (anti-HCV) who received HCV-negative kidney[39]. In this study, HCV became detectable around 30 d with the viremia peaking on d 62 post-transplantation. Similarly, approximately 18% of bone marrow recipients who were HCV seropositive prior to transplantation became reactive to HCV RNA after receiving a transplant from an apparently HCV-negative donor[40].

CONCLUDING REMARKS

The availability of research assays capable of detecting HCV RNA at sensitivities superior to those offered by clinical assays significantly contributed to the recent discovery of occult HCV infection in individuals years after having been clinically deemed to have cleared the virus. Interestingly, when HCV reactivity in both plasma/serum and peripheral lymphomononuclear cells is taken into consideration, nearly all individuals with apparent complete resolution of hepatitis C can be found to carry low levels of HCV RNA. The fact that the HCV RNA replicative strand and viral proteins are detectable in immune cells, as well as that certain HCV variants are unique to immune cells, lends strong support to the existence of an extrahepatic compartment of HCV replication. Overall, the data accumulated in recent years highlight not only the need for development and implementation of more sensitive HCV RNA diagnostic assays but also the importance of screening both serum and peripheral immune cells for HCV RNA[8]. At present, the data pertinent to pathogenic and epidemiological consequences of occult HCV infection remain very sparse. Further research in this area is of significant clinical relevance in which, as works from our and other groups have shown, an involvement of the infected immune system should not be neglected.

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