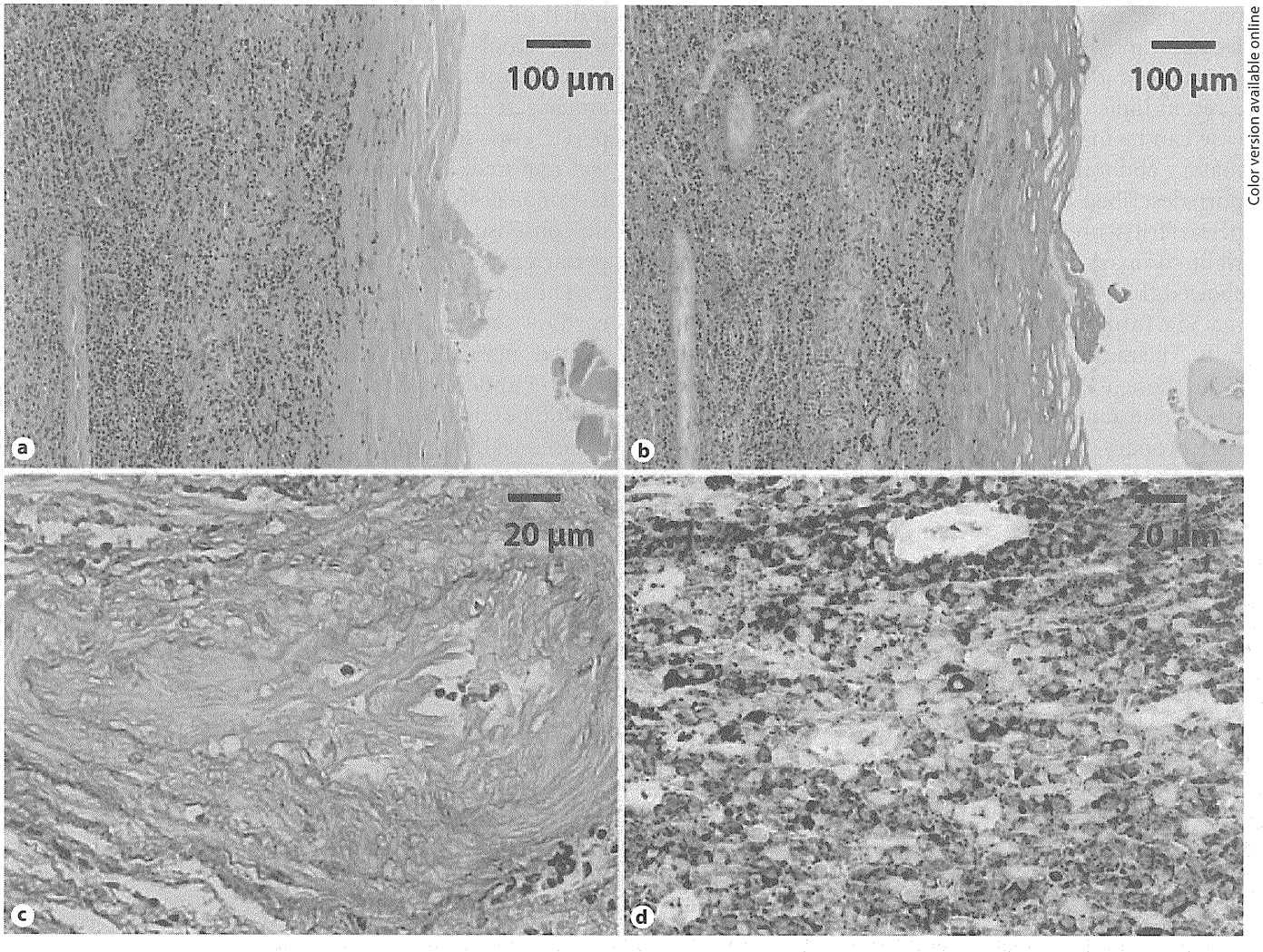

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Fig. 5. Histological examination of the resected mass. **a**, **b** Massive lymphoplasmacytic infiltrate and fibrosis were shown mainly in the adventitia as demonstrated by hematoxylin-eosin staining (**a**) and elastin van Gieson staining (**b**). **c** The small vessels in the adventitia were hypertrophied and some of them were occluded by the thrombus. **d** Immunohistological analysis using an anti-IgG4 antibody revealing that a majority of infiltrated cells were IgG4-positive plasma cells.

onary artery still remained. We performed percutaneous coronary intervention with placement of two drug-eluting stents in the left anterior descending artery. He was discharged without symptoms, taking prednisone at a dose of 15 mg per day.

Four months later, follow-up angiography demonstrated that the ectatic lesion of the right coronary artery was further dilated (fig. 1c) and the surrounding tumorous tissue enlarged from 40 × 24 to 48 × 29 mm (fig. 2b–d). Considering the risk of rupture, the patient underwent surgical resection of the aneurysm and coronary artery bypass grafting by attaching a saphenous vein graft sequentially to the left circumflex artery and the distal portion of the right coronary artery. He was angina free with prednisone at a dose of 7.5 mg per day at the last follow-up visit 4 months after surgery.

Histological examination of the resected mass showed a prominent lymphoplasmacytic infiltrate and fibrosis predominantly in the adventitia, with destruction of normal structures of the intima and the media (fig. 5a, b). The small vessels in the adventitia were severely hypertrophied, and some of them were occluded by the thrombus (fig. 5c). Immunohistological analysis revealed that a majority of infiltrated cells were IgG4-positive plasma cells, thus confirming the diagnosis (fig. 5d).

Discussion

Since elevated serum IgG4 levels in patients with autoimmune pancreatitis were first reported in 2001 [3], there are rapidly emerging evidences that a novel clinico-

pathological entity, IgG4-related systemic disease, can affect multiple organs including the pancreas, bile duct, salivary glands and retroperitoneum [1–6]. Further, it is a recent topic of interest that IgG4-related systemic disease can be manifested as periarteritis, often as inflammatory abdominal aortic aneurysm [7–10]. A recent report described the first reported case of IgG4-related periarteritis presenting with a tumorous lesion of the right coronary artery [11]. The surgically resected mass showed the pathological features of diffuse lymphoplasmacytic infiltration and numerous IgG4-positive plasma cells. The radiological and histological findings are similar to those of our case. Thus, our case is considered to be the second reported case of IgG4-related periarteritis of the coronary arteries with severer manifestations. It is tempting to envision the possibility that concomitant familial hypercholesterolemia influenced these quite unusual radiological findings. Our patient was treated with steroids, and autoimmune pancreatitis and parotitis were ameliorated. However, on the other hand, the effect on periarteritis seemed to be limited, and surgical resection was required. While IgG4-related systemic disease often responds dramatically to corticosteroid therapy, there is no consensus on the optimal treatment of IgG4-related periarteritis, and further studies are needed.

The spectrum of IgG4-related systemic disease in the cardiovasculature is not fully understood, and IgG4-related periarteritis involving the coronary arteries may be underdiagnosed. IgG4-related systemic disease should be considered in any patient with abnormally increased wall thickness or ectatic lesions in the coronary arteries.

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Peripheral B cells as reservoirs for persistent HCV infection

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Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus of approximately 9.6 kb that belongs to the Flaviviridae family (Suzuki et al., 2007). HCV infection is a global health problem affecting nearly 200 million people (Lauer and Walker, 2001). The infection causes prolonged and persistent disease in over half of viral carriers that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Afdhal, 2004). Recent studies have suggested that HCV infects not only hepatocytes but also peripheral mononuclear lymphocytes, particularly B cells, which express CD81, a widely expressed tetraspanin molecule. CD81 has been shown to interact with the E2 region of HCV envelope proteins (Pileri et al., 1998) and is thus regarded as one of the key molecules involved in HCV infection. HCV infection of B cells is the likely cause of various B cell dysregulation disorders. Herein, we propose that HCV uses peripheral B cells as reservoirs for persistent infection, which are in turn responsible for HCV pathogenesis.

Although the liver is considered the primary and main target of HCV infection, extrahepatic manifestations such as mixed cryoglobulinemia, a systemic immune complex-mediated disorder characterized by B cell proliferation that may evolve into overt B cell non-Hodgkin's lymphoma (B-NHL), are often recognized among patients persistently infected with HCV (Agnello et al., 1992; Zuckerman et al., 1997). Epidemiological evidence strongly suggests a close association between chronic HCV infection and B-NHL occurrence (Turner et al., 2003; de Sanjose et al., 2008). A pathogenic role of HCV in B cell disorders has been further demonstrated by studies in which the clinical resolution of B cell dysfunctions was observed after successful regimens of anti-HCV treatment (Mazzaro et al., 1996; Agnello et al., 2002). Based on the aforementioned data, Antonelli et al. (2008) postulated a role of B cells in HCV pathogenesis. In accordance with this notion, our recent study clearly demonstrated that HCV infects and may replicate in the peripheral $CD19^+$ B cells of chronic hepatitis C (CHC) patients (Ito et al., 2010a). In order to determine how HCV evades antiviral innate immune responses that are normally induced in B cells, we analyzed expression levels of IFN-β in peripheral B cells of CHC patients because type I IFN plays a critical role in the antiviral innate immune response. We found that HCV infection failed to trigger antiviral immune responses, such as IFN-β production, in B cells of CHC patients (Ito et al., 2010b). This suggests that HCV evades antiviral innate immune responses in peripheral B cells and uses these cells as reservoirs for its persistent infection in the host.

The idea that B cells may serve as HCV reservoirs was advocated by Muller et al. (1993). Several subsequently published papers also favored the notion of HCV lymphotropism (Ducoulombier et al., 2004; Blackard et al., 2006; Pal et al., 2006). **Figure 1** illustrates the possible process of HCV infection in B cells based on previous studies using human hepatocyte-derived cell lines (Burlone and Budkowska, 2009; Georgel et al., 2010). Among B cell subsets, memory B cells are assumed to be the main reservoirs of HCV infection primarily because of their long lifespans. In support of this notion, our recent study indicated that $CD19^+$ $CD27^+$ cells (i.e., memory B cell phenotype) express a high amount of CXCR3, a chemokine receptor, and are recruited to the inflammatory site in the liver of CHC patients where IFN-γ-inducible protein-10, a CXCR3 ligand, is highly produced (Mizuochi et al., 2010; **Figure 1**). This unique strategy seems to

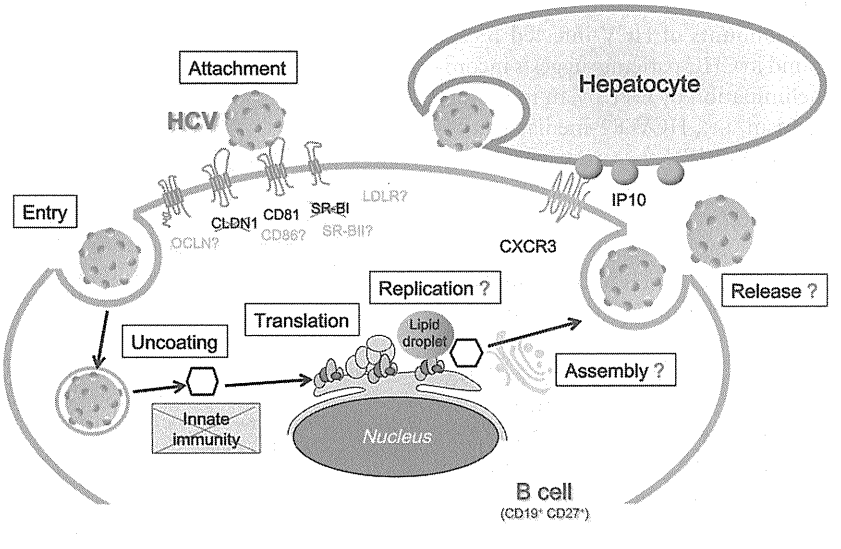

FIGURE 1 | Proposed lifecycle of HCV in B cell. The life cycle of the hepatitis C virus (HCV) has several specific steps. Virus entry is mediated by the direct interaction of envelope glycoproteins with co-receptors, presumably E2–CD81 binding. CLDN1 and SR-BI are not expressed in B cell. Innate immunity response against HCV is suppressed by HCV infection. Some HCV-encoding proteins, i.e., core and NS3, have been proven to be expressed in B cells. HCV RNA replication was examined by nested PCR and *in situ* hybridization. Mechanisms of virus assembly, vesicle fusion, and virion release in B cell remain unknown. LDLR, low-density lipoprotein receptor; SR-BI, scavenger receptor class B type I; SR-BII, scavenger receptor class B type II; CLDN1, claudin-1; OCLN, occludin; CXCR3, chemokine (C-X-C motif) receptor 3; IP-10, interferon gamma-induced protein-10.

be beneficial for HCV in securing sites for persistent infection. HCV may search for reservoir sites in cellular compartments other than hepatocytes in case the liver becomes unsuitable for HCV replication, perhaps because of cellular destruction caused by the host immune response and/or by irrelevant conditions for successful virus replication, such as the development of cirrhosis or hepatocellular carcinoma.

At least two important issues remain to be investigated. First, how do HCV-infected B cells evade "acquired/adaptive" immune responses represented by cytotoxic T cells (CTL)? In peripheral blood, the frequencies of HCV-specific $CD8^+$ lymphocytes with persistent HCV infection are lower than those with acute HCV infection. Furthermore, the CTL response to the HCV antigen is impaired in chronic HCV patients (Lechner et al., 2000). Interestingly, the percentage of CTL in peripheral blood is lower than that in the liver, which may be advantageous for persistent HCV infection in B cells. Because the peripheral B cells of CHC patients express the HCV core as well as NS3 antigens (Ito et al., 2010a), both of which encode functional CTL epitopes (Hiroishi et al., 2010), it is possible that HCV-infected B cells are eliminated by CTL to some extent. However, the fact that substantial amounts of HCV-infected B cells are found in CHC patients suggests incomplete elimination by CTL by an inhibitory mechanism, i.e., HCV E2-mediated inhibition of IL-2/IFN-γ secretion (Petrovic et al., 2011). Second, do HCV-infected B cells produce infectious HCV? Stamataki et al. (2009) demonstrated that the infectious JFH-1 strain of HCV can bind B cells but fails to establish productive infection. On the other hand, Inokuchi et al. (2009) recently demonstrated the presence of negative-stranded HCV RNA, a marker of viral replication, in B cells from 4 of 75 (5%) CHC patients. These results support the notion that HCV replicates in B cells and suggest that infectious HCV are produced in B cells. We have currently been investigating this intriguing issue by using an *in vitro* assay system.

In conclusion, lymphoid reservoirs of HCV infection may play a role in viral persistence and thereby be involved in its pathogenesis. Infection and replication of HCV in peripheral B cells should be regarded as a considerable impediment to the treatment of CHC patients undergoing various antiviral regimens. From a therapeutic viewpoint, it may be beneficial to eliminate peripheral B cells in CHC patients by administering anti-B cell antibodies, such as rituximab, along with combination chemotherapy of peg-IFN-α and ribavirin, which eliminate circulating HCV in the blood. Together, this could lead to a synergistic effect on HCV clearance in CHC patients.

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Review Article

HCV Infection and B-Cell Lymphomagenesis

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Hepatitis C virus (HCV) has been recognized as a major cause of chronic liver diseases worldwide. It has been suggested that HCV infects not only hepatocytes but also mononuclear lymphocytes including B cells that express the CD81 molecule, a putative HCV receptor. HCV infection of B cells is the likely cause of B-cell dysregulation disorders such as mixed cryoglobulinemia, rheumatoid factor production, and B-cell lymphoproliferative disorders that may evolve into non-Hodgkin's lymphoma (NHL). Epidemiological data indicate an association between HCV chronic infection and the occurrence of B-cell NHL, suggesting that chronic HCV infection is associated at least in part with B-cell lymphomagenesis. In this paper, we aim to provide an overview of recent literature, including our own, to elucidate a possible role of HCV chronic infection in B-cell lymphomagenesis.

1. Introduction

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus that belongs to the *Flaviviridae* family [1]. HCV infection is a worldwide problem affecting nearly 200 million people [2] and causes prolonged and persistent diseases in virus carriers, often leading to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [3]. Although the liver is considered to be the primary target of HCV infection, extrahepatic manifestations, such as mixed cryoglobulinemia, which is a systemic immune complex-mediated disorder characterized by B-cell proliferation that may evolve into overt B-cell non-Hodgkin's lymphoma (B-NHL), are often recognized among patients persistently infected with HCV [4, 5]. In fact, epidemiological evidence strongly suggests a close link between chronic HCV infection and B-NHL [6, 7]. The pathogenic role of HCV in B-cell disorders has been suggested in reports wherein a clinical resolution of the B-cell dysfunctions, stated above, was observed after successful anti-HCV treatment using interferon (IFN) [8–10]. Based on such evidences, a possible role of B cells in HCV pathogenesis has been postulated but not yet conclusively demonstrated.

The objective of this paper is to summarize recent literature focused on the possible involvement of HCV infection in B-cell lymphomagenesis, which could offer new insights into the role of B cells in the pathogenesis of HCV infection.

2. Does HCV Infect and Replicate in Peripheral B Cells of Chronic Hepatitis C (CHC) Patients?

HCV, as the name indicates, has been regarded as a hepatotropic virus. However, the possibility that HCV infects cells other than hepatocytes cannot be excluded. In the early 1990s, the existence of HCV RNA was demonstrated by PCR in not only serum/plasma [11] and liver tissues [12] but also in peripheral blood mononuclear cells (PBMCs) of patients infected with HCV [13, 14]. Muller et al. first reported in 1993 that HCV RNA could be found in B cells [15]. They predicted that PBMC, particularly B cells, could be sites for HCV replication and may serve as reservoirs of HCV infection. Moldvay et al. demonstrated that negative-strand HCV RNA, a replicative intermediate of HCV, was observed in PBMC of

patients with CHC (6 of 11) by *in situ* hybridization [16]. Muratori et al. reported negative-strand HCV RNA within PBMC detected by fluorescein-tagged *in situ* RT-PCR (12 of 14 patients with CHC) [17]. Further evidence suggested that HCV replicates in B cells. For example, Morsia et al. demonstrated the replication of HCV in $CD19^+$ B cells by detecting the negative-strand RNA although their sample size was very small (1 of 3 patients with CHC was positive) [18]. Around the same time, Pileri et al. demonstrated that the HCV envelope protein E2 binds the CD81 molecule that is expressed on not only hepatocytes but also various cell types including B cells [19]. This finding thus provided a rationale for the notion that HCV infects and replicates in B cells. Several years later, Gong et al. confirmed the existence of negative-strand HCV RNA in PBMC of patients with CHC (14 of 35) [20]. Some argued that the negative-strand HCV RNA in PBMC may be due to mere contamination or passive absorption by circulating HCV in peripheral blood. They successfully excluded this possibility by demonstrating the expression of HCV-encoding protein, NS5, which indicates that HCV not only replicates but also produces HCV protein in PBMC. Their results are in agreement with an earlier study by Sansonno et al. in which HCV core and NS3 proteins were detected in PMBC of patients with CHC [21].

Occult HCV infection is characterized by the presence of HCV RNA in the liver and the absence of both HCV RNA and anti-HCV antibodies in serum. Castillo et al. detected HCV RNA in PBMC of 40 of 57 (70%) patients with occult HCV infection [22]. In a subsequent report, they confirmed the replication of HCV in PBMC of patients with occult HCV infection by detecting both positive and negative strands of HCV RNA using a strand-specific RT-PCR and *in situ* hybridization techniques [23]. Meanwhile, Januszkiewicz-Lewandowska et al. demonstrated the presence of HCV RNA in PBMC of patients who underwent antiviral chemotherapy and therefore were HCV-serum negative [24]. Collectively, these findings not only favor the notion that PBMC, particularly B cells (discussed later), infected with HCV can serve as reservoirs for persistent HCV infection but are also an alert that PBMC of patients with CHC, including patients with occult CHC, could be potentially infectious even when HCV RNA is negative in their sera. There has been a debate over which cell population in PBMC is the main target for HCV infection. An array of evidence suggests that HCV replicates in various cell types of PBMC, including peripheral dendritic cells, monocytes, and macrophages [25–27]. A recent study by Kondo et al. demonstrated that lymphotropic HCV (SB strain) could infect not only established T-cell lines and B-cell lines but also primary naïve $CD4^+$ T cells, suggesting that HCV replication in such T cells suppressed their proliferation and development in Th1 commitment [28]. Under these circumstances, a number of reports have indicated that HCV infects CD81-positive lymphocytes, preferentially B cells [18, 29–31]. Our recent study also clearly demonstrated that HCV RNA and HCV core and NS3 proteins are detected in $CD19^+$ but not in $CD19^-$ PBMC [32]. Furthermore, Inokuchi et al. confirmed that negative-strand HCV RNA, regarded as a marker of viral replication, was detected in B cells of patients with CHC [33]. Considering this evidence, it can be concluded that HCV infects and replicates in PBMC, particularly in the $CD19^+$ B-cell subset, of patients with CHC. An intriguing question has emerged as to whether different HCV variants or B-tropic HCV cause HCV infection in the $CD19^+$ B cells of patients with CHC or not. When cDNA sequences derived from RNA isolated from plasma and $CD19^+$ B cells of randomly selected patients with CHC were compared, limited variations were found in the internal ribosome entry site (IRES) region (our unpublished data). However, as predicted by a computer program named mfold, these nucleotide substitutions did not affect RNA secondary structure or thermodynamic stability of IRES region [34]. Furthermore, the amino acid sequences in the hypervariable region 1 (HVR1), which directly reflect clonal variations of HCV, did not show any distinct differences between plasma and $CD19^+$ B cells of patients with CHC. These results indicate that HCV RNA isolated from CHC B cells is indistinguishable from RNA isolated from plasma of the same patient with CHC (our unpublished data). Sequence polymorphisms located at IRES and HVR1 of E2 were observed in lymphoid cells of individuals with persistent HCV infection, strongly favoring the concept of HCV lymphotropism. Recently, HCV variants observed in B cells showed poor translational activity in hepatocytes but not in B-cell lines, indicating that adaptive mutations had occurred in B cells [35]. However, our results do not support the concept of lymphotropism or B-tropism of HCV in patients with CHC [30] but instead are in good agreement with studies by Muller et al. in which the PCR products obtained from serum and PBMC specimens of an HCV-positive individual were found to have nearly identical sequences [15]. Although the number of clones analyzed was limited, our conclusion that HCV RNA isolated from $CD19^+$ B cells is indistinguishable from RNA isolated from the plasma of the same patient with CHC is inconsistent with the concept of B-tropic HCV RNA. Further investigation involving a large number of HCV patients would be necessary to support this conclusion.

Overall, the data accumulated to date strongly suggest that HCV infects and replicates in the peripheral B cells of patients with CHC. However, currently it is not known whether a novel HCV strain, B-tropic HCV RNA, preferentially infects peripheral B cells or not. The role of B cells in the pathogenesis of HCV infection is examined in the next section.

3. Peripheral B Cells May Serve as Reservoirs for Persistent Infection of HCV

As described in the previous section, evidence indicates that peripheral B cells in patients with CHC were infected with HCV and thus may serve as HCV reservoirs. This evidence posed a logical question as to how HCV evades the innate antiviral immune responses in B cells. However, this important issue has so far not been formally investigated.

Sensing mechanisms for invading viruses in host immune cells consist of toll-like-receptor (TLR-) mediated [36] as well as retinoic-acid-inducible-gene-I-(RIG-I-) mediated [37] pathways. Both pathways culminate in

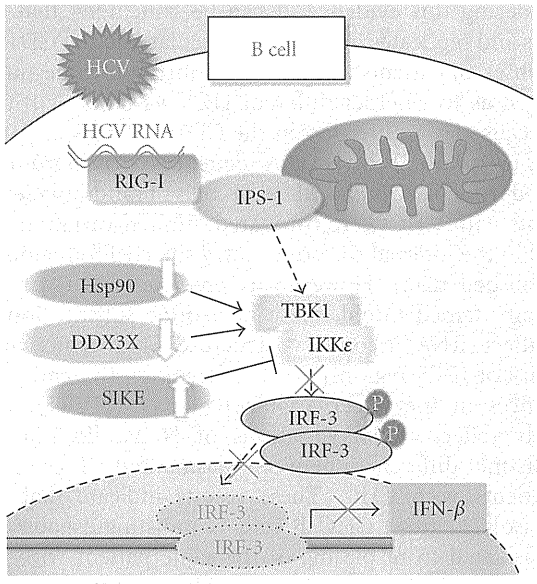

FIGURE 1: Impaired innate antiviral immunity in B cells of patients with chronic hepatitis C.

the translocation of IFN regulatory factor-3 (IRF-3) to the nucleus to transcribe the IFN-β gene. Type-I IFN, for example, IFN-β, plays a critical role in the innate antiviral immune response [38, 39]. In our recent study, it was found that the expression levels of RIG-I and its adaptor molecule, IFN promoter-stimulator 1 (IPS-1), were substantially enhanced in CHC B cells. However, dimerization and the subsequent nuclear translocation of IRF-3 were almost undetectable in CHC B cells. It has been demonstrated that TANK-binding kinase-1 (TBK1) and IκB kinase-ε (IKKε) are essential for the phosphorylation of IRF-3 [40]. The constitutive expression levels of both kinases were found to be markedly enhanced in CHC B cells. However, the reduced expression of TBK1 stabilizers, including Hsp90 [41] and DDX3X [42], and the enhanced expression of the IKK suppressor SIKE [43], were observed in CHC B cells, suggesting that IRF-3 phosphorylation was downregulated. Hence, transcription of the IFN-β gene was not augmented. These results strongly suggest that HCV infection circumvents innate antiviral immune responses, that is, type I IFN production in B cells, and (Figure 1) thus, takes advantage of B cells for persistent infection.

It can be assumed that, among B-cell subsets, memory B cells are the main reservoirs of HCV infection primarily because of their long lifespans. Supporting this notion, our recent study indicated that $CD19^+$ $CD27^+$ cells (memory B cells [44]) are recruited to the liver of patients with CHC through the interaction between CXCR3 expressed on $CD19^+$ $CD27^+$ cells and IP-10 (IFN-γ-inducing protein 10 kD) produced in the liver [45]. This strategy would be beneficial for HCV in securing sites for long-lasting infection. HCV infection of hepatocytes has long been considered an a *priori* assumption. However, this assumption does not necessarily mean that hepatocytes are the exclusive target of HCV infection. HCV may search for reservoir sites in other cellular compartments if the liver becomes unsuitable for replication, perhaps due to cellular destruction caused by the host immune response and/or by the development of conditions such as cirrhosis and hepatocellular carcinoma.

Lymphoid reservoirs of HCV infection could play a role in viral persistence [29, 46–48]. Several maneuvers are employed for persistent infection of HCV [49]. Viral modulation is an effective strategy to escape host immune responses [50]. Another strategy is the suppression of the innate immunity of host by viral components. These components include HCV E2 protein, which acts as a decoy target of protein kinase R (PKR) [51]; HCV NS3/4A protein, which cleaves the adaptor molecules TRIF and IPS-1 and thereby blocks TLR3 and RIG-I signaling, respectively [52, 53]; HCV NS5A protein, which inhibits IFN-stimulated genes expression [54] and PKR function [55]; HCV core protein, which interferes with JAK/STAT signaling [56, 57]. Regardless of the mechanisms, the infection and replication of HCV in peripheral B cells should be considered barriers to the treatment of patients with CHC with antiviral regimens. Based on the notion that peripheral B cells serve as reservoirs for persistent HCV infection and from a therapeutic perspective, it may be beneficial to eliminate peripheral B cells in patients with CHC by the administration of anti-B-cell antibodies, such as rituximab, along with combination therapy with peginterferon and ribavirin to eliminate circulating HCV in the blood, leading to a synergistic effect on HCV clearance in patients with CHC.

4. HCV Infection and B-Cell Lymphomagenesis

The striking association between HCV infection and type II mixed cryoglobulinemia (MC) has been well documented [4, 58, 59]. MC is a benign lymphoproliferative disorder and is regarded as a variant of low-grade B-NHL. Therefore, lymphotropism of HCV suggests that HCV could play a pathogenic role in the clonal proliferation of B cells [60, 61]. Because HCV RNA genomic sequences are not able to integrate into the host genome, indirect mechanisms of malignant transformation should be considered. In this regard, the persistent stimulation of B cells by viral antigens and/or the enhanced expression of lymphomagenesis-related genes could be responsible for leading to polyclonal and later to monoclonal expansion of B cells. Furthermore, the occurrence of a subsequent transformation may lead to B-NHL.

A number of epidemiological studies regarding the association between HCV infection and the occurrence of B-NHL have been carried out [5, 7, 62–65]. A substantial geographic as well as demographic variation exists in the association between HCV infection and risk of B-NHL. A positive association was found in Italy, Japan, and USA. A recent case-control study with a large number of subjects from the International Lymphoma Epidemiology Consortium based in Europe, North America, and Australia further confirmed the association between HCV infection and NHL and specific B-NHL subtypes, that is, diffuse large B-cell lymphoma (DLBCL), marginal zone lymphoma, and lymphoplasmacytic lymphoma [6]. In contrast, other studies from Northern Europe, UK, and Canada failed to show the

association. Geographic differences in HCV genotype have been thought to cause these discrepancies [66] although this remains controversial. Large-scale, population-based, well-controlled studies are necessary to reach a robust conclusion. It can be concluded that, at least in areas with a high prevalence of HCV carriage, HCV is an important risk factor for B-cell lymphomagenesis.

In this paper, we propose a novel hypothesis that peripheral B cells serve as reservoirs for persistent HCV infection. We also suggest that long-lasting HCV infection in B cells may induce lymphoproliferative disorders that may eventually evolve into B-cell NHL, although little is known about the mechanism responsible for B-cell lymphomagenesis. In the remainder of this section, the possible mechanisms of B-NHL tumorigenesis induced by HCV infection will be discussed based on current knowledge of lymphomagenesis-related genes.

Activation-induced cytidine deaminase (AID) is essential for somatic hypermutation (SHM) and class switch recombination of immunoglobulin genes in B cells [67–69]. Recently, it has been proposed that AID may be instrumental in the initiation and progression of B-NHL. This is because a malfunction in either of the two processes stated above is apparently responsible for chromosomal translocations and aberrant SHM, which are the two main causes of genetic lesions associated with B-NHL [70, 71]. Several oncogenes have been demonstrated to be targets for SHM with immunoglobulin genes. In many cases, these anomalies activate the DNA damage response system that either allows DNA repair or eliminates the aberrant B-cell clones [72]. Failure of these repair systems may be a cause of B-cell malignancies. Specific features of SHM are the predominance of single-based substitution, the preference for transitions over transversion, and the specific targeting of the RGYW/WRCY motif. Pasqualucci et al. showed that hypermutation of proto-oncogenes exists in DLBCL [71]. However, in HCV-associated NHL, the number of mutations in some proto-oncogenes was lower than that already found in HCV-negative B-cell NHL patients [73]. Because there is a close association between HCV infection and the incidence of B-NHL, as described above [6, 7], analyzing the expression levels of AID in CHC B cells is of great interest.

Lai et al. established a B-cell line (SB) from HCV-infected B-NHL cells. The virus particles produced from SB cell culture could infect primary human hepatocytes, Raji cells, and PBMC in vitro [74]. They examined the expression of AID in Raji cells and PBMC after HCV infection in vitro. It was found that HCV infection activated the expression of AID in Raji cells. AID expression level was also higher in PBMC of patients infected with HCV than in uninfected individuals [75]. However, their study did not assess which cell population showed an enhancement of AID expression. It was observed in our recent study that expression levels of AID mRNA were markedly enhanced in the $CD19^+$, but not in the $CD19^-$ subset of patients with CHC [32]. Furthermore, the enhanced expression of AID protein was detected in the $CD19^+$ B-cell subset of patients with CHC [32]. The fact that this enhancement of AID expression is confined to the B-cell subset is extremely intriguing because several reports have demonstrated the augmented expression of AID in B-NHL [26, 76, 77].

Using an AID-deficient mouse model, Pasqualucci et al. concluded that AID is required for germinal center-derived lymphomagenesis [78]. They addressed the issue of errors in AID-mediated antigen receptor gene modification processes being the principal contributors to the pathogenesis of human B-NHL. Increasing epidemiological evidence has highlighted the close correlation between HCV infection and B-NHL [6, 7, 79]. Thus, it is tempting to hypothesize that the enhancement of AID in CHC B cells is at least partly responsible for the initiation of lymphomagenesis. In fact, several recent studies suggest that AID is deeply involved in tumorigenesis [80–84]. Notably, HCV enhanced AID expression by NF-κB activation through the expression of viral core proteins. Furthermore, NF-κB expression was upregulated and activated by HCV NS2 proteins in HepG2 cells [85]. These findings suggest that inappropriate expression of AID acts as a DNA mutator that enhances genetic susceptibility to mutagenesis [86].

Additionally, enhanced expression of other lymphomagenesis-related genes including cyclin D1, cyclin D2, BAL, STK15, and galectin-3 in CHC B cells is worth considering [32]. Overexpression of CCND1, which alters cell-cycle progression, is frequently observed in various tumors and may contribute to tumorigenesis [87, 88], whereas CCND2 is known to be expressed at constitutively high levels in B-NHL [89]. BAL is a novel risk-related gene in DLBCL, a typical B-NHL [90], while STK15 is a gene highly expressed in a histologically aggressive type of NHL [91]. Galectin-3 is an antiapoptotic protein, highly expressed in DLBCL [92]. Presumably, the enhanced expression of these genes in CHC B cells [32] may also correlate with B-cell lymphomagenesis.

Tumor necrosis factor alpha-induced protein 3, also called A20, was first identified in 1990 as a TNF-induced cytoplasmic protein with zinc finger motifs [93], which thereafter has been described as a key player in the negative regulation of inflammation by terminating NF-κB signaling [94–96]. Recently, A20 has gained much attention as a novel tumor suppressor [97, 98]. Honma et al. first reported that A20 is frequently inactivated or even deleted in mantle cell lymphoma and DLBCL, and they raised the possibility that inactivation of A20 may be at least partly responsible for lymphomagenesis [99, 100]. Other investigators have subsequently supported their findings [101, 102]. Moreover, A20 also regulates antiviral signaling as well as programmed cell death [103–105]. Currently, the expression, biological activities, and mechanisms of action of A20 have been the focus of attention on a wide scale [106]. Interestingly, Ngueyn et al. reported [107] that the HCV core protein induced an increased expression of A20 in the human hepatocyte cell line HepG2, which has generated a genuine interest in the expression of A20 in peripheral B cells of patients with CHC. Our preliminary data suggests that the A20 molecule is partially cleaved in CHC B cells (Kusunoki et al.; in preparation). An intriguing possibility is that the A20 gene interacts with and is mutated by AID, the expression of which is dramatically enhanced in CHC B cells [32]. In

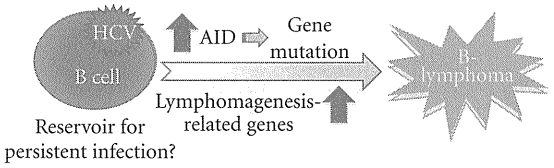

FIGURE 2: Role of HCV infection in B-cell lymphomagenesis, a hypothesis.

this regard, the expression levels of A20 in patients with B-NHL suffering from chronic HCV infection are worth investigating.

5. Conclusion

In this paper, we summarized recent studies illuminating the possible role of HCV infection in B-cell lymphomagenesis. We proposed a hypothesis that HCV utilizes B cells as reservoirs for persistent infection, which could result in the enhanced expression of lymphomagenesis-related genes, particularly AID, which is thought to be crucial for the initiation and progression of B-NHL (Figure 2). Elimination of HCV in plasma by antiviral reagents as well as in peripheral B cells by specific antibodies would be beneficial for patients with CHC to achieve a complete viral clearance. Finally, although a positive association between HCV infection and B-NHL occurrence is still being debated [108–111], it is worthwhile to investigate the possible mechanisms by which B-cell lymphoproliferative disorders, which may evolve into B-NHL, are induced in patients with CHC.

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Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders

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Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including metabolic disorders. Chronic HCV infection is often associated with type 2 diabetes. However, the precise mechanism underlying this association is still unclear. Glucose is transported into hepatocytes via glucose transporter 2 (GLUT2). Hepatocytes play a crucial role in maintaining plasma glucose homeostasis via the gluconeogenic and glycolytic pathways. We have been investigating the molecular mechanism of HCV-related type 2 diabetes using HCV RNA replicon cells and HCV J6/JFH1 system. We found that HCV replication down-regulates cell surface expression of GLUT2 at the transcriptional level. We also found that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells. HCV infection transcriptionally up-regulated the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. Gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with the FoxO1-dependent increased gluconeogenesis. This paper will discuss the current model of HCV-induced glucose metabolic disorders.

Keywords: HCV, diabetes, gluconeogenesis, GLUT2, FoxO1, JNK, NS5A

INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus that belongs to the genus *Hepacivirus* of the family *Flaviviridae*. The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein of about 3,000 amino acids, which is cleaved by cellular signalases and viral proteases to generate at least 10 viral proteins, such as core, envelope 1 (E1) and E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Choo et al., 1991; Lemon et al., 2007).

Hepatitis C virus is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. More than 170 million people worldwide are chronically infected with HCV (Poynard et al., 2003). Persistent HCV infection causes not only liver diseases but also extrahepatic manifestations. It is well established that HCV perturbs the glucose metabolism, leading to insulin resistance and type 2 diabetes in predisposed individuals. Several epidemiological, clinical, and experimental data suggested that HCV infection serves as an additional risk factor for the development of diabetes (Mason et al., 1999; Negro and Alaei, 2009; Negro, 2011). HCV-related glucose metabolic changes and insulin resistance and diabetes have significant clinical consequences, such as accelerated fibrogenesis, increased incidence of hepatocellular carcinoma, and reduced virological response to interferon (IFN)-α-based therapy (Negro, 2011). Therefore, it is very important to clarify the molecular mechanism of HCV-related diabetes. However, the precise mechanisms are poorly understood.

Experimental data suggest a direct interference of HCV with the insulin signaling pathway. Transgenic mice expressing HCV core gene exhibit insulin resistance (Shintani et al., 2004; Koike, 2007). In this transgenic mice model, both tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 and IRS-2 are decreased. These decreases are recovered when the proteasome activator PA28γ is deleted, suggesting that the HCV core protein suppresses insulin signaling through a PA28γ-dependent pathway (Miyamoto et al., 2007). Several other reports also showed a link of the HCV core protein with insulin resistance (Kawaguchi et al., 2004; Pazienza et al., 2007).

Hepatocytes play a crucial role in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways, respectively. Gluconeogenesis is mainly regulated at the transcriptional level of the glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, whereas glycolysis is mainly regulated by glucokinase (GK). Gluconeogenesis and glycolysis are coordinated so that one pathway is highly active within a cell while the other is relatively inactive. It is well known that increased hepatic glucose production via gluconeogenesis is a major feature of type 2 diabetes (Clore et al., 2000).

To identify a novel mechanism of HCV-related diabetes, we have been investigating the effects of HCV on glucose production in hepatocytes using HCV RNA replicon cells (Lohmann et al., 1999) and HCV J6/JFH1 cell culture system (Lindenbach et al., 2005; Wakita et al., 2005; Bungyoku et al., 2009). We previously reported that HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporter 2 (GLUT2; Kasai et al., 2009). Furthermore, we

recently reported that HCV promotes hepatic gluconeogenesis via an NS5A-mediated, forkhead box O1 (FoxO1)-dependent pathway, resulting in increased cellular glucose production in hepatocytes (Deng et al., 2011). This paper discusses our current model for HCV-induced glucose metabolic disorders.

HCV REPLICATION DOWN-REGULATES CELL SURFACE EXPRESSION OF GLUT2

The uptake of glucose into cells is conducted by the facilitative glucose carrier, glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (Wu and Freeze, 2002; Macheda et al., 2005; Godoy et al., 2006). Glucose is transported into hepatocytes by GLUT2. We previously reported that HCV J6/JFH1 infection suppresses hepatocytic glucose uptake through down-regulation of surface expression of GLUT2 in human hepatoma cell line, Huh-7.5 cells (Kasai et al., 2009). We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was significantly lower than in those from patients without HCV infection. Our data suggest that HCV infection down-regulates GLUT2 expression at transcriptional level. We are currently analyzing transcriptional control of human GLUT2 promoter in HCV replicon cells as well as in HCV J6/JFH1-infected cells.

HCV INFECTION PROMOTES HEPATIC GLUCONEOGENESIS

Then we analyzed hepatic glucose production and expression of transcription factors using HCV replicon cells and HCVcc system in order to clarify a role of HCV infection in glucose metabolic changes. Hepatic glucose production is usually regulated by gluconeogenesis and glycolysis. Therefore, we examined whether HCV infection induces gluconeogenesis or glycolysis. We found that the PEPCK and G6Pase genes were transcriptionally up-regulated in J6/JFH1-infected cells (**Figure 1**). On the other hand, the GK gene was transcriptionally down-regulated in HCV-infected cells. We obtained similar data in HCV replicon cells (both in subgenomic replicon cells and full-genomic replicon cells). When HCV replication was suppressed by IFN treatment, the up-regulation of PEPCK and G6Pase gene expression as well as the down-regulation of GK gene expression were canceled. From these results, HCV infection selectively up-regulates PEPCK and G6Pase genes, whereas HCV infection down-regulates GK gene (Deng et al., 2011).

Both HCV replicon cells and HCV-infected cells produced greater amounts of glucose than the control cells. IFN treatment canceled the enhanced glucose production in HCV replicon cells as well as in HCV-infected cells. G6P is an important precursor molecule that is converted to glucose in the gluconeogenesis pathway (**Figure 1**). Our metabolite analysis showed that a significantly higher level of G6P was accumulated in HCV-infected cells than in the control cells, suggesting that HCV indeed promotes hepatic gluconeogenesis to cause hyperglycemia. There is a trend toward an increase in gluconeogenesis in HCV-infected cells (**Figure 1**).

HCV SUPPRESSES FoxO1 PHOSPHORYLATION AT Ser319, LEADING TO THE NUCLEAR ACCUMULATION OF FoxO1

It has been reported that G6Pase, PEPCK, and GK are regulated by certain transcription factors, including FoxO1 (Hirota et al., 2008), hepatic nuclear factor 4α (HNF-4α; Hirota et al.,

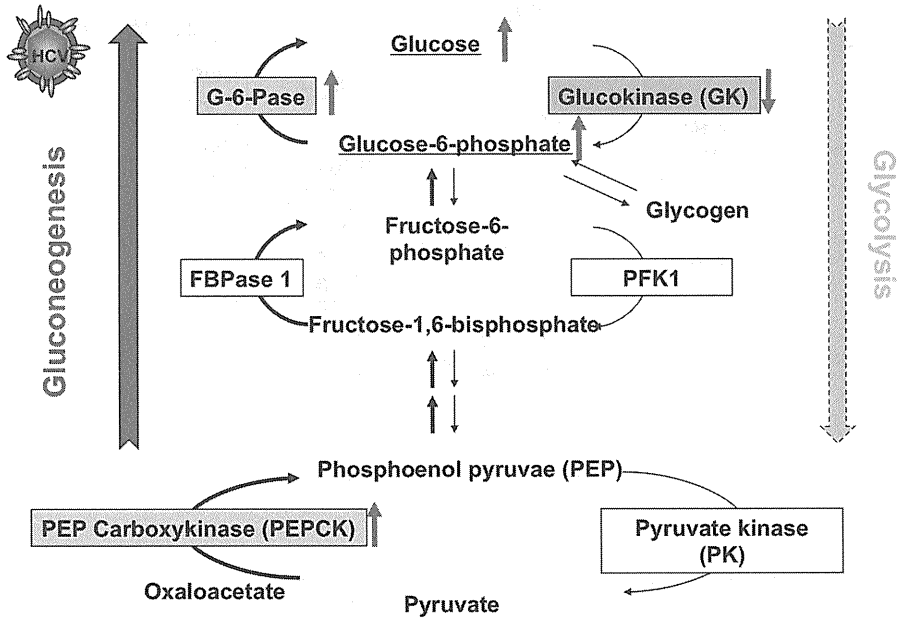

FIGURE 1 | Regulation of Gluconeogenesis and Glycolysis in the HCV-infected cells. HCV infection promotes gluconeogenesis via transcriptional up-regulation of the genes for PEPCK and G6Pase, the rate-limiting enzymes for hepatic gluconeogenesis, and transcriptional down-regulation of the gene for GK, the rate-limiting enzyme for hepatic glycolysis.

2008), Krüppel-like factor 15 (KLF15; Takashima et al., 2010), and cyclic AMP (cAMP) response element binding protein (CREB; Rozance et al., 2008). While we were analyzing these factors in both HCV replicon cells and HCV J6/JFH1-infected cells, we found the involvement of the FoxO1 in the transcriptional activation of G6Pase and PEPCK (Deng et al., 2011). It is known that the FoxO1 enhances gluconeogenesis through the transcriptional activation of various genes, including G6Pase and PEPCK (Gross et al., 2008). The function of FoxO1 is regulated by post-translational modifications, including phosphorylation, ubiquitylation, and acetylation (Tzivion et al., 2011). The phosphorylated form of FoxO1 is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (**Figure 2**). Phosphorylation status of FoxO1 at Ser319 is critical for FoxO1 nuclear exclusion (Zhao et al., 2004). Although the total amounts of FoxO1 protein were unchanged, FoxO1 phosphorylation at Ser319 was markedly suppressed in HCV-infected cells compared to that in the mock-infected cells. It is known that the FoxO1 is phosphorylated by the protein kinase Akt and is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (Tzivion et al., 2011). The majority of FoxO1 was accumulated in the nuclear fraction in HCV-infected cells, whereas in control cells FoxO1 was distributed in both the nuclear and cytoplasmic fractions. Akt phosphorylation was enhanced in HCV-infected cells, although the protein levels of total Akt protein were comparable, which is consistent with the report by Burdette et al. (2010). Our findings suggest an interesting scenario in which the HCV-mediated suppression in FoxO1 phosphorylation is caused by an unknown mechanism independent of Akt activity.

HCV-INDUCED JNK ACTIVATION IS INVOLVED IN THE SUPPRESSION OF FoxO1 PHOSPHORYLATION

It is known that the stress-sensitive serine/threonine kinase JNK regulates FoxO at multiple levels (van der Horst and Burgering, 2007; Karpac and Jasper, 2009). We demonstrated that HCV infection induces phosphorylation and activation of JNK in a time-dependent manner, which is similar to that observed for the suppression of FoxO1 phosphorylation. As a result, c-Jun, a key substrate for JNK, got phosphorylated and activated in HCV-infected cells. The JNK inhibitor SP600125 clearly prevented the phosphorylation of c-Jun, and concomitantly recovered the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that HCV activates the JNK/c-Jun signaling pathway, resulting in the nuclear accumulation of FoxO1 by reducing its phosphorylation status. The detailed mechanisms of HCV-induced suppression of FoxO1 phosphorylation via the JNK/c-Jun signaling pathway remain to be explored. There are at least two possibilities. The JNK/c-Jun signaling pathway (1) suppresses a protein kinase, or (2) activates a protein phosphatase to reduce phosphorylation of FoxO1.

HCV-INDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION IS INVOLVED IN INCREASED GLUCOSE PRODUCTION THROUGH JNK ACTIVATION

Hepatitis C virus infection increases mitochondrial reactive oxygen species (ROS) production (Deng et al., 2008). *N*-acetyl cysteine (NAC; a general antioxidant) clearly prevented the phosphorylation of JNK, and concomitantly canceled the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that

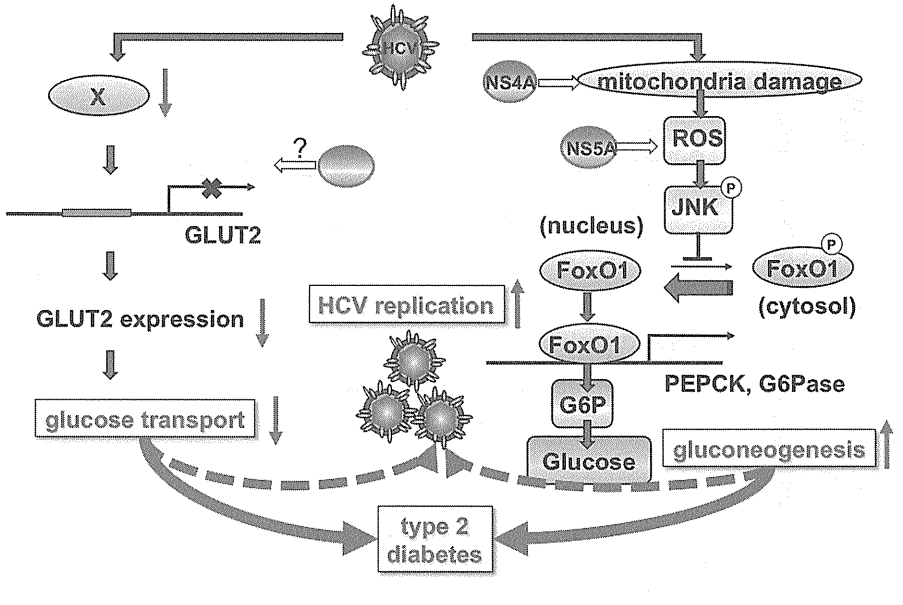

FIGURE 2 | A proposed mechanism of HCV-induced glucose metabolic disorders. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. HCV infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage. HCV NS5A protein is involved in ROS production. HCV-induced ROS production causes JNK activation, resulting in the decreased phosphorylation and nuclear accumulation of FoxO1. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis. High glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

HCV-induced ROS production is involved in the JNK activation. There was no significant difference in HCV RNA replication or infectious virus release between SP600125- or NAC-treated HCV-infected cells and non-treated HCV-infected cells. These results suggest that ROS-mediated JNK activation plays a key role in the suppression of FoxO1 phosphorylation, nuclear accumulation of FoxO1, and enhancement of glucose production in HCV-infected cells (Deng et al., 2011).

HCV NS5A IS INVOLVED IN THE ENHANCEMENT OF GLUCOSE PRODUCTION

Then we sought to determine which HCV protein(s) is involved in the enhancement of glucose production. Transient expression of NS5A protein in Huh-7.5 cells significantly promoted the gene expression levels of G6Pase and PEPCK determined by real time quantitative RT-PCR. Promoter assay revealed that the level of PEPCK promoter activity was significantly higher in NS5A-expressing cells than in the control cells. Our results suggest that NS5A activate both the PEPCK promoter and the G6Pase promoter, leading to an increase in glucose production (Deng et al., 2011). The study by Banerjee et al. (2010) suggests that the HCV core protein modulates FoxO1 and FoxA2 activation and affects insulin-induced metabolic gene regulation in human hepatocytes. Our results, however, suggest that the HCV core protein is not significantly involved in the increased gluconeogenesis (Deng et al., 2011). The difference between these two studies needs to be explored.

There were previous reports suggesting that ROS production is induced in NS5A-expressing cells (Dionisio et al., 2009) or in hepatocytes of NS5A transgenic mice (Wang et al., 2009). We therefore sought to determine whether NS5A contributes to increased hepatic gluconeogenesis through the induction of ROS production. NS5A-expressing cells displayed a much stronger signal of ROS than in control cells. NS5A-expressing cells promoted phosphorylation level at Ser63 of c-Jun and suppressed FoxO1 phosphorylation at Ser319, suggesting that NS5A mediates JNK/c-Jun activation and FoxO1 phosphorylation suppression. These results suggest that NS5A play a role in the HCV-induced enhancement of hepatic gluconeogenesis through JNK/c-Jun activation and FoxO1 phosphorylation suppression.

CONCLUSION AND FUTURE PERSPECTIVES

Taken together, we propose a model of HCV-induced glucose metabolic disorders as shown in **Figure 2**. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. As GLUT2 is a facilitative GLUT, it ensures large bidirectional fluxes of glucose in and out the cell due to its low affinity and high capacity (Leturque et al., 2009). Down-regulated cell surface expression of GLUT2 results in disruption of bidirectional transport of glucose in hepatocytes. Even in the fasting state, down-regulation of GLUT2 may result in low glucose uptake of hepatocytes, causing hyperglycemia. In the fed state, glucose secretion from hepatocytes may be suppressed due to low level cell surface expression of GLUT2, as GLUT2 is a bidirectional transporter.

Hepatitis C virus infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage (Nomura-Takigawa et al., 2006). HCV NS5A protein is involved in ROS production (Dionisio et al., 2009; Wang et al., 2009; Deng et al., 2011). HCV-induced ROS production causes JNK activation, which results in the decreased phosphorylation and nuclear accumulation of FoxO1 by an unidentified mechanism. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis (Deng et al., 2011).

These two pathways, HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis, may contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis may result in high concentration of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentration in the hepatocytes inhibits HCV replication (Nakashima et al., 2011). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

Our understanding of HCV-induced glucose metabolic disorders will require much more work to fully unfold this pathway. Further investigation including the mechanism of HCV-induced GLUT2 downregualtion, JNK-mediated decreased phosphorylation of FoxO1, and the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle and host cells are currently under way.

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Original article

Generation of a recombinant reporter hepatitis C virus useful for the analyses of virus entry, intra-cellular replication and virion production

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Abstract

The lack of a culture system that efficiently produces progeny virus has hampered hepatitis C virus (HCV) research. Recently, the discovery of a novel HCV isolate JFH1 and its chimeric derivative J6/JFH1 has led to the development of an efficient virus productive culture system. To construct an easy monitoring system for the viral life cycle of HCV, we generated bicistronic luciferase reporter virus genomes based on the JFH1 and J6/JFH1 isolates, respectively. Transfection of the J6/JFH1-based reporter genome to Huh7.5 cells produced significantly greater levels of progeny virus than transfection of the JFH1 genome. Furthermore, the expression of dominant-negative Vps4, a key molecule of the endosomal sorting complex required for transport machinery, inhibited the virus production of JFH1, but not that of J6/JFH1. These results may account for the different abilities to produce progeny virus between JFH1 and J6/JFH1. Using the J6/JFH1/Luc system, we showed that the two polyanions heparin and polyvinyl sulfate decreased the infectivity of J6/JFH1/Luc virus in a dose-dependent manner. We also analyzed the function of microRNA on HCV replication and found that miR-34b could affect the replication of HCV. The reporter virus generated in this study will be useful for investigating the nature of the HCV life cycle and for identification of HCV inhibitors.

Keywords: HCV; Reporter virus; Virus production; ESCRT; microRNA

1. Introduction

Hepatitis C virus (HCV) is an enveloped virus and has a positive-stranded RNA genome of about 9.6 kb [1,2]. HCV persistently infects hepatocytes, and the persistent infection can lead to liver cirrhosis and hepatocellular carcinoma. Considering that approximately 170 million people are infected with HCV worldwide [3], HCV is a major public health problem throughout the world. A combination therapy of pegylated interferon-α and ribavirin has been established as the standard of care for treating HCV infection [3,4]. Nonetheless, approximately 50% of individuals with chronic HCV infection are still unable to resolve infection [4,5]. For this reason, more effective therapies are greatly needed against the disease caused by HCV infection [6].

The HCV genome encodes a 3000 amino acid polyprotein which is cleaved by host and viral proteases to yield the mature structural proteins, composed of core and glycoprotein E1 and E2, and the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [1–3]. Translation of the HCV open reading frames is mediated via the 5′ untranslated region and a part of the core coding region carrying the internal ribosome entry site (IRES) [1,7].

In 1999, Bartenschlager and his colleagues produced the HCV replicon system, a tissue culture system that recapitulated the RNA replication of HCV in a human hepatoma cell line [8]. In the initial subgenomic replicon system, genes

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unessential for RNA replication that contained the core, E1, E2, p7 and NS2 of the HCV genome were replaced with a genetic cassette carrying an antibiotics resistance gene and IRES from encephalomyocarditis virus (EMCV). The development of a subgenomic replicon system became a driving force for the studies on the mechanism of HCV replication, and these studies revealed numerous biological features of HCV replication. However, the resulting systems were unable to produce progeny virus. Therefore, the nature of the HCV, i.e., the virus production and virus entry, remained unclear for a long while.

Wakita and his colleagues isolated a full-length HCV genome from the sera of a patient with fulminant hepatitis [9]. The HCV strain, designated JFH1, belongs to genotype 2a. The transfection of the Huh7 hepatoma cell line with the JFH1 genome yields a progeny virus called HCVcc that is infectious both *in vivo* and *in vitro*. The HCVcc system allowed us to perform virological studies to investigate the nature of HCV [9,10]. However, the analyses using HCVcc have not been suitable for carrying out high-throughput screening due to the labor-intensive quantitative reverse transcription-PCR methods used in screening and the difficulties presented by the low signal-to-noise ratios.

In this study, to develop a robust tool for use in the screening of HCV replication, we have constructed a genome-length luciferase reporter HCV derived from the JFH1 and J6/JFH1 strains, and used it to analyze the intra-cellular RNA replication and extra-cellular progeny virus production. We demonstrated here that our recombinant reporter HCV system was useful for studying viral genome replication, virus entry, and virion production of HCV.

2. Materials and methods

2.1. Plasmids

The plasmid pFGR-JFH1/Luc, which encodes bicistronic constructs of HCV IRES-driven firefly luciferase reporter genes and the EMCV IRES-driven full-genomic JFH1 genome, was constructed by insertion of the JFH1 full genome of pJFH1 [9] into pSGR-JFH1 [11]. The plasmid pFL-J6/JFH1, which contains a chimeric full-genome composed of the 5′NCR to NS2 region derived from J6 and NS3 to the 3′NCR region from JFH1 [10], was kindly supplied by C.M. Rice of the Center for the Study of Hepatitis C, Rockefeller University. To yield the bicistronic luciferase reporter construct composed of full-length J6/JFH1, the JFH1 full genome of pFGR-JFH1/Luc was replaced with the J6/JFH1 full genome of pFL-J6/JFH1 by digestion with BstZ17I, and the resultant plasmid was designated as pFGR-J6/JFH1/Luc. As a negative control for the HCV replication, a non-synonymous mutation at NS5B (GDD to GND), which disrupts NS5B polymerase activity, was introduced into the pFGR-J6/JFH1/Luc NS5B region by site-directed mutagenesis, and the resultant plasmid was designated pFGR-J6/JFH1/Luc (GND).

2.2. Cell culture and indirect immunofluorescence

All experiments described in this study were performed by using Huh7.5 human hepatoma cells, a highly HCV-susceptible subclone of Huh7 cells. The cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 0.01% streptomycin, and were subcultured twice weekly. Huh7.5 cells electroporated with JFH1/Luc or J6/JFH1/Luc RNA were subjected to indirect immunofluorescence analysis as previously reported [12]. The primary antibody used was derived from an HCV-infected patient's serum. The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (MBL, Nagoya, Japan).

2.3. In vitro transcription and electroporation

Plasmid DNA was linearized with XbaI, extracted with phenol and chloroform, precipitated with ethanol, and dissolved in RNase-free water. The purified DNA was used for *in vitro* RNA transcription using a T7 Megascript kit (Ambion, Austin, TX) following the manufacturer's protocols. The concentration was determined by measurement of the optical density at 260 nm, and the RNA integrity was checked by agarose gel electrophoresis. The *in vitro*-transcribed RNA (10 μg) was transfected into Huh7.5 cells by means of electroporation (975 μF, 270 V) using a Gene Pulser (Bio-Rad, Hercules, CA). The cells were then cultured in complete medium. The culture fluid of transfected cells was harvested and cleared by passing through 0.45-μm-pore-size filters and stored at −80 °C until use.

2.4. Luciferase assay

The *firefly* luciferase activity was measured by a luciferase assay system (Promega, Madison, WS). The cells were harvested, washed twice with dication-free phosphate buffered saline (PBS), and lysed in a passive lysis buffer supplied by the manufacturer. A 20-μl sample of the lysate was subjected to a luciferase assay. The luminescence was measured at 10 s after an initial 2 s delay according to the manufacturer's instructions, using a Lumat LB9501 luminometer (Berthold, Freiburg, Germany). The assays were performed in duplicate at least three times, and the mean and standard error were computed.

2.5. Vectors of ESCRT family proteins and DNA transfection

The cDNA of the endosomal sorting complex required for transport (ESCRT) family proteins was amplified from Huh7.5 cells by RT-PCR and cloned into pcDNA3.1-FLAG [13], an expression vector containing a CMV promoter and FLAG tag sequence in pcDNA3.1 (Invitrogen, Carlsbad, CA). For the expression of each ESCRT family protein, Huh7.5 cells were transfected with each ESCRT expression vector by using TransIT LT1 transfection reagents (Takara, Kyoto, Japan). The expression levels of the three ESCRT family proteins in