Mutations That Alter Use of Hepatitis C Virus Cell Entry Factors Mediate Escape From Neutralizing Antibodies

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BACKGROUND & AIMS: The development of vaccines and other strategies to prevent hepatitis C virus (HCV) infection is limited by rapid viral evasion. HCV entry is the first step of infection; this process involves several viral and host factors and is targeted by host-neutralizing responses. Although the roles of host factors in HCV entry have been well characterized, their involvement in evasion of immune responses is poorly understood. We used acute infection of liver graft as a model to investigate the molecular mechanisms of viral evasion. **METHODS:** We studied factors that contribute to evasion of host immune responses using patient-derived antibodies, HCV pseudoparticles, and cell culture–derived HCV that express viral envelopes from patients who have undergone liver transplantation. These viruses were used to infect hepatoma cell lines that express different levels of HCV entry factors. **RESULTS:** By using reverse genetic analyses, we identified altered use of host-cell entry factors as a mechanism by which HCV evades host immune responses. Mutations that alter use of the CD81 receptor also allowed the virus to escape neutralizing antibodies. Kinetic studies showed that these mutations affect virus–antibody interactions during postbinding steps of the HCV entry process. Functional studies with a large panel of patient-derived antibodies showed that this mechanism mediates viral escape, leading to persistent infection in general. **CONCLUSIONS: We identified a mechanism by which HCV evades host immune responses, in which use of cell entry factors evolves with escape from neutralizing antibodies. These findings advance our understanding of the pathogenesis of HCV infection and might be used to develop antiviral strategies and vaccines.**

Keywords: Virology; Liver Disease; Tissue Culture Model; Immunity.

Hepatitis C virus (HCV) infection is a major cause of liver disease.[1] A vaccine is not available and antiviral treatment is limited by resistance and adverse effects.[2] HCV-induced liver disease is a leading indication for liver transplantation (LT).[3] A major limitation of LT is the universal reinfection of the liver graft with accelerated recurrence of liver disease. A strategy to prevent reinfection is lacking.[3] Thus, there is an urgent unmet medical need for the development of efficient and safe antivirals and vaccines.

HCV entry is required for initiation, maintenance, and dissemination of infection. Viral entry is a key target for adaptive host responses and antiviral strategies.[4,5] Functional studies in clinical cohorts highlight that viral entry and escape from antibody-mediated neutralization play an important role in viral persistence and liver disease.[6–12] HCV entry is a highly orchestrated process mediated by viral envelope glycoproteins E1 and E2 and several host factors including heparan sulfate, CD81, scavenger receptor BI (SR-BI), claudin-1 (CLDN1), occludin (OCLN) (reviewed by Zeisel et al[5]), and kinases.[13] Although the role of E1E2 in antibody-mediated neutralization has been studied intensively,[4,5,14] the role of host factors for viral evasion in vivo is only poorly understood.

Acute graft infection is an established in vivo model to study viral evasion because viral infection and host-neutralizing responses can be monitored precisely.[8] Viral entry and escape from host-neutralizing responses are important determinants allowing the virus to rapidly infect the liver during transplantation.[8] However, the molecular mechanisms by which the virus evades host immunity to persistently reinfect the liver graft are unknown.

To uncover viral and host factors mediating enhanced viral entry and escape, we functionally analyzed genetically closely related prototype variants derived from a well-char-

Abbreviations used in this paper: CLDN, claudin; HCV, hepatitis C virus; HCVcc, cell culture-derived HC; HCVpp, hepatitis C virus pseudoparticles; HMAb, human monoclonal antibody; HVR, hypervariable region; LT, liver transplantation; mAb, monoclonal antibody; OCLN, occludin SR-BI, scavenger receptor class B type I; VA, variant A; VC, variant C; VL, variant L.

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acterized patient undergoing LT.[8] In one variant, P01VL, reinfecting the liver graft was characterized by high infectivity and escape from neutralizing antibodies present in autologous pretransplant serum.[8] The other closely related variants, P01VA and P01VC, were not selected during LT and were characterized by lower infectivity and high sensitivity to neutralization by autologous pretransplant serum.[8] Previous studies had indicated that an E2 region comprising amino acids 425–483 most likely contained mutations responsible for the phenotype of enhanced entry and viral evasion of variants reinfecting the liver graft.[8]

Materials and Methods

Patients

Evolution and functional analysis of viral variants of patient P01 have been described.[8] Anti-HCV–positive serum samples from patients undergoing transplantation and chronic HCV infection were obtained with approval from the Strasbourg University Hospital Institutional Review Board (ClinicalTrial.gov Identifiers NCT00638144 and NCT00213707).

Plasmids

Plasmids for HCV pseudoparticle (HCVpp) production of variants VL, VA, and VC have been described.[8] E1E2-encoding sequences were used as templates for individual and combinations of mutations using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Massy, France). Mutations were confirmed by DNA sequence analysis (GATC Biotech, Mulhouse, France) for the desired mutation and for exclusion of unexpected residue changes in the full-length E1E2 encoding sequences. Mutated constructs were designated X#Y, where # is the residue location in H77c,[15] X is the mutated amino acid, and Y is the original amino acid.

Antibodies

Monoclonal anti-E1 (11B7) and anti-E2 (AP33, IGH461, 16A6); human anti-HCV IgG[10,16]; human monoclonal antibodies (HMAbs) CBH-2, CBH-5, CBH-23, and HC-1 have been described.[9,17] Anti-CD81 (JS-81) was from BD Biosciences (Heidelberg, Germany), AP33 was from Genentech (San Francisco, CA), and 11B7, IGH461, and 16A6 were from Innogenetics (Ghent, Belgium).

Cell Lines

HEK 293T and Huh7.5.1 cells were cultured as described.[10,13,16] Huh7.5.1 cells overexpressing HCV entry factors were created by stable lentiviral gene transfer of CLDN1, OCLN, SR-BI, or CD81.[18] Huh7.5 stably transduced with retroviral vectors encoding for CD81- and CD13-specific short hairpin (sh) RNAs have been described.[19] Receptor expression was assessed by flow cytometry.[13]

HCV Pseudoparticle and Cell Culture-Derived HCV Production, Infection, and Neutralization

Lentiviral HCVpp bearing patient-derived envelope glycoproteins were produced as described.[8,10,20] The amount of HCVpp was normalized after quantification of human immunodeficiency virus p24 antigen expression (Innotest Human Immunodeficiency Virus Antigen mAb Kit; Innogenetics) and HCVpp entry was performed as described.[8,10,11,16] Chimeric HCVcc expressing patient-derived structural proteins were constructed and produced as described in the Supplementary Materials and Methods section. HCVcc infectivity was measured by determining the tissue culture infectious dose 50% ($TCID_{50}$)[21] or intracellular HCV-RNA levels as described.[13,21,22] HCVpp and HCVcc neutralization were performed as described.[8,10,11,16]

Kinetic Assays

HCVpp kinetic assays were performed in Huh7.5.1 cells using anti-CD81 (JS-81) and anti-E2 (CBH-23) monoclonal antibodies (mAbs) as described.[16,23]

Statistical Analysis

Statistical analysis (repeated-measures analysis of variance) was performed using SPSS 16.0 software for Windows (SPSS, Inc, Chicago, IL).

Results

HCV E2 Residues at Positions 447, 458, and 478 Confer Enhanced Viral Entry of a High-Infectivity Variant Reinfecting the Liver Graft

To investigate the molecular mechanism of enhanced entry of the variant VL reinfecting the liver graft, we first introduced individual mutations of region $E2_{425-483}$[8] of the low-entry and neutralization-sensitive mutant VC into HCVpp expressing envelope glycoproteins of the highly infectious escape variant VL (Figure 1*A*). Previous studies indicated that this region most likely contains the mutations responsible for the high-infectivity phenotype of VL.[8] After normalization of HCVpp levels by p24 antigen expression, viral entry was quantified relative to the escape variant VL. The entry level of the nonselected variant VC was 5% compared with the escape variant VL (Figure 1*B*). By introducing the mutations S458G and R478C into VC, chimeric HCVpp showed similar viral entry level as the paternal variant VL whereas introduction of individual or a combination of other mutations only had a partial effect (Figure 1*B*, Supplementary Figure 1). To explore the impact of other positions on viral entry we introduced mutations from another nonselected variant termed VA into VL (Figure 1*A*) and identified position F447 as an additional residue relevant for enhanced entry of the escape variant VL (Figure 1*C*). These results show that residues F447L, S458G, and R478C are largely responsible for the high infectivity of the escape variant VL.

Enhanced Viral Entry by Mutations F447L, S458G, and R478C of the Escape Variant Is the Result of Altered Use of CD81

To address whether the mutations affect viral entry by different use of cell entry factors SR-BI, CD81, CLDN1, and OCLN, we studied viral entry of HCVpp derived from parental and chimeric variants in Huh7.5.1 cells stably overexpressing the 4 main entry factors individually (Figure 2*A*). Overexpression of either SR-BI, CD81, CLDN1, or OCLN did not affect the stability or proportion of other cell-surface HCV receptors (Figure 2*B* and data not shown).