Hepatitis C virus superinfection of liver grafts: a detailed analysis of early exclusion of non-dominant virus strains

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Liver transplantation (LT) of hepatitis C virus (HCV)-infected grafts into HCV-infected recipients leads to superinfection with two different virus strains. To characterize the virological outcomes of HCV superinfection immediately after LT, we performed phylogenetic analysis of a fragment of the NS5B gene in donor and recipient serum samples prospectively collected before and after LT, starting on day 1. In four of six cases, the donor strain finally prevailed, while in the remaining two cases, the native recipient strain overtook the donor quasispecies. Clonal sequence analysis showed that, in three cases, the expelled strain was undetectable 1 day after LT. Our study shows that superinfection with a different HCV strain can lead to the exclusion of one strain by the other as soon as the first day after LT. This would suggest that competition might not be limited to the replication level, but could also take place during virus entry.

Hepatitis C virus (HCV) infects an estimated 170 million individuals worldwide, with approximately 3 million individuals newly infected each year (Lavanchy, 2009). HCV displays a high rate of genetic variability and has been classified into six major genotypes (genotypes 1–6) and numerous subtypes (Kuiken & Simmonds, 2009). In addition, in a single infected individual, HCV circulates as a complex population of closely related genomes referred to as quasispecies (Forns et al., 1999; Martell et al., 1992).

Chronic infection develops in 50–80% of infected individuals; HCV chronic carriers are at increased risk of developing cirrhosis and hepatocellular carcinoma. Thus, HCV is the leading indication of liver transplantation (LT) in the Western world and Japan. Regrettably, the demand for liver transplants exceeds the organ supply. One approach to expand the pool of organs for transplantation is to use grafts from extended-criteria donors, such as HCV-positive donors. Several studies have reported no differences in outcomes with the use of HCV-positive grafts in comparison with the use of non-infected grafts (Marroquin et al., 2001; Vargas et al., 1999; Velidedeoglu et al., 2004), although donor age has recently been recognized to play an important role after transplantation with HCV-positive grafts (Khapra et al., 2006). In addition, it is important to note that grafts from genotype 1 donors should not be allocated to recipients infected with genotypes 2 or 3, in which antiviral therapy is significantly more effective than for genotype 1, and the introduction of the latter genotype may be detrimental for the patient’s outcome.

HCV superinfection refers to infection with a different HCV strain in an individual already infected with HCV. This phenomenon has been reported in experimentally infected chimpanzees (Farci et al., 1992; Okamoto et al., 1994; Prince et al., 1992) and in individuals at a very high risk for infection, such as intravenous drug users, haemophiliacs or patients on haemodialysis, and multitransfused patients before the screening of HCV in the blood donor population (Eyster et al., 1999; Herring et al., 2004; Kao et al., 1993; Laskus et al., 2001). In humans as well as in chimpanzees, superinfection with two different HCV strains leads to the dominance of one strain over the other and, eventually, to the exclusion of the non-dominant strain. This observation is in agreement with the competitive exclusion principle, a concept from classical genetic evolution. The competitive exclusion principle states that when two species compete for limited resources, one species will eventually outcompete the other and become dominant in the population (Gause, 1971). To date, there is no information about the very early
kinetics of HCV superinfection in the setting of LT. In the present study, we report a detailed analysis of virological outcomes of donor and recipient strains, since the first day after LT. Extensive phylogenetic analysis of direct and clonal sequences has been used to detect the donor and recipient strains in serum samples prospectively collected at multiple time points after LT. As peripheral blood mononuclear cells (PBMC) have been proposed as an independent viral compartment, we also examined the presence of these strains in PBMC samples before and after LT in selected cases.

Six patients who underwent LT for HCV-related cirrhosis and received liver grafts from HCV-RNA positive donors from 2004 to 2007 were included in the study. The Transplant Program and Ethical Committee of our Institution approved a protocol that conformed to the ethical guidelines of the 1975 Declaration of Helsinki. In this protocol, patients infected with HCV genotype 1 could receive a graft from an HCV-infected donor. To use such grafts, a liver biopsy excluding liver fibrosis was mandatory and written consent from the recipient acknowledging the implantation of an HCV-infected graft was always required. Baseline characteristics of patients involved in the study are summarized in Table 1. Standard immunosuppressive therapy consisted of steroids and tacrolimus (cases 2, 4 and 6) or steroids plus cyclosporine A (cases 1, 3 and 5). Donor–recipient pairs from cases 1, 3, 4, 5 and 6 were infected with the same HCV genotype and subtype (1b), whereas in case 2, the donor was infected with genotype 1a and the recipient with subtype 1b. Supplementary Table S1 (available in JGV Online) provides a detailed summary of serum sample collection and sequence analysis performed at each time point. Full methods for all experiments and definitions for histological assessment of liver fibrosis are available in the Supplementary Methods in JGV Online.

We quantified HCV RNA by real-time PCR in serum samples collected before LT (donor and recipient, see Table 1) and at days 1 and 2, week 1 and months 1 and 4 after LT (Supplementary Fig. S1, available in JGV Online). In cases 1, 3 and 5, pre-LT viral loads were higher in the donors than in the recipients, whereas in cases 2, 4 and 6, the recipients had higher viral loads than their donors. Overall, viral loads rose quickly after LT, exceeding pre-LT values between months 1 and 4. In case 4, in which the donor strain was transiently detected as dominant during the first week after LT, and excluded by the recipient native strain thereafter, viral titre rose abruptly 1 month after LT. Liver biopsy during the first months after LT showed severe hepatitis C recurrence in three (50 %) patients and mild recurrence in the other three (50 %) patients (Table 1). Disease recurrence was classified based on the stage of fibrosis and portal pressure increase (Blasco et al., 2006; Neumann et al., 2004; Scheuer, 1995; see Supplementary Methods for precise definitions). The origin of the dominant strain after LT was determined by direct sequencing analysis of a fragment of the NS5B gene from donors and recipients (Table 2). Phylogenetic analysis revealed that the viruses present in all serum samples recovered after LT were closely related to the donor strain in cases 1, 2, 3 and 5, whereas cases 4 and 6 retained their original viral strains. Interestingly, case 4 harboured a donor-related strain during the first week, which was replaced by the native recipient strain 2 weeks after LT. Indeed, a final dominance from either the donor or the recipient strain was observed, demonstrating that the competitive exclusion principle is fulfilled in HCV populations in the LT setting. These results are in

### Table 1. Baseline characteristics of patients included in the study

<table>
<thead>
<tr>
<th>Case</th>
<th>Donor/recipient</th>
<th>MELD*</th>
<th>Immune suppression</th>
<th>Fibrosis† (months after LT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (years)</td>
<td>Sex</td>
<td>Viral load‡ [log10(IU ml⁻¹)]</td>
<td>Genotype</td>
</tr>
<tr>
<td>1</td>
<td>54/61</td>
<td>F/M</td>
<td>6.46/4.97</td>
<td>1b/1b</td>
</tr>
<tr>
<td>2</td>
<td>31/44</td>
<td>M/M</td>
<td>5.38/5.97</td>
<td>1b/1a</td>
</tr>
<tr>
<td>3</td>
<td>67/57</td>
<td>F/F</td>
<td>5.47/5.08</td>
<td>1b/1b</td>
</tr>
<tr>
<td>4</td>
<td>79/52</td>
<td>M/M</td>
<td>4.43/5.00</td>
<td>1b/1b</td>
</tr>
<tr>
<td>5</td>
<td>55/51</td>
<td>M/M</td>
<td>5.43/4.35</td>
<td>1b/1b</td>
</tr>
<tr>
<td>6</td>
<td>51/45</td>
<td>M/M</td>
<td>3.29/5.66</td>
<td>1b/1b</td>
</tr>
</tbody>
</table>

*MELD score is a continuous function of bilirubin, international normalized ratio (INR) and creatinine to predict short-term survival rates in patients with decompensated liver cirrhosis. The higher the MELD score, the poorer the chance of survival for the patient.

†Fibrosis was scored as absent (F0), restricted to the portal tract (F1), perportal or portal–portal septa with intact architecture (F2), bridging fibrosis with architectural distortion but no obvious cirrhosis (F3) and cirrhosis (F4).

‡Viral loads are from donors/recipients at the moment of LT.
Table 2. Detection of dominant strains by direct sequencing analysis in serum and PBMC samples obtained after LT

<table>
<thead>
<tr>
<th>Case</th>
<th>Serum</th>
<th>PBMC (weeks after LT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>1</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>2</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>5</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*In case 1, the last serum sample was obtained 6 months after LT.

acCORDANCE WITH PREVIOUS STUDIES REPORTING THAT BETWEEN 30 AND 60% OF THE PATIENTS RETAINED THEIR ORIGINAL STRAIN (Fan et al., 2003; Laskus et al., 1996; Vargas et al., 1999).

We also analysed the sequences recovered from the PBMC samples and compared them with those present in the serum (Table 2). If the recipient strain finally prevailed in the serum (case 4), the recipient’s PBMC samples obtained before and after LT contained the virus of recipient origin. However, if the donor strain overtook the recipient, a change in the virus strain was also found in PBMC, revealing that changes in the serum-dominant strain were closely mirrored in the PBMC (cases 1, 2, 3 and 5).

In order to study further the possible coexistence of minor donor and recipient variants after LT, clonal sequence analysis was performed on serum samples obtained before LT (donor and recipient) and 1 day, 1 week and 1 month after LT. In case 4, an additional sample collected 2 weeks after LT was also analysed. A minimum of 16 clones and a maximum of 47 clones per sample were sequenced. Phylogenetic trees constructed with clonal sequences confirmed the results obtained by direct sequencing regarding the dominant strain after LT (Fig. 1 and Supplementary Fig. S2, available in JGV Online). For cases 1, 2 and 5, all the sequences obtained at any time after LT clustered exclusively with clones from the donor quasispecies, while for case 6, all the sequences clustered with recipient variants. All these homogeneous clusters were supported by significant bootstrap values (>600). In case 3, viral variants obtained after LT (day 1, week 1 and month 1) formed a uniform cluster that included clonal sequences derived from the donor and a few clones of the recipient, indicating coexistence of donor and recipient variants. Very dynamic changes were observed in case 4. While sequences obtained 1 day after LT clustered with both donor and recipient pre-LT variants, viral clones isolated at week 1 grouped exclusively with the donor. Thereafter, all the clones obtained at week 2 and month 1 clustered only with the recipient native strain.

To characterize donor and recipient circulating quasispecies before LT, we determined the complexity of the quasispecies (normalized Shannon entropy). Complexity of the quasispecies was higher in the donor quasispecies from cases 1, 2, 3 and 5, while in cases 4 and 6, the recipient quasispecies was more complex when compared with those of the donors (Supplementary Table S2, available in JGV Online).

Our data show that competition between the two strains starts during the first 24 h after LT. In this regard, studies on virus dynamics during chronic HCV infection have demonstrated that the turnover of plasma virions is extremely rapid, <3 h (Neumann et al., 1998; Ramratnam et al., 1999). Moreover, several studies on HCV kinetics after LT have shown that reinfection of the graft and selection of virus variants are very dynamic processes, with replication starting shortly after reperfusion (Feliz et al., 2004; Fukumoto et al., 1996; Garcia-Retortillo et al., 2002; Gretchen et al., 1996; Hughes, et al., 2004; Powers et al., 2006). In particular, in case 4, the day 1 quasispecies was composed of a population of dual variants (donor and recipient origin), whereas at week 1, all of the clones clustered with the donor virus. At this time point, the frequency of minor variants of recipient origin fell under our detection limit, emerging again at week 2, when variants from the donor could no longer be detected. The emergence of recipient clones may indicate that some of these variants infected the graft and competed successfully with the donor strain at the replication level, and were not merely residual virions commonly detected during the first 24 h after LT (Garcia-Retortillo et al., 2002).

In cases 1, 2 and 5, competition was resolved within the first 24 h after LT (donor prevailed) as no coexistence was detected by extensive phylogenetic analysis of clonal sequences. The possibility that the native virus from the recipient was unable to infect the graft is highly unlikely, since infection of the graft after LT is universal and occurs even in patients with very low circulating HCV RNA levels. It is possible, however, that the competition between the donor and the recipient strains occurred at the early steps of the HCV life cycle, such as virus entry. In order to clarify
this hypothesis, in vitro studies based on HCV pseudoparticles or cell culture-derived HCV would be of interest to investigate the infectivity properties of the competing strains and the role of host neutralizing antibody responses in the overtake phenomenon.

PBMC have extensively been proposed as an extrahepatic compartment for virus replication (Lerat et al., 1998; Pal et al., 2006). In order to investigate whether the exclusion also took place in this compartment, we analysed PBMC samples obtained before and after LT. In agreement with a

Fig. 1. Representative phylogenetic trees from case 1 (donor strain prevails) and case 4 (recipient strain prevails). Sequences obtained before LT are depicted by ▲ (donor) and △ (recipient). Post-LT clonal sequences are depicted by ● (day 1), ○ (week 1), □ (week 2) and ◼ (month 1). Bootstrap values >600 (based on 1000 replicates) are represented. Trees were rooted with a reference sequence from genotype 1b (HCV-J). Phylogenetic trees from cases 2, 3, 5 and 6 are shown in Supplementary Fig. S2, available in JGV Online.
previous study by Radkowski et al. (2001), we observed that the dominance of one strain over the other occurs both in the serum and in the PBMC at about the same time. The rapid replacement of the HCV dominant variant in PBMC, together with the fact that antiviral therapy is able to eradicate HCV from this compartment (Ramirez et al., 2009), suggest that PBMC may not act as major long-lived extrahepatic reservoirs of HCV following spontaneous or drug-induced plasma clearance.

An intriguing problem in the field of HCV superinfection is the demonstration of the coexistence of virus variants. This problem may be due, in part, to the timing of the sampling and to the techniques used. In this sense, the prospective design with early and frequent sampling after LT and the use of extensive phylogenetic analysis are important aspects to underline in the current study.

Early sampling allowed us to show the dynamics of the competition process from the first day after LT. We have observed that exclusion of non-dominant quasispecies (including minor variants) may occur immediately after LT, be delayed until 2 weeks or may even last longer than 1 month. The molecular mechanism of the exclusion of one strain by the other remains unknown. It could be argued that the donor strain represents a mutant escape for the host immune system and that this could be highly advantageous in the competition with the native strain. It should also be considered that the donor strain might be well adapted to the graft, in which chronic infection has already been established. However, despite these initial advantages for the donor quasispecies, many competitions end with the exclusion of the donor strain. Quasispecies competition has been extensively studied in the vesicular stomatitis virus (VSV) model, in which Clarke et al. (1994) demonstrated that in vitro VSV quasispecies coexisted for many generations, until highly advantageous mutations occurred stochastically in variants of one of the two competing quasispecies, upsetting the equilibrium and leading to a sudden exclusion of the other.

An interesting observation was that, in all cases, the pre-LT quasispecies from the strain that finally prevailed showed greater complexity (using normalized Shannon entropy) than the excluded quasispecies. A higher number of different variants within the population could represent some advantage in the competition, as a greater number of different variants may lead to a better opportunity for the virus to finally persist (Ray et al., 1999). Nevertheless, further studies are required to confirm these results.

A report by Vargas et al. (1999) including 18 cases showed that patients whose predominant strain belonged to the donor were significantly less likely to develop recurrent hepatitis. In our study, only one out of four patients in whom the donor strain prevailed developed severe recurrence, while the two patients who retained their original strains (cases 4 and 6) developed severe infection recurrence after LT. On the other hand, we did not find any correlation between the final outcome of superinfection and relevant clinical parameters such as age, sex, aminotransferase level and immune suppression regimen. Viral load was also analysed and we observed that, in both cases retaining their own strain, the recipient’s viraemia at the moment of LT was higher than the donor’s, while in three out of four cases in which the donor strain took over, the donor’s viral load was higher than the recipient’s. However, it is not clear whether viral load determines the outcome of superinfection, because our cohort is small and a previous study with 14 patients showed that the final outcome of the infection was independent of the viral load (Laskus et al., 1996).

In conclusion, this study provides evidence of HCV superinfection and exclusion in the setting of LT. We have demonstrated that exclusion of non-dominant variants is a rapid and dynamic process that can take place as early as 1 day after LT. Further studies with larger cohorts of patients are needed to clarify which variables (infectivity and replication capacity of each of the quasispecies, specific virus–host interactions) lead to the dominance or exclusion of one of the competing HCV strains during superinfection.

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