APÊNDICES

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POST-TRANSPLANT RECURRENT HEPATITIS C - IMMUNOHISTOCHEMICAL DETECTION OF HCV CORE ANTIGEN AND POSSIBLE PATHOGENIC IMPLICATIONS

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Abbreviation used:
bDNA: branched DNA assay; EIA: enzyme-linked immunoassay; HCV: hepatitis C virus;
HVR: hypervariable region; PCR: polymerase chain reaction; FFPE: formalin-fixed, paraffin embedded.

ABSTRACT

The mechanisms by which severe cholestatic hepatitis develops after liver transplantation are not fully understood. Reports on immunohistochemical distribution of HCV antigens are still scarce, but recently, HCV immunostaining was suggested for early diagnosis of cholestatic forms of recurrent hepatitis C in liver grafts. After purification, Rb246 pab anti-core (aa1-68) yielded specific, granular cytoplasmic staining in hepatocytes. Signal amplification through Envision-Alkaline Phosphatase System avoided endogenous biotin and peroxidase. Rb246 was applied to liver samples of explants of 12 transplant recipients, 6 with the most severe form of post-transplantation recurrence, severe cholestatic hepatitis (group 1) and 6 with mild recurrence (group 2). We also assessed immuno-reactivity at 2 time-points post-transplantation (7 mos and 17 mos) in both groups. HCV-core Ag was semi-quantified from 0 to 3+ in each time-point. Serum HCV-RNA was also measured on the different time-points by branched DNA. In the early post-transplant time-point, 1 patient had a mild staining (1+) and 2 patients had a moderate staining (2+) in group 1, compared to 5 patients with no staining (0) and 1 patient with mild staining (1+) in group 2. Late post-transplant liver samples were available in 9 patients, and 2 out of 4 samples in group 1 showed a mild staining, compared with no staining patients in 5 patients in group 2. Strikingly, on the explant samples, HCV immunostaining was strongly positive in group 1, and mildly positive in group 2. Two out of 5 samples showed 3+ staining, and 3 samples showed 2+ staining in group 1; 2 out of 5 samples showed no staining, 2 samples showed 1+ staining and 1 sample showed 2+ staining in group 2. Serum HCV-RNA was significantly higher in group 1, on both time-points post-transplantation. HCV-core Ag was not directly associated to serum HCV-RNA on the different time-points. These preliminary results suggest that strong HCV immunostaining in the explant is predictive of more severe disease recurrence.

INTRODUCTION

Chronic HCV infection is the leading indication for liver transplantation, estimated at 35% to 45% [1]. In that population, recurrent infection post-transplantation is almost

universal, with a 10- to 20-fold increase in levels of viremia[2]. The majority of transplant recipients will develop histological evidence of disease due to recurrent HCV infection, if followed for up to 5 years, with a significant proportion progressing to chronic hepatitis and cirrhosis[3,4,5,6,7]. The natural history of HCV infection post-transplantation is now beginning to be delineated, and we have limited understanding of factors that influence disease progression. After transplantation, the majority of patients evolve to a slowly progressive chronic hepatitis over a period of months to years. Few patients however, develop severe cholestasis and progressive hepatitis, frequently resulting in liver failure in a few months from the diagnosis, associated with high mortality if no re-transplantation occurs, similar to fibrosing cholestatic hepatitis B virus infection[8,9,10]. Cholestatic HCV seems to be a disease of direct HCV cytopathic injury in the setting of extreme virus levels [11,12], and lack of a specific HCV-directed response to acute hepatitis, induced by high levels of immunosuppressors[13].

Immunohistochemical detection of HCV antigens in liver tissue could potentially provide important pathological information, making possible the correlation between viral replication sites and tissue injury. However, mostly due to the scarcity of reliable antibodies for detection of HCV antigens in formalin-fixed paraffin-embedded (FFPE) sections, only limited studies have been performed on post-transplant recurrent HCV disease. In a recent study, intrahepatic HCV antigen expression was detected as early as 10 days post-transplantation in 25% of biopsy specimens and within 3 weeks in 50% of specimens[14]. By the time histological acute hepatitis is clinically overt, HCV core antigens can be detected in more than 90% of biopsy specimens[14,15,16]. Doughty et al., utilizing immunohistochemical staining, with the commercial monoclonal antibody Tordji-22 (BioGenex, San Ramon, CA) had demonstrated initially a strong correlation between the location of HCV antigens and areas of liver injury, particularly regions of hepatocyte ballooning. However, further testing controlling for the level of tissue damage showed cross-reaction of this antibody with host epitopes present in non-HCV liver transplant tissue with hepatocyte ballooning[17].

The aim of this study was to examine whether HCV antigenic expression in liver tissue of patients with recurrent HCV disease post-transplantation could correlate with severity of the recurrence, comparing patients with severe cholestatic hepatitis post-transplantation with matched controls with mild recurrent disease post-transplantation. Immunohistochemical staining was performed using an Rb246

polyclonal anti-core antibody developed by InnoGenetics, Belgium on FFPE liver tissue sections, and previously reported by Alves et al.[18].

SUBJECTS AND METHODS

Study Groups*:*

Six patients who underwent liver transplantation for HCV-related end-stage liver disease at University of California, San Francisco between 1988 and 1993 fulfilled the diagnostic criteria for severe cholestatic hepatitis and were included in this study. These patients evolved with the most severe form of post-transplantation recurrence, severe cholestatic hepatitis (group 1), diagnosed by presence of jaundice in the absence of extrahepatic obstruction and liver histology showing severe centrizonal hepatocyte cholestasis and ballooning, confluent hepatocyte necrosis, interface hepatitis, and bile ductular proliferation[8]. Five of these 6 patients subsequently died of complications related to liver failure. Other six patients evolved with mild recurrent disease post-transplantation (group 2), also evidenced by their liver histology, showing grade 1 or 2 inflammation and stage 0 fibrosis[19], and served as the comparison group. The two previous groups were matched by genotype (all genotype 1), type of immunosupression and length of follow-up. A single pathologist (LF) evaluated all the biopsies. All patients had recurrent HCV infection after transplantation, defined by the presence of viremia detected by polymerase chain reaction (PCR) amplification and by abnormal liver histology. All patients received triple immunusuppression with ciclosporine, azathioprine, and prednisone. The number of rejection episodes was similar between the 2 groups (median {range} of 1 {0-2} for mild and 1 {0-2} for severe). All the patients were anti-HCV and HCV-RNA positive pre and post-transplantation. All patients were negative for HBsAg, anti-HBc IgM and anti-HIV and none of them received any type of anti-viral treatment during the participation in the study. FFPE liver tissue specimens were tested for HCV antigens using a polyclonal anti-core antibody (Rb246) at three time points: pre-transplantation (the day of transplantation), and at two time-points post-transplantation, initial (mean ± SD of 7 ± 4 and 7 ± 3 months for mild and severe recurrence, respectively), and delayed (21 ± 7 and 26 ± 10 months for mild and severe recurrence, respectively). Serum samples at the same three time points were tested for quantitation of HCV-RNA (by bDNA) for each patient.

Methods:

HCV RNA Extraction and Amplification:

HCV RNA was extracted from 50µl of serum using phenol chloroform extraction method as previously described[20]. Reversed transcription and polymerase chain reaction (RT-PCR) were done in a single tube reaction of 50 µl using the primers selected from the E2/HVR region (Sense *5'GGTGGCTCACTGGGGAGGTCCT3'* 1366-1387), (Anti-sense *5'CATTGCAGTTCAGGGCAGTCCTG3'* 1587-1610)[21]**.** This round of PCR yielded a 244-bp product. A second round of amplification was done using 5 µl of the first reaction and the following primers: (Sense *5'TCCATGGTGGGGAACTGGGC3'* 1406-1426), (Anti-sense *5'TGCCAAC TGCCATTGGTGTT3'* 1561-1581). The final product (approximately 176 bp) was detected by electrophoresis on 2% agarose gel (Sigma, St. Louis, MO) containing ethidium bromide at a concentration of 10 µg/mL.

HCV RNA Quantitation:

The level of HCV RNA was determined by employing branched DNA (b-DNA) signal amplification assay (Quantiplex HCV-RNA b-DNA v.1.0, Chiron Corp. Emeryville CA.). This assay is a sandwich hybridization oligonucleotide probe assay, which targets 5' untranslated and Core regions of HCV RNA. The viral quantitation limit for this assay is 350,000 HCV RNA Eq./ml. The manufacturer's standard b-DNA assay protocol was followed.

HCV Genotyping:

HCV was genotyped by Restriction Fragment Length Polymorphism (RFLP) analysis of the 5' non-coding region (NCR) of the genome as previously described[22]. Genotype classification was assigned as recommended by Simmonds et al.[23].

Immunohistochemical Staining:

Each FFPE sample was submitted to immunohistochemical assays for the HCV core antigen, through a rabbit antiserum directed to a cocktail of synthetic peptides encompassing immuno-dominant epitopes of the initial 68-aminoacid sequences of the core region. After absorption with normal human liver powder, Rb246 pab anti-core (aa1-68) yielded specific, granular cytoplasmic staining in hepatocytes. Signal amplification through the Envision Alkaline Phosphatase Method avoided endogenous biotin and peroxidase. Rb246 was applied to liver samples of explants of 12 transplant recipients, 6 with severe cholestatic hepatitis (group 1) and 6 with

mild recurrence (group 2). Immuno-reactivity for HCV core Ag was also assessed at 2 time-points post-transplantation: all 12 patients had liver biopsies at 6 months, representing “early post-transplant period”, whereas 4 patients from group 1 and 5 patients from group 2 had available samples from liver biopsies at 2 years, representing “late post-transplant period”. HCV-core Ag was semi-quantified by a single pathologist (VAFA) from grade 0 to 3 in each time-point (1 +, <25% of the hepatocytes in the section; 2 +, 26-50% and 3 +, >50%).

STATISTICAL ANALYSIS:

Continuous variables were described as median and range, and compared using the Mann *U* Whitney test. Fischer’s exact test was used to compare categorical samples, when appropriate. A *P* value of 0.05 was considered to be statistically significant.

RESULTS:

1-Study population characteristics:

Biochemical, virological and histological characteristics of the study population are shown in Table 1. Liver histology closest to the time of available serum is shown. In the group with severe recurrence, liver histology evolved further such that with delayed follow-up, injury was scored as a median grade 3 (range of 2-4) and stage 2 (range of 1-4). In three cases fibrosis scores were less marked, presumably because the patients died before there was sufficient time to evolve to end-stage fibrosis. At this time, peak bilirubin and ALT median levels were 29 mg/dl (range of 12.5-72 mg/dl) and 192 IU/L (range of 92 to 933 IU/L). Serum samples and histology from these delayed time points were not analyzed since most patients had begun antiviral therapy, which would confound the interpretation of results.

2- HCV-RNA Levels:

HCV-RNA levels are shown at different time-points in both groups in table 2. HCV RNA levels were similar at the time of liver transplantation in both groups. HCV-RNA levels were significantly higher in the group with severe recurrent hepatitis compared to the group with mild recurrence at early post-transplantation follow-up (p= 0.01). HCV RNA levels were similar in the late post-transplant follow-up in group 1 compared to group 2.

3- Immunohistochemistry:

Core Antigen of HCV was immunostained as fine granules in the cytoplasm of hepatocytes. No nuclear staining was observed in any liver tissue specimen. The results of immunohistochemical staining are shown in table 3. In the early post-transplant time-point, 3 patients had no staining (0), 1 patient had a mild staining (1+) and 2 patients had a moderate staining (2+) in group 1, compared to 5 patients with no staining (0) and 1 patient with mild staining (1+) in group 2. Therefore, patients in the severe cholestatic group presented, early in their post-tansplant period, more positivity for HCV core antigens, although it did not reach statistical significancy (*P*=0.09). Late post-transplant liver samples were available in 9 patients; a mild staining was found in 2 out of 4 samples available in group 1, compared with no staining found in samples from 5 patients in group 2. Strikingly, on the pre-transplant samples (explants), HCV immunostaining was strongly positive in group 1 (who developed post-transplant severe cholestatic hepatits) and mildly positive in group 2 (who developed mild recurrent disease). Two out of 5 samples showed 3+ staining, and 3 samples showed 2+ staining in group 1; 2 out of 5 samples showed no staining, 2 samples showed 1+ staining and 1 sample showed 2+ staining in group 2. The presence of HCV core antigens in the explant was significantly correlated with the severe outcome post-transplantation (P= 0.02), but not correlated with pre-transplantation serum HCV-RNA quantitation, that was similar in both groups.

Figure 1a shows the explant from one patient with severe recurrence; with numerous hepatocytes presenting HCV core antigen granules in the cytoplasm (3+). Figure 1b shows a detail depicting stronger staining in peri-septal hepatocytes. Figure 1c shows the liver from the same patient with severe recurrence, 6 months post-transplantation; immunohistochemical reaction for HCV core antigen was negative. Figure 2a shows the explant from a patient with mild recurrence; with few hepatocytes presenting HCV core antigen granules in the cytoplasm (1+). Figure 2b shows liver biopsy from the same patient with mild recurrence, 21 months post-transplantation; although morphology depicts chronic hepatitis, immunohistochemical reaction for HCV core antigen was negative.

Discussion

The identification of HCV antigen in the liver was first reported in 1990[24]. That preliminary report was extended by specificity studies, which established the association of HCV infection with hepatocellular HCV antigen. Several antibodies have been described for the detection of HCV in liver biopsies. HCV antigen was detected exclusively in the cytoplasm of hepatocytes in specimens obtained from livers of patients with chronic HCV infection, with a detection rate of 23 to 100%[25;26]. In most of the liver specimens, the number of positive hepatocytes and the intensity of staining were low.

The detection of immunodominant epitopes of the core antigen was reliably confirmed in routine FFPE liver samples. Similar to what some of us had previously reported in patients with chronic hepatitis C[18], 29 cases (43,9%) of our HCV samples were found positive, with no staining of any of the negative controls.

In the explants of patients who further developed severe cholestatic hepatitis post-transplantation, HCV core antigen immunostaining was significantly higher compared to matched controls, raising the possibility of a pre-transplant viral factor predisposing to a severe post-transplant recurrence. Deshpande et al. had demonstrated that high HCV RNA levels in native livers correlate with the development of cholestatic hepatitis in liver allografts and a poor outcome[27]. The most significant finding in their study was the ability to predict the development of cholestatic hepatitis by measuring HCV RNA levels in the explanted livers. Six of 8 patients who developed cholestatic hepatitis had levels greater than 5,000 copies/µg compared with only one patient in the control group with non-cholestatic recurrence. In a previous report from our group, the rate of detection of HCV core antigen was parallel to the stage of liver disease[18]. Although less sensitive, immunostaining of HCV core antigen was also a marker of viral replication, since immunostaining was found in 27 of 67 (40.3%) HCV-RNA positive patients, but only in 4 of 18 HCV-RNA negative patients. Although we did not measure HCV-RNA in the liver, the detection of HCV core antigen in tissue may represent a less expensive and time-consuming method of detection of HCV-RNA. These results support our findings comparing HCV core antigen in the explants of patients with severe cholestatic disease to controls, where the presence of a high HCV core antigen immunostaining seems to identify patients with poor post-transplant outcome.

The HCV core antigen immunostaining was less sensitive and weakly related to the serum HCV-RNA quantitation post-transplantation, which was much higher in the group with severe cholestatic recurrence. In the early post-transplant follow-up, there was a trend toward a higher HCV core antigen immunostaining in the severe cholestatic group, compatible to their greater HCV RNA levels in serum. In the late post-transplant follow-up, although a higher HCV RNA level was present in serum, we did not observe higher HCV core antigen immunostaining. However, the small sample size at this time-point makes it difficult to derive any conclusion on the lack of correlation between post-transplant HCV RNA levels in serum and HCV core antigen in tissue.

In our series, pre-transplant HCV RNA levels in serum from patients who developed severe cholestatic recurrence were not significantly higher compared to matched controls with mild recurrence. However, post-transplantation, HCV RNA levels were progressively higher in the group with severe cholestatic hepatitis compared to controls. Several studies have investigated the relationship between pre- and post-transplant serum HCV RNA levels and the development of graft recurrent hepatitis in the graft. Charlton et al.[6]. showed that patients with pre-transplant HCV RNA levels in serum greater than $1x\ 10^6$ Eq/mL, had a poor post-transplant graft survival. Additionally, Doughty et al.[11]. and Zervos et al.[28]. both had shown greater serum pre- and post-transplant HCV RNA levels in patients who developed cholestatic hepatitis post-transplantation compared to controls. The last two studies supported our results of progressive HCV RNA levels in patients who developed recurrent severe cholestatic hepatitis in the graft, suggesting cytopathic mechanisms in the pathogenicity of this syndrome.

In conclusion, this preliminary study suggests that strong HCV immunostaining in the explant is predictive of more severe disease recurrence post-transplantation, and can earlier identify patients suitable for antiviral therapy, thus potentially preventing the development of severe recurrence.

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Table 1. Biochemical, histological and virological features of study groups

Patients	**Severe Recurrence Group 1 (N=6)**	**Mild Recurrence Group 2 (N=6)**
Genotype	1a,1a, 1a 1b,1b, 1b	1a, 1a, 1 1b,1b, 1b
Histology		
Early follow-up		
Grade of inflammation	1 (0-2)	1 (0-1)
Stage of fibrosis	0 (0-1)	0
Late follow-up*		
Grade of inflammation	3 (0-4)	1 (1-2)
Stage of fibrosis	1 (0-3)	0
Liver biochemistry		
Pre-transplantation		
Bilirubin (mg/dL)	3.2 (1.3-31.5)	2.0 (0.8-2.6)
Serum ALT (IU/L)	72 (21-300)	156 (31-257)
Post-transplantation		
Late follow-up*		
Bilirubin (mg/dL)	3.4 (1.1-13.3)	0.9 (0.5-1.6)
Serum ALT (IU/L)	126 (61-276)	78 (41-244)

Abbreviations: NA = not available; all values are presented as the median and range

*Histology and biochemical analysis prior to any antiviral therapy is shown. Further histological and biochemical progression was observed in patients with severe recurrence (see text).

Table 2. HCV-RNA Quantitation in different groups

Patients	Severe Recurrence Group 1 (N=6)	Mild Recurrence Group 2 (N=6)
HCV RNA (MEq/mL)		
Pre-transplantation	0.53 (0.2-9.1)	0.76 (0.2-2.9)
Early post-transplantation follow-up	24.8 (3.9-1446)*	3.5 (0.4-3.7)*
Late post-transplantation follow-up	11.9 (0.2-12.7)	14.3 (0.2-41.0)

Comparison of HCV RNA at the early post transplantation follow-up - mild versus severe disease, *P*=0.01.

Table 3. HCV core antigens distribution in liver tissue among groups

	Severe Recurrence Group 1 (N=6)				**Mild Recurrence Group 2 (N=6)**			
Time-Point \ Grade*	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Explant (N)	0	0	3	2	2	2	1	0
Early post-transplantation follow-up (N)♣	3	0	3	0	5	1	0	0
Late post-transplantation follow-up (N)	2	2	0	0	5	0	0	0

*Grade 0 meaning no HCV core Ag detected; grade 1 ≤ 25% of the hepatocytes in the section; grade 2 = 26-50%; and grade 3 > 50%.

P= 0.02

♣*P*=0.06