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Christophe Duvoux
Anh Tran Ngoc
Liliane Intrator
Christophe Hézode
Georgios Germanidis
Jean-Michel Pawlotsky
Daniel Cherqui
Daniel Dhumeaux

Hepatitis C virus (HCV)-related cryoglobulinemia after liver transplantation for HCV cirrhosis

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C. Duvoux (✉) · A. Tran Ngoc
C. Hézode · D. Dhumeaux
Service d'Hépatologie et
de Gastroentérologie,
Hôpital Henri Mondor,
Université Paris XII, 51, avenue du
Maréchal de Lattre de Tassigny,
94010 Créteil, France
E-mail: cduvoux@easynet.fr
Tel.: +39-1-49812353
Fax: +39-1-49812352

L. Intrator
Laboratoire d'Immunologie Biologique,
Hôpital Henri Mondor-AP-HP,
Université Paris XII, Créteil, France

G. Germanidis · J.-M. Pawlotsky
Laboratoire de Virologie et
de Bactériologie,
Hôpital Henri Mondor-AP-HP,
Université Paris XII, Créteil, France

D. Cherqui
Service de Chirurgie Générale et Digestive,
Hôpital Henri Mondor-AP-HP,
Université Paris XII, Créteil, France

Abstract The aim of this study was to analyze the clinical impact of hepatitis C virus (HCV)-related cryoglobulinemia in patients that had received liver transplants after HCV cirrhosis. Thirty patients who had received transplants between 1990 and 1996 for HCV cirrhosis and who had a follow-up longer than 1 year were studied. Serum HCV RNA levels, HCV genotype, cryoglobulinemia, rheumatoid factor, serum C3 and C4, IgA, IgG, IgM levels, liver tests, and liver histology were studied 30 ± 16 months post-transplant. Cryoglobulinemia was found in 9 of 30 patients (30.0%) and was symptomatic in 4 of the 9 cases (glomerulonephritis, 1 case; palpable purpura, 3 cases). Age, sex distribution, alanine aminotransferase (ALAT) activity, and Knodell score did not differ, whether cryoglobulinemia was present or not. Rheumatoid factor (209.5 ± 70.4 IU/l vs 12.0 ± 4.4 IU/l, $P = 0.004$) and IgM levels (3.2 ± 0.5 g/l vs 1.6 ± 0.9 g/l, $P = 0.0001$) were significantly higher, and C4 levels (0.16 ± 0.16 g/l vs 0.30 ± 0.10 g/l, $P = 0.009$) were significantly lower in patients with cryoglobulinemia. One patient died from cryoglobulin-related renal failure. We concluded that, after liver transplantation (LT) for HCV cirrhosis, cryoglobulinemia was frequent and often symptomatic. Cryoglobulinemia did not seem to be associated with more severe graft damage. Cryoglobulinemia-associated morbidity must be taken into account in the management of post-transplant HCV infection.

Keywords Liver transplantation · Hepatitis C virus · Cryoglobulinemia · Vasculitis

Abbreviations *Anti-LKM1* · Anti-liver and -kidney microsome type 1

Introduction

Hepatitis C virus (HCV)-related cirrhosis is currently one of the main indications of liver transplantation (LT) worldwide [3]. However, HCV recurrence is almost constant after LT [5, 9, 10, 12, 30, 34], and HCV RNA can be detected by PCR in the serum of at least 95% of patients after liver transplantation (LT) for HCV-related cirrhosis. On the other hand, it is now established that HCV is the main agent of mixed cryoglobulinemia (CG) [2, 14, 20]. Contrasting with the well-known natural history of HCV recurrence on the liver graft [3, 5, 9, 10, 12, 30, 34], information regarding the prevalence and clinical relevance of post-transplant HCV-related CG is scarce. A few clinical reports have suggested that post-transplant HCV-related CG might account for a significant morbidity and even mortality [13, 26, 27]. The aim of this study was to assess the frequency and clinical impact of HCV-related CG in a series of patients that had received liver transplants after HCV cirrhosis.

Patients and methods

Patients

From January 1990 to September 1996, 36 patients received liver transplants for HCV-related cirrhosis in our institution. Diagnosis of HCV-related cirrhosis in these patients was based on the combination of histologically proven cirrhosis, serological markers of HCV infection, positivity of serum HCV RNA by polymerase chain reaction (PCR), and exclusion of other causes of chronic liver disease. Among these 36 patients, 30 patients with a post-LT follow-up longer than 1 year and evidence of chronic HCV graft reinfection were studied. There were 20 men and 10 women, with a mean age of 52.7 ± 7.1 years (range 37–63) at LT. Other characteristics are summarized in Table 1. Informed consent was obtained from all patients before participation in the study.

Diagnosis of HCV graft reinfection

Post-transplant chronic HCV graft reinfection was established on the following criteria: (a) detection of HCV RNA in post-transplant sera, with or without elevation of serum aminotransferase activity, for more than 6 months post-transplant; (b) histologic features of chronic hepatitis on the liver graft biopsy specimens; and (c) no other cause of graft dysfunction.

Immunosuppressive regimen

Our standard immunosuppressive regimen has been previously described [8] and is summarized as follows: (a) In the case of normal post-operative renal function, initial immunosuppression consisted of continuous intravenous infusion of cyclosporine A (CsA; Sandimmun, Sandoz, Rueil-Malmaison, France) targeting a whole-blood monoclonal concentration plateau of 400 ng/ml, in association with steroids and azathioprine (Imurel, Wellcome, Paris, France). By postoperative day 15, intravenous cyclosporine was switched to oral cyclosporine and tapered to target trough blood monoclonal levels of 200 ng/ml by the end of the 1st post-operative month. Azathioprine was systematically withdrawn by the end of the 3rd postoperative month. (b) In the case of post-operative renal failure, as defined by serum creatinine above 180 μmol/l on day 1, CsA and azathioprine were stopped and a 10-day course of antithymocyte globulins (Thymoglobuline; Pasteur Mérieux, Lyon, France), 100 mg/day, was administered. In such cases, CsA was reintroduced 48 h before completion of antithymocyte globulins treatment. (c) By November 1995, oral tacrolimus (Prograf, Fujisawa, Nanterre, France) was used in most patients as a part of the initial and maintenance immunosuppressive regimens; steroids were used in combination with tacrolimus until the 6th postoperative month and were withdrawn thereafter.

In the case of histologically-confirmed rejection episode, patients received 2 to 3 pulses of 15 mg/kg of methylprednisolone; steroid-resistant rejections were treated with an additional 10-day course of OKT3.

Studied parameters

The following clinical data were collected: age at transplant; sex; type of initial immunosuppression (CsA, tacrolimus, or antithymocyte globulins); maintenance immunosuppressive regimens as assessed by the CsA or tacrolimus trough blood levels and steroid daily dose on the day of serum sampling; and pre- and post-transplant extrahepatic clinical manifestations possibly related to HCV. In addition, the following investigations were performed after a median time of 24.0 months (range 12–60 months) post-transplant: measurement of CG and rheumatoid factor; total serum hemolytic complement (CH50) titer; C3 and C4 complement fractions and IgG, IgA, IgM levels; liver biochemical tests (aspartate aminotransferase, ASAT, alanine aminotransferase, ALAT, alkaline phosphatase and γ-glutamyl transpeptidase serum activities, serum bilirubin and prothrombin time); quantification of serum HCV viral load; and HCV genotyping. Measurement of monoclonal immunoglobulins and detection of anti-tissue antibodies, including anti-smooth muscle, anti-nuclear, anti-LKM1 and anti-mitochondrial antibodies were also undertaken. Finally, liver biopsy specimens were obtained simultaneously to serum sampling in 27 of the 30 patients.

Laboratory procedures

Standard laboratory tests were performed using well-validated, routine procedures used in the clinical laboratories of our institution.

Detection, quantification, and genotyping of HCV RNA

Sera from all patients were sampled for HCV RNA testing, aliquoted, and frozen at –80°C. HCV RNA was tested by a qualitative RT-PCR technique and quantified using the second-generation bDNA assay (Quantiplex HCV RNA 2.0; Chiron Diagnostics, Emeryville, Calif., USA). HCV genotype was determined in patients with positive PCR with the Inno-LiPA HCV kit (Innogenetics, Gent, Belgium).

Detection of cryoglobulins

Venous blood samples were taken from fasting subjects in a warm room at 37°C and allowed to clot at 37°C for two h. Serum was obtained after centrifugation at 37°C. After centrifugation, serum supernatant was removed and stored with 0.1% sodium azide at 4°C in Felix tubes. Tubes were examined for cryoprecipitation after 8 days. When present, cryoglobulin was quantified by cryocrit determination.

Table 1 Preoperative characteristics of the 30 candidates receiving liver transplants for hepatitis C virus-associated liver disease. *ASAT* Aspartate aminotransferase, *ALAT* alanine aminotransferase, *Ig* immunoglobulin

Number of patients	30
Sex (M/F)	20/10
Age (years)	52.7 ± 7.1
Indications of liver transplantation	
Endstage cirrhosis	24 80%
Hepatocellular carcinoma complicating cirrhosis	6 20%
Child-Pugh score	9.4 ± 2.3
Prothrombin time (%)	51.5 ± 15.1
Serum bilirubin (μmol/l)	49.4 ± 30.7
ASAT (IU/l)[a]	105.0 ± 66.9
ALAT (IU/l)[b]	80.2 ± 48.1
IgG (g/l)[c]	24.0 ± 9.5
IgM (g/l)[d]	2.0 ± 0.8
IgA (g/l)[e]	3.9 ± 1.4

[a]Normal values less than 40 IU/l
[b]Normal values less than 45 IU/l
[c]Normal values less than 14.0 g/l
[d]Normal values less than 2.4 g/l
[e]Normal values less than 4.1 g/l

Detection of rheumatoid factor

Rheumatoid factor was measured with a nephelometer analyzer (BNA, Behring, Marburg, Germany). Normal values were less than 15 IU/ml.

Total serum hemolytic complement (CH50) titer

CH50 was determined using the kinetic method (Chromo Timer System; BNA, Behring, Marburg, Germany). Results were expressed as a proportion of time required to lyse a fixed amount of sheep red blood cells (normal values 80–120%).

Levels of C3 and C4 complement fractions

C3 and C4 fractions were measured with a nephelometer analyzer. The results were expressed as grams per liter (normal range: C3, 0.75–1.4 g/l; C4, 0.15–0.42 g/l).

Detection of monoclonal immunoglobulins

Detection of monoclonal immunoglobulins was performed by an immunofixation technique (Peragon SPE Kit; Beckman Instruments), using monospecific antisera against γ, κ, and λ light chains,

Detection of serum auto-antibodies

Anti-nuclear, anti-smooth muscle, anti-LKM1 and anti-mitochondrial antibodies were detected by indirect immunofluorescence using air-dried cryostat sections from rat or mouse livers and kidneys and Hep-2 cells (Kallestad, Chaska, Minnesota) as substrates. The serum specimen were tested undiluted for anti-nuclear antibodies and at a 1:10 dilution for other antibodies. The titers were established using increasing dilutions up to 1:2560.

Pathological analysis of the graft

The HCV-related liver graft changes were graded by histology index of both Knodell [17] and METAVIR [4] scoring systems.

Statistical analysis

Results are presented as mean ± 1 SD or prevalence rates. Comparison of categorical parameters were peformed using the χ^2-test, or Fischer exact test when appropriate. Comparison of continuous parameters were performed using the Student's *t*-test or the non-parametric Mann-Whitney test when appropriate. A *P*-value less than 0.05 was considered significant.

Results

In 27 out of 30 patients, HCV graft reinfection was diagnosed on flare-up in aminotransferase activity, which occurred 4.3 ± 3.6 months after LT (range 1–13 months) and was confirmed in all these cases by the detection of serum HCV RNA. In the 3 remaining patients, aminotransferase activity remained normal and HCV recurrence was diagnosed only on the detection of serum HCV RNA.

Post-transplant CG was found positive in 9 of 30 patients, for a cumulative incidence of 30%. In all of these 9 patients, CG was detected with a cryocrit below 1%, making the typing of cryoglobulin not possible. Four of the nine patients with positive CG presented with clinical manifestations consistent with CG-related vasculitis. Such manifestations were not observed in patients without CG. The clinical features of patients with symptomatic CG are presented in Table 2. Clinical manifestations consisted of isolated, recurrent lower limb palpable purpura in three cases, and nephropathy consistent with membranous proliferative glomerulonephritis associated wih lower limb palpable purpura in the other case. In five patients, CG was asymptomatic. However, in one of these patients, lymphoproliferative disorder was diagnosed 39 months post-transplant. No evidence of Epstein-Barr virus infection was found on the tumor, but bone marrow morphology showed lymphoid aggregates as reported in nontransplanted, HCV-infected patients presenting with CG-associated, low-grade B-cell non-Hodgkin's lymphoma [28].

The clinical and biological features of the patients with or without CG were compared (Table 3). C4 levels were significantly lower and rheumatoid factor activity, and IgM levels were significantly higher in patients with CG when compared with patients without CG. The presence of serum monoclonal immunoglobulin was screened in 28 of the 30 studied patients and was found positive in 6 of 28 cases (21.4%). The monoclonal immunoglobulin was an IgG of the λ type in 2 cases, an IgG of the κ type in 2 cases, and consisted of 2 two IgGs of λ and κ type in 2 cases. The proportion of monoclonal immunoglobulins did not differ whether CG was positive or negative: 2 of 9 vs 4 of 19. Age, sex distribution, liver biochemical tests, and prevalence of serum autoantibodies, HCV viral load, and HCV genotype distribution were similar in both groups. In addition, initial and maintenance immunosuppression did not differ significantly between the two groups of patients, although CG tended to be observed more frequently in CsA- than in tacrolimus-treated patients (9 of 22 vs 0 of 8, $P = 0.07$).

Post-transplant chronic hepatitis C biochemical activity as assessed on ASAT and ALAT activity did not differ between the patients with or without CG. In addition, the mean Knodell score (6.5 ± 2.9 vs 6.9 ± 3.5, $P = 0.9$), and the distribution of the activity and fibrotic indexes according to the METAVIR scoring system (data not shown) were similar in both groups of patients.

Patients with detectable CG were retested for CG after a median time of 36 months (range 12–60 months) following the first evaluation. In seven of nine cases, CG was still positive, with a cryocrit below 1%. However, in two cases, CG was no more detectable: in one case, CG disappeared spontaneously in a previously symptomatic patient (patient *n*°3, Table 2); in the other case, CG

Table 2 Features of the four patients who received liver transplants for HCV-associated liver disease and presenting with post-transplant symptomatic cryoglobulinemia. *LT* liver transplantation, *CG* cryoglobulinemia, *C4* complement fraction 4, *MP* methylprednisolone, *RF* rheumatoid factor

	Age at LT (years)	Sex	Time from LT to symptoms (months)	Symptoms	CG	RF activity[a]	C4	Treatment	Outcome
Case 1	53	F	27	Palpable purpura + glomerulonephritis	+	26	0.04	MP + Cyclophosphamide	Renal + heart failure + death
Case 2	63	F	27	Recurrent lower limb palpable purpura	+	112	0.07	Steroids (1st episodes)	Resolution, survival
Case 3	48	M	15	Lower limb palpable purpura	+	10	0.30	None	Ribavirin (4th episode) Resolution, alive
Case 4	54	F	34	Lower limb palpable purpura	+	647	0.50	None	Resolution, survival

[a]Normal values less than 15 IU/l
[b]Normal values less than 0.15–0.42 g/l

disappeared during a treatment with interferon and ribavirin, in a previously asymptomatic patient in whom antiviral treatment had been initiated for chronic active hepatitis.

Discussion

Although the natural history of HCV recurrence on the liver graft has been largely described [3, 5, 9, 10, 12, 30, 34], post-transplant extrahepatic consequences of HCV recurrence have been little studied. Several case reports have pointed out the potential clinical relevance of such manifestations, which sometimes could lead to death [13, 27]. We show in this work that the detection of HCV-related CG is a relatively frequent event post-transplant, with a cumulative incidence of 30%, close to that observed in chronically HCV-infected patients who have not received transplants [19, 23].

Moreover, in spite of immunosuppression, four of nine (44%) patients with CG experienced clinical manifestations consistent with HCV-related vasculitis, a prevalence which could be higher than the 10% prevalence of symptomatic HCV-related CG usually reported in nontransplanted, HCV-infected patients [23]. Overall, post-transplant CG accounted for a significant morbidity in 4 of 30, i.e., 13% of the patients in this series, and post-transplant CG-related death occurred in 1 of 30, i.e., 3% of our patients. This suggests that the clinical impact of post-transplant HCV-related CG could be far from negligible. Such results are consistent with those of two other studies [1, 11], in which the frequency of CG after LT for HCV-related cirrhosis was 18% and 19%, with clinical vasculitis observed in 5 of 15 (33%) [11] and four of six (66%) [1] patients with CG, respectively.

Testing for CG pre-LT would have been of interest in order to clarify the natural history of CG in patients that received liver transplants for HCV-related cirrhosis; however, the relationship between HCV and CG in nontransplant patients was first established in 1992 [2, 20], and we could not systematically look for CG pre-transplant, since most of the patients in this study had received transplants between 1990 and 1994.

The reason why CG is present in some patients post-transplant remains unknown. In the present study, we could not identify any relevant factors significantly associated with CG. Patient demographics, as well as virological factors such as genotype distribution or HCV viral load, did not differ between patients with or without CG. In addition, CG was not associated with more severe damage on the graft in contrast with what has been suggested in a preliminary report [11]. Although CG tended to be observed more frequently in CsA-treated patients, this difference did not reach statistical significance. However, whether CsA-based immunosuppressive regimens are associated with a higher incidence of post-transplant CG will have to be clarified by further studies. Indeed, such a finding could have a significant impact on the management of immunosuppression after LT for HCV cirrhosis.

It is currently accepted that HCV infection can induce clonal B cell expansion. This could be facilitated by a lowering of B lymphoid cell stimulating threshold through binding to CD81, a receptor which has recently been identified as a putative HCV membrane cell receptor [25]. The inhibition of apoptosis by HCV [21, 29] might also play a role, resulting in the promotion of B cell expansion. In transplant patients, the usual, dramatic increase in HCV viral load [7, 9, 10] could overstimulate B lymphoid cells' expansion and anti-HCV antibodies production, thus enhancing the production of immune complexes. In our study, HCV viral load was similar whether CG tested positive or negative, and this does not support such an hypothesis. However, HCV

Table 3 Features of the 30 patients who received liver transplants for HCV-related liver disease, with respect to the presence or absence of cryoglobulinemia after transplantation. *ATG* antithymocyte globulins, *ASAT* aspartate aminotransferase, *ALAT* alanine aminotransferase, *CsA* cyclosporin A, *CG* cryoglobulinemia, *HCV* hepatitis C virus, *C3* complement fraction 3, *C4* complement fraction 4

	Normal value	Patients positive for CG (n = 9)	Patients negative for CG (n = 21)	P
Age (years)		56.1 ± 4.2	51.3 ± 7.7	0.09
Sex (M/F)		4/9 44.4%	16/21 76.2%	0.2
ATG induction immunosuppression		2/9 22.2%	11/21 52.4%	0.26
Acute, treated rejection episodes[a]		1 11.1%	9 42.8%	0.20
Maintenance CsA		9 100%	13 1361.9%	0.07
Maintenance tacrolimus		0 0	8 38.1%	
Steroids (mg/day) at 1 year		5.9 ± 3.9	3.4 ± 2.9	0.07
Prothrombin time (%)	80–100	91.0 ± 10.6	88.7 ± 13.2	0.6
Bilirubin (μmol/l)	< 17	29.3 ± 19.1	21.3 ± 17.5	0.3
ASAT (IU/l)	< 40	54.8 ± 46.2	41.5 ± 36.9	0.4
ALAT (IU/l)	< 45	106.7 ± 114.4	78.7 ± 75.5	0.6
Creatinine (μmol/l)	< 130	132.7 ± 21.2	116.5 ± 23.4	0.08
C3	0.5–0.9 g/l	0.57 ± 1.62	0.76 ± 0.20	0.015
C4	0.1–0.4 g/l	0.16 ± 0.16	0.30 ± 0.10	0.009
CH50	80–120%	106.8 ± 51.5	134.9 ± 44.8	0.3
Rheumatoid factor	< 15 IU/l	209.5 ± 70.4	12.0 ± 4.4	0.004
IgG (g/l)	< 14.0	13.8 ± 7.2	12.0 ± 4.4	0.54
IgM (g/l)	< 2.4	3.2 ± 0.5	1.6 ± 0.9	0.0001
IgA (g/l)	< 4.1	2.3 ± 0.9	2.2 ± 1.2	0.8
Anti-nuclear antibodies	> 1/20	2/8 25.0%	9/19 47.4%	0.3
Anti-smooth muscle antibodies	> 1/20	2/8 25.0%	5/19 26.3%	0.9
HCV viral load (mEq/ml)		7.7 ± 7.1	17.4 ± 29.2	0.4
HCV genotype				
1a		1/9 11.1%	4/21 19.0%	
1b		7/9 77.8%	10/21 47.6%	
2a		1/9 11.1%	4/21 19.0%	0.7
Others		0	3/21 14.4%	

[a]One episode of rejection required OKT3 and was observed in a CG-negative patient

viral load might have been underestimated in patients with CG, since HCV RNA is known to be concentrated in cryoprecipitate [2, 6, 18, 33]. Sequential measurements of HCV viral load could be more useful in assessing the relationship between viral load and the occurrence of CG.

Mixed CG is currently considered as a pre-lymphomatous state [14, 28], and HCV infection has been involved in the onset of some types of lymphoma [31, 32, 35]. In addition, we recently described an increased incidence of post-transplant lymphoproliferative disorders in patients who received liver transplants

for HCV-related cirrhosis [15]. A facilitating role of HCV-related CG in the occurrence of some late, Epstein-Barr virus (EBV)-negative, post-transplant lymphoproliferative disorders could therefore be considered, as observed in one of our patients in this study.

The trend to a higher serum creatinine level in patients with CG suggests a deleterious effect of HCV-associated CG on renal function after transplantation, as already reported [16, 22]. However, in the present series, this trend was due to an increase in serum creatinine in only one patient (i.e., patient 1), in whom CG was responsible for glomerulonephritis, which later evolved toward end-stage renal failure and death. No firm conclusion can thus be drawn from our work, regarding the impact of CG on post-transplant renal function.

Interferon therapy in combination with ribavirin has recently been proposed for the treatment of CG-related symptoms in nontransplant patients. In addition, a beneficial effect of interferon and/or ribavirin has also been observed after LT, in patients with symptomatic, HCV-related CG [1, 24]. In patients 1 and 2 of our study, CG-related symptoms were diagnosed before the effect of interferon-ribavirin therapy had been demonstrated; these patients were therefore treated by increasing the immunosuppressive therapy (patients 1 and 2) and/or plasmapheresis (patient 1; Table 2). However, in the case of disabling manifestations of HCV-related CG in LT recipients, ribavirin, alone [24] or in combination with interferon therapy, must certainly be tested first.

In conclusion, this study shows that, after LT for HCV cirrhosis, the detection of CG associated with post-transplant HCV recurrence is a relatively frequent event which accounts for a significant clinical impact. Its clinical spectrum usually ranges from cutaneous vasculitis to glomerulonephritis. However, the role of HCV-associated CG in the occurrence of some EBV-negative lymphoproliferative disorders is conceivable [15, 28], and further studies are required to confirm such an association. Pathophysiological factors responsible for post-transplant CG have to be clarified as well. Meanwhile, specific therapeutic strategies must be tested to limit the clinical consequences of post-transplant extrahepatic manifestations of HCV infection.

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