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Persistence of Cryoglobulinemia in Patients with Chronic Hepatitis C after Successful Treatment with Direct-acting Antivirals

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Abstract: Hepatitis C virus (HCV) infection can cause chronic liver disease; it has also been associated with lymphoproliferative disorders (LPDs), such as cryoglobulinemia and B-cell non-Hodgkin's lymphoma. Our previous studies suggested that cryoglobulinemia, high titer of rheumatoid factor (RF), and hypocomplementemia are immunological markers of LPDs. In addition, recent therapies with direct-acting antivirals (DAAs) have achieved high rates of sustained virological response (SVR) in patients with chronic hepatitis C (CH-C). This study analyzed the efficacy of DAA therapy in CH-C patients with cryoglobulinemia, and the association of biochemical and other immune markers for LPDs with persistence of cryoglobulinemia in patients after DAA therapy. Of 226 patients tested, 31 (13.7%) had cryoglobulinemia prior to receiving DAAs, and these individuals showed lower complement 4 levels, decreased complement hemolytic activity, and higher IgM than patients without cryoglobulinemia. Of the 24 cryoglobulinemia-positive patients (83%) who could be followed for 24 weeks, 20 became cryoglobulinemia negative after the therapy. The remaining four patients retained the abnormal LPD markers, indicating the possibility of long-term LPD persistence even following successful eradication of HCV in CH-C patients. Thus, long-term follow-up is recommended to avoid exacerbation of extrahepatic manifestations as well as new events.

Key words: cryoglobulinemia, HCV, DAA, lymphoproliferative disorders

Introduction

Hepatitis C virus (HCV) infects 71 million people worldwide, causing chronic hepatitis (CH), liver cirrhosis (LC), and eventually hepatocellular carcinoma[1, 2)]. Chronic HCV infection causes considerable morbidity and mortality worldwide due to the high percentage of patients progressing to cirrhosis and end-stage liver disease[3, 4)]. Recent studies show that treatment with direct-acting antivirals (DAAs) achieves higher rates of sustained virological response (SVR) in

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CH-C patients than interferon (IFN)-based therapies[5–8].

HCV infection is also associated with extrahepatic manifestations in some patients, including mixed cryoglobulinemia[9–11] and B-cell non-Hodgkin's lymphoma (NHL)[12]. Our group previously reported a high prevalence of abnormal markers for lymphoproliferative disorders (LPDs) in patients with CH-C[13], and in particular, an association with HCV infection and / or B cell adsorption. Cryoglobulinemia, high levels of rheumatoid factor (RF), low complement levels, and clonal expansion of B cells were also frequently observed in the patients with CH-C; approximately 74% showed RNA positivity for HCV in B cells isolated from those patients. These results suggested that HCV infection is important in the manifestation of both liver disease and immunological disorders in affected patients. Indeed, a recent study also demonstrated abnormal activation of B cells in association with both CH-C and chronic infection with hepatitis B (CH-B)[14].

The extrahepatic manifestations of hepatitis viral infections have been associated with the presence of immune complexes comprising HCV virions and / or viral protein, immunoglobulin [IgM with rheumatoid factor (RF) activity], and complement proteins[15]. Indeed, antibodies to HCV (anti-HCV) and HCV RNA are detected more frequently in patients with non-Hodgkin's lymphoma than in the general population (30% vs. 1.3%)[12], while cryoglobulinemia represents the oligoclonal proliferation of B cells and occurs in 19–56% of patients infected with HCV[11, 16–18]. HCV has also been implicated in relapsed cryoglobulinemia after treatment with DAAs in short-term follow-ups[19]. Based on these observations, cryoglobulinemia is considered a strong LPD marker. In addition, rheumatoid factor (RF) in high titers and hypocomplementemia (low levels of C3, C4, or CH50) are regarded as immunological markers for autoimmune disease and lymphoproliferation, such as in Sjögren's syndrome[20].

In the last 5 years, antiviral therapies against CH-C have advanced remarkably from IFN-based therapies to DAAs, with improved SVR ratios. In terms of mechanisms, IFN therapies enhance the host defense responses in patients by stimulating the immune system, whereas DAAs directly target viral proteins needed for viral replication, processing, and assembly. Thus, we can analyze the direct effects of viral eradication after DAAs and associated changes in extrahepatic manifestations without over-stimulation of the immune system that occurs with IFN treatment. In this study, we investigated the prevalence of abnormal LPD markers, such as cryoglobulinemia in CH-C patients before and during DAAs administration.

Patients and methods

Study subjects

A total of 226 Japanese patients (CH-C, n = 165; LC, n = 61) admitted to Showa University Hospital and Showa University Koto Toyosu Hospital were enrolled in this study. The 159 patients infected with HCV genotype 1, were treated with SOF / LDV (n = 86), DCV / ASV (n = 48), OBV / PTV / r (n = 9), EBR + GZR (n = 9), and GCR / PBV (n = 7), respectively, and the 67 patients infected with HCV genotype 2 were treated with SOF + RBV (n = 57) and GCR / PBV (n = 10). Table 1 details the profiles of DAAs used in this study. Briefly,

NS3 is a protease inhibitor, while NS5B is the polymerase inhibitor of HCV, and NS5A is the nonstructural protein 5A, which plays a key role in viral replication and virion assembly. Ritonavir is one of the anti-HIV protease inhibitors which inactivates cytochrome P450 3A4 (CYP3A4) and boosts HCV NS3 inhibitors[21].

SVR was defined as undetectable HCV RNA levels 24 weeks after the end of the treatment. Viral information (genotype of HCV and titer of HCV RNA in serum), host factors [age, gender, platelet counts, serum levels of alanine transaminase (ALT), immunological markers (IgG, IgA, and IgM) and markers for LPD (cryoglobulinemia, high levels of RF, hypocomplementemia)] were analyzed. All variables including HCV-RNA level were evaluated before treatment, 1 and 8 weeks after the start of treatment, at the end of treatment, and 8 and 24 weeks after the end of treatment. Clinical characteristics of the CH-C patients are shown in Table 1. This was a retrospective observational study of patients with chronic HCV infection who received different DAAs therapies and who achieved SVR between February 2012 and November 2018. Each participant provided written informed consent, and this study was approved by the ethics committees of Showa University Hospital (approval number: 660) and Showa University Koto Toyosu Hospital (approval number: 14H007), both of which are suitably constituted according to the 1975 Declaration of Helsinki.

Markers of lymphoproliferative disorders

Semi-quantitative centrifugation was used to detect cryoglobulinemia, wherein blood samples were centrifuged at 600 × g for 20 min at 37℃. The serum was then cooled to 4℃ and allowed to stand for 48 hours, and then centrifuged again at 2,500 × g for 10 min at 4℃. The appearance of cryocrit at 4℃ and its disappearance after warming to 37℃ for 20 min was positive for cryoglobulins, while appearance of a small amount of cryocrit at 4℃ and disappearance of cryocrit after warming at 37℃ for 20 min was judged as weakly positive for cryoglobulins: no cryocrit at 4℃ was considered negative for cryoglobulins. RF was measured by latex nephelometry, and C3, C4 or CH50 activities were measured by nephelometry or Mayer's method, respectively.

Table 1. Profile of direct-acting antivirals used in this study

HCV Genotype	DAAs	Number of cases	NS3 inhibitor	NS5A inhibitor	NS5B inhibitor	Booster of NS3 inhibitor	other antiviral
1	DCV + ASV	48	asunaprevir	daclatasvir			
	LDV + SOF	86		ledipasvir	sofosbuvir		
	OBV + PTV/r	9	paritaprevir	ombitasvir		ritonavir*	
	EBR + GZR	9	grazoprevir	elbasvir			
	GCR/PBV	7	glecaprevir	pibrentasvir			
2	SOF + RBV	57			sofosbuvir		ribavirin
	GCR / PBV	10	glecaprevir	pibrentasvir			

*Ritonavir inactivates CYP3A4

Statistical analysis

Median values of continuous variables without normal distribution were compared using the Mann-Whitney U test. Discontinuous variables were compared using the chi-square test or Fisher's exact test. P values < 0.05 were judged to be statistically significant. The values of the normal distribution were expressed as mean ± standard deviation (SD). JMP Pro 14 software (SAS Institute, Cary, NC) was used for statistical revision.

Results

Abnormality of LPD markers in CH-C patients

Table 2 shows the clinical characteristics of HCV-infected patients. Cryoglobulinemia was identified in 13.7% of CH-C patients who enrolled in this study, and this prevalence was lower than that previously reported (24%)[22]. A high prevalence of hypocomplementemia [low C3 (33.6%), low C4 (10.6%), low CH50 (59.3%)], and high levels of RF (37.6%) were detected in the patients before DAAs administration, and interestingly, these findings were identical to the previous report.

Host and viral markers associated with cryoglobulinemia in CH-C patients

Among the 226 patients with CH-C, 31 cases (13.7%) were positive for Cg. We next analyzed the associated markers for cryoglobulinemia in this cohort. Table 3 indicated that no viral marker was identified, while a lower level of albumin and higher level of AFP were correlated with a Cg-positive status. Among the LPD markers, higher IgM, low C4, and low CH50 were associated with Cg positivity in CH-C patients.

Table 2. Clinical characteristics of HCV-infected patients (n = 226)

Age (years; median)	67 (24–88)
Gender (Male / Female)	104 (50.4%) / 122 (29.6%)
Outcome of DAAs therapy (SVR / non SVR)	220 (97.3%) / 6 (2.7%)
HCV RNA (log IU/ml)	5.9 ± 0.1
HCV Genotype (1 / 2)	159 (70.4%) / 67 (29.6%)
CH / LC	165 (73.0%) / 61 (26.9%)
ALT (IU/l)	54.1 ± 3.1
Platelets ($\times 10^4/mm^3$)	16.4 ± 0.4
Albumin (g/dl)	4.1 ± 0.4
FIB4 index	3.6 ± 0.2
AFP (ng/ml)	9.9 ± 1.1
Cryoglobulinemia	31 / 226 (13.7%)
IgG (mg/dl)	1,810 ± 34
IgA (mg/dl)	252 ± 11
IgM (mg/dl)	120 ± 4.9
C3 (< 86 mg/dl)	76 / 226 (33.6%)
C4 (< 10 mg/dl)	24 / 226 (10.6%)
CH50 (< 20 U/ml)	134 / 226 (59.3%)
Rheumatoid factor (> 15 IU/ml)	85 / 226 (37.6%)

The changing status of cryoglobulinemia after treatment with DAAs

We next analyzed the cryoglobulinemia response in patients treated with DAAs. Circulating Cgs are thought to comprise HCV and / or viral protein, IgM with RF activity, and complements, and thus eradicating the HCV component might lead to Cg destruction[15]. Among the 24 patients observed for the full 24 weeks after DAA treatment, 20 (83%) became Cg-negative. Further follow-up of more than 6 months after the end of treatment showed that the remnant circulating Cgs disappeared in two more patients (Fig. 1). These results suggest that clearing the Cg complex completely could take a long time, possibly more than 1 year after the end of treatment.

Status of the other LPD markers in patients not cleared of cryoglobulinemia

Tables 4 and 5 describe the clinical characteristics and LPD markers both before and after DAA therapy in patients who retained some level of cryoglobulinemia. Nine patients were still Cg-positive at 8 weeks after therapy (Table 4), and four remained positive at 24 weeks after therapy (Table 5). Table 4 showed that low CH50 (CH50 < 20 U/ml) was measured in 8 of 9 patients before therapy, and 6 of the 8 patients (75%) retained the low-CH50 status. High RF (RF > 15 IU/ml) levels were observed in 6 of 9 patients before therapy, and that status remained unchanged in these patients. High IgG (IgG > 1,800 mg/dl) and high IgM (IgM > 200 mg/dl) were retained in 3 of 5 patients (60%) and 2 of 4 patients (50%), respectively, at 24 weeks after therapy. All the patients showed improved ALT and AFP. The FIB4 index, which was associated with hepatic fibrosis (F3-F4 > 3.25), was improved in all but one patient (No. 4). These results indicate that liver injury and markers of HCC and fibrosis were improved at 8

Table 3. Univariable analysis for Cg(+) status in 220 CH-C patients

	Cg positive (n = 31)	Cg negative (n = 189)	*P*-value
Age (years; median)	69 (24-83)	67 (33-88)	N.S.
Gender (Male / Female)	10 (32.3%) / 21 (67.7%)	93 (49.2%) / 96 (50.8%)	N.S.
HCV RNA (log IU/ml)	5.8 ± 0.2	5.9 ± 0.1	N.S.
HCV Genotype (1 / 2)	21 (67.7%) / 10 (22.3%)	135 (71.4%) / 54 (28.6%)	N.S.
CH / LC	22 (73.3%) / 8 (26.7%)	140 (79.1%) / 37 (20.9%)	N.S.
ALT (IU/l)	50.8 ± 8.4	55.2 ± 3.4	N.S.
Platelets ($\times 10^4/mm^3$)	16.5 ± 1.1	16.4 ± 0.4	N.S.
Albumin (g/dl)	3.9 ± 0.1	4.1 ± 0.0	0.032
FIB4 index	3.9 ± 0.5	3.6 ± 0.2	N.S.
AFP (ng/ml)	16.9 ± 3.0	8.9 ± 1.2	0.0137
IgG (mg/dl)	1,908 ± 90	1,809 ± 36	N.S.
IgA (mg/dl)	240 ± 30	254 ± 28	N.S.
IgM (mg/dl)	149 ± 13	116 ± 5.4	0.0194
C3 (< 86 mg/dl)	12 / 31 (38.7%)	64 / 189 (33.9%)	N.S.
C4 (< 10 mg/dl)	8 / 31 (25.8%)	16 / 189 (8.5%)	0.0041
CH50 (< 20 U/ml)	26 / 31 (83.9%)	108 / 189 (57.1%)	0.0047
RF (> 15 IU/ml)	13 / 31 (41.9%)	71 / 189 (37.6%)	N.S.

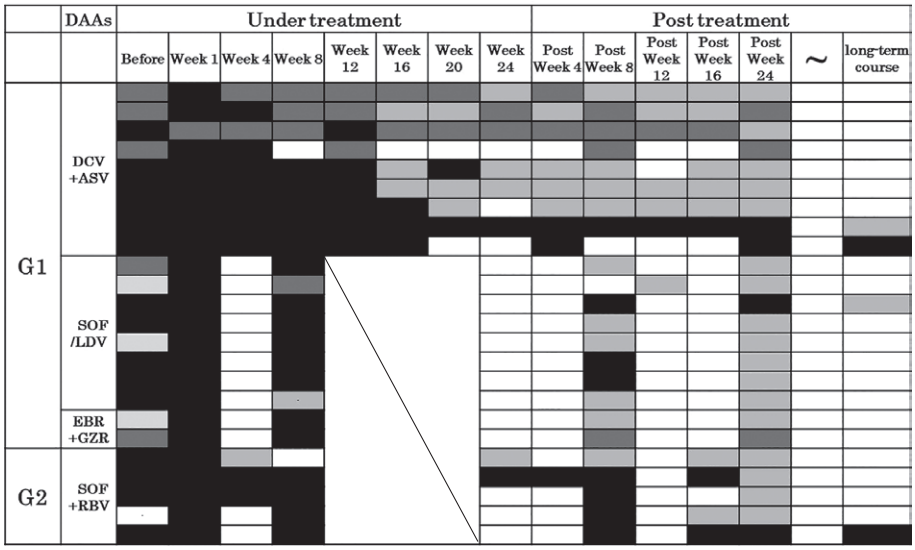

Fig. 1. Changing cryoglobulinemia status during and after the therapy with DAAs in CH-C patients. Cryoglobulin (Cg) status in 24 patients who were Cg positive before the DAA therapy is distinguished by colored boxes. Cg positive is portrayed as a black box, weakly positive as a dark gray box, and negative as a light gray box.

weeks after the end of DAA therapy, while the abnormal LPD markers persisted.

At 24 weeks after the end of DAA therapy, 3 of the 4 patients who remained Cg-positive also retained abnormal LPD markers, indicating that several immune disorders persisted even after 24 weeks in the CH-C patients who showed no evidence of HCV (Table 5).

Discussion

It has been proven epidemiologically that HCV causes a variety of extrahepatic manifestations, including cardiovascular diseases and dysfunction of the central nervous system[15]. In these manifestations, LPDs are most closely associated with HCV infection[22–24]. Zignego *et al*[25] reported the t (14; 18) translocation and overexpression of bcl-2 in lymphoid cells of patients with a lymphoproliferative disorder associated with HCV infection. It has been proposed that binding of HCV E2 protein to CD 81 and / or infection of B cells with HCV promotes B-cell clonality[26], and after the discovery of HCV, it was recognized that the majority of patients with mixed cryoglobulinemia were also infected with HCV[9–11]. Subsequent studies confirmed that up to 90% of patients with mixed cryoglobulinemia have chronic HCV infection[9, 27–30], although while circulating Cgs were present in 40–60% of HCV-infected patients, only 5–10% of these patients were symptomatic[31]. There is also substantial data indicating that HCV infects B cells and replicates[13, 32], but it is not completely clear whether HCV infection with B cells is required to develop mixed cryoglobulinemia.

The strongest support for the relationship between HCV infection and cryoglobulinemia is the response to antiviral therapies. Eradication of interferon therapy was reported to be effective partly for remission of cryoglobulinemia vasculitis on treatment, but some patients still relapsed

Table 4. Clinical characteristics of patients who remained positive for cryoglobulinemia at 8 weeks after the end of therapy

No.	Gender (F / M)	Age (y)	CH / LC	HCV RNA (IU/ml)	Genotype	ALT (Pre)	ALT	T-Bil (Pre)	T-Bil	Alb (Pre)	Alb	AFP (Pre)	AFP
1	F	81	CH	6.1	1	43	12	0.7	0.7	3.5	4	20.6	7.5
2	M	69	CH	6.3	1	78	23	1.6	1.0	4.1	4.2	7.2	5.1
3	F	79	CH	7.2	1	18	11	0.6	0.4	4	4.2	3	4
4	F	78	CH	5.8	1	27	20	0.4	0.5	4	4.2	1.5	1.2
5	M	77	LC	5.6	1	28	26	2.4	1.1	2.9	2.9	6.4	5.5
6	F	67	CH	5.3	2	14	6	0.4	0.3	4.3	4.1	3	-
7	F	77	CH	4.8	2	27	15	0.7	0.8	4.2	4.3	3.8	3.6
8	M	56	CH	5.3	2	153	21	0.9	0.9	4.6	4.8	6.2	4.8
9	M	68	LC	4.5	2	86	31	1.2	0.5	3.2	4.1	32.7	10.4

No.	IgG (Pre)	IgG	IgM (Pre)	IgM	C3 (Pre)	C3	C4 (Pre)	C4	CH50 (Pre)	CH50	RF (Pre)	RF	FIB-4 index (Pre)	FIB-4 index
1	1,875	1,449	169	81	71	74	9.3	11.6	5	29	72	34	6.2	4.2
2	1,736	1,518	293	197	87	82	13.7	13.8	26	26	1,352	473	5.6	4.1
3	1,433	1,422	490	417	74.9	109	2	2.8	6	15	1,244	1,071	2.6	2.8
4	2,681*	2,257	173	133	120	101	25.2	23.2	5	10	54	48	3.2	3.5
5	2,827	2,305	92	49	49	52	3.8	5.6	5	5	13	10	5.2	4
6	1,780	1,593	141	117	158	141	16.1	16.3	5	5	194	146	1.1	1.1
7	1,185	1,067	251	193	78	70	16.8	16.3	5	5	43	29	2.4	2.5
8	2,367	1,563	105	84	121	96	18.7	16	5	5	18	12	1.7	1.2
9	1,918	2,014	224	217	85	112	13.2	14.6	5	28	15	16	3.4	2.3

*Dark box indicates that the abnormal LPD markers status was not cured.

after the end of the therapy[33]. Antiviral therapy using DAAs enables a high rate of SVR in CH-C patients, thus promising potential complete remission for HCV-related LPDs such as cryoglobulinemia.

In this study, 31 of 220 CH-C patients (17.3%) being Cg-positive presented a slightly lower prevalence than that reported previously[13], while the prevalence of other LPD markers was almost identical as in previous study groups. In the 24 Cg-positive patients who were observed for 24 weeks after DAAs treatment, 83% of them became negative, indicating that eradication of HCV improved immunological abnormalities including HCV infection-related cryoglobulinemia. Nevertheless, Figure 1 indicates that some patients (8.3%) retained cryoglobulinemia even after more than 6 months. Our proposed model for developing cryoglobulinemia in CH-C patients is as follows[15]. At the first step, HCV viral particles and / or viral core protein bind to the B cells, leading to stimulation of B-cell activators. These stimulated B cells are then clonally proliferated, and they could release the IgM with RF activity. The IgM-RF molecules could form a cold precipitate of immune complexes comprising HCV particles and the C1q protein. The HCV immune complexes then can bind to vascular endothelial cells, stimulate

Table 5. Clinical characteristic of patients who remained positive for cryoglobulinemia at 24 weeks after the end of therapy

No.	Gender (F / M)	Age (y)	CH / LC	HCV RNA (IU/ml)	Group	ALT (Pre)	ALT	T-Bil (Pre)	T-Bil	Alb (Pre)	Alb	AFP (Pre)	AFP
1	F	81	CH	6.1	1	43	13	0.7	0.8	3.5	4.1	20.6	6.7
2	M	69	CH	6.3	1	78	21	1.6	1.6	4.1	4.2	7.2	3.2
3	F	79	CH	7.2	1	18	11	0.6	0.6	4	4.2	3	4
9	M	68	LC	4.5	2	86	15	1.2	0.6	3.2	3.9	32.7	6.8

No.	IgG (Pre)	IgG	IgM (Pre)	IgM	C3 (Pre)	C3	C4 (Pre)	C4	CH50 (Pre)	CH50	RF (Pre)	RF	FIB-4 Index (Pre)	FIB-4 index
1	1,875	1,542	169	79	71	81	9.3	12.5	5	33	72	30	6.2	3.3
2	1,736	1,324	293	137	87	85	13.7	21.1	26	32	1,352	273	5.6	3.6
3	1,433	1,447	490	392	74.9	−*	2	−	6	−	1,244	−	2.6	2.9
9	1,918	1,784	224	174	85	129	13.2	22.5	5	47	15	12	3.4	1.7

*The mark of – indicates no data.

the complement system to produce vasoactive peptides, and recruit neutrophils to cause leukocytoclastic vasculitis. Indeed, our results showed that cryoglobulinemia could persist even in SVR patients lacking serum HCV virions. Also, those patients who were Cg-positive before the DAA therapy showed a higher prevalence of immunological abnormalities (hypocomplementemia, high IgM) and hepatic fibrosis (low albumin and high FIB-4 index), than the Cg-negative patients. In a few cases still showing cryoglobulinemia at 24 weeks after the DAAs treatment, some immunologic abnormalities also remained. Together, the present data indicate that the immunological abnormalities accompanying cryoglobulinemia could persist for a long period after HCV eradication. The B cells in such patients also could potentially incur somatic mutations, such as the t (14; 18) translocation and overexpression of bcl-2 described above, as well as overt malignant lymphoma. Long-term observation is therefore recommended to monitor relapse or new onset of lymphoproliferative disease in CH-C patients.

Conflict of interest disclosure

Potential conflicts of interest: The authors have no commercial or other association that might pose a conflict of interest.

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