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Leukocytes are the major target of hepatitis C virus infection: Possible mechanism of multiorgan involvement including the heart

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Summary
Chronic hepatitis C virus infection has been associated with several extrahepatic manifestations, among these, renal and cardiac involvement. However, morphological evidence of hepatitis C virus localization in various organs remains to be clarified.
We used immunohistochemistry to analyze the hepatitis C virus core and nonstructural antigens. Peripheral blood mononuclear cells were obtained from four patients with hepatitis C virus infection. Liver, kidney, heart, and bone marrow were taken from autopsy specimens from nine patients with hepatocellular carcinoma and three patients with cardiomyopathies with positive hepatitis C virus infection.
Antibody against hepatitis C virus core antigen stained peripheral blood mononuclear cells, and the majority of positive staining was seen in CD68-positive macrophages. Hepatitis C virus core and NS4 antibodies stained mostly infiltrating cells in the liver, heart, kidney, and bone marrow, but not hepatocytes, myocytes, or globular cells. Serial sections stained by CD68, CD3, and CD20 antibodies showed that most of the hepatitis C virus-positive cells were CD68-positive macrophages.
We demonstrate for the first time clear distribution of hepatitis C virus antigen in mononuclear cells in various organs from patients with hepatitis C virus infection. This study suggests that macrophages are the major target of hepatitis C virus infection.

Introduction

Hepatitis C virus (HCV) has infected an estimated 170 million individuals worldwide and, in the next few years, the number of annual deaths from HCV-related liver disease and cancer in the US may exceed the number of deaths

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caused by the human immunodeficiency virus [1]. A screening test developed in 1990 has nearly eliminated the spread of HCV through blood transfusions in industrial countries, and the sharing of contaminated needles is now by far the most common mode of infection. As a result, the US Centers for Disease Control and Prevention estimates that new infections have decreased from approximately 230,000/year in the 1980s to fewer than 36,000 in the US in 1996. However, because most individuals infected in earlier decades are alive, it is estimated that 1.8% of the US population harbors the virus [2]. As these patients become older, HCV-related liver disease, now accounting for 8000–10,000 deaths/year in the US, and the single most common indication for liver transplants, is likely to increase [1].

HCV has been associated with several extrahepatic manifestations, among which the best characterized are mixed cryoglobulinemia associated with a risk of developing B-cell non-Hodgkin's lymphoma, and glomerulonephritis [3], which has been thought to be probably related to inflammatory disease of the small vessels [4]. Porphyris cutanea tarda is commonly associated with HCV infection, and other disorders have been loosely associated with the virus, including secondary Sjögren's syndrome, Moorthen's corneal ulcer, hyper-and hypo-thyroidism, and myositis [5]. However, the pathogenesis of these extrahepatic complications is not well understood. The importance of HCV in patients with myocarditis and cardiomyopathies has recently been noted [6–12].

Although the liver is considered the primary site of HCV replication *in vivo*, results from *in vitro* studies indicate that HCV is capable of low-efficiency replication in hematopoietic cells under special conditions [13–15]. Numerous investigators have sought evidence of hematopoietic HCV replication *in vivo* by studying peripheral blood mononuclear cells (PBMC) for the viral replicative intermediate RNA using reverse-transcription polymerase chain reaction (RT-PCR) methods. However, results using this approach have yielded conflicting results, possibly because of the problem of non-specific priming during RT-PCR of the HCV 5′ noncoding region of HCV. Thus the question of HCV *in vivo* hematotropism remains controversial [16,17].

In the current study, we tested the hypothesis of extrahepatic HCV expression *in vivo* using a highly sensitive and specific immunohistochemistry, which was designed to detect structural and nonstructural HCV proteins.

Methods

Patients

Nine consecutive patients with hepatocellular cancer and three patients with cardiomyopathies with HCV infection who were autopsied between October 2001 and December 2006 at Kyoto University, Kyoto, Japan, were studied.

Blood samples were obtained from four patients (a women and three men; mean age, 46 years) with circulating HCV RNA. Samples from five patients with hypertension with negative anti-HCV-antibody served as negative controls. These samples were obtained with informed consent.

Preparation of cells

PBMC were prepared from heparinized venous blood by Ficoll–Hypaque density gradient centrifugation (Ficoll–Hypaque, Pharmacia, Uppsala, Sweden). The PBMC pellet was washed three times by centrifugation in RPMI 1640 medium (Gibco, Grand Island, NY, USA) and the cells were counted in a hematocytometer. The cells ($0.5–0.7 \times 10^6$/mL) were fixed with 10% neutral-buffered formalin for 10 min and embedded in paraffin.

Immunohistochemistry

Slides of PBMC or tissue sections were fixed in 10% neutral-buffered formalin, and 5 μm sections of formalin-fixed paraffin blocks subjected to immunohistochemistry. The sections were deparaffinized with xylen and rehydrated by passage through gradually more diluted ethanol solutions, finishing with water. Endogenous peroxides were suppressed by treatment with 1% hydrogen peroxide in methanol for 30 min. Non-specific background staining was suppressed by preoccupation with 10% normal horse serum for 30 min. Mouse monoclonal antibodies against HCV-core [18] were used at 1:500 dilution and incubated at 4 °C overnight. Sections were incubated with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) for 30 min at room temperature and revealed by using DAB solution for 2 min.

HCV-core positive cells were semiquantitatively counted and classified into four groups: 1+, 0–25%; 2+, 26–50%; 3+, 51–75%; 4+, 76–100% of the total number of cell infiltrations in a blinded fashion. One thousand infiltrating cells were counted, and the number of cells with positive staining for HCV-core antigen was counted.

The specificity of the reaction for the detection of HCV-specific antigens was confirmed by examination of uninfected liver tissue and preadsorption of the antisera with their specific synthetic peptides before immunostaining. Preadsorption of HCV-core antibody (20 μg/ml) was performed by intubation with 10 μg/ml of HCV-core peptide (39–74 amino acid) for 2 h at room temperature. Immunostaining for clustering of differentiation (CD) markers [CD68 for macrophage (Dako Cytomation, Carpinteria, CA), CD3 for helper T (Calbiochem, Damstadt, Germany), and CD20 for B lymphocytes (Lab Vision, Fremont, CA)] was also performed.

For the double staining, CD3, CD20, and CD68 were stained first using the TSA Biotin Systemkit (NEN Life Science Products, Boston, MA) to enhance the reaction, and then the Vector SG substrate kit was used. Then, the sections were treated with 1% hydrogen peroxide in methanol for 30 min. Non-specific background was blocked by preincubation with 10% normal horse serum for 30 min. The sections were incubated at 4 °C overnight with anti-HCV-core antibody (1:500). Biotinylated anti-mouse IgG (Vector Laboratories) was used as the secondary antibody at room temperature for 30 min, and the sections were able to undergo color development by DAB solution for 2 min.

To confirm the specificity of the staining with HCV antigen, we also stained PBMC and the tissues from patients with or without HCV using a monoclonal antibody against nonstructural (NS) protein 4, which was kindly provided by

Dr. Isao Fuke of the Research Foundation for Microbial Diseases at Osaka University, because the presence of NS4 protein indicates that HCV replicates in the tissues [19,20].

Results

Fig. 1 demonstrates positive staining for HCV-core antigen in various organs from patients with hepatocellular cancer and cardiomyopathies with HCV infection. Unexpectedly the majority of HCV-core antigen positive cells in the liver (Fig. 1Ab), heart (Fig. 1Bb), kidney (Fig. 1Cb), and bone marrow (Fig. 1Db) were mononuclear cells. After adsorption of antisera with specific core peptide, most of the positive signals disappeared (Fig. 1Ac—Dc). Prevalence of positive staining in various organs was variable (Table 1). In cardiomyopathic patients, the number of HCV-positive cells tended to be increased in the heart. HCV-positive cells were most abundant in bone marrow. Double staining by CD68, CD4, or CD20 antibody showed that most of the HCV antigen positive cells were CD68-positive macrophages. Immunohistochemical data implicate macrophages as the primary site of HCV replication in the liver, heart, kidney, and bone marrow. Fig. 2 shows staining of peripheral blood mononuclear cells. Most of positive cells for HCV-core were CD68-positive macrophages (Table 2). Monoclonal NS4 antibody demonstrated positive staining in the liver, heart, kidney, bone marrow, and PBMC from HCV-positive patients, but no staining was found from those without HCV infection (Fig. 3).

Discussion

HCV infection is a major cause of hepatocellular carcinoma and the primary reason for liver transplantation in the United States. While hepatocytes have been reported to be the

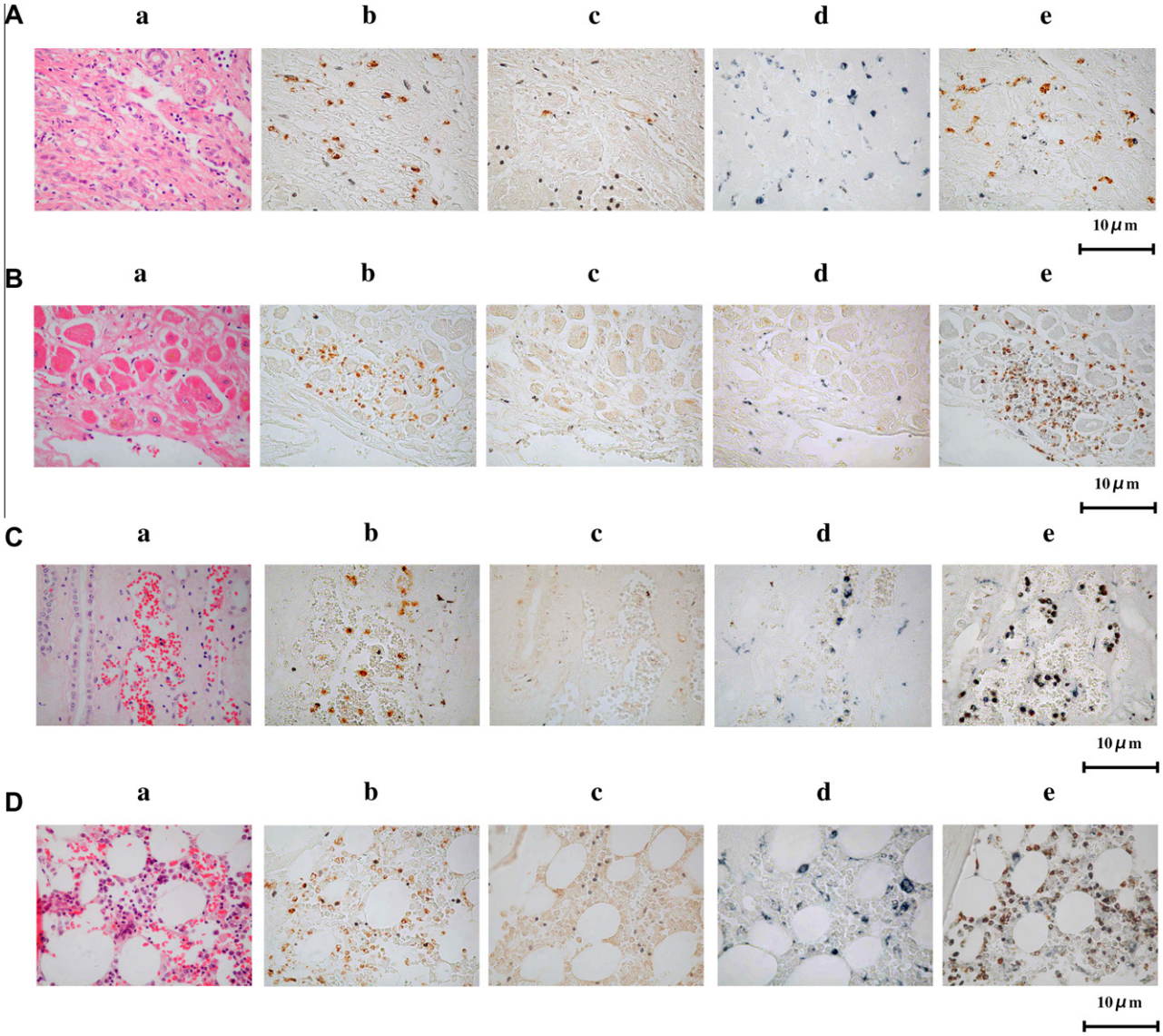

Fig. 1 Identification of HCV-core antigens in the liver (A), heart (B), kidney (C), and bone marrow cells (D) by immunohistochemistry. (a) Hematoxylin and eosin stain. (b) Immunostained with anti-HCV-core antibody. (c) Positive immunostaining became negative after adsorption with HCV-core antigen. (d) Immunostaining of anti-CD68 antibody. (e) Immunostaining of anti-HCV-core antibody and anti-CD68 antibody.

Table 1 HCV-core positive cells in patients with hepatocellular cancer and cardiomyopathics.

Age	Sex	Diagnosis	Liver	Heart	Kidney	Bone marrow
54	F	HCC	1	1	1	1
58	M	HCC	2	1	1	4
65	M	HCC	2	1	1	3
66	M	HCC	1	1	1	2
68	M	HCC	1	1	1	1
70	M	HCC	2	3	2	4
73	M	HCC	2	2	2	3
76	F	HCC	1	1	1	1
81	M	HCC	1	1	1	3
73	F	CM	2	2	1	3
81	M	CM	1	2	1	2
84	F	CM	2	4	1	2

HCC: hepatocellular cancer.
CM: cardiomyopathies.

major site of viral replication, a broad clinical spectrum of extrahepatic complication and diseases are associated with chronic HCV infection. We found positive and negative strands of HCV RNA from the hearts of many types of cardiomyopathies and myocarditis, and HCV was found to be an important cause of these diseases.

Recently, we were able to identify anti-HCV antibodies, HCV RNA, NT-proBNP, and cardiac troponin I and T in sera stored for up to 17 years and found the antibodies to be more prevalent in patients with myocarditis than in the general US population. The amounts of HCV RNA recovered varied widely among patients. Furthermore, whereas the prevalence of HCV infection in patients with active myocarditis tended to be higher than in patients without active myocarditis, the latter also had a higher prevalence of infection than the general population. It appears that, as observed with liver disease, the severity and time course of myocarditis associated with HCV infection is highly variable [10].

Immunohistochemistry has been used to detect HCV antigens. In paraffin-embedded tissue, however, conflicting results have been reported with the use of the commercially available antibody. One group of investigators found it to be a very specific and fairly sensitive test for HCV in fixed liver tissue [21], while others found it to be non-specific [22]. In a recent study, the majority of stained cells were hepatocytes, with granular cytoplasmic reaction [23]. Previous studies applying non-commercial monoclonal and polyclonal antibodies to either NS proteins or to HCV-core identified a similar immunohistochemical staining pattern [24]. These were seen to correlate well with HCV status as identified by nested PCR and by *in situ* hybridization [25]. Occasional positive staining of lymphocytes was identified [24]. Associated bile duct epithelial staining was identified in a small percentage [21,23,24]. Other investigators observed occasional nuclear reaction and identified constant nuclear staining of hepatocytes in a study [26]. These were almost always in association with the cytoplasmic staining of hepatocytes. Actually there is no available explanation for these occasionally identified staining patterns other than possible cross reactivity, or non-specific reaction that needs further investigation.

In the present study, antibody against HCV-core antigen stained PBMC, and the majority of positive staining was seen in CD68-positive macrophages. HCV-core antibody stained mostly mononuclear cells in the liver, heart, kidney, and bone marrow, but not hepatocytes, myocytes, or globular cells. Positive staining was found in PBMC and mononuclear cells of various tissues by antibody against NS4 protein, which also supports that HCV replicates in mononuclear cells. Therefore, mononuclear cells may be a primary target of HCV infection. The notion that HCV is a lymphotropic virus is supported by findings of replicating HCV found in B-cells [27], T cells [28], and PBMCs from patients with chronic hepatitis C [29]. Recently, occult HCV infection, defined as the presence of low levels of HCV genomes in serum, PBMCs, and/or liver in the absence of clinically evident liver disease, was identified in patients years after apparent complete resolution of hepatitis C [30]. More recently, immune cells have been shown to be reservoirs of HCV in symptomatic and occult infections [31,32].

The question of whether or not HCV is capable of replication in hematopoietic tissues remains controversial, despite a large number of published studies on the topic. Hematopoietic reservoirs of HCV infection could potentially play an important role in viral persistence through mechanisms such as immune escape and viral modulation of host immune responses. From a clinical perspective, hematopoietic infection may help explain the diverse clinical biology of chronic hepatitis C, including the existence of multiple extrahepatic disease syndromes, the association with non-Hodgkin's lymphoma, resistance to anti-viral therapy, relapse successful

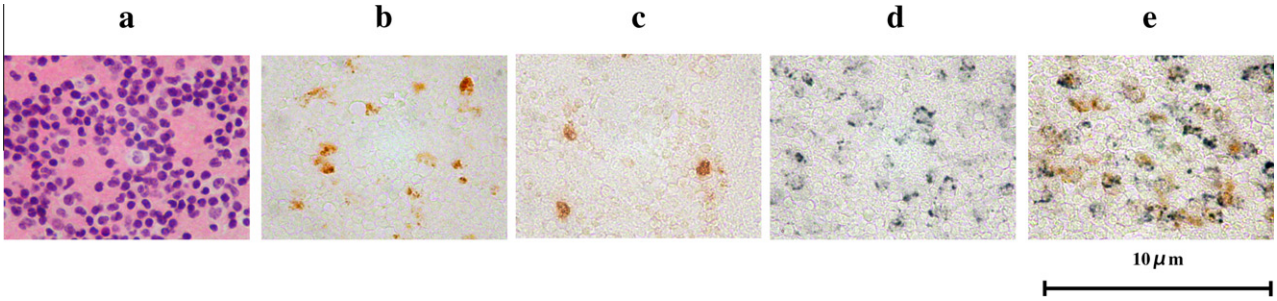

Fig. 2 Identification of HCV-core antigen in the peripheral blood mononuclear cells. (a) Hematoxylin and eosin stain. (b) Immunostained with anti-HCV-core antibody. (c) Positive immunostaining became negative after adsorption with HCV-core antigen. (d) Immunostaining of anti-CD68 antibody. (e) Immunostaining of anti-HCV-core antibody and anti-CD68 antibody.

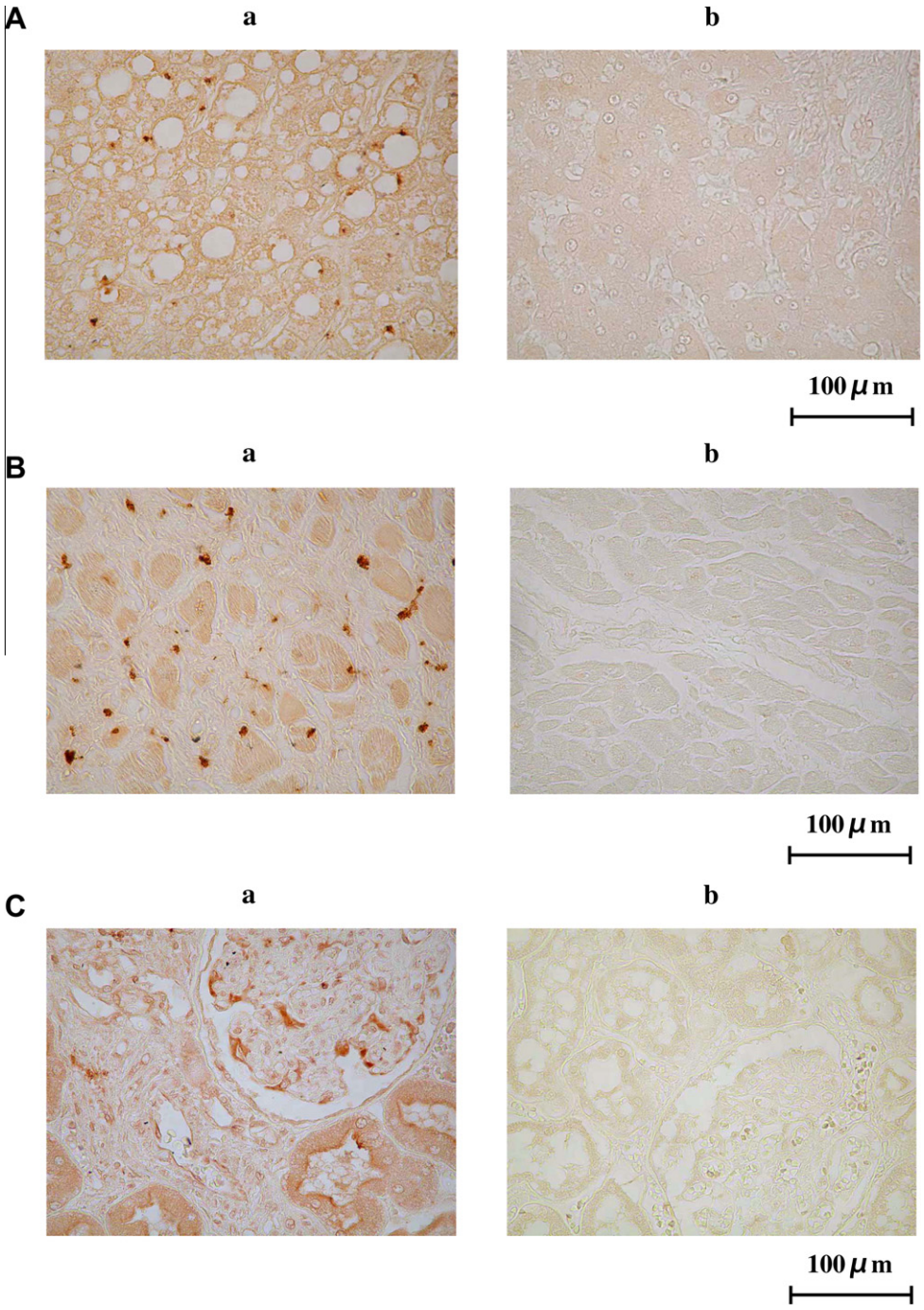

Fig. 3 Identification of HCV NS4 antigen in the liver (A), heart (B), kidney (C), bone marrow (D), and peripheral blood mononuclear cells (E). (a) Immunostained with anti-NS4 antibody with HCV-positive patients. (b) Immunostained with anti-NS4 antibody with HCV negative patients.

therapy, and recurrent infection after liver transplantation. Recent study showed that HCV commonly infects and replicates in perihepatic lymph nodes *in vivo*, documenting a new viral reservoir during natural infection in man [27]. All models to date have been based on the assumption that productive HCV replication occurs in extrahepatic reservoirs at an insignificant level compared with the liver [33,34].

The current results suggest a new model for extension of HCV infection in hepatitis C. Monocytes may become infected in blood or in bone marrow. Another possibility is that HCV infection may spread locally through the lymphatics to perihepatic lymph nodes, at which time peripheral immune cells might become productively infected prior to recirculation. In our study, bone marrow cells are positive in all patients, suggesting that bone marrow may be the primary site of HCV infection. Shimizu et al. [15] provided strong evidence of HCV hematotropism in both cell culture and the chimpanzee model. Based on previous studies the

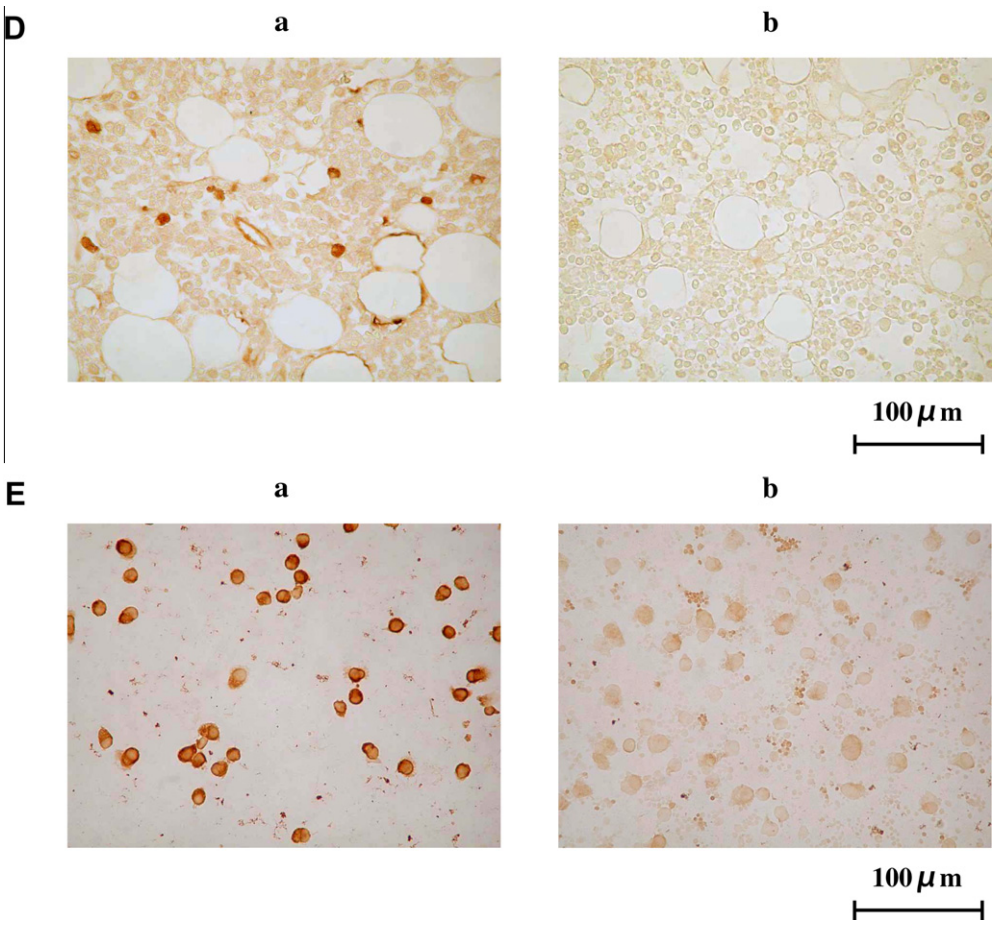

Fig 3. (*continued*)

evidence supporting tissue compartmentalization of HCV quasispecies replication *in vivo* is overwhelming. The previous studies have found that perihepatic lymphadenopathy is a common finding in patients with chronic hepatitis C [35,36]. Although it was originally assumed that perihepatic lymphadenopathy was a direct result of liver inflammation, recent findings raise the possibility that viral infection may play a direct role in perihepatic lymph node hyperplasia, possibly contributing to HCV-associated non-Hodgkin's lymphoma and other B-cells lymphoproliferative disorders [37–39], although this association is also controversial [40,41]. A recent study by Sung et al. [42] provided a strong link between HCV infection and lymphoma by demonstrating productive HCV replication in patient-derived lymphoma cells *in vitro*. Finally, the highly significant association between hepatitis C and immune complex disorders may be a direct consequence of HCV infection of B-cells in perihepatic lymph nodes, a question that deserves further study. It is also possible that lymph node and/or PBMC infection is a barrier to successful treatment with interferon regimens.

The present study suggests that CD68 monocytes/macrophages are the major target of HCV, and that CD3-positive T cells or CD20-positive B-cells are not major targets. Pharmacologic preparations targeting leukocytes infection might be a reasonable approach. Clinical applications to test this new concept include evaluating effects of anti-viral agents *ex vivo* using patient's mononuclear cells before treatment. Or, the effects of new therapeutic agents can be assessed *in vitro* by cultured mononuclear cells. Also, apheresis of mononuclear cells can be a possible treatment of HCV infection.

Table 2 Relationship between HCV-core positive cells and CD68, and CD3, and CD20 in peripheral blood mononuclear cells.

Patient	HCV-core + cells fin PBMC	Percentage in HCV-core + cells		
		CD68	CD3	CD20
1	32.5	52.3	0.0	0.0
2	46.5	66.7	0.0	0.0
3	26.5	1.9	0.0	0.0
4	77.0	4.5	0.0	0.0
5	52.0	82.7	0.0	0.0
6	44.5	15.7	0.0	0.0
7	12.5	24.0	0.0	0.0
%, Mean ± SEM	41.6 ± 7.8	35.4 ± 12.1	0.0 ± 0.0	0.0 ± 0.0

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References

[1] Cohen J. The scientific challenge of hepatitis C. Science 1999;285:26—30.
[2] Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, et al. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med 1999;341:556—62.
[3] Kasuno K, Ono T, Matsumori A, Nogaki F, Kusano H, Watanabe H, et al. Hepatitis C virus-associated tubulointerstitial injury. Am J Kidney Dis 2003;41:767—75.
[4] Johnson RJ, Gretch DR, Yamabe H, Hart J, Bacchi CE, Hartwell P, et al. Membranoproliferative glomerulonephritis associated with hepatitis C virus infection. N Engl J Med 1993;328:465—70.
[5] Nakamura K, Matsumori A, Kusano KF, Banba K, Taniyama M, Nakamura Y, et al. Hepatitis C virus infection in a patient with dermatomyositis and left ventricular dysfunction. Jpn Circ J 2000;64:617—8.
[6] Matsumori A. Cardiomyopathies and heart failure. In: Matsumori A, editor. Biomolecular, infectious and immune mechanisms. Boston: Kluwer Academic Publishers; 2003. p. 1—15.
[7] Matsumori A. Hepatitis C virus induces cardiomyopathies. Circ Res 2005;96:144—7.
[8] Matsumori A, Matoba Y, Sasayama S. Dilated cardiomyopathy associated with hepatitis C virus infection. Circulation 1995;92:2519—25.
[9] Matsumori A, Ohashi N, Hasegawa K, Sasayama S, Eto T, Imaizumi T, et al. Hepatitis C virus infection and heart diseases: a multicenter study in Japan. Jpn Circ J 1998;62:389—91.
[10] Matsumori A, Shimada T, Chapman NM, Tracy SM, Mason JW. Myocarditis and heart failure associated with hepatitis C virus infection. J Card Fail 2006;12:293—8.
[11] Matsumori A, Yutani C, Ikeda Y, Kawai S, Sasayama S. Hepatitis C virus from the hearts of patients with myocarditis and cardiomyopathy. Lab Invest 2000;80:1137—42.
[12] Okabe M, Fukuda K, Arakawa K, Kikuchi M. Chronic variant of myocarditis associated with hepatitis C virus infection. Circulation 1997;96:22—4.
[13] Kato N, Nakazawa T, Mizutani T, Shimotohno K. Susceptibility of human T-lymphotropic virus type I infected cell line MT-2 to hepatitis C virus infection. Biochem Biophys Res Commun 1995;206:863—9.
[14] Morsica G, Tambussi G, Sitia G, Novati R, Lazzarin A, Lopalco L, et al. Replication of hepatitis C virus in B lymphocytes (CD19+). Blood 1999;94:1138—9.
[15] Shimizu YK, Igarashi H, Kiyohara T, Shapiro M, Wong DC, Purcell RH, et al. Infection of a chimpanzee with hepatitis C virus grown in cell culture. J Gen Virol 1998;79(Pt 6):1383—6.
[16] Boisvert J, He XS, Cheung R, Keeffe EB, Wright T, Greenberg HB. Quantitative analysis of hepatitis C virus in peripheral blood and liver: replication detected only in liver. J Infect Dis 2001;184:827—35.
[17] Lanford RE, Chavez D, Chisari FV, Sureau C. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. J Virol 1995;69:8079—83.
[18] Takahashi K, Okamoto H, Kishimoto S, Munekata E, Tachibana K, Akahane Y, et al. Demonstration of a hepatitis C virus-specific antigen predicted from the putative core gene in the circulation of infected hosts. J Gen Virol 1992;73(Pt 3):667—72.
[19] Bermudez-Aguirre AD, Padilla-Noriega L, Zenteno E, Reyes-Leyva J. Identification of amino acid variants in the hepatitis C virus non-structural protein 4A. Tohoku J Exp Med 2009;218:165—75.
[20] De Francesco R. Molecular virology of the hepatitis C virus. J Hepatol 1999;31(Suppl. 1):47—53.
[21] Brody RI, Eng S, Melamed J, Mizrachi H, Schneider RJ, Tobias H, et al. Immunohistochemical detection of hepatitis C antigen by monoclonal antibody TORDJI-22 compared with PCR viral detection. Am J Clin Pathol 1998;110:32—7.
[22] Vartanian RR, Dupuis BNR. Nonspecificity of the novel paraffin-reactive hepatitis C virus, clone TORDJI-22. Lab Invest 1996;74:138A.
[23] Shiha GE, Zalata KR, Abdalla AF, Mohamed MK. Immunohistochemical identification of HCV target antigen in paraffin-embedded liver tissue: reproducibility and staining patterns. Liver Int 2005;25:254—60.
[24] Nouri-Aria KT, Sallie R, Mizokami M, Portmann BC, Williams R. Intrahepatic expression of hepatitis C virus antigens in chronic liver disease. J Pathol 1995;175:77—83.
[25] Sansonno D, Cornacchiulo V, Iacobelli AR, Gatti P, Di Stasi M, Dammacco F. Demonstration and distribution of HCV RNA sequences by in situ hybridization and HCV-related proteins by immunohistochemistry in the liver tissue of patients with chronic HCV infection. Pathobiology 1995;63:239—48.
[26] Nayak NC, Sathar SA. Immunohistochemical detection of hepatitis C virus antigen in paraffin embedded liver biopsies from patients with chronic liver disease. Acta Histochem 1999;101:409—19.
[27] Pal S, Sullivan DG, Kim S, Lai KK, Kae J, Cotler SJ, et al. Productive replication of hepatitis C virus in perihepatic lymph nodes in vivo: implications of HCV lymphotropism. Gastroenterology 2006;130:1107—16.
[28] MacParland SA, Pham TN, Gujar SA, Michalak TI. De novo infection and propagation of wild-type hepatitis C virus in human T lymphocytes in vitro. J Gen Virol 2006;87:3577—86.
[29] Kao JH, Chen PJ, Lai MY, Wang TH, Chen DS. Positive and negative strand of hepatitis C virus RNA sequences in peripheral blood mononuclear cells in patients with chronic hepatitis C: no correlation with viral genotypes 1b, 2a, and 2b. J Med Virol 1997;52:270—4.
[30] Pham TN, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV, Michalak TI. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. J Virol 2004;78:5867—74.
[31] Pham TN, King D, Macparland SA, McGrath JS, Reddy SB, Bursey FR, et al. Hepatitis C virus replicates in the same immune cell subsets in chronic hepatitis C and occult infection. Gastroenterology 2008;134:812—22.
[32] Carreno V, Pardo M, Lopez-Alcorocho JM, Rodriguez-Inigo E, Bartolome J, Castillo I. Detection of hepatitis C virus (HCV) RNA in the liver of healthy, anti-HCV antibody-positive, serum HCV RNA-negative patients with normal alanine aminotransferase levels. J Infect Dis 2006;194:53—60.
[33] Herrmann E, Lee JH, Marinos G, Modi M, Zeuzem S. Effect of ribavirin on hepatitis C viral kinetics in patients treated with pegylated interferon. Hepatology 2003;37:1351—8.
[34] Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, et al. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science 1998;282:103—7.

[35] Cassani F, Valentini P, Cataleta M, Manotti P, Francesconi R, Giostra F, et al. Ultrasound-detected abdominal lymphadenopathy in chronic hepatitis C: high frequency and relationship with viremia. J Hepatol 1997;26:479—83.
[36] Dietrich CF, Lee JH, Herrmann G, Teuber G, Roth WK, Caspary WF, et al. Enlargement of perihepatic lymph nodes in relation to liver histology and viremia in patients with chronic hepatitis C. Hepatology 1997;26:467—72.
[37] Ferri C, Caracciolo F, Zignego AL, La Civita L, Monti M, Longombardo G, et al. Hepatitis C virus infection in patients with non-Hodgkin's lymphoma. Br J Haematol 1994;88:392—4.
[38] Pioltelli P, Zehender G, Monti G, Monteverde A, Galli M. HCV and non-Hodgkin lymphoma. Lancet 1996;347:624—5.
[39] Silvestri F, Pipan C, Barillari G, Zaja F, Fanin R, Infanti L, et al. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. Blood 1996;87:4296—301.
[40] Mele A, Pulsoni A, Bianco E, Musto P, Szklo A, Sanpaolo MG, et al. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. Blood 2003;102:996—9.
[41] Udomsakdi-Auewarakul C, Auewarakul P, Sukpanichnant S, Muangsup W. Hepatitis C virus infection in patients with non-Hodgkin lymphoma in Thailand. Blood 2000;95:3640—1.
[42] Sung VM, Shimodaira S, Doughty AL, Picchio GR, Can H, Yen TS, et al. Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: the apoptotic effects of virus infection. J Virol 2003;77:2134—46.