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Nuclear localization of nucleocapsid-like particles and HCV core protein in hepatocytes of a chronically HCV-infected patient

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Abstract

Little is known about the life cycle of hepatitis C virus. Determination of the subcellular localization of HCV proteins may contribute to our understanding of the in vivo functions of the viral proteins. HCV core protein regulates multiple functions in host cells and it has been detected both in the cytoplasm and in the nucleus using different expression systems. In this study, nucleocapsid-like particles were observed in the nucleus of hepatocytes from a chronically HCV-infected patient. They were similar in size and shape to those of HCV core-like particles purified from recombinant *Pichia pastoris* cells. In addition the HCV core protein was detected not only in the cytoplasm but also in the nucleus and nucleolus of hepatocytes by immunoelectron microscopy. This is the first report showing nuclear localization of HCV core protein and nucleocapsid-like particles in hepatocytes during in vivo HCV infection.

Keywords: HCV; Core protein; Virus-like particles; Transmission electron microscopy; Immunoelectron microscopy

Hepatitis C virus (HCV) infection represents a major problem of public health with around 350 millions of chronically infected individuals worldwide [1]. The HCV genome is a 9.5 kb single-stranded positive sense RNA that contains a single open reading frame encoding a polyprotein of 3010–3030 amino acid (aa) residues. Upon its synthesis, this polyprotein is processed into at least 10 mature cleavage products, including the core (HCcAg), the envelope E1 and E2, and p7 structural proteins, as well as the NS2, NS3, NS4A, NS4B, NS5A, and NS5B non-structural proteins [2,3].

HCcAg is believed to be the viral capsid protein. Several additional functions, such as transformation and modulation of host cells, have been suggested for HCcAg [4–6]. Besides, HCcAg has been detected either in the cytoplasm or in the nucleus depending on the construct [7–10]. However, nuclear localization of HCcAg has not been proved during the natural HCV infection.

Previous reports had described the presence of HCV-related virus-like particles (VLPs) in different models [11–20]. In addition, it has been suggested that transmission electron microscopy (TEM) and Immunoelectron microscopy (IEM) might be particularly useful for diagnosis in clinical situations where serological markers and HCV RNA in serum are negative [11]. However, the limited sensitivity of the applied methods, cross-reactivity of employed antibodies with host-derived antigens, and the low levels of HCV particles in sera, plasma, and liver preparations indicated that additional experimental data are necessary before these techniques can be generalized.

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On the other hand, detection and localization of hepatitis C virus (HCV) antigens in the liver could be important to study the host–viral interactions at the cellular level. The presence of unenveloped VLPs resembling nucleocapsid-like structures with 20–30 nm in diameter and HCcAg in the nuclei of some hepatocytes was the most interesting finding of this work

Materials and methods

Patient. A liver needle biopsy sample was taken at the time of routine diagnostic biopsy from a patient with chronic HCV infection. This patient was selected based upon the criteria previously described [20]. It was not seropositive for markers of hepatitis B virus, hepatitis A virus, and human immunodeficiency virus by enzyme immunoassays (Tecnosuma International, Havana, Cuba). In addition, a liver needle biopsy sample was taken from an HCV-uninfected healthy donor liver for transplantation purpose as negative control.

Antibodies. The following mouse monoclonal antibodies (mAbs) were used for IEM studies: anti-HCcAg CBSS-HepC.1 mAb recognizing 5–35 aa, anti-HCcAg CBSS-HepC.4 mAb recognizing 95–100 aa, and anti-HCcAg CBSS-HepC.5 mAb recognizing 1–120 aa [20].

HCV-core like particle purification. HCV-core like particles were obtained as previously described [21]. In brief, recombinant *Pichia pastoris* MP-36 strain expressing HCcAg and the first 148 aa of the HCV E1 protein was grown in minimal glycerol medium (MYG) (1.3% yeast nitrogen base, 1% glycerol, and 0.4 μg/mL biotin) at 30 °C for 48 h. HCcAg expression from the methanol oxidase promoter was induced by replacing MYG with minimal methanol medium (MM) (1.3% yeast nitrogen base, 0.5% methanol, 0.4 μg/mL biotin) and further incubation at 30 °C for 96 h. At the end of the yeast cell culture, cells were harvested and washed twice in Ten buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl). Cell disruption was performed using glass beads in Ten buffer. The lysate was clarified by centrifugation at 12,000*g* for 20 min and supernatant and pellet fractions were obtained. The yeast cellular debris was treated with 0.5% sarkosyl in Ten buffer (a ratio of 10 mL for each g of cellular debris was used). The suspension was incubated for 120 min at room temperature with gentle agitation and then centrifuged at 12,000*g* for 20 min. Then, the supernatant containing HCcAg (1 mg/mL) was applied to a column (90×1.5 cm diameter) of Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden). It was run at a flow rate of 0.5 mL/min. Fractions containing HCcAg with more than 80% of purity were pooled.

Transmission electron microscopy. The hepatic tissue samples were fixed for 1 h at 4 °C in 1% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde, rinsed in 0.1 M sodium cacodylate (pH 7.4), post-fixed for 1 h at 4 °C in 1% OsO4, and dehydrated in increasing concentrations of ethanol. The embedding was done as previously described with minor modification [22]. Briefly, ultrathin sections (400–500 Å) made with an ultramicrotome (NOVA, LKB) were placed on 400 mesh grids, stained with saturated uranyl acetate and lead citrate, and examined with a JEOL/JEM 2000 EX transmission electron microscope (JEOL, Japan). In order to avoid sample bias, 100 microphotographs were analyzed in this study.

Immunoelectron microscopy. Samples of hepatic tissue were fixed with 4% (v/v) paraformaldehyde containing 0.2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at 4 °C for 3 h and washed with 0.1 M phosphate buffer, pH 7.3. Fixed cells were dehydrated as described above, embedded in Lowicryl, and polymerized by exposure to ultraviolet light at room temperature (RT) for 72 h. Ultrathin sections of liver biopsies were incubated with a mixture of anti-HCcAg mAbs (CBSS-HepC.1, CBSS-HepC.4, and CBSS-HepC.5) in phosphate buffer, for 45 min at RT. The sections were rinsed three times for 30 min at RT with 0.1% bovine serum albumin in phosphate-buffered saline, pH 7.3 (BSA-PBS), and incubated for 1 h at RT with gold-labeled (15 nm) anti-mouse IgG (Amersham, UK) diluted 1:100 in BSA-PBS. As control the primary antibody was substituted by normal mouse serum. All sections were stained and analyzed with a transmission electron microscope as mentioned above.

Indirect immunofluorescence analysis. Samples of hepatic tissue were fixed in 2% paraformaldehyde in PBST (0.1% Tween 20, phosphate-buffered saline). The fixed cells were blocked with 1% skimmed milk and incubated with a mixture of anti-HCcAg mAbs (CBSS-HepC.1, CBSS-HepC.4, and CBSS-HepC.5) in phosphate buffer. After washing twice, the cells were incubated with FITC-conjugated goat anti-mouse IgG (Sigma). The cells were then mounted and observed by confocal laser scanning microscopy.

Results and discussion

The determination of the subcellular localization of HCV proteins and VLPs may contribute to our understanding of the in vivo functions of the viral proteins. In the liver biopsy sample studied, ultrastructural cell damages typical of acute viral hepatitis and enveloped virus-like particles (VLPs) were observed. They were similar to those recently detected in liver biopsies from chronically HCV-infected patients [20]. However, the presence of unenveloped VLPs resembling nucleocapsid-like structures with 20–30 nm in diameter in the nuclei of some hepatocytes was the first interesting finding of this work (Fig. 1A). Note that in some cases VLPs co-localized with the nuclear chromatin. These particles were also observed in close contact with the nuclear membranes of some hepatocytes (Fig. 2).

In addition, these VLPs were similar in size and shape to the HCV core-like particles purified from recombinant *P. pastoris* cells (Figs. 1A and B), but smaller and more homogeneous than the naturally occurring core particles found in serum of HCV-infected individuals and those obtained in the baculovirus expression system [19].

On the other hand, using various anti-HCcAg mAbs (CBSS-HepC.1, CBSS-HepC.4, and CBSS-HepC.5) the presence of the HCcAg in the nucleus of hepatocytes was confirmed in this patient by IEM. As shown in Fig. 3B, HCcAg was detected in the nuclei and nucleoli of hepatocytes. It is noteworthy that (as was observed for VLPs) HCcAg also co-localized with the nuclear chromatin. HCcAg-specific immunolabeling was also observed in the cytoplasm and endoplasmic reticulum. However, in liver biopsies from normal controls (Fig. 3A) and sections where the first antibody was omitted, no HCcAg was detected.

The localization of HCcAg in the nucleus of hepatocytes from one HCV-infected patient may be of great biological significance. Since HCcAg has been shown to regulate cellular growth, a number of cellular and viral promoters are involved in hepatocarcinogenesis in transgenic mice [4–6,23–27].

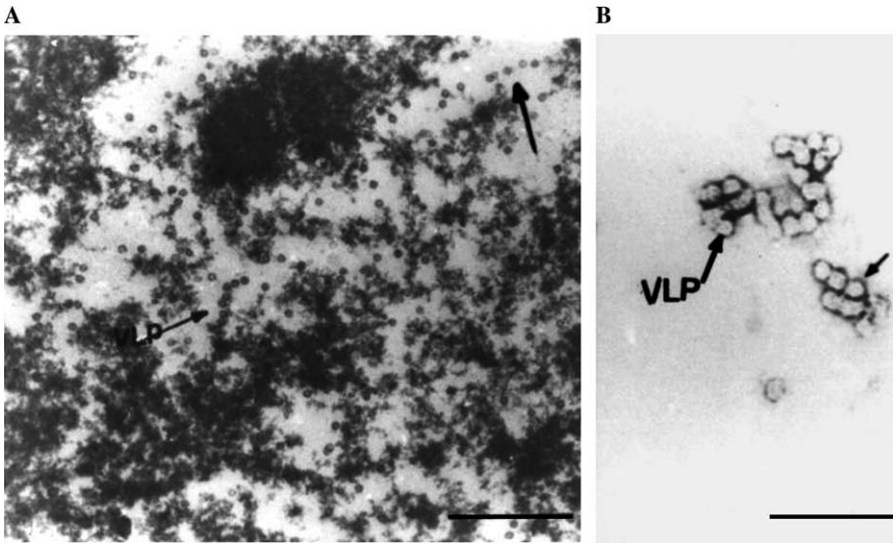

Fig. 1. Electron micrograph of nucleocapsid-like particles in the nuclei of hepatocytes. (A) Fragment of a hepatocyte nuclei from a chronically HCV-infected patient showing abundant nucleocapsid-like particles of 20–30 nm in diameter. (B) Negatively stained nucleocapsid-like particles 20–30 nm in diameter purified from recombinant *P. pastoris* cells. (Bar = 400 nm in A; 250 nm in B.)

HCcAg can acquire either nuclear or cytoplasmic localization, probably depending on the genotype of HCV and the size of the processed protein [8,9,28]. Unfortunately, the genotype of the HCV species present in the studied patient was not studied. However, the HCcAg nuclear localization in hepatocytes of one HCV-infected patient strongly suggests that the same cleavage effects to described in previous experiments [4,7–9,28–30] may take place during viral replication in vivo. Those studies indicated that truncated forms of HCcAg led to its localization in the nucleus, with a possible involvement in the regulation of transcription of host and viral genes. The finding of VLPs and HCcAg co-localized with the nuclear chromatin suggests that these proteins may play similar functions in vivo.

The nuclear localization of nucleocapsid-like particles, probably containing HCcAg, suggests that this finding may be of relevance in the HCV life cycle. This raises the interesting possibility of nuclear HCcAg assembly in vivo. Recently, both enveloped and unenveloped VLPs have been observed in the cytoplasm and in the endoplasmic reticulum but not in the nucleus of hepatocytes from four chronically HCV-infected patients [20]. In addition to the genotype of HCV and the size of the processed HCcAg, other factors may regulate either HCV nucleocapsid-like particles or HCcAg nuclear transport. A previous report using a hepatoma cell line stably expressing HCV structural proteins demonstrated that just 10–20% of the HCcAg expressing cells were stained in the nucleus by an HCcAg specific mAb

Fig. 2. Electron micrograph of nucleocapsid-like particles in close proximity to the nuclear membrane of hepatocytes. An hepatocyte nucleus from a chronically HCV-infected patient showing abundant nucleocapsid-like particles of 20–30 nm in diameter in close proximity to the nuclear membrane. (Bar = 200 nm.)

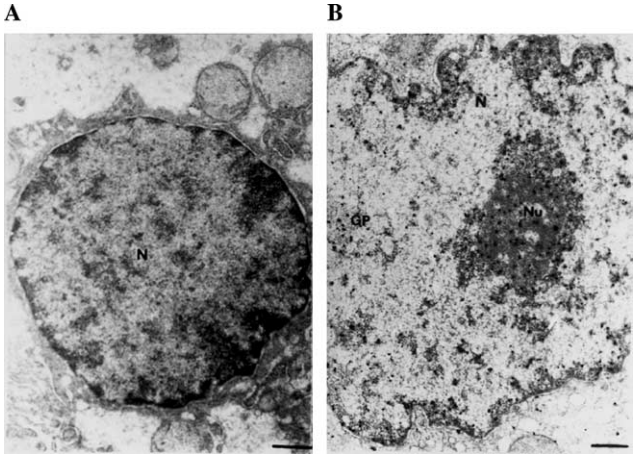

Fig. 3. Immunolabeling of HCcAg in the nuclei of hepatocytes by IEM. (A) Liver biopsies from healthy subjects, no HCcAg was immunolabeled in the nucleus (N). (B) Abundant identification of HCcAg in the nucleus (N) with enrichment in nucleoli (Nu) from hepatocytes of a chronically HCV-infected patient with a mixture of anti-HCcAg mAbs (CBSS-HepC.1, CBSS-HepC.4, and CBSS-HepC.5) and gold-labeled anti-mouse IgG (GP). (Bar = 250 nm in A, 200 nm in B.)

[30]. Similarly, in this work, the analysis using confocal laser scanning microscopy showed that 10% of the hepatocytes of the HCV-infected patient were stained in the nucleus and cytoplasm while 90% were stained just in the cytoplasm by the anti-HCcAg mAbs (not shown). Besides, we have recently shown that just a minor fraction of core-like particles temporarily assembled in the cell nucleus when expressing HCcAg in *P. pastoris* cells [31]. These studies suggest that while the mechanism of HCcAg migration into the nucleus is not clear it seems to be regulated. A more profound study in this field could further contribute to clarify the life cycle of HCV.

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References

[1] G.L. Davis, Current therapy for chronic hepatitis C, Gastroenterology 118 (2000) S104–S114.
[2] M. Hijikata, N. Kato, Y. Ootsuyama, M. Nakagawa, K. Shimotohno, Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis, Proc. Natl. Acad. Sci. USA 88 (1991) 5547–5551.
[3] Y. Matsuura, T. Miyamura, The molecular biology of hepatitis C virus in mammalian and insect cells, J. Virol. 66 (1993) 1425–1431.
[4] R.B. Ray, L.M. Lagging, K. Meyer, R. Steele, R. Ray, Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein, Virus Res. 37 (1995) 209–220.
[5] R.B. Ray, L.M. Lagging, K. Meyer, R. Ray, Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype, J. Virol. 70 (1996) 4438–4443.
[6] R.B. Ray, R. Steele, K. Meyer, R. Ray, Hepatitis C virus core protein represses p21WAF1/Cip1/Sid1promoter activity, Gene 208 (1998) 331–336.
[7] T. Miyamura, Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted, J. Gen. Virol. 76 (1995) 53–61.
[8] S.Y. Lo, F. Masiarz, S.B. Hwang, M.C. Lai, J.H. Ou, Differential subcellular localization of hepatitis C virus core gene products, Virology 213 (1995) 455–461.
[9] Q. Liu, C. Tackney, R.A. Bhat, A.M. Prince, P. Zhang, Regulated processing of hepatitis C virus core protein is linked to subcellular localization, J. Virol. 71 (1997) 657–662.
[10] G. Barba, F. Harper, T. Harada, M. Kohara, S. Goulinet, Y. Matsuura, G. Eder, Zs. Scaff, Zs.M.J. Chapman, T. Miyamura, C. Brechot, Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets, Proc. Natl. Acad. Sci. USA 94 (1997) 1200–1205.
[11] C. Bosman, M.B. Valli, L. Bertolini, A. Serafino, R. Boldrini, M. Marcellini, G. Carloni, Detection of virus-like particles in liver biopsies from HCV-infected patients, Res. Virol. 149 (1998) 311–314.
[12] M. Kaito, S. Watanabe, K. Tsukiyama-Kohara, K. Yamaguchi, Y. Kobayashi, M. Konishi, M. Yokoi, S. Ishada, S. Suzuki, M. Kohara, Hepatitis C virus particle detected by immunoelectron microscopic study, J. Gen. Virol. 75 (1994) 1755–1760.
[13] Y.K. Shimizu, S.M. Feinstone, M. Komara, R.H. Purcell, H. Yoshimura, Hepatits C virus: detection of intracellular virus particles by electron microscopy, Hepatology 23 (1996) 205–209.
[14] S. Laccovacci, A. Manzin, S. Barca, M. Sargiacomo, A.L. Serafino, M.B. Valli, G. Macioce, J. Hassan, A. Ponzetto, M. Clementi, C. Peschle, G. Carloni, Molecular characterization and dynamics of hepatitis C virus replication in human fetal hepatocytes infected in vitro, Hepatology 26 (1997) 1328–1337.
[15] A. Serafino, M.B. Valli, A. Alejandrini, A. Ponzetto, G. Carloni, L. Bertolini, Ultrastructural observations of viral particles within hepatitis C virus-infected human B lymphoblastoid cell line, Res. Virol. 148 (1997) 153–159.
[16] V. Falcón, C. Garcia, M.C. de la Rosa, I. Menendez, J. Seoane, J.M. Grillo, Ultrastructural and immunocytochemical evidences of core-particle formation in the methylotrophic *Pichia pastoris* yeast when expressing HCV structural proteins (core-E1), Tissue Cell 31 (1999) 117–125.
[17] T.F. Baumert, S. Ito, D.T. Wong, T. Liang, Hepatitis C virus structural proteins assemble into virus-like particles in insect cells, J. Virol. 72 (1998) 3827–3836.
[18] E. Blanchard, D. Brand, S. Trassard, A. Goudeau, P. Roingeard, Hepatitis C virus-like particle morphogenesis, J. Virol. 76 (2002) 4073–4079.
[19] P. Maillard, K. Krawczynski, J. Nitkiewicz, C. Bronnert, M. Sidorkiewicz, P. Gounon, J. Dubuisson, G. Faure, R. Crainic, A. Budkowska, Nonenveloped nucleocapsids of Hepatitis C virus in the serum of infected patients, J. Virol. 75 (2001) 8240–8250.
[20] V. Falcon, N. Acosta-Rivero, G. Chinea, J. Gavilondo, M.C. de la Rosa, I. Menéndez, S. Dueñas-Carrera, A. Viña, W. García, V. Gra, M. Noa, E. Reytor, M.T. Barceló, F. Alvarez, J. Morales-Grillo, Ultrastructural evidences of HCV infection in hepatocytes of chronically HCV-infected patients, Biochem. Biophys. Res. Commun. 305 (2003) 1085–1090.
[21] N. Acosta-Rivero, J.C. Aguilar, A. Musacchio, V. Falcon, A. Vina, M.C. de la Rosa, J. Morales, Characterization of the HCV core virus-like particles produced in the methylotrophic yeast *Pichia pastoris*, Biochem. Biophys. Res. Commun. 287 (2001) 122–125.
[22] A.R. Spurr, A slow viscosity epoxy resin embedding medium for electron microscopy, J. Ultrastruct. Res. 26 (1969) 31–43.
[23] C.M. Shih, S.Y. Lo, T. Miyamura, S.Y. Chen, Y.H. Lee, Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in Huh-7 cells, J. Virol. 67 (1993) 5823–5832.
[24] C.M. Shih, C.M. Chen, S.Y. Chen, Y.H. Lee, Modulation of the trans-suppression activity of hepatitis C virus core protein by phosphorylation, J. Virol. 69 (1995) 1160–1171.
[25] K. Moriya, H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, K. Koike, The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice, Nat. Med. 4 (1998) 1065–1067.
[26] H. Marusawa, M. Hijikata, T. Chiba, K. Shimotohno, Hepatitis C virus core protein inhibits fas- and tumor necrosis factor alpha-mediated apoptosis via NF-κB activation, J. Gen. Virol. 73 (1999) 4713–4720.
[27] M.M. Lai, Hepatitis C virus proteins: direct link to hepatic oxidative stress, steatosis, carcinogenesis and more, Gastroenterology 122 (2002) 568–571.
[28] E. Santolini, G. Migliaccio, N. La Monica, Biosynthesis and biochemical properties of the hepatitis C virus core protein, J. Virol. 68 (1994) 3631–3641.

[29] R. Suzuki, Y. Matsuura, T. Suzuki, A. Ando, J. Chiba, S. Harada, I. Saito, T. Miyamura, Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted, J. Gen. Virol. 76 (1995) 53–61.

[30] T. Suzuki, T. Matsuura, T. Harada, R. Suzuki, I. Saito, T. Miyamura, Molecular basis of subcellular localization of HCV core protein, Liver 16 (1996) 221–224.

[31] N. Acosta-Rivero, V. Falcon, C. Alvarez, A. Musacchio, G. Chinea, M.C. de la Rosa, A. Rodriguez, S. Dueñas-Carrera, V. Tsutsumi, M. Shibayama, I. Menéndez, J. Luna-Munoz, M.M. Miranda-Sanchez, J. Kouri, J. Morales. Structured HCV nucleocapsids composed of P21 core protein assemble primary in the nucleus of *P. pastoris* yeast, Biochem. Biophys. Res. Commun. 309 (2003) 48–53.