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Detection of HCV Components and Pathological Reactions in Apoptotic Hepatocytes from Chronically HCV-infected Patients

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Abstract: Analysis of Hepatitis C Virus (HCV)-infected hepatocytes at the cellular level may contribute to elucidate the mechanisms of HCV pathogenesis. In this work, the presence of HCV components and pathological reactions in apoptotic hepatocytes from chronic HCV-infected patients were studied by electron microscopy and confocal microscopy. Eight samples of liver biopsies from patients with chronic hepatitis C were studied by laser scanning confocal microscopy, Transmission Electron Microscopy (TEM) and Immunoelectron Microscopy (IEM). Data provide evidence for apoptosis of hepatocytes from HCV-infected liver biopsies during chronic HCV infection. Confirmation of this process was based on the morphological data by TEM including cell shrinkage; chromatin condensation; formation of apoptotic bodies; phagocytosis by neighbouring cells; and the presence of DNA fragmentation by TUNEL assay and caspase 3 activation. Interestingly, Hepatitis C core protein (HCcAg) was specifically immunolabeled in the rough endoplasmic reticulum, mitochondria as well as in the nucleus of apoptotic hepatocytes. In addition, E1 was specifically immunostained in the cytoplasm and in the mitochondria of some hepatocytes. The presence of Crystalloid Bodies (CB) similar to those observed in recombinant *P. pastoris* expressing HCcAg was observed in the cytoplasm of some hepatocytes. Immunogold labelling showed that HCcAg co-localized with these CB. In addition, structures forming a paracristalline array and particles with a diameter of 50 nm appeared in the mitochondria of some apoptotic hepatocytes. Moreover, unstructured large aggregates containing HCcAg similar to those detected at late stages of HCcAg expression in recombinant *P. pastoris* cells were frequently observed in damaged hepatocytes. Of note, these aggregates were specifically immunostained with anti-HCcAg. Data suggest the possibility for a direct role of these HCV-related structures as well as HCcAg and E1 in apoptosis and pathogenicity.

Key words: HCV, liver biopsy, core protein, apoptosis, electron microscopy, confocal microscopy

INTRODUCTION

Hepatitis C Virus (HCV) infection is considered a major health problem affecting an estimated 170 million people worldwide[1]. Approximately 70 to 80% of HCV patients develop chronic hepatitis, which may be complicated by cirrhosis and/or hepatocellular carcinoma[2]. At present, there is no vaccine available to prevent HCV infection and current therapies are not optimal[3]. HCV is a member of the Flaviviridae family with a positive strand RNA of 9.6 kb. The viral genome is translated into a single polyprotein of 3,000 amino acids in host cells. A combination of host and viral proteases are involved in polyprotein processing to give at least nine different proteins[4,5]. The structural proteins of HCV are believed to comprise the core protein (HCcAg) and two envelope glycoproteins: E1 and E2[5].

Due to the lack of cell culture systems supporting the replication of HCV, viral proteins have been studied using different gene expression systems as well as transgenic mice[6-13]. In addition, it has been shown that HCcAg processing, morphogenesis and localization in yeast cells is similar to that observed in mammalian cells[9,14-17]. These studies indicate that recombinant yeast cells could be used as a useful system to study HCcAg-cell interactions.

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Several studies indicate that HCcAg has numerous functional activities. They have been shown that HCcAg regulate cellular growth, affect nuclear trafficking, modulate apoptosis, lipid metabolism, a number of cellular and viral promoters and is involved in hepatocarcinogenesis in transgenic mice[10,11,16,18-28]. These properties suggest that HCcAg, in concert with cellular factors, may contribute to pathogenesis during persistent HCV infection[19]. However, it should be note that many of those experiments have been performed using reverse genetic technology, various expression systems and experimental conditions. Thus, the relevance of these results under physiological conditions remains to be re-evaluated.

Multiple factors may influence the host-virus interaction in patients infected with HCV and these may result in diverse disease presentations. Since mechanisms of HCV infection and pathogenesis remain unclear, characterization of these mechanisms is now a major issue for the development of new strategies for anti-HCV treatment and prevention. It has been suggested that apoptosis of liver cells may play a significant role in the pathogenesis of hepatitis C[29]. However, the role of virus-related apoptosis in chronic HCV infection is unclear. It is unknown whether HCV induces apoptosis directly or whether cellular injury is immunologically mediated.

Detection and localization of HCV components in the liver would be important to study the host-viral interactions at the cellular level. In this work, the presence of HCV components and pathological reactions in apoptotic hepatocytes from chronic HCV-infected patients were studied by electron microscopy and confocal microscopy.

MATERIALS AND METHODS

Patients and samples: Patients with chronic HCV infection hospitalized for hepatitis in the Institute of Gastroenterology, Havana, Cuba were recruited after informed consent in writing was obtained. Liver needle biopsies samples were taken at the time of routine diagnostic biopsy from all patients. Eighth chronically HCV-infected patients (5 females and 3 males, aged 21-49 years) were selected based upon they were serologically positive to third-generation HCV enzyme immunoassays (Tecnosuma International, Havana, Cuba) and that the anti-HCV positive sera were confirmed by Ortho HCV 2.0 ELISA (Ortho Diagnostic Systems, Rariton, NJ). They also showed positive detection of serum HCV RNA by Reverse-Transcription Nested Polymerase Chain Reaction (RT-PCR) (Amplicor HCV Amplification Kit 2.0, Roche Diagnostic Systems,Inc), were histologically confirmed as bearing chronic hepatitis and had abnormal serum alanine aminotransferase levels for at least six month before the biopsy was performed. None were seropositive for markers of hepatitis B virus, hepatitis A virus and human immunodeficiency virus by enzyme immunoassays (Tecnosuma International, Havana, Cuba). None have suffered from non-viral liver diseases such us: drug toxicity, alcoholic liver disease, autoimmunity and metabolic and genetic liver disorders. In addition, liver needle biopsies samples were taken from two HCV-uninfected healthy donor livers for transplantation purpose as negative controls.

Antibodies: The following mouse monoclonal antibodies (mAbs) were used: anti-HCcAg SS-HepC.1 mAb recognizing aa 5 to 35 of HCcAg, SS-HepC.4 mAb recognizing aa 1-95 of HCcAg, SS-HepC.5 mAb recognizing aa 1-120 of HCcAg and anti-E1 SS-HepC.2 mAb recognizing aa 190 to 219 of E1. They have been described elsewhere[30,31].

Growth conditions for *pichia pastoris* cells: The *P. pastoris* strain MP-36/C-E1.339.12, transformed with pNAO.COE1.339 plasmid coding for the entire HCcAg and the first 148 aa of the HCV E1 protein has been previously described[9,14]. The MP-36 strain was used as a negative control[9,14]. The MP36/CE1.339.12 transformant and the MP-36 strain were grown using conditions already established[9,14]. They were harvested at 25°h after methanol induction.

Primers: The following synthetic probes corresponding to the highly conserved 5' noncoding region of HCV were used in this work:

- To detect the HCV-RNA of positive-strand the biotin-labeled HCV-1 antisense probe (5'- biotin-GTTTATCCAAGAAAGGACCC-3', position 188-207) was used.
- To detect the HCV-RNA of negative-strand the biotin-labeled HCV-2 sense probe (5'-biotin-TTCACGCAGAAAGCGTCTAG-3', position 63-82) was used.
- For controls, an antisense biotin-labeled probe for rat prolactin mRNA (5'-biotin-ACATATCTGTATACAGGGTAG-3', position 63-82) was used.
- These probes were synthesized and purified using conditions previously described[32].

***In Situ* Hybridization (ISH):** Samples were immediately fixed with 4% paraformaldehyde in Phosphate-Buffered Saline (PBS) buffer at 4ºC and then mounted on gelatine-coated glass slides and stored for 2 days at -200 ºC. Mounted samples were hydrated for 10 minutes in PBS and then incubated with dako Biotein Blocking System[33,34]. The tissue sections were

incubated with avidin solution for 10 minutes. Afterwards, the avidin solution was rinsed off and the slides incubated with biotin solution for 10 minutes. After three washes with PBS-Tween 5% (PBS-T), samples were incubated overnight at 4 ºC with the biotin-labelled probes (dilution 1:2000). Incubations were followed by washes with PBS-T. The second incubations were accomplished with fluorescein isothiocyanate (FITC)-labeled avidin (dilutions 1:100 in PBS-T, Vector laboratories, Inc., Burlingame, CA., USA) for 1 hour at Room Temperature (RT). After three washes with PBS-T the sections from all samples were counterstained with propidium iodide (dilution 1:1000, Vector laboratories, Inc. Burlingame CA., USA) from 5 to 10 minutes, followed by extensive washing in PBS-T. Stained samples were coverslipped in Vectashield mountaing medium (Vector Laboratories, Inc. Burlingame, CA., USA), sealed with nail polish and viewed on a confocal laser scanning microscope. The specificity of the ISH assay for HCV-RNA genome of both polarities was confirmed by the absence of signals when an unrelated probe was used or when HCV-specific probes were omitted from the hybridization mixture. In addition, the absence of signals was evident after predigestion of biopsy sections with RNase A (0.2 mg/mL) for 2 hours at 37 ºC before hybridization.

Determination of *in situ* cell death (TUNEL reaction): Samples were fixed with 4% paraformaldehyde in PBS solution. Afterwards, they were incubated for 10 minutes with PBS solution. DNA fragmentation was determined using a fluorescein *in situ* cell death detection kit, exactly as directed by the manufacturers (Boehringer, Manheim, Germany). Two controls were performed: the first one consisted of omitting the enzyme deoxi-nucleotidyl transferase (negative control) and the second one of adding DNA-asa I to the samples (positive control). Later on, samples were washed with PBS-T three times. Then, the sections from all samples were counterstained with propidium iodide (dilution 1:1000, Vector laboratories, Inc. Burlingame CA., USA) from 5 to 10 minutes, followed by extensive washing in PBS-T. Stained samples were coverslipped in Vectashield mountaing medium (Vector Laboratories, Inc. Burlingame, CA., USA), sealed with nail polish and viewed on a confocal laser scanning microscope.

Detection of caspase activation: Samples were immediately fixed with 4% paraformaldehyde in Phosphate-Buffered Saline (PBS) buffer at 4ºC and then mounted on gelatine-coated glass slides and stored for 2 days at -200 ºC. Mounted samples were hydrated for 10 minutes in PBS and then incubated with 0.2% Triton X in PBS during 10 minutes. To block non-specific antibody reaction, best results were obtained by incubating the sections with 0.2% Bovine Serum Albumin (BSA) (free of IgG) (Sigma Chemical Co. St. Louis, Mo.USA), for 10 minutes at RT. After two washes with PBS-T, samples were incubated overnight at 4ºC with a mAb specific for activated caspase 3 (dilution 1:60, Sigma Chemical, St. Louis Mo. USA). The incubations were followed by washes with PBS-T. The second incubations were accomplished with FITC-conjugated anti-mouse IgG (dilutions 1:60 in PBS-T, Vector laboratories, Inc., Burlingame, CA., USA) for 1 hour at RT. After three washes with PBS-T the sections from all samples were counterstained with propidium iodide (dilution 1:1000, Vector laboratories, Inc. Burlingame CA., USA) from 5 to 10 minutes, followed by extensive washing in PBS-T. Immunostained samples were coverslipped in Vectashield mountaing medium (Vector Laboratories, Inc. Burlingame, CA., USA), sealed with nail polish and viewed on a confocal laser scanning microscope. Negative controls were performed by substituting the primary antibodies with normal mouse serum. No labels were observed in the negative control preparations.

Laser confocal scanning imaging and serial section collection: Samples were viewed with a 60x (NA 1.4) objective on a Nikon microscope with attached laser confocal scanning system MRC 600 (BioRad, Watfod). Ten to twelve fields were imaged from each sample. Four to fifteen serial optical z-sections (0.2-0.5 µm thick) were collected from each observed field using the dual channel imaging with filter (554 nm) for propidium iodide excitation and the filter (494 nm) for the fluorescein channel. Each series of confocal optical sections was scanned through a total of 25 µm. The resulting optical sections were fully projected onto two-dimensional planes using the imaging processing system of the microscope (Camos package).

Transmission Electron Microscopy (TEM): Samples of yeast cells were fixed and analyzed by transmission electron microscopy as previously described[9,15]. The liver tissue samples were fixed for 1 h at 4 ºC 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 ºC in 1% OsO4 and dehydrated in increasing concentrations of ethanol. The embedding was done as previously described with minor modification[30]. 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).

Immunoelectron Microscopy (IEM): The yeast cells were fixed and dehydrated as previously described[9,15]. Grids or ultrathin sections of yeast cells were incubated for 1 hour with the SS-HepC.1 and then incubated for 1 h at RT with gold-labeled anti-mouse IgG (Amersham, England) diluted 1:100 in BSA-PBS. As control the primary antibody was substituted by normal mouse serum. After washing with distillated water the samples were stained and analyzed with a transmission electron microscope as mentioned above. Samples of liver 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 ºC for 3 h and washed with 0.1 M phosphate buffer. Fixed cells were dehydrated as described above, embedded in Lowicryl and polymerized by exposure to ultraviolet light at room temperature for 72 h. Ultrathin sections of liver biopsies were incubated with either a mixture of anti-HCcAg mAbs or anti-E1 mAb in phosphate buffer, for 45 min at RT. The sections were rinsed three times for 30 min at RT with 0.1% BSA in PBS (BSA-PBS) and incubated for 1 h at RT with gold-labeled anti-mouse IgG (Amersham, England) 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.

RESULTS

Firstly, the presence of genomic and anti-genomic (negative replicative intermediate strand) HCV-RNA strands in the liver biopsies from patients with chronic HCV infection was analyzed. By using an *in situ* hybridization (ISH) assay followed by confocal microscopy, the HCV RNA of either positive (Fig. 1B, C) or negative (Fig. 1D) polarity was detected in the cytoplasm of some hepatocytes. No hybridization signals were observed in the liver biopsy specimens from the negative control subjects (Fig. 1A). The specificity of the ISH assay for HCV-RNA genome of both polarities was confirmed by the absence of signals when an unrelated probe was used or when the HCV-specific probes were omitted from the hybridization mixture. In addition, the absence of signals was evident after predigestion of biopsy sections with RNase A (not shown).

The presence of apoptotic hepatocytes in the liver biopsy specimens was analyzed using the TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick end labelling) assay. Figure 1F shows a representative experimental demonstrating that some hepatocytes from HCV-infected patients were undergoing apoptosis as indicated by dUTP incorporation. However, no sign of apoptosis was detected in hepatocytes from uninfected patients (Fig. 1E). In order to verify the detection of apoptotic hepatocytes, the activation of caspase 3 was investigated. Almost no immunoreativity was evident in healthy liver tissue using antibodies specific for activated caspase 3 (Fig. 1G). In contrast, liver tissue from patients with chronic HCV infection clearly showed hepatocytes that stained positively for active caspase 3 (Fig. 1H).

To confirm the presence of the apoptotic process in the liver biopsies, morphological data by electron microscopy was analyzed. As shown in Fig. 2 shrinkage of the apoptotic cells was significant. They were half the original size of the normal cells (Fig. 2A). Apoptotic nuclei characterized by chromatin condensation were found sporadically in the hepatocytes. Interestingly, HCcAg was specifically immunolabeled in the endoplasmic reticulum, mitochondria as well as in the nucleus of apoptotic hepatocytes (Fig. 2B). Note that HCcAg was mainly immunolabeled in the nuclear heterochromatin which appeared condensed and margined. Nuclear fragments with highly condensed chromatin were also observed in the cytoplasm (Fig. 2B, arrowhead). Apoptotic bodies (Councilman-like bodies) were also observed at ultrastructural level. These small and sharply defined bodies lied free among other hepatocytes (Fig. 2C inset). Apoptotic bodies were also frequently incorporated in the neighbouring cells, such as hepatocytes, Kupffer and endothelial-like cells (Fig. 2C,D).

It is interesting to note that HCcAg was specifically immunostained in the mitochondria of some hepatocytes (including apoptotic hepatocytes) (Fig. 3B). In addition, E1 was specifically immunostained in the cytoplasm and occasionally in the mitochondria of hepatocytes (Fig. 3C). However, no immunogold staining was observed in liver sections from uninfected individuals (Fig. 3A) or in sections incubated with normal mouse immunoglobulins as primary antibodies (not shown).

Various structures were detected both in the cytoplasm and in the mitochondria of some hepatocytes (including apoptotic hepatocytes) from HCV-infected liver biopsies. The presence of crystalloid bodies was observed in the cytoplasm of some hepatocytes (Fig. 4A,B). These structures were not detected in hepatocytes from normal controls. Immunogold labelling showed that HCcAg was specifically detected in these crystalloid bodies (Fig. 4B). However, no immunogold labelling was detected in sections incubated with either the anti-E1 mAb or normal mouse immunoglobulins as primary antibodies (Fig. 4A).

In addition, structures forming a paracristalline array and particles with a diameter of 50 nm appeared in the mitochondria of some apoptotic hepatocytes (Fig. 4C,D). These structures were not observed in liver

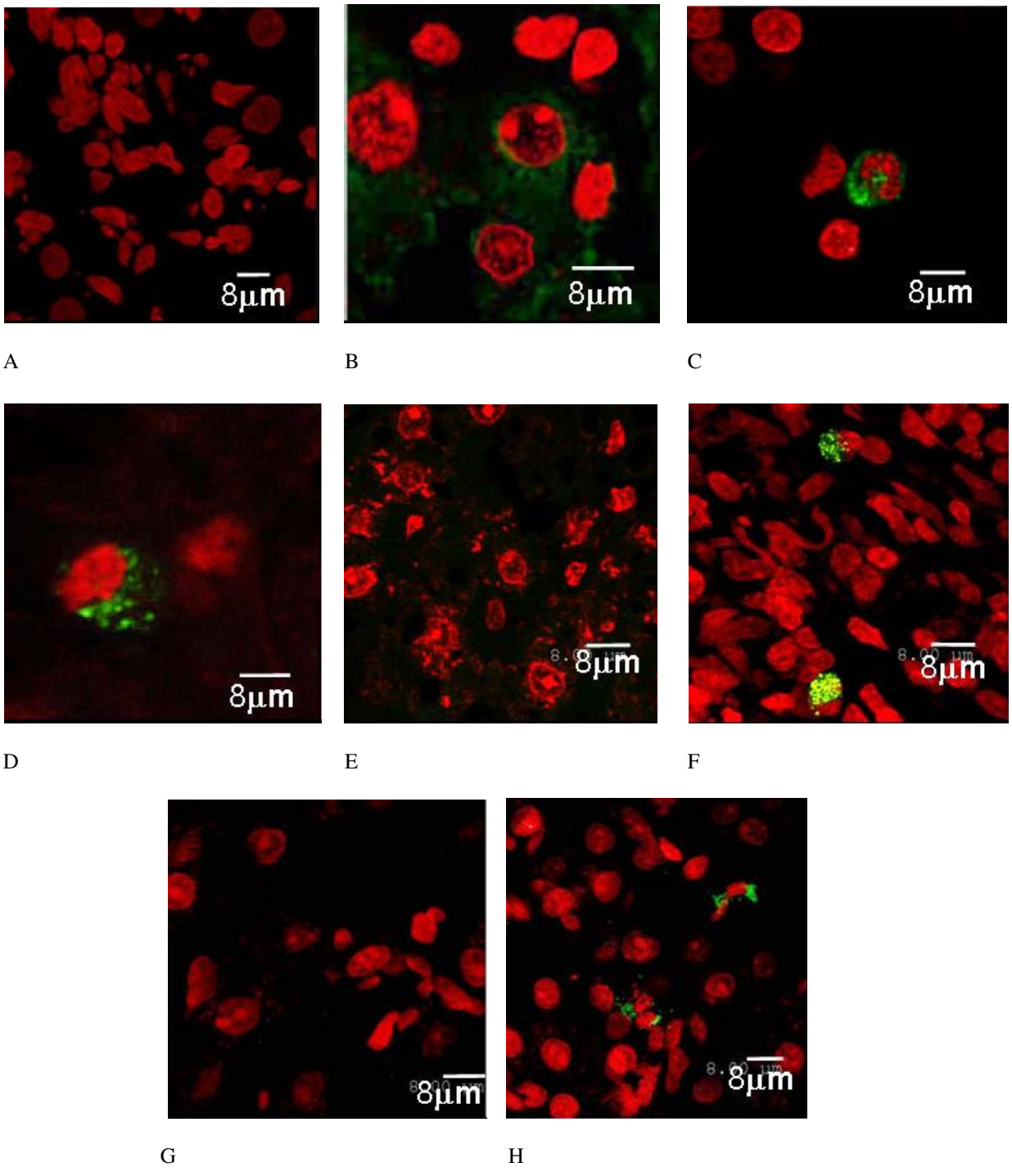


Fig. 1: Laser scanning confocal microscopy analysis of HCV-infected hepatocytes and apoptotic hepatocytes. B-D) Fluorescent *in situ* hybridization of the HCV-RNA of positive (B,C) and negative (D) polarity, in liver biopsies from a patient with HCV infection. No hybridization signals were observed in the liver biopsy specimens from the negative control subjects (A). E-H) Fluorescent staining of DNA fragmentation by TUNEL assay (E,F) and active caspase 3 (G,H) in liver biopsies from HCV-infected patients (F,H) and from healthy individuals (E,G) (Bar=8 µm)

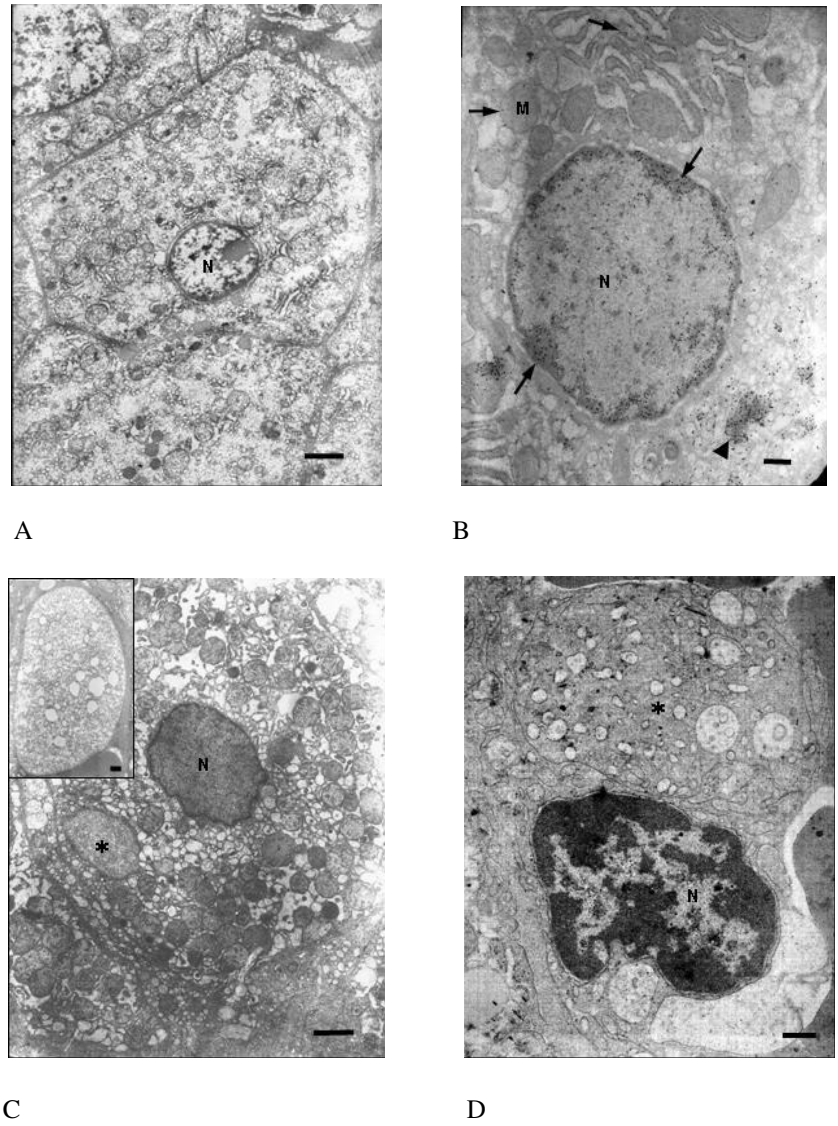
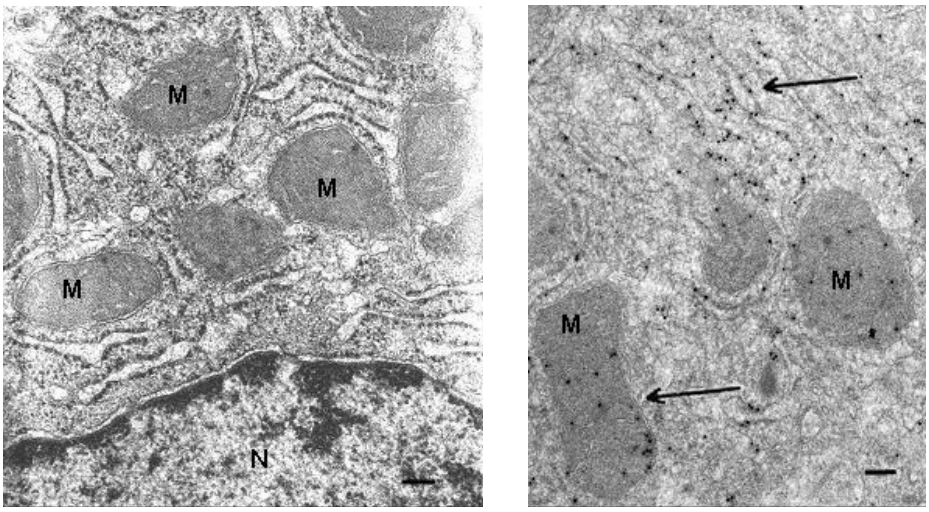


Fig. 2: TEM and IEM analysis of apoptotic hepatocytes. A) Apoptotic hepatic cell showing shrinkage; B) Immunostaining with a mixture of anti-HCcAg mAbs and gold-labeled anti-mouse IgG (arrows), revealed HCcAg in the nucleus (N), along the rough endoplasmic reticulum and mitochondria (M). Fragments of heterochromatin were observed in the cytoplasm (arrowhead) C) Presence of apoptotic bodies (*) lying free among hepatocytes (inset) and endocyted by adjacent hepatocytes. D) Apoptotic body endocyted by an endothelial-like cell (Bar=2 um in A; 500 nm in B; 2 um in C (inset:500 nm); 500 nm in D)

sections from uninfected individuals. Neither the paracristalline structures nor the particles could be immunostained with anti-HCcAg and anti-E1 mAbs. Moreover, unstructured large aggregates were frequently observed in damaged hepatocytes (Fig. 4E). These aggregates were specifically immunostained with anti-HCcAg mAbs (Fig. 4E) but not with the anti-E1 mAb (not shown).

P. pastoris cells have shown to be a suitable system to study HCcAg processing and morphogenesis[9,14,15,17]. Some characteristics of HCcAg expression in this system are similar to those observed in HCV infection. As shown in Fig. 5B, a fraction of HCcAg was specific immunolabeled in the membrane of mitochondria at late stages of expression in recombinant *P. pastoris* cells. On the other hand, crystalloid bodies were predominantly produced in the cytoplasm of recombinant *P. pastoris* cells (Fig. 5C,D). Interestingly, HCcAg was immunodetected in these crystal arrays by IEM (Fig. 5D). However, no immunolabelling was observed in sections incubated with either the anti-E1 mAb or normal mouse serum as



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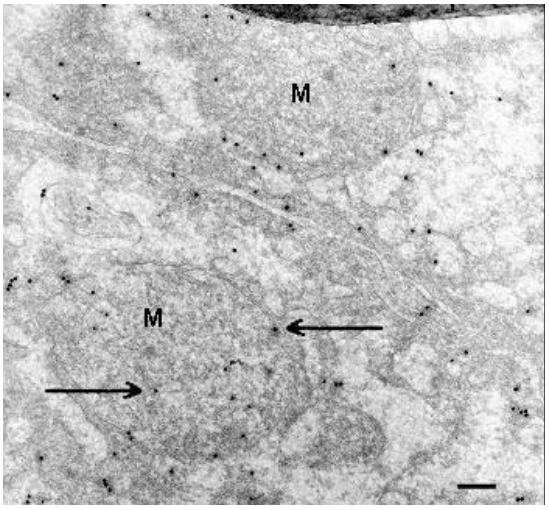

C

Fig. 3: Immunolabeling of HCcAg and E1 by IEM. A) Liver biopsies from healthy subjects, no immunostaining was observed in the rough endoplasmic reticulum and cytoplasm using either a mixture of anti-HCcAg mAbs or the E1-specific mAb, respectively. B) Immunostaining with a mixture of anti-HCcAg mAbs and gold-labeled anti-mouse IgG, detected HCcAg in mitochondria and along the rough endoplasmic reticulum. C) Immunostaining with the anti-E1 mAb and gold-labeled anti-mouse IgG revealed E1 in mitochondria and cytoplasm. Nucleus (N); Mitochondria (M) (Bar=200 nm)

primary antibodies (Fig. 5C). Protein aggregates were also detected at late stages of HCcAg expression in recombinant *P. pastoris* cells (Fig. 5E,F). Of note, these aggregates were immunolabeled with anti-HCcAg mAbs (Fig. 5F) but not with the anti-E1 mAb (Fig. 5E). No immunolabelling was observed in MP-36 cells used as negative control (Fig. 5A).

DISCUSSION

Analysis of HCV-infected hepatocytes at the cellular level may contribute to elucidate the mechanisms of HCV pathogenesis. The HCV-RNA of positive strand was specifically detected in some hepatocytes from the liver biopsies analyzed suggesting

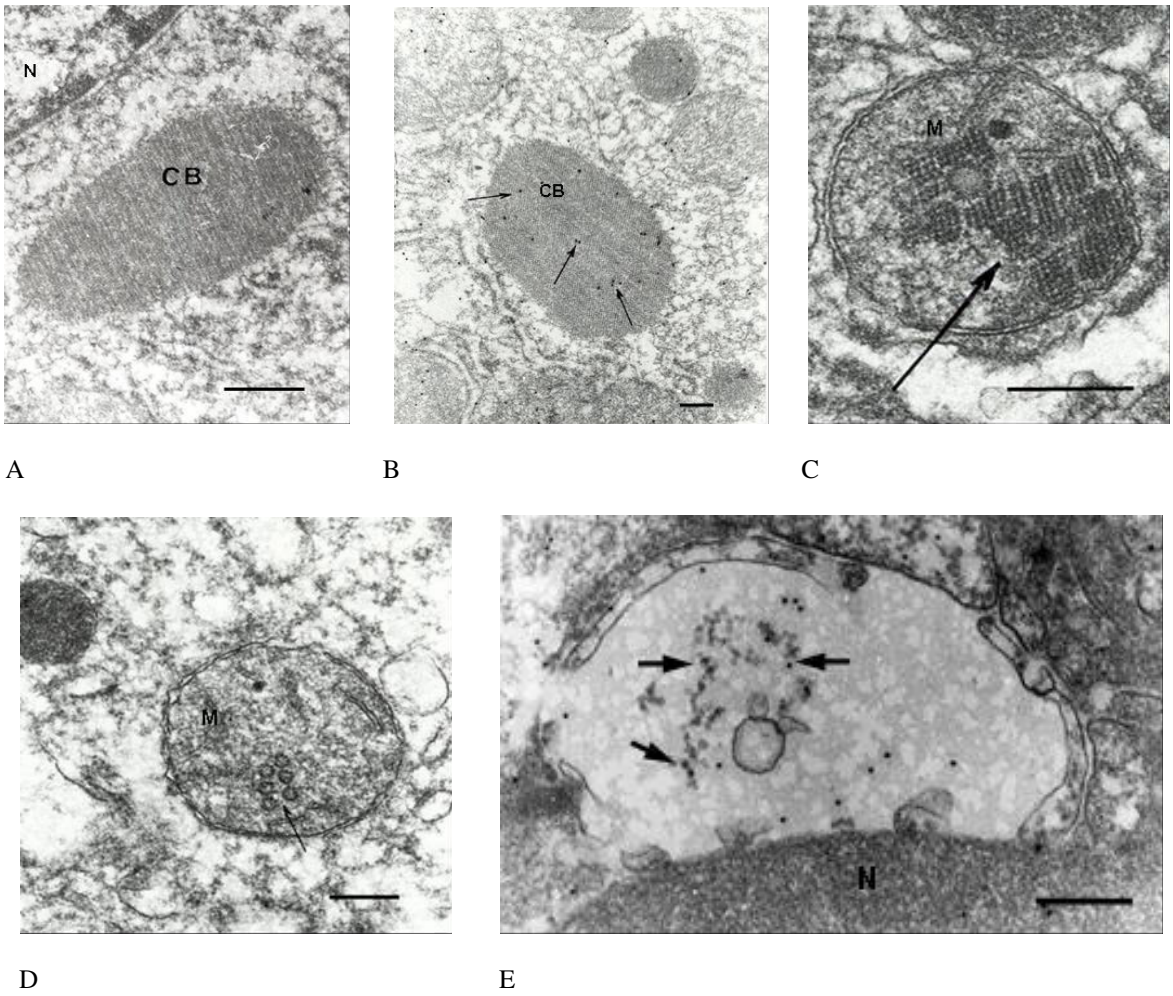

Fig. 4: TEM and IEM analysis of various structures from HCV-infected liver biopsies. A.B) Part of hepatic cells from HCV-infected liver biopsies showing the presence of crystalloid bodies (CB); (A) No immunostaining was observed in a CB using a normal mouse serum as primary antibodies, (B) Specific immunostaining was observed in a CB using a mixture of anti-HCcAg mAbs and gold-labeled anti-mouse IgG. C,D) Part of hepatic cells from liver biopsies of HCV-infected patients showing the presence of either a paracrystaline inclusion (C) or particles in the mictochondria (D); E) Immunolabeling of HCcAg in protein aggregates present in a damaged hepatocyte. Nucleus (N); Mitochondria (M) (Bar=200 nm in A,C,D,E; 500 nm in B)

the infection of these hepatocytes by HCV. On the other hand, detection of negative strand HCV-RNA in the cytoplasm of positive hepatocytes suggests the presence of ongoing viral replication in the liver of HCV-infected patients.

Mechanisms leading to liver cell injury, inflammation and fibrosis in chronic hepatitis C are not fully understood. Both immune-mediated reactions and direct cytopathic effects of HCV may be involved in its pathogenesis[19]. Although it is unclear which cellular and molecular mechanisms participate in this process, there is evidence to suggest that apoptosis of liver cells may play a significant role in the pathogenesis of hepatitis C[29].

A standard test for apoptosis is the TUNEL assay, which detects fragmented DNA[35]. Physiologically, TUNEL positivity represents a criterion by which to identify apoptosis. Pathologically, a TUNEL-positive reaction can appear in both apoptosis and necrosis[35]. It is known that the caspase activation assay detects apoptosis at an early stage before DNA fragmentation occurs. Recently, it has been shown that caspases are activated in human liver biopsies specimens of chronic hepatitis C patients[29]. Importantly, activation of caspase 3 and caspase 7 correlated significantly with inflammatory activity[29].

The complexity of measuring apoptosis involves the difficulty of distinguishing apoptosis from necrosis.

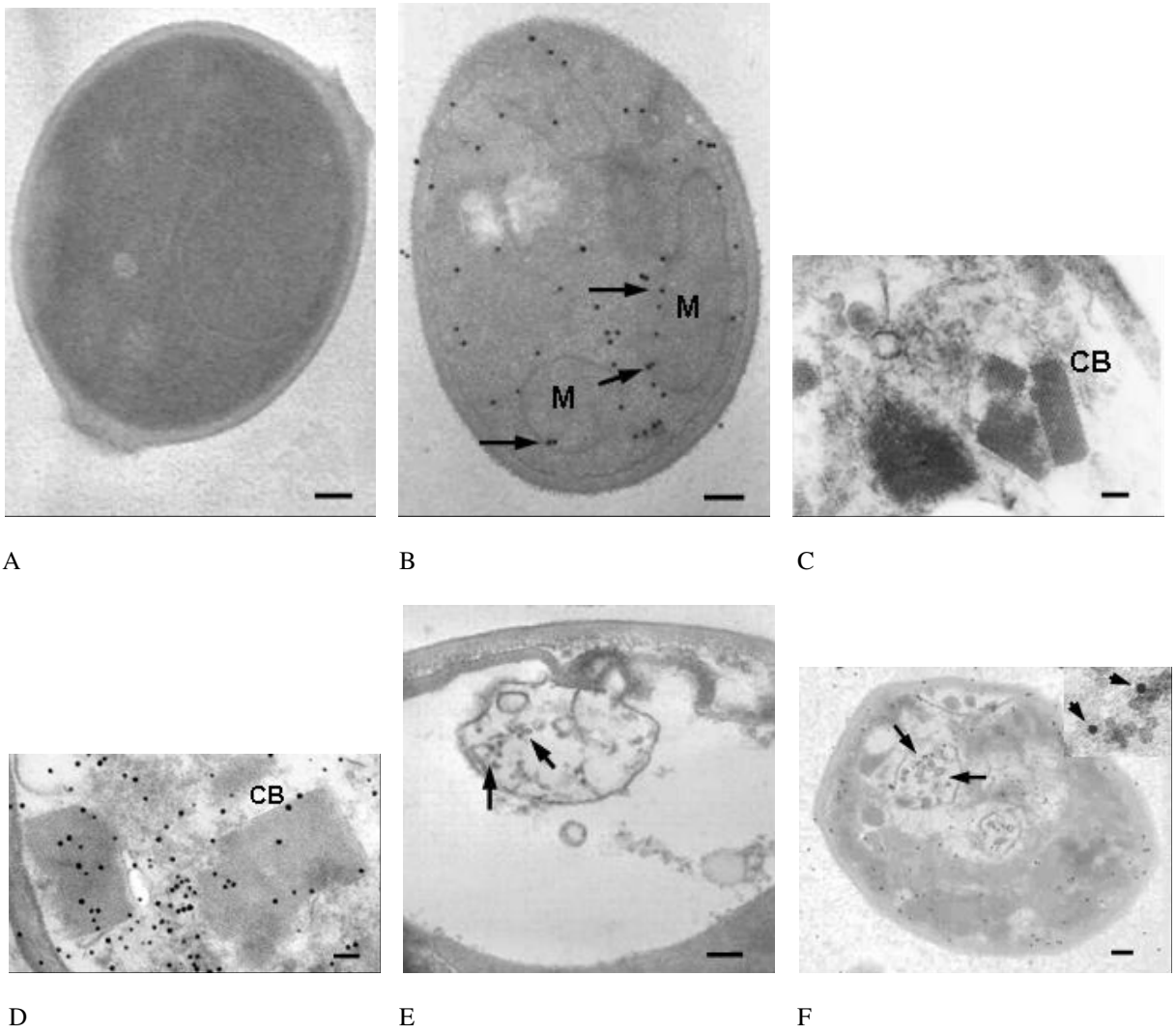

Fig. 5: TEM and IEM analysis of recombinant *P. pastoris* cells. A) MP-36 *P. pastoris* cells, no immunostaining was observed in the cells using a mixture of anti-HCcAg mAbs; B) Immunostaining with a mixture of anti-HCcAg mAbs and gold-labeled anti-mouse IgG, revealed HCcAg in cytoplasm and in the membrane of mitochondria. C,D) Presence of crystalloid bodies (CB) in the cytoplasm of recombinant cells, the cells were incubated with either normal mouse serum (C) or a mixture of anti-HCcAg mAbs (D) as primary antibodies. E,F) Presence of protein aggregates in vesicles of recombinant cells, the cells were incubated with either normal mouse serum (E) or a mixture of anti-HCcAg mAbs (F) as primary antibodies. (Bar=200 nm in A,B,F; 100 nm in C,D,E)

A fundamental difference between the two mechanisms of cell death is the morphological alteration of the cell. The nuclear modification of the apoptotic cells is accompanied by a preservation of the cytoplasmic structures of the cell. In contrast, immediate loss of membrane integrity occurs in the necrotic cells. This distinction thus far has made the electron microscopic evaluation of morphological changes a most reliable tool for determination of apoptosis.

The observations of the present study provide evidence for apoptosis of hepatocytes during chronic HCV infection. Confirmation of this process was based on the morphological data by TEM including cell shrinkage; chromatin condensation; formation of apoptotic bodies; phagocytosis by neighbouring cells; and the presence of either DNA fragmentation by TUNEL asay or caspase 3 activation. Overall, these results indicate that apoptosis may be an important

component of liver lesion progression during chronic HCV infection.

Apoptosis mediated by Fas or tumor necrosis factor (TNF) is a major pathway associated with liver injury and chronicity of HCV infection[36,37]. In addition, HCcAg could serve as a versatile signal to induce or inhibit apoptosis[20,23,38]. It is thought that the inhibition of apoptosis may allow the HCV to establish persistent infection and contribute to oncogenesis, while the promotion of apoptosis may explain the occurrence of hepatitis. Modulation of apoptosis may involve binding of HCcAg to the intracellular signal transducing portion of death receptors such as TNF, Fas or lymphotoxin B[21,38-41] and displacement of signaling molecules such as TNF-receptor-associated factor-2 (TRAF-2) and TNF-receptor-associated death domain (TRADD)[42]. Besides, HCcAg has been shown to inhibit or activate NF-κB and to induce variable effects in modulating the sensitivity to cytokines[20].

On the other hand, it has been reported that HCcAg expressed in various cell lines localized in the mitochondria and causes mitochondrial injury, leading to oxidative stress[12]. In addition, an increase in the citochrome c content of cytosolic fractions from HCcAg-expressing cells was observed[12]. Transgenic mice that express either HCV structural proteins (HCcAg, E1, E2 and p7) or just HCcAg and have incidence of hepatic steatosis are vulnerable to oxidant stress and also showed a tendency to develop hepatic lipid peroxidation[12,43]. In addition, HCcAg has been shown to affect the lipid metabolism[10,44]. These studies suggest that HCV proteins, particularly HCcAg, contribute to oxidative stress, steatosis and apoptosis.

It is known that oxidative stress perturbs lipid metabolism, in addition to myriad other effects, thus contributing to steatosis and apoptosis. Several studies have suggested that oxidative stress is a potentially important pathologic mechanism in hepatitis C. The presence of lipid-peroxide aducts, morphologic changes in mitochondria and evidence of glutation depletion have been shown in liver tissue from HCV-infected patients[30,45,46].

Data from this work suggest that under certain conditions HCcAg and E1 protein may localize in the mitochondria of hepatocytes during *in vivo* HCV infection. Although direct interaction of E1 with mitochondria could not be discarded it is possible that HCcAg-E1 interaction may lead to E1 mitochodrial localization[47]. It is interesting to note that HCcAg was detected not only in the ER and mitochondria but also in the nucleus of apoptotic hepatocytes. The fact that HCcAg localized in damaged mitochondria and in the nucleus of apoptotic hepatocytes from HCV-infected patients suggest a possible pathogenic role of this viral protein. Specifically, this interaction may contribute to the apoptotic process in virus infected hepatocytes.

In condition of cellular stress, mitochondria have been shown to be a source, as well as the target, of reactive oxygen species[48]. Mitochondrial dysfunction is an important factor in cytotoxicity, which may cause the cell to undergo apoptosis. Mitochondrial dysfunction leads to a release of proapoptotic factors such as cytochrome c and Apoptosis-Inducing Factor (AIF) from the mitochondria[49]. These factors promote activation of caspase proteases, which cause proteolytic cleavage of death substrates, culminating in cytotoxicity[49,50]. Thus, both direct effects of viral proteins (especially HCcAg) on mitochondria or induction of an intracellular oxidative stress by these viral proteins may induce mitochondrial dysfunction and lead to apoptosis.

Different HCV-related structures were observed in hepatocytes of HCV-infected patients (including apoptotic hepatocytes). Interestingly, HCcAg co-localized with crystalloid bodies in the cytoplasm of hepatocytes. The presence of crystalline inclusions in hepatocytes is a pathological reaction of several liver pathologies. Giant mitochondria containing paracrystalline inclusions are especially common after taking oral contraceptives[51] and in hyperbilirubinemias[52,53]. Variations in the size and appearance of paracrystalline inclusions in the mitochondria and living free in the cytoplasm often occur in the alcoholic liver[51] and are a characteristic alteration in Wilson's disease[54].

Remarkably, none of studied patients have suffered from non-viral liver diseases such us: drug toxicity, alcoholic liver disease, autoimmunity and metabolic and genetic liver disorders. Moreover, cytoplasmic paracrystalline inclusions containing HCcAg similar to those observed in HCV-infected hepatocytes are characteristic of late stages of HCcAg expression in *P. pastoris* cells. So, it is possible that formation of paracrystalline inclusions in these patients may be a pathological reaction of HCV infection.

On the other hand, structures forming a paracristalline array also appeared in the mitochondria of some hepatocytes. In addition, particles with a diameter of 50 nm were observed in the mitochondria. It is noteworthy that similar particles have been detected in the mitochondria of apoptotic chondrocytes[55].

The presence of these HCV-related structures and of HCcAg and E1 in the mitochondria of hepatocytes may be of relevance for HCV pathogenesis. In addition, the assembly of these new structures in the cell may alter or displace host-cell components and lead to cytopathic effects. Data suggest the possibility for a direct role of these HCV-related structures as well as HCcAg and E1 in apoptosis and pathogenicity.

Unstructured large aggregates containing HCcAg were observed in damage hepatocytes from

HCV-infected patients. These aggregates were similar to those found in recombinant *P. pastoris* cells at late stages of HCcAg expression. Previously, it has been reported that *in vitro* assembly of HCcAg under non-reducing conditions leads to unstructured large aggregates formation[56]. It was suggested that the use of reducing conditions contributed to the correct folding of HCcAg *in vitro* and the assembly of homogeneous particles.

It is known that oxidative attack on proteins results in site specific amino acid modifications that may lead to aggregation of cross-linked reaction products. Sulphur containing amino acids and thiol groups specifically, are very susceptible sites. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cros-link to a second thiyl radical to form disulphide bridges. Thus, it is possible that HCcAg aggregates under conditions favouring unbalanced redox potential of the infected cell.

We speculate that HCcAg may assemble into viral capsids under normal physiological conditions of the cell. On the other hand, HCcAg may forms large aggregates instead of structured capsids under pathological conditions (Ex. unbalanced redox potential of the cell) thus affecting the HCV virion production and the rate of HCV replication. Thus this hypothesis predicts the relationship between nucleocapsid assembly and the pathogenesis induced by HCV infection.

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