World Journal of Gastroenterology

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DOI: 10.3748/wjg.v21.i47.13225

World J Gastroenterol 2015 December 21; 21(47): 13225-13239
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Basic Study

Hepatitis B and C virus-induced hepatitis: Apoptosis, autophagy, and unfolded protein response

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Supported by University of Manitoba Start-up funds and an award from the Manitoba Medical Service Foundation to Ghavami S; University of Manitoba Start-up Funds to Alizadeh J.

Institutional review board statement: This retrospective study was approved by local research ethics committee of Health policy research Center (Protocol number HP-101-91). All patients were informed about the study and gave verbal informed consent prior to enrollment.

Conflict-of-interest statement: All authors do not have any conflict of interest.

Data sharing statement: This is an open access work and the researcher can use the data for any further investigations.

Open-Access: This article is an open-access article which was

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Received: August 29, 2015
Peer-review started: August 30, 2015
First decision: September 29, 2015
Revised: October 14, 2015
Accepted: November 13, 2015
Article in press: November 13, 2015
Published online: December 21, 2015

Abstract

AIM: To investigate the co-incidence of apoptosis, autophagy, and unfolded protein response (UPR) in hepatitis B (HBV) and C (HCV) infected hepatocytes.

METHODS: We performed immunofluorescence confocal microscopy on 10 liver biopsies from HBV and HCV patients and tissue microarrays of HBV positive liver samples. We used specific antibodies for LC3β, cleaved caspase-3, BIP (GRP78), and XBP1 to detect autophagy, apoptosis and UPR, respectively. Anti-HCV NS3 and anti-HBs antibodies were also used to confirm infection. We performed triple blind counting of events to determine the co-incidence of autophagy (LC3β punctuate), apoptosis (cleaved caspase-3), and unfolded protein response (GRP78) with HBV and HCV infection in hepatocytes. All statistical analyses were performed using SPSS software for Windows (Version 16 SPSS Inc, Chicago, IL, United States). *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed with Mann-Whitney test to compare incidence rates for autophagy, apoptosis, and UPR in HBV- and HCV-infected cells and adjacent non-infected cells.

RESULTS: Our results showed that infection of hepatocytes with either HBV and HCV induces significant increase ($P < 0.001$) in apoptosis (cleavage of caspase-3), autophagy (LC3β punctate), and UPR (increase in GRP78 expression) in the HCV- and HBV-infected cells, as compared to non-infected cells of the same biopsy sections. Our tissue microarray immunohistochemical expression analysis of LC3β in HBV^{Neg} and HBV^{Pos} revealed that majority of HBV-infected hepatocytes display strong positive staining for LC3β. Interestingly, although XBP splicing in HBV-infected cells was significantly higher ($P < 0.05$), our analyses show a slight increase of XBP splicing was in HCV-infected cells ($P > 0.05$). Furthermore, our evaluation of patients with HBV and HCV infection based on stage and grade of the liver diseases revealed no correlation between these pathological findings and induction of apoptosis, autophagy, and UPR.

CONCLUSION: The results of this study indicate that HCV and HBV infection activates apoptosis, autophagy and UPR, but slightly differently by each virus. Further studies are warranted to elucidate the interconnections between these pathways in relation to pathology of HCV and HBV in the liver tissue.

Key words: Cell fate; Cell death; Hepatocyte; Viral infection; Endoplasmic reticulum stress

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Core tip: For the first time, the current study has addressed the co-incidence of apoptosis, autophagy, and unfolded protein response in the liver tissue of hepatitis B and hepatitis C (HBV, and HCV) infected patients. The results showed that all of these events are activated at the same time by HBV and HCV infection in the liver. All of these pathways probably are involved in replication and pathogenesis of HBV and HCV infection; therefore their modulation would probably be beneficial for new therapeutic approaches in these diseases.

Yeganeh B, Rezaei Moghadam A, Alizadeh J, Wiechec E, Alavian SM, Hashemi M, Geramizadeh B, Samali A, Bagheri Lankarani K, Post M, Peymani P, Coombs KM, Ghavami S. Hepatitis B and C virus-induced hepatitis: Apoptosis, autophagy, and unfolded protein response. *World J Gastroenterol* 2015; 21(47): 13225-13239 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i47/13225.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i47.13225

INTRODUCTION

The liver is the primary organ infected by, and in which both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate. HBV and HCV are structurally unrelated and responsible for infection of huge numbers of individuals in the world.

It has been reported that around 2 billion people are infected with HBV, and more than 350 million are chronic carriers according to clinical definitions that identify individuals who have continuous viral and subviral particles in their blood for more than six months[1,2]. Several studies have determined that HBV infection in adulthood might lead to a carrier stage in about 5%-10% of infected individuals. In addition,

in up to 30% of these individuals hepatitis, fibrosis, cirrhosis, and finally hepatocellular carcinoma (HCC) can develop and it is usually considered a progressive chronic liver disease (CLD)[3]. Several groups have investigated the risk of HCC development in different populations and shown that HCC development can be increased up to 100-fold in carrier infected individuals compared with uninfected individuals, which depends on the population and different markers. The risk of developing HCC among carriers with CLD ranges from 10-fold to 100-fold greater compared to uninfected people[2,4]. Approximately 85% of acute HCV infection leads to a chronic situation and 50% of individuals who suffer from HCV chronic infections (about 170 million worldwide) can develop CLD. Approximately 5%-20% of this population can progress to liver cirrhosis in 5-20 years after their infection, and 1%-2% of these patients will probably develop HCC per year[3]. It is strongly believed that this can be considered one of the closest relationships between an environmental agent and a cancer that has so far been identified[3].

Autophagy is a tightly regulated catabolic process, which is essential in many cellular events including development, differentiation, survival, and homeostasis[5-7]. In the past few years, many investigators have focused on the involvement of autophagy in different human diseases and many aspects of this relationship have been described[8-10]. Autophagy is usually considered a very important step for numerous virus life cycles[11], including HBV and HCV[12].

Apoptosis is programmed cell death initiated *via* two different pathways (1) extrinsic which is activated by ligation of death receptors; and (2) intrinsic which is activated by mitochondrial death-related proteins. These two distinct pathways crosstalk and potentiate each other to ultimately activate the caspase cascade and facilitate controlled proteolysis of cellular components[13-15].

Synthesized and secretory proteins are correctly folded and assembled in the endoplasmic reticulum (ER)[16]. During cellular stress, the ER loses its capacity to correct protein folding which results in the accumulation of unfolded and misfolded proteins. Following this, the unfolded protein response (UPR) targets the degradation of the accumulated proteins in the ER, inhibits global protein translation and also activates the transcription of genes that increase the protein folding capacity of the ER including lectins, chaperones, and calcium pumps[17]. Three ER membrane sensors mediate signals from the ER upon activation of the UPR including activating transcription factor 6 (ATF6), inositol-requiring enzyme 1α (IRE1α), and protein kinase RNA (PKR)-like ER-localized kinase (PERK)[16]. Each of these molecules activates independently distinct signaling pathways to provide an integrated response to ER stress[18]. Unfolded and misfolded proteins in the ER disrupt binding of the binding immunoglobulin protein (BIP)/glucose-regulated protein 78 (GRP78) with ER stress sensors, leading to their activation. PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) which results in a decrease in mRNA translation with concurrent translation increase of several mRNAs like activating transcription factor 4 (ATF4) and the CCAAT-enhancer-binding protein homologous protein (CHOP) (ATF4 downstream target)[16].

Several previous investigations have shown that HBV[19-24] and HCV[25-27] infection can modulate apoptosis, autophagy, and UPR in different *in vitro* and non-human *in vivo* models. However, most of these studies did not use human samples and also have not simultaneously investigated apoptosis, autophagy, and UPR in the same infected tissue or organ. To address these gaps, we used tissue microarray, and fluorescence immunohistochemistry (IHC) in the present study to evaluate apoptosis, autophagy and UPR in human biopsy samples from patients who were infected with HBV or HCV. This study, for the first time, provides an evaluation of these events at the same time in HBV and HCV liver biopsies of infected patients.

MATERIALS AND METHODS

Materials and antibodies

The following antibodies were used in this study for immunoflourescence or IHC, or both: LC3β antibody was obtained from Proteintech (18725-1-AP, Chicago, IL, United States). Antibody for hepatitis B surface antigen (HBsAg) was obtained from Novus Biologicals (NBP1-22568, Littleton, CO, United States). Cleaved caspase-3 (Asp175) antibody was purchased from Cell Signaling Technology (#9661, MA, United States). Anti-Hepatitis C virus NS3 (ab49486), anti-BIP (GRP78) (ab21685) and anti-XBP1 (ab37152) antibodies were purchased from Abcam (Cambridge, MA, United States). Biotin conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, United States). VECTASTAIN Peroxidase ABC kit was purchased from Vector Laboratories (Vector Laboratories, Burlingame, CA, United States). Fluorescent-conjugated (Alexa Fluor 488 and 546) secondary antibodies were purchased from Molecular Probes (Eugene, OR, United States). Type B hepatitis and normal liver tissue array (IC03001) was purchased from United States BioMax (Rockville, MD, United States).

Ethical protocol

This retrospective study was approved by local research ethics committee of Health policy research Center (Protocol number HP-101-91). All patients were informed about the study and gave verbal informed consent prior to enrollment.

Selection criteria for HBV and HCV patients

HBV- and HCV-infected individuals (10 in each group)

were diagnosed and selected based on the following criteria from the patients who had been referred to Namazi Hospital (Shiraz, Iran): (1) HBV-infected patients: Hepatitis virus surface antigen positive and HBV DNA > 2000 IU/mL in the serum; and (2) HCV-infected patients: HCV antibody positive, which was confirmed by detecting HCV RNA in the serum.

All cases had abnormal levels of liver enzymes including alanine transaminase; 2 × the upper limit of normal range (Normal Range is 7-56 IU/L) in two measurements that were performed three months apart. In all cases other diagnoses were ruled out with appropriate work up and no case had mixed cause of liver function abnormality in this series. DNA was extracted using Qiagen kit (Qiagen, Hiden, Germany), as per manufacturer's instructions. HBV and HCV genome copy numbers were measured using Qiagen kit (Germany). Patients with other causes of chronic liver disease such as Wilson's hemochromatosis, drug induced liver disease, alpha-1 antitrypsin deficiency, or alcoholic liver diseases were excluded. Patients were also excluded if pregnant, or if presented with comorbid conditions including diabetes mellitus, congestive heart failure, or chronic kidney disease.

Liver biopsy and sample preparation

Biopsy of the liver was performed by the radiologist under the guide of ultrasound or computerized tomography (CT) scan using Tru-cut® needles (standard biopsy needle). Biopsies were fixed in formalin, and then processed as routine pathology specimens.

Histology and immunohistochemistry

Paraffin-embedded liver tissues were used for histology and IHC. Briefly, paraffin sections of 4 μm thickness were prepared, deparaffinized, rehydrated and used for staining. Following unmasking of antigens using Heat-Induced Epitope Retrieval and citrate buffer (0.1 mol/L citric acid, 0.1 mol/L sodium citrate, pH 6.0) in a Coplin Jar for 30 min, IHC was performed by means of detecting antigens with corresponding primary antibodies followed by biotinylated secondary antibodies (Jackson ImmunoResearch Labs) and an avidin-biotin peroxidase complex technique using ABC kit. The slides were then counterstained with Mayer' s hematoxylin (Sigma, H9627), dehydrated and mounted with Permount (Thermo Fisher Scientific, Ottawa, ON, Canada). For Immunofluorescence staining, after primary antibody incubation, sections were covered with fluorescent-conjugated (Alexa Fluor 488 and 546) secondary antibodies followed by nuclear staining using ProLong® Gold Antifade Mountant with 4',6-diamidino-2-phenylindole DAPI (Molecular Probes, Eugene, OR, United States).

As a negative control, sections were processed as above but addition of primary antibody was omitted. Images were captured using a Leica CTRMIC 6000 confocal microscope equipped with a Hamamatsu C910013 spinning disc camera (Leica Microsystems, Inc., Concord, ON, Canada). Laser intensity and detector sensitivity settings remained constant for all image acquisitions within each experiment. Images were later analyzed with Volocity software (Perkin-Elmer, Woodbridge, ON, Canada).

Liver tissue microarray and scoring

IHC analysis was performed on commercially available tissue microarray (TMA) (cat. No. IC03001; Biomax Inc., Rockville, MD, United States) consisting of 30 cases of normal liver tissue and 10 cases of HBV-induced hepatitis. For evaluation of IHC staining, semi-quantitative scoring (H-scores) was used to assess positive staining for LC3β protein expression in TMAs according to the method described previously[28]. The H-score was calculated by a semi-quantitative assessment of both staining intensity (scale 0-3) and the percentage of positive cells (0%-100%), which, when multiplied, generated a score ranging from 0 to 300. The intensity score was made on the basis of the average intensity of staining where 0 = negative, 1 = weak, 2 = intermediate and 3 = strong. Statistical analysis was carried out on the H-score data obtained. Scoring of the sections was performed blindly by three independent individuals.

Classification of HBV and HCV patients based on the liver disease stage and grade

HBV and HCV patient liver biopsies were carefully assessed by two pathologists and the stage and grade of their liver disease were classified based on the following definitions:

Grade (necroinflammation grade): 0 = none, 1-6 = mild, 7-12 = moderate, 13-18 = severe; Stage (Ishak Fibrosis Score): 0 = No fibrosis, 1-2 = Fibrous expansion of some and most portal areas (+/-) short fibrous septa, 3-4 = Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging and Fibrous expansion of portal areas with marked bridging (P-P) as well as portal to central (P-C), 5-6 = Marked bridging (P-P and/or P-C), with occasional nodules (incomplete cirrhosis), and Cirrhosis, probable or definite. The results of HBV and HCV patient classifications are shown in Table 1 (stage) and Table 2 (grade).

Statistical analysis

All statistical analyses were performed using SPSS software for Windows (Version 16 SPSS Inc, Chicago, IL, United States). *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed with Mann-Whitney test for comparing autophagy marker (punctate LC3β), apoptosis marker (cleaved caspase-3), and UPR marker (BIP) between HBV- and HCV-infected cells and adjacent non-infected cells. Statistical analyses were performed with Kruskal-Wallis test for relation of stage (ordinal variable)

Table 1 Stage of liver disease in hepatitis B and C virus infected patients

Stage	None	Mild	Moderate	Severe
HBV (*n*)	5	5	0	0
HCV (*n*)	3	5	1	1

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Grade of liver disease in hepatitis B and C virus infected patients

Grade	None	Mild	Moderate	Severe
HBV (*n*)	4	3	3	0
HCV (*n*)	4	2	1	3

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

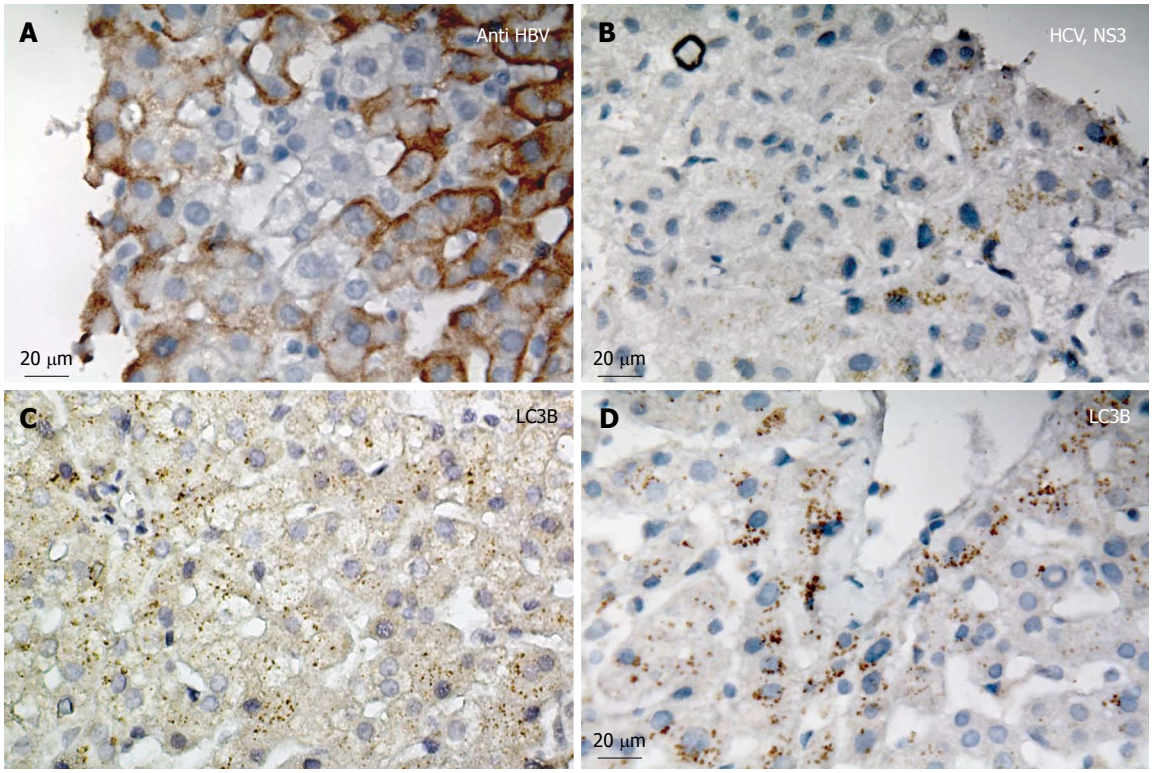

Figure 1 Confirmation of hepatitis B and C virus infection and LC3β expression in liver tissues. A: Immunohistochemistry (IHC) confirms hepatitis B virus (HBV) infection in the liver tissue; the image is representative of IHC for HBV infection in all patients; B: IHC confirms hepatitis C virus (HCV) infection (Anti NS3 HCV) in the liver tissue; the image is representative of IHC for HCV infection in all patients; C: IHC confirms LC3β expression in HBV infected liver tissue; the image is representative of IHC for all patients; D: IHC confirms LC3β expression in HCV infected liver tissue; the image is representative of IHC for all patients.

and grade (ordinal variable) liver biopsies of HBV and HCV patients with autophagy marker (punctate LC3β), apoptosis marker (cleaved caspase-3), and UPR marker (BIP). Because of our small sample size, we used the non-parametric Kruskal-Wallis test. It is noteworthy to mention that Kruskal-Wallis test is equivalent nonparametric test of "one-way analysis of variance".

RESULTS

HBV and HCV infection induces autophagy in human liver tissue

IHC analyses confirmed HBV and HCV infection in different liver tissue samples (Figure 1A and B). LC3β expression was later investigated in different HBV- and HCV-infected liver tissues and LC3β expression was confirmed in all samples (Figure 1C and D). It has been previously shown that lipidated LC3β serves as a reliable marker of autophagy in ICC and IHC[5,29-31]. Because the previous immunochemistry analyses were meant to primarily confirm infection status and globally explore LC3β expression, we also used fluorescent IHC to directly identify autophagy in the liver biopsy cells from HBV and HCV positive patients (Figure 2A and B). Our results show accumulation of autophagosomes in HBV- (Figure 2A) and HCV- (Figure 2B) infected hepatocytes as determined by significantly higher number of LC3β-positive puncta in both HBV- (Figure 2C) and HCV- (Figure 2D) infected cells compared to adjacent non-infected hepatocytes ($P < 0.001$).

Tissue microarray immunohistochemical profile of LC3β in HBV^{Neg} and HBV^{Pos} hepatocytes

Immunostaining for LC3β was performed on the commercially available TMA slide (see Materials and Methods). A total of 40 samples were analyzed. Ten of the samples were type B hepatitis and 30 were normal

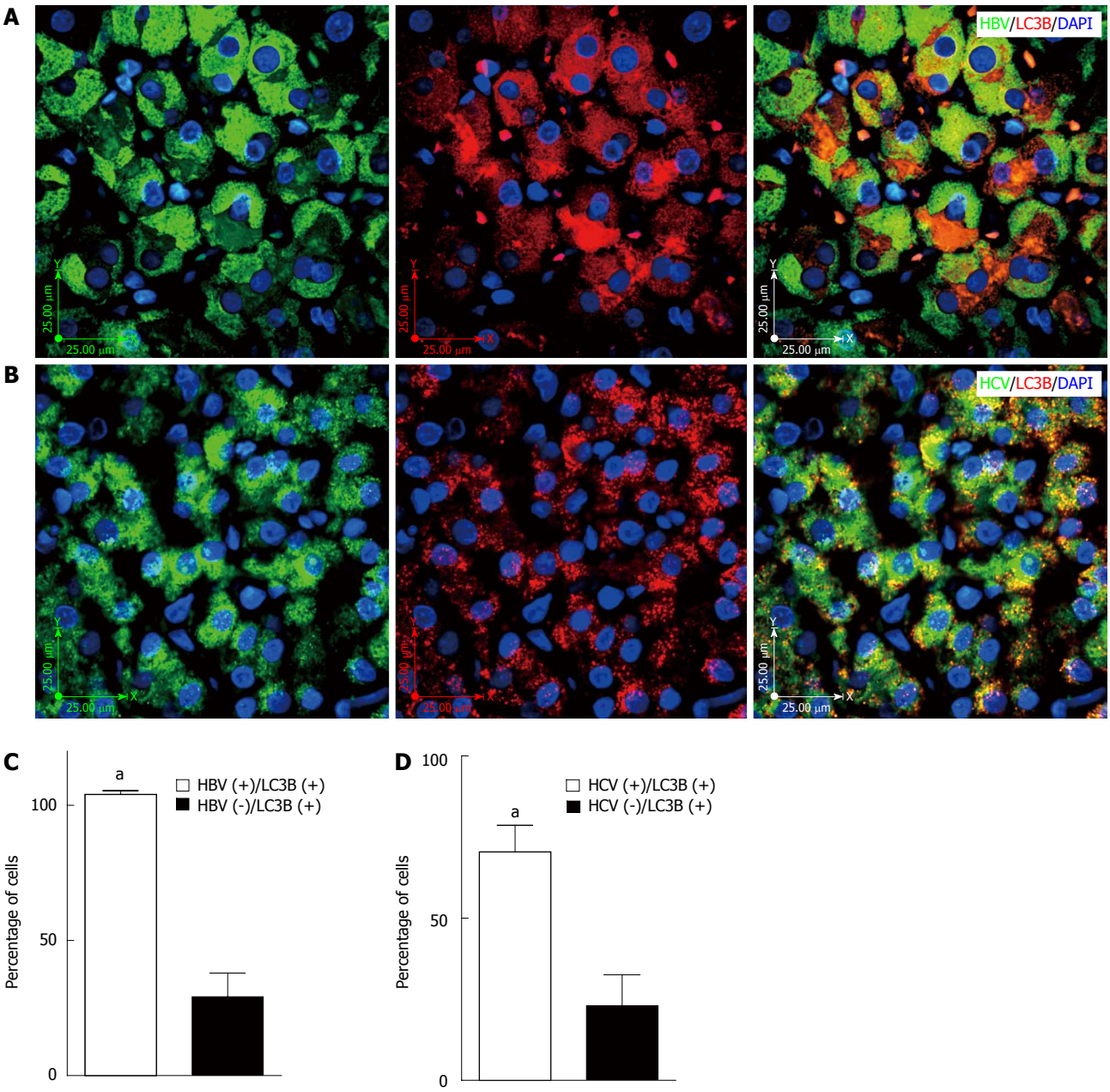

Figure 2 Hepatitis B and C virus infection induces autophagy in liver tissues. A: Fluorescent immunohistochemistry (IHC) confirms co-localization of hepatitis B virus (HBV) and punctate LC3β in the HBV-infected liver tissue; the image is representative of fluorescence IHC for all patients; B: Fluorescencent IHC confirms co-localization of hepatitis C virus (HCV) and punctate LC3β in the HCV infected liver tissue; the image is representative of fluorescence IHC for all patients; C: HBV infection significantly ($^aP < 0.001$ *vs* HBV) induces autophagy in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HBV infection and punctuated LC3β) in at least 50 cells counted in four different microscopic fields of view from each patient's sample. D: HCV infection significantly ($^aP < 0.001$ *vs* HCV) induces autophagy in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HCV infection and punctuated LC3β) in at least 50 cells counted in four different microscopic fields of view from each patient's sample.

liver tissue. Normal liver cells, as well as adjacent non-infected cells in the HBV-infected samples, displayed weak LC3β staining (Figure 3A). However, the majority of HBV-infected liver cells exhibit strong positive staining of LC3β (Figure 3B). Evaluation of LC3β protein expression by IHC using H-score showed a significant ($P < 0.001$) cytoplasmic staining of LC3β compared with the normal liver samples (Figure 3C).

HBV and HCV infection induces apoptosis in human liver tissue

Previous reports have shown that caspase-3 cleavage is one of the most important hallmarks of apoptosis activation in different models[6,32,33]. The results of immunofluorescence staining showed that both HBV and HCV infection induce caspase-3 cleavage in infected hepatocytes (Figure 4A and B). In addition,

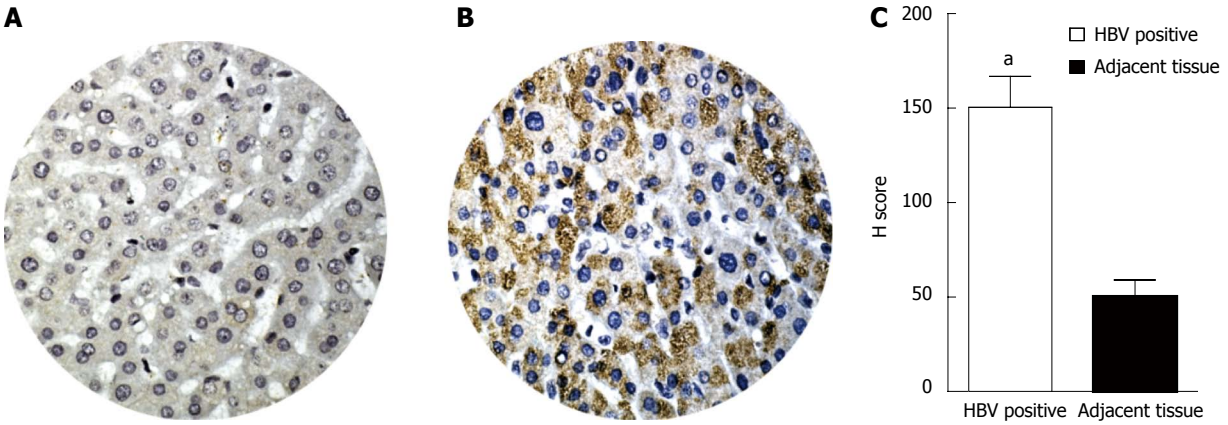

Figure 3 Comparison of LC3β expression, determined by immunohistochemistry in HBV^{Pos} and HBV^{Neg} liver tissue microarrays. Immunohistochemistry was performed as described in Materials and Methods. H-scores were derived from semi-quantitative assessments of both staining intensity (scale 0-3) and the percentage of positive cells (0%-100%) and, when multiplied, generate a score ranging from 0 to 300. A: Representative image of tissue core from HBV^{Neg} liver showing low expression of LC3β (H-score of 50); B: HBV^{Pos} liver tissue showing higher expression of LC3β (H-score of 150); C: The bar graphs show expression levels (H-score) of LC3β in HBV^{Pos} and HBV^{Neg} liver tissue microarrays. The bars represent the mean ± SEM. $^{a}P < 0.001$ *vs* the normal HBV^{Neg}. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

caspase-3 cleavage was significantly higher in HBV- and HCV-infected cells compared to non-infected adjacent cells (Figure 4C and D) ($P < 0.001$).

HBV and HCV infection induces UPR in human liver tissue

UPR induces expression of genes encoding proteins, such as BIP, to restore ER homeostasis[29,34]. Therefore, higher expression of BIP is the major biochemical marker of the activation of UPR. Analysis of IHC staining showed significantly higher expression of BIP in HBV (Figure 5A and C) and HCV (Figure 5B and D) infected hepatocytes compared to non-infected adjacent cells ($P < 0.001$).

HBV infection induces XBP1 splicing in human liver tissue

IRE1α is a bifunctional enzyme which possesses kinase and RNase activity. One of the primary targets of RNase activity of IRE1α is XBP1, leading to production of spliced XBP1 (sXBP1) during the UPR[13,34]. In our present investigation, we showed that both HBV and HCV infection induced XBP1 expression in infected hepatocytes (Figure 6A and B). Interestingly, HBV infection significantly induced XBP1 splicing (nuclear localized XBP1) compared to adjacent non-infected hepatocytes (Figure 6C) ($P < 0.001$) while HCV infection increased XBP splicing which is not statistically different compared to non-infected adjacent cells (Figure 6D) ($P > 0.05$).

Autophagy, apoptosis, and UPR do not correlate with the stage and the grade of liver disease in HBV and HCV patients

Our statistical analysis showed that autophagy, apoptosis, and UPR do not significantly correlate with the stage and the grade of the liver disease in both HBV and HCV patient groups (Tables 1 and 2) ($P > 0.05$).

DISCUSSION

In the current study we showed that HBV or HCV infection can significantly induce apoptosis, autophagy, and UPR in infected human patient hepatocytes compared to non-infected adjacent cells. This study importantly represents real clinical sample evaluation of these events in the context of authentic HBV and HCV infection.

HCV infection has a striking tendency towards chronicity, often of significant liver diseases like chronic hepatitis, cirrhosis, and hepatocellular carcinoma[35]. Apoptosis (programmed cell death) is a cellular process in which cells systematically kill themselves through activating intracellular death pathways in response to different kinds of stimuli[36]. Although apoptosis has been observed as a crucial mechanism in viral clearance[37], the exact mechanisms of HCV pathogenesis have not yet been fully delineated. There is an accumulating body of evidence that highlights the significant role of hepatocyte apoptosis regulation in HCV pathogenesis[38] and a variety of apoptotic pathways were proposed that might be involved in this mechanism[39]. The HCV core protein alone can greatly affect cellular functions. It can either induce or inhibit the apoptosis process. Apoptosis promotion by the core protein may be the reason for occurrence of hepatitis and liver damage and apoptosis inhibition. On the other hand, core might provide conditions for HCV to establish persistent infection[40].

HBV is also a causative agent of chronic hepatitis and represents one of the major risk factors for development of HCC[41-43]. The hepatitis B virus X protein (HBx) has recently garnered much attention

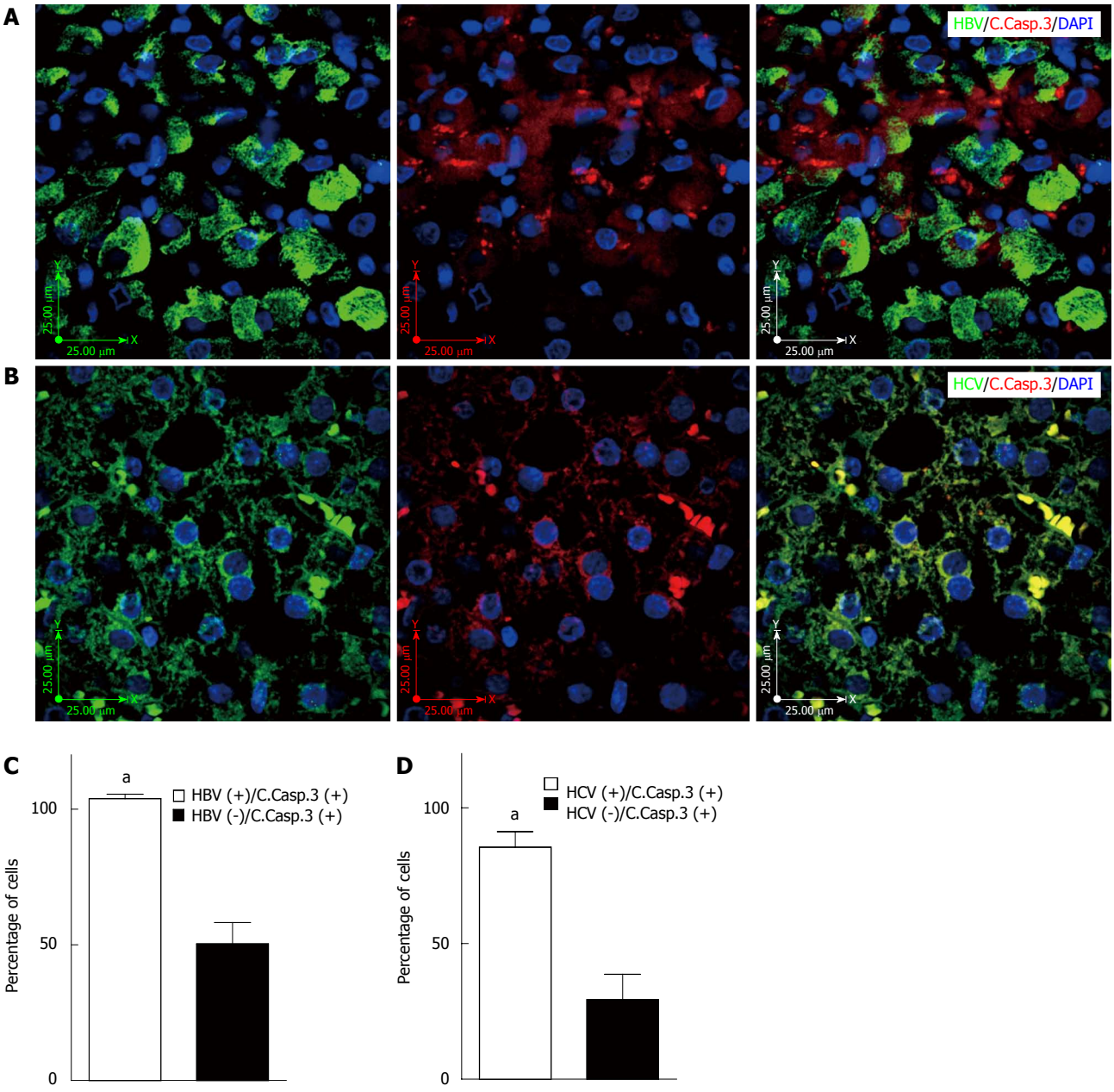

Figure 4 Hepatitis B and C virus infection induces apoptosis in the infected liver tissue. A: Immunofluorescence double-labeling shows co-localization of hepatitis B virus (HBV) and cleaved caspase-3 in the HBV infected liver tissue; the image is representative of fluorescence immunohistochemistry (IHC) for all patients; B: Immunofluorescence double-staining shows co-localization of hepatitis C virus (HCV) and cleaved caspase-3 in the HCV infected liver tissue; the image is representative of fluorescence IHC for all patients; C: HBV infection significantly ($^aP < 0.001$ *vs* HBV') induces apoptosis in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HBV infection and cleaved caspase-3) in at least 50 cells counted in four different microscopic fields from each patient's sample; D: HCV infection significantly ($^aP < 0.001$ *vs* HCV') induces apoptosis in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HCV infection and cleaved caspase-3) in at least 50 cells counted in four different microscopic fields from each patient's sample.

regarding its effect on cellular functions, especially apoptosis during HBV infection. Being the smallest protein encoded by HBV genome, HBx is expressed in 70% of patients with HBV-related HCC[44,45], highly conserved in mammalian hepadnaviruses and essential for infection in mammals[46]. HBx has various functions that may participate in HBV pathogenesis[47] and different studies have investigated the role of HBx in multifaceted aspects of apoptosis process. One of the main mechanisms of HBx protein during HBV infection and that contributes to HCC development is its influence on apoptosis. Since the pro-apoptotic functions of HBx were first described[47], a wide range of different studies have evaluated the effect of HBx expression on apoptotic pathway regulation; however, results of such studies are variable. According to studies in multiple cellular contexts, HBx might induce[19,20,47-53], inhibit[54-57] or have no effect[58] on apoptosis.

As previously mentioned, autophagy is often

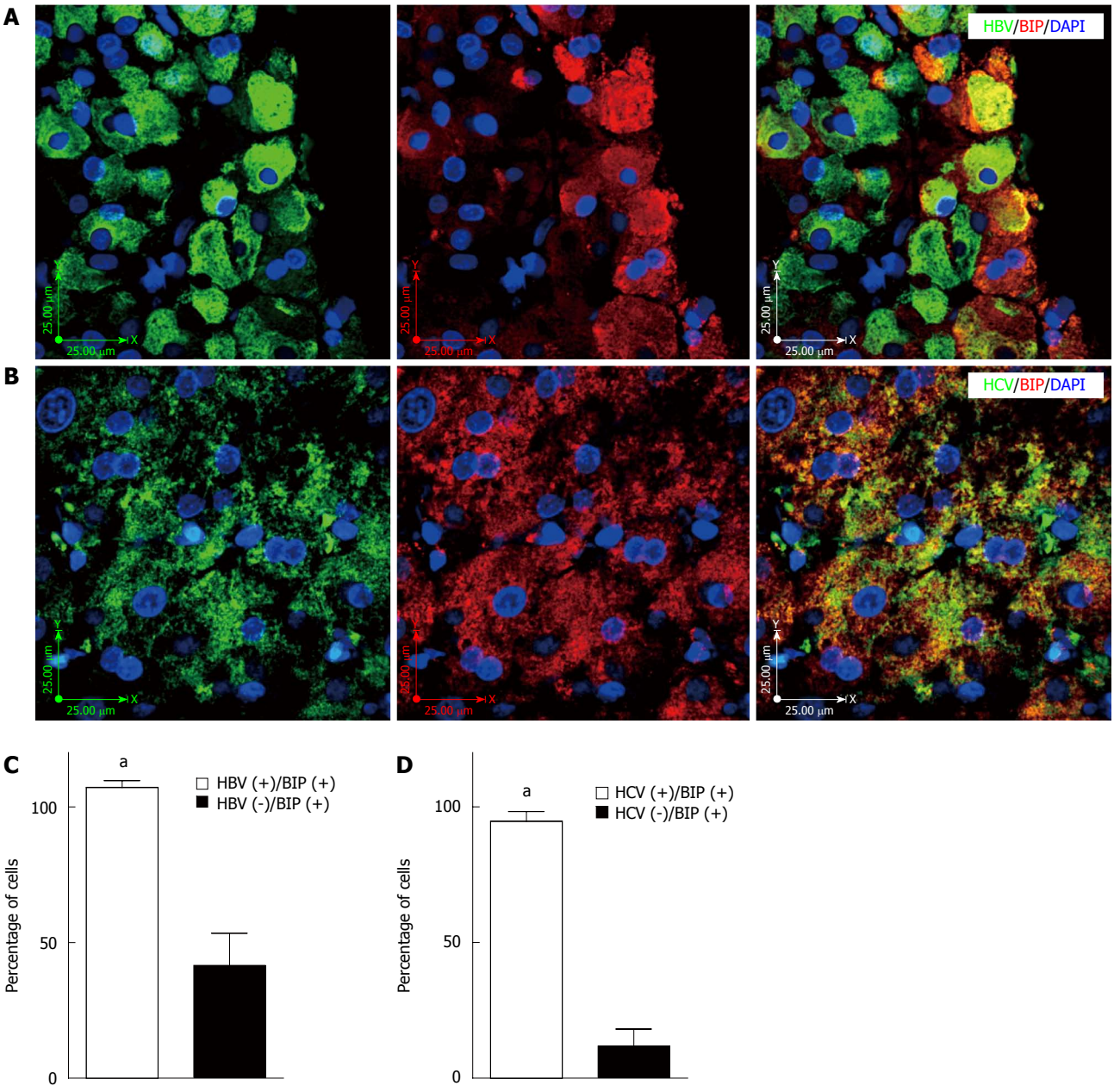

Figure 5 Hepatitis B and C virus infection induces unfolded protein response in the infected liver tissue. A: Fluorescence immunohistochemistry (IHC) confirms co-localization of hepatitis B virus (HBV) and BIP (GRP78) expression in the HBV-infected liver tissue; the image is representative of fluorescence IHC for all patients; B: Fluorescence IHC confirms co-localization of hepatitis C virus (HCV) and BIP (GRP78) expression in the HCV-infected liver tissue; the image is representative of fluorescence IHC for all patients; C: HBV infection significantly ($^{a}P < 0.001$ *vs* HBV') induces UPR in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event,HBV infection and BIP (GRP78) expression in at least 50 cells counted in four different views of fluorescence IHC in each patient's sample; D: HCV infection significantly ($^{a}P < 0.001$ *vs* HCV') induces UPR in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HCV infection and BIP (GRP78) expression) in at least 50 cells counted in four different views of fluorescence IHC in each patient's sample. UPR: Unfolded protein response.

defined as an evolutionarily conserved process that catabolizes intracellular components by delivering them into lysosomes[59-61]. It has been shown that some viruses utilize and induce autophagy in favor of their own replication and survival using their proteins[62-64]. To contribute to different steps of its life cycle like replication, translation, assembly, and lipo-viro-particle release, HCV takes advantage of the autophagic process. However, these effects are partially indirect[65]. Similarly, several studies have been performed aimed at clarifying the involvement of autophagy in establishment of chronic HCV infection[26,66,67] and findings from these studies suggest that autophagy is involved in HCV infection. A subgenomic replicon, corresponding to the HCV NS3-NS5B coding region of HCV genotype 1b, can trigger autophagy[68] which implies autophagy induction *via* viral nonstructural proteins. In addition, siRNA-targeting ATGs, that inhibit autophagy, abrogates HCV replication[69]. Moreover, HCV infection activates various pattern recognition

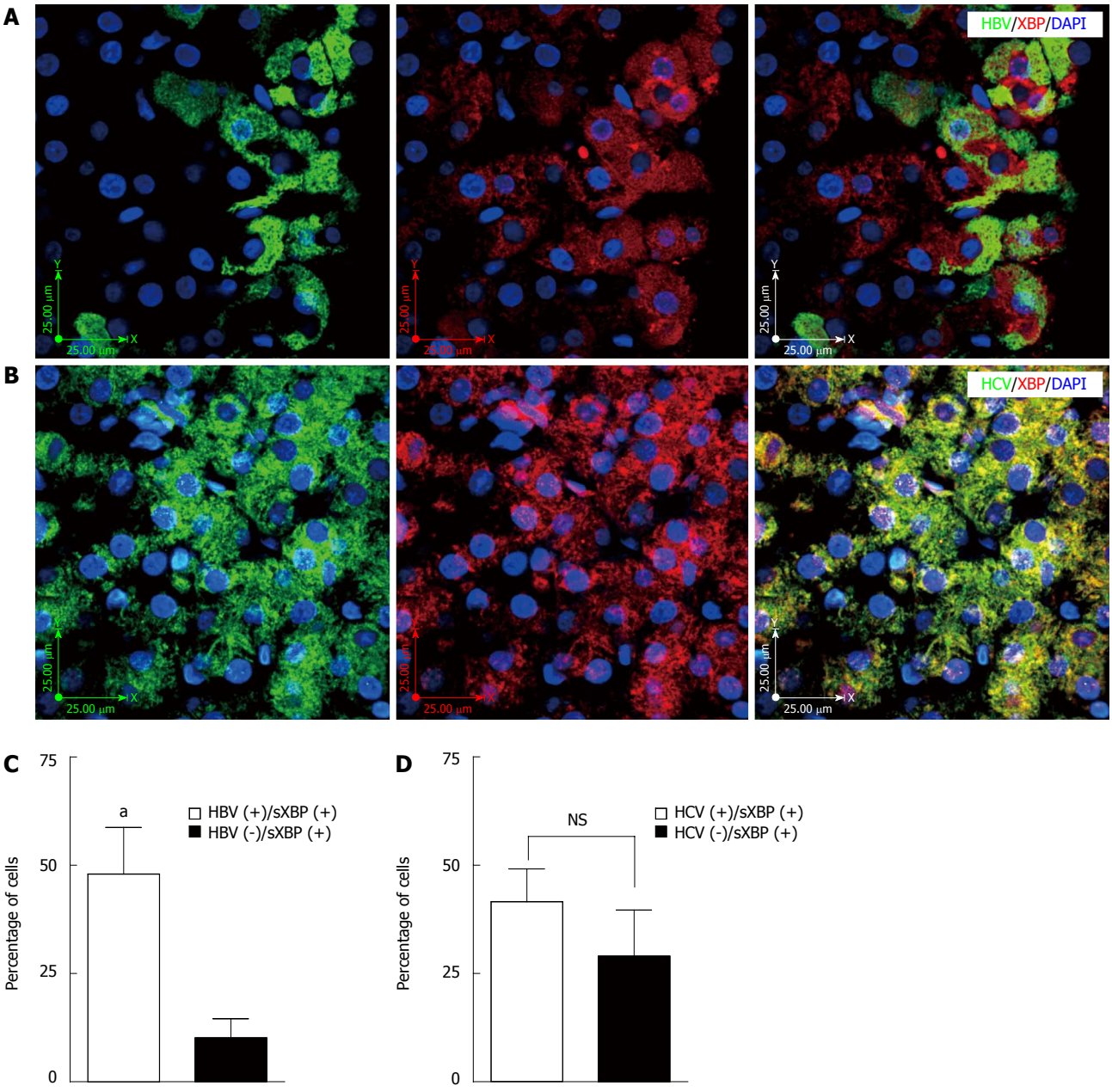

Figure 6 Hepatitis B virus infection induces XBP splicing in the infected liver tissue while hepatitis C virus infection does not induce XBP splicing. A: Fluorescence immunohistochemistry (IHC) confirms co-localization of hepatitis B virus (HBV) and spliced XBP (sXBP) (spliced XBP localized in the nucleus) in the HBV-infected liver tissue; the image is representative of fluorescence IHC for all patients; B: Fluorescence IHC shows that co-localization of hepatitis C virus (HCV) and spliced XBP (sXBP) (spliced XBP localized in the nucleus) is not a dominant event in the HCV-infected liver tissue; the image is representative of fluorescence IHC for all patients; C: HBV infection significantly ($^{a}P < 0.001$ *vs* HBV^{-}) induces XBP splicing (spliced XBP localized in the nucleus) in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event [HBV infection and spliced XBP (sXBP) (spliced XBP localized in the nucleus)] in at least 50 cells counted in four different views of fluorescence IHC in each patient's sample; D: HCV infection does not significantly ($P > 0.05$) induce XBP splicing in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event [HCV infection and spliced XBP (sXBP) (spliced XBP localized in the nucleus)] in at least 50 cells counted in four different views of fluorescence IHC in each patient's sample.

receptors[70], being able to trigger autophagy[71]. Nevertheless, their exact roles in autophagy induction during HCV infection remains poorly understood.

Some recent studies investigated the interaction between HBV chronic infection and autophagy[72,73]. Autophagy is involved in different stages of HBV development and infection; however, its exact impacts are not fully known. Presumably, autophagy can either increase the replication of HBV DNA or contribute to HBV envelopment[22,74]. Results from electron microscopy, confocal microscopy, and biochemical assays have demonstrated that HBV can enhance autophagy during infection in cell cultures and mouse liver[72]. There is compelling evidence that HBV acts on the early step of phagosome formation and that the degradation rate of autophagic protein is not increased while HBV triggers the formation of early phagosomes[74,75]. Different HBV proteins are involved

in the autophagy process. HBx not only is involved in apoptosis induction[19] but also in autophagy process in the course of HBV infection[73]. HBV usurps cellular activities such as autophagy and proliferation in favor of virus replication[22]. Nevertheless, the involved mechanisms for the induction of autophagy and the step of HBV replication affected by autophagy are not completely understood and open to interpretation[72,74].

The UPR has recently been identified as a novel mechanism involved in a wide range of human diseases including viral infections[76,77]. In fact, it has been revealed that a number of viruses utilize UPR to help attenuate anti-viral responses and establishment of infection[78-82]. HCV uses the membranous compartment of the ER as the biogenesis site for its envelope protein and particle assembly[83]. Therefore, there is general agreement on the induction of ER stress by HCV infection which can interfere with the ER function in host cells and upon sensing ER stress, cells activate the UPR signaling pathway[18]. Moreover, there are some well documented data that HCV induces ER stress and UPR in both *in vitro* and *in vivo* experimental models[27,84-86]. Virus-induced UPR is demonstrated to trigger apoptosis of the infected hepatocytes and also overwhelming evidence indicates the crucial role of UPR in the HCV replication[68,84,85] and life cycle[27,85-92]. HCV infection apparently activates all three UPR sensors[27,60,84,93]. It was observed in a cell culture system that HCV-induced UPR plays a positive role in RNA replication and efficient propagation of HCV as HCV replication was suppressed when one of three UPR pathways was significantly abrogated[68]. A study by Zheng *et al*[92] showed that HCV NS4B can activate UPR by induction of XBP1 mRNA splicing and ATF6 cleavage. Our finding shows that there is no significant difference in XBP splicing between HCV-infected and non-infected cells which does not correlate with previous reports that highlighted the role of XBP splicing in HCV infection in hepatocytes. We assume that this difference could be the result of the difference in genetic background of the population study or the lack of enough samples in our study.

Different studies have shown that HBx protein of HBV can trigger UPR. Meanwhile, a major drawback that has prevented us from broadening our in-depth knowledge in a natural infection system has been the lack of a strong and efficient *in vitro* infectivity model[94]. Cho *et al*[24] observed that HBx downregulates the cellular ATP level and mitochondrial membrane potential; then ATP reduction induces ER stress. In addition, ATF4, which is a UPR marker, was demonstrated to up-regulate COX2 expression. This kind of UPR induction by HBx might trigger the development of HCC as well as liver inflammation[24]. Moreover, HBx and S proteins both can activate the IRE1/XBP1 branch of the UPR. Li *et al*[23] demonstrated that the transiently-expressed HBx protein in Hep3B and HepG2 cells caused a high increase (up to 7-fold) in XBP1 promoter activity and also ATF6 cleavage in a dose-dependent manner. This mechanism of HBx in inducing UPR was determined as a potential mechanism, contributing to the replication of HBV in liver cells[23]. Similarly, in another study conducted by Li *et al*[74], they over-expressed the S protein in Huh7 hepatoma cells and observed the presence of the XBP1 mRNA (both precursor and spliced forms) which suggests the ability of S protein to trigger UPR by the IRE1/XBP1 pathway. XBP1, as a crucial sensor of UPR pathway, is a key protein in the growth and differentiation of hepatocytes as shown by XBP1 knockout mice[95]. It is supposed that HBx might act as a kinase activator that increases the phosphorylation level of the ER stress sensor IRE1, thereby activating the IRE1-XBP1 pathway. On the other hand, HBx is able to interact with the bZIP class of transcription factors and both ATF6 and XBP1 belong to this family so that HBx can activate or co-activate them to enhance trans-activating activities[95]. Furthermore, HBx might be involved in the expression and persistence of HBV since the ATF6 pathway associates with ER chaperone expression and helps the unfolded proteins to refold[91,96]. It should be noted that studies on the UPR at early stages of HBV infection have not yet been addressed and this may be due to the difficulty in investigating HBV infection[97].

In conclusion, our studies confirm previous *in vivo* and *in vitro* studies which showed HBV and HCV infection is associated with the induction of apoptosis, autophagy, and UPR in hepatocytes. In the present study, we showed, for the first time, simultaneous induction of apoptosis, autophagy, and UPR after HBV or HCV infection in human liver tissue. Our study highlights the co-incidence of all of these events in the human liver after natural HBV or HCV infection. As apoptosis, autophagy, and UPR are linked together *via* different regulatory proteins, it will be very important to address which events (apoptosis, autophagy, or UPR) are induced first after HBV or HCV induction. This understanding would be beneficial to design new strategies to control these pathways after HBV or HCV infection to ameliorate the process of liver injury after viral infection. As our study has been done in human samples, these results have great potential impact in HBV- and HCV-infection-initiated liver diseases. Further long term and follow up studies are in process to highlight the effect of these events in the progression of liver diseases.

ACKNOWLEDGMENTS

This study is a complementary and Side Project of the PhD thesis of Dr. Payam Peymani (Thesis Number: 92-6907).

COMMENTS

Background

Hepatitis caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) infection has emerged as a major public health problem throughout the world.

Dysregulation of apoptosis, autophagy and the unfolded protein response (UPR) has been implicated in a wide spectrum of human diseases including viral infection. These pathways are tightly interconnected and their crosstalk is a key factor for cell-fate determination in response to different stimuli. However, their interplay during HBV and HCV infection remains unclear. In this study the authors investigated induction of autophagy, UPR, and apoptosis in HBV- and HCV-infected human liver tissues to examine the importance of these pathways in HBV- and HCV-induced liver damage. In addition, these studies will pave the way for the development and application of therapeutics that modulate these pathways to affect HBV and HCV replication and the progress of liver damage in patients.

Research frontiers

The authors investigates the importance of apoptosis, autophagy, and UPR in health and diseases. They modulate these pathways to provide new approaches in treatment of different diseases.

Innovations and breakthroughs

The current research showed the co-incidence of apoptosis, autophagy, and UPR in HBV and HCV infected hepatocytes.

Applications

The next step of this research would be to target the modulation of apoptosis, autophagy, UPR in the hepatocytes of HBV/HCV infected patients to prevent the progress of liver damage in these patients.

Peer-review

This is an interesting manuscript about cell fate in hepatocytes after hepatitis B and C virus infection. Also, the manuscript was clearly written and organized.

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ISSN 1007-9327

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