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Human hepatocytes apoptosis induced by replication of hepatitis B virus subgenotypes F1b and F4: Role of basal core promoter and preCore mutations

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ABSTRACT

In the context of pathogenesis of HBV infection, HBV genotypes and mutants have been shown to affect the natural course of chronic infection and treatment outcomes. In this work, we studied the induction of apoptosis by the replication of HBV subgenotypes F1b and F4, and the naturally occurring mutants BCP and preCore. Both subgenotypes F1b and F4 HBV genome transfections induced cell death by apoptosis in human hepatocytes. The BCPdm (A1762T/G1764A) and preCore (G1896A) mutants induced higher levels of apoptosis than the wt virus. This increase in apoptosis was not associated with the enhanced viral replication of the variants. HBV-mediated apoptosis was independent of viral subgenotypes, and associated with the modulation of members of the regulatory Bcl-2 family proteins expression in the mitochondrial apoptotic pathway. Finally, the apoptosis induction increase observed for the preCore mutants suggests that HBeAg might have an anti-apoptotic effect in human hepatocytes.

1. Introduction

Hepatitis B virus (HBV) is an important human pathogen that chronically infects approximately 257 million people worldwide (WHO|Hepatitis B, 2017). HBV chronic infection is associated with the development of severe liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC).

The chronic infection outcome relies on a finely balanced and complex interaction between the virus and the host immune system (McMahon, 2009). It has been widely accepted that liver damage is mainly caused by the host immune response (Baumert et al., 2007). The mechanisms of cell damage are generally defined as the result of a cytotoxic-T lymphocyte (CTL) mediated immune response against the viral infection (Chisari et al., 2010). Moreover, apoptosis, has also been involved in HBV pathogenesis (Lamontagne et al., 2016).

Apoptosis, or programmed cell death, is a highly regulated process that can be initiated by a large variety of stimuli, both extra and intracellular, and trigger two major signaling pathways (Siddiqui et al., 2015). Particularly, the intrinsic or mitochondrial pathway is regulated by the Bcl-2 family proteins, which includes both pro-apoptotic and anti-apoptotic members (Chipuk et al., 2010). The deregulation of apoptosis is involved in a wide range of pathological processes, including development of HCC (Schattenberg et al., 2011).

HBV viral proteins, such as the X protein (HBV-X), have been proved able to induce apoptosis (Chirillo et al., 1997; Kim et al., 2008; Lu and Chen, 2005; Miao et al., 2006). Conversely, it was shown that HBeAg can inhibit p53-mediated apoptosis; implying that intracellular HBeAg exerted an anti-apoptotic effect in hepatoma cells (Liu et al., 2016). Therefore, in addition to host factors, viral factors could be involved in the progression of HBV-related liver diseases.

Epidemiological data have increasingly associated HBV genotypes and subgenotypes (sgts) with differences in clinical and virological characteristics, such as severity of liver disease and response to antiviral therapies (Lin and Kao, 2011; Liu and Kao, 2013; Tong and Revill, 2016; Zhang et al., 2016).

HBV has been classified in ten genotypes (A-J) and multiple subgenotypes (Kramvis, 2014). The genotypes and sgts have a different ethnic-geographical distribution (Sunbul, 2014). In Argentina sgts F1b

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and F4, likely originated in Amerindian populations, are the most prevalent (Ledesma et al., 2015; Pezzano et al., 2011). Moreover, growing evidence has shown a close association of sgt F1b with more severe course of chronic HBV infections (Ching et al., 2016; Gounder et al., 2016; Livingston et al., 2007).

On the other hand, mutations in the preCore, as well as basal core promoter (BCP) regions, have been associated with the progression of HBV chronic infection (Parekh et al., 2003). HBV variants carrying the double 1762T/1764A mutation in the BCP region and/or the 1896A mutation in the preCore region have been presumed to be strictly associated with progressive forms of liver disease (Chen and Yang, 2011; Lin et al., 2005; Wei et al., 2017).

The 1762T/1764A mutations reduce preCore RNA transcription, HBeAg expression levels and increase the rate of virus replication. In addition to 1762T/1764A, mutations at nearby positions such as 1753, 1757, 1766, and 1768 can also be detected. Site-directed mutagenesis experiments have suggested that the additional mutations at 1753, and 1766 further reduce HBeAg expression and enhance genome replication 10-fold, compared to wild type (wt) virus (Parekh et al., 2003).

Regarding the preCore region, the most common mutation is 1896A, which converts the penultimate (28th) precore codon from TGG to TAG and abolishes HBeAg expression (Lin and Kao, 2015).

In this work, we studied and characterized the induction of apoptosis in human hepatocytes by HBV sgts F1b and F4, and its BCP and preCore variants.

2. Materials and methods

2.1. Cell culture

Human hepatoma cell line Huh-7 was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (Internegocios), 1 mM nonessential aminoacids (GIBCO), 0.15% sodium bicarbonate, 100 UI/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Plasmids

Vector pUC19 containing full-length HBV genomes of sgts F1b and F4 with the wt sequence, the double BCP (A1762T/G1764A; BCPdm) and the preCore (G1896A) mutations were analyzed in this study. Additionally, a quadruple BCP (T1753C/A1762T/G1764A/C1766T; BCPqm) sgt F1b mutant was included. The generation of these plasmids was previously described (Sevic et al., 2017).

2.3. Transient transfection

In order to simulate viral variability, a mix of 10–20 clones (pUC19-full-length HBV genomes) of each viral variant, was used in each experiment.

As previously described, full-length linear HBV genomes were excised from the plasmid by restriction enzyme digestion with 5 U of BspQI (New England Biolabs, USA) at 50 °C (Sevic et al., 2017). The 3.2-kb fragments were gel purified with the PureLink Quick Gel Extraction Kit (Invitrogen, USA), according to the manufacturer's instructions and the DNA was quantified spectrophotometrically.

For transfections, cells were seeded in 24 or 6 well plates and grown to 60–70% confluence. Transfections were carried out using X-tremeGene 9 transfection reagent (Roche, Germany), according to the manufacturer's recommendations. Empty vector pUC19 was used as control. Cells were maintained at 37 °C in 5% CO_2 atmosphere. After 6 h incubation, mixtures were replaced with fresh medium and incubated for 72 h.

2.4. Acridine orange and ethidium bromide staining (AO-EB)

Approximately 5×10^5 transfected cells (adherent and detached cells) were harvested, washed with cold phosphate-buffered saline (PBS) and resuspended in 0.1 ml of PBS, containing 4 μg/ml of acridine orange (Sigma, USA) and 4 μg/ml of ethidium bromide (Sigma, USA). Cell suspension was immediately dispensed onto slides, viewed under a fluorescent microscope (Olympus BX50) and photographed. Image analysis was performed with ImageJ software (Wayne Rasband, NIH, USA). For each sample, at least 300 cells were counted and the percentage of early and late apoptotic cells was determined.

Viable cells were identified by the bright green fluorescent nuclei with an organized structure; early apoptotic cells, presented nuclei containing highly condensed or fragmented green chromatin; late apoptotic cells showed bright orange highly condensed nuclei and fragmented chromatin and necrotic cells contained diffuse orange nuclei.

2.5. Flow cytometry

The annexin V–FITC Apoptosis Detection Kit (BD Pharmingen, USA) was used to detect phosphatidilserine (PS) translocation from the inner to the outer leaflet of the plasma membrane. The assay was carried out according to the manufacturer's instructions. Briefly, approximately 5×10^5 adherent and detached transfected cells were harvested, washed twice with cold PBS and stained with Annexin V and Propidium Iodide (PI) for 15 min at room temperature in the dark. Apoptosis was determined by measuring the red and/or green fluorescence of the cells with a flow cytometer (Pas-III, Partec, Germany). Data analysis was performed using the WinMDI 2.9 software (The Scripps Research Institute, La Jolla, CA, USA).

2.6. Caspase-3 activity assay

Caspase-3 activity in total cell lysates was measured using the EnzChek caspase-3 assay kit (Molecular Probes, USA), according to the manufacturer's instructions. Briefly, 72 h post-transfection, cells were harvested, washed with PBS and resuspended in Cell Lysis Buffer. Lysates were centrifuged and supernatants were harvested. The protein concentration of the samples was determined by the Bradford protein assay. Cell lysates were added to standard black 96 well plates, mixed with 2X Reaction Buffer containing 200 μM of caspase-3 substrate Z-DEVD-AMC and incubated for 1 h at room temperature in the dark. Fluorescence was measured in a fluorescence microplate reader (Beckman Coulter DTX 880, USA) using 370/465 ± 20 nm excitation/emission wavelengths, and calculations were done by normalizing the fluorescence to 50 μg of proteins.

2.7. Western Blot analysis

For total protein extraction, approximately 2×10^6 adherent and detached cells were harvested, washed with PBS and resuspended in lysis buffer [20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% Triton, and protease inhibitor cocktail (Sigma, USA)]. The cells were lyzed by 3 freeze/thawing cycles. Lysates were centrifuged and supernatants were harvested. Total protein concentration was determined using the Bradford protein assay. Equal amounts of proteins were loaded on 15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond, GE Healthcare, UK) by electroblotting at 100 V for 60 min. The membranes were blocked for 1 h in 5% non-fat milk in Tris-buffered saline (20 mM Tris and 150 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBST) for 1 h at room temperature, followed by incubation with the specific primary antibody: mouse monoclonal anti-Bax (1:500, Santa Cruz Biotechnology), rabbit polyclonal anti-Bcl-X (1:500, Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin (0.5 μg/ml, Sigma, USA), overnight at 4 °C. The

membranes were washed 3 times with TBST and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody: goat anti-mouse (1:10000, Santa Cruz Biotechnology) or goat anti-rabbit (1:10000, Santa Cruz Biotechnology) for 1 h at room temperature. Protein specific bands were visualized using an enhanced chemiluminescence (ECL) system (GE Healthcare, UK) by autoradiography. The quantification was performed using ImageJ analysis software (Wayne Rasband, NIH, USA). β-actin detection was used as the total protein loading control.

2.8. Determination of HBeAg levels

HBeAg levels in cell culture supernatants were determined using the ARCHITECT system (Abbot, USA), which is chemiluminescent microparticle immunoassay (CMIA). Results were expressed in Sample/Cut off value (S/CO).

2.9. Statistical analysis

All experiments were independently performed three times. Statistical significance was determined using a two-tailed Student *t*-test. A value of $p < 0.05$ was considered to be statistically significant. Results were expressed as mean ± standard deviation. All analyses were performed using GraphPad Prism 5.01 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Apoptosis induction

Apoptosis induction was evaluated after full-length HBV genome transfections with sgts F1b and F4 wt sequences and BCP/preCore mutants.

The observation of cellular morphology by contrast phase microscopy revealed that full-length HBV genome transfections induced morphological changes compatible with programmed cell death, including cell shrinkage, loss of contact with neighboring cells, chromatin condensation and margination at the nuclear membrane, plasma membrane blebbing and apoptotic bodies; while control cells showed the typical morphology of the cell line (Supplementary Fig. S1).

To further analyze apoptosis induction, AO-EB staining (Fig. 1) and flow cytometry analysis (Fig. 2) were performed. Significant increases in early and late apoptotic cells were revealed by both methods, in cells transfected with sgts F1b and F4 wt full-length HBV genomes. In addition, an increased level of apoptosis induction was observed with the BCPdm variant, compared to the wt, for both tested sgt.

In order to determine whether the increase in apoptosis induced by the BCPdm was due to a higher replication capacity of these variants, apoptosis was then studied in a sgt F1b BCPqm variant, with higher replication capacity than both wt and BCPdm. The BCPqm also increased the levels of apoptotic cells compared to control, however, no differences were observed when induced apoptotic levels were compared to the wt virus or the BCPdm (Fig. 2). The induction of apoptosis was also evaluated in cells transfected with sgt F1b and sgt F4 preCore variants. As seen in Fig. 2, the viruses with the preCore mutation significantly increased apoptosis induction compared to control cells. Furthermore, these variants induced significantly higher percentages of early and late apoptotic cells when compared to the wt viruses, for both tested sgts.

No significant differences were observed in apoptosis induction levels when comparing between sgts F1b and F4, for each analyzed variant.

A hallmark of apoptosis is the activation of caspase-3, the predominant effector caspase responsible for many of the characteristic downstream events associated with apoptosis. To confirm the occurrence of apoptosis in HBV transfected cells by another methodology, the activation of caspase-3 was analyzed. Caspase-3 activity was significantly increased in all cells transfected with sgts F1b and F4 full-length HBV genomes, compared to control transfected cells (Supplementary Fig. S2).

3.2. Expression of Bcl-2 family proteins

To identify the possible apoptotic signaling pathway involved in HBV-induced apoptosis, the expression of anti (Bcl-X) and pro-apoptotic (Bax) Bcl-2 family members was analyzed by Western Blot (Fig. 3).

Expression of the anti-apoptotic Bcl-X was down-regulated in all the transfected cells, although not significantly for sgt F1b wt. There were no differences in Bcl-X expression levels between viral variants.

On the other hand, expression of the pro-apoptotic Bax increased in all HBV full-genome transfected cells, but not significantly in sgt F1b wt. Furthermore, in sgt F1b variants, Bax expression was significantly higher in BCPdm and preCore mutants, compared to the wt virus. However, no significant differences were observed in Bax levels between sgt F4 wt and its variants.

3.3. Detection of HBeAg antigen

The secretion for HBeAg into the supernatant of HBV transfected cells was quantified (Fig. 4).

A significant difference in HBeAg expression was observed between the wt constructs of sgts F1b and F4 (58.84 ± 10.8 vs 95.41 ± 2.23 S/Co). For the mutants in the BCP region, no differences were observed compared to wt in sgt F1b, whereas in sgt F4, the BCPdm expressed significantly lower levels than the wt (60.11 ± 2.13 vs 22.29 ± 6.3 S/Co). Finally, as expected, the preCore mutants did not show expression of HBeAg.

4. Discussion

In this study, we report evidence of apoptosis induced by HBV sgts F1b and F4 after full-genome transfection in Huh-7 cells. These sgts, native from Latin America, are responsible for most of the new acute and chronic infections in our country (Ledesma et al., 2015), and have been scarcely studied so far.

Although it is generally acknowledged the non-cytopathic nature of HBV, several studies have suggested a new paradigm where HBV replication is associated with cell death (Lu et al., 2007, 2006; Yeganeh et al., 2015).

Chronic HBV infection is considered one of the main causes of hepatic cirrhosis and HCC (Iavarone and Colombo, 2013; Liu and Kao, 2007; Pollicino et al., 2011). Considering the high regeneration capacity of liver cells, it is possible that extensive apoptosis would result in a higher level of liver cell proliferation in order to regenerate hepatocytes. Such increase in cell division may perturb the normal cell cycle control, resulting in an accumulation of mutations in the genome of progeny cells, which may lead to the selection of premalignant hepatic cells and ultimately contribute to HCC development.

In the present work, we showed that full-length HBV genome transfection of sgts F1b and F4 induces apoptosis. These results are in agreement with previous studies reporting that HBV full-genome transfection of genotypes A, B, C and D induces apoptosis in HepG2 and Huh-7 cells (Bhoola and Kramvis, 2016; Lu et al., 2007, 2006). Therefore, our results confirm previous in vitro studies and show that genotype F, one of the less characterized HBV genotypes, behaves as the other genotypes in relation to its capacity to induce apoptotic cell death.

In our study, no significant differences were observed in the induction of apoptosis between sgts F1b and F4, which might indicate that HBV-mediated apoptosis is independent of the viral subgenotype in human hepatocytes.

The BCP and preCore are the most common mutants selected during

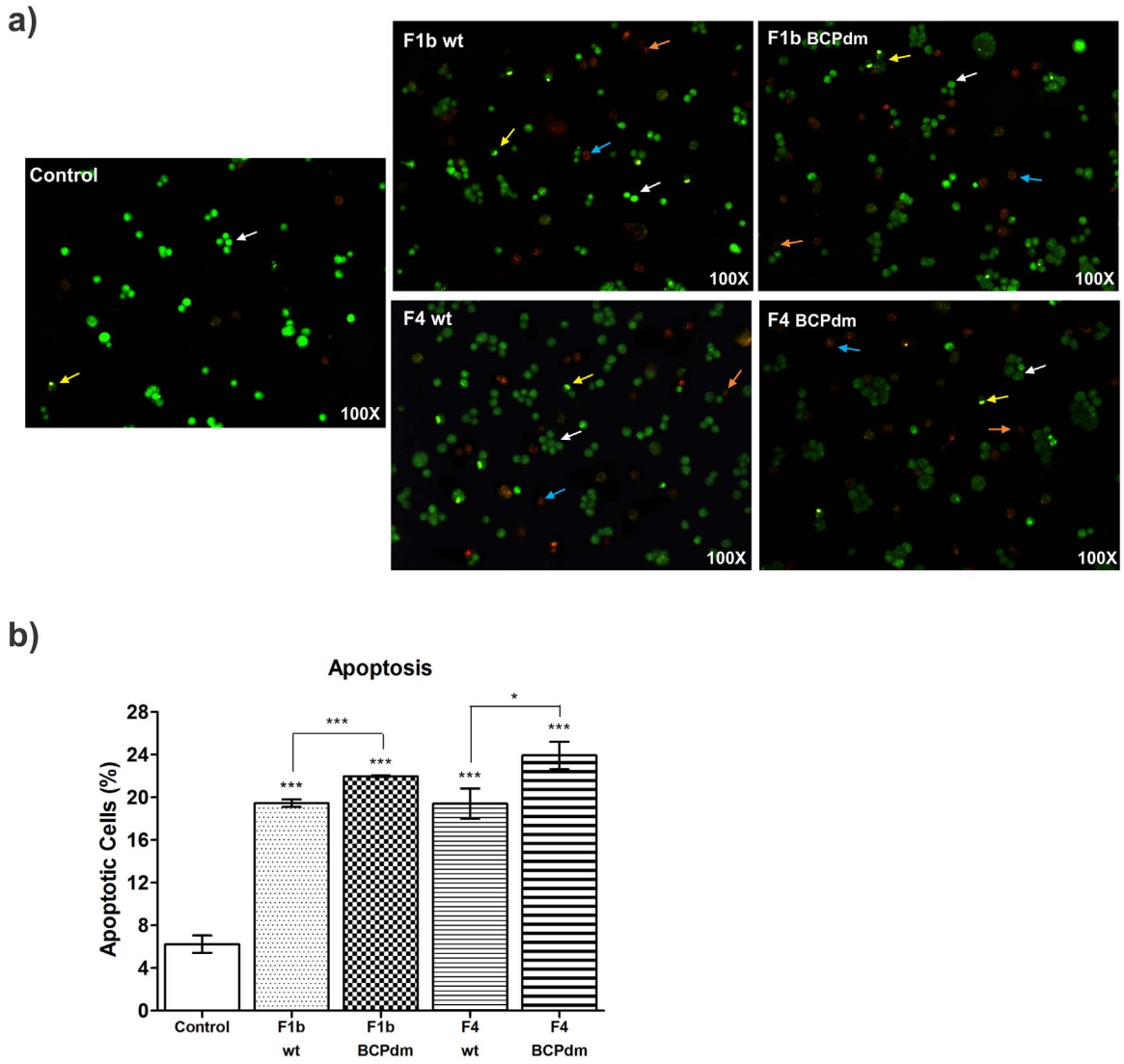

Fig. 1. Apoptosis detection in Huh-7 cells transfected with full-length HBV genomes by acridine orange and ethidium bromide staining. a) Huh-7 cells were transfected with pUC 19 empty vector (control), F1b wt, F1b BCPdm, F4 wt and F4 BCPdm variants. Seventy-two hours post-transfection, cells were stained with acridine orange and ethidium bromide and observed under fluorescent microscopy (100X magnification). White arrows: viable cells; yellow arrows: early apoptotic cells; blue arrows: late apoptotic cells; orange arrows: necrotic cells. b) Quantification of early and late apoptotic cells. Shown values represent the mean ± standard deviation of three independent experiments. Statistically significant changes compared to control cells are indicated with asterisks above the bars and statistically significant changes between the viral variants are indicated with asterisks above the brackets. * $p < 0.05$ and *** $p < 0.0001$.

the course of HBV chronic infection, and its emergence is associated with progressive forms of liver disease (Liu et al., 2004; Parekh et al., 2003). Several studies have demonstrated that BCP and preCore mutations are present in high frequency in patients with advanced liver disease, and there is a high correlation between the occurrence of these mutations and the later development of cirrhosis and HCC in chronic patients (Kao et al., 2003; Milich and Liang, 2003; Yang et al., 2016).

In this study, BCPdm as well as preCore mutants significantly increased apoptosis in comparison to the wt genomes, for both studied sgts. This enhancement of apoptosis might be one of the factors contributing to the higher frequency of liver cirrhosis and HCC observed in patients carrying these mutations.

Although it has been reported that HBV replication can induce apoptosis (Bhoola and Kramvis, 2016; Lu et al., 2007; Yeganeh et al., 2015), this is the first report analyzing the effect that BCP and preCore mutations exert on this process. The results obtained in this work indicate that two highly frequent naturally occurring mutations in chronic patients converge to the same result in Huh-7 cells, the enhancement in apoptosis induction.

It has been postulated that BCP mutations are associated with a more severe form of chronic liver disease since it enhances viral replication (Milich and Liang, 2003). However, in vitro experiments have provided controversial results; while some studies reported increased viral replication (Buckwold et al., 1996; Moriyama et al., 1996), others observed no difference between viral variants bearing the BCPdm and their wt counterparts (Jammeh et al., 2008; Liu et al., 2011). A less frequently observed mutation is the BCPqm, which enhanced viral replication in a higher degree than BCPdm (Parekh et al., 2003; Tong et al., 2005).

In a previous study, we have observed that sgt F1b virus with mutations at the BCP region exhibited a higher replication capacity than wt virus (González López Ledesma, personal communication). While BCPdm showed less than a two-fold increase in virus replication, BCPqm increased virus replication by more than three folds. The fact that sgt F1b BCPqm did not show differences in apoptosis induction compared to both wt and BCPdm, suggests that viral replication rate is not directly associated to an increase in apoptosis in Huh-7 cells.

It has been widely described that HBV proteins may affect different

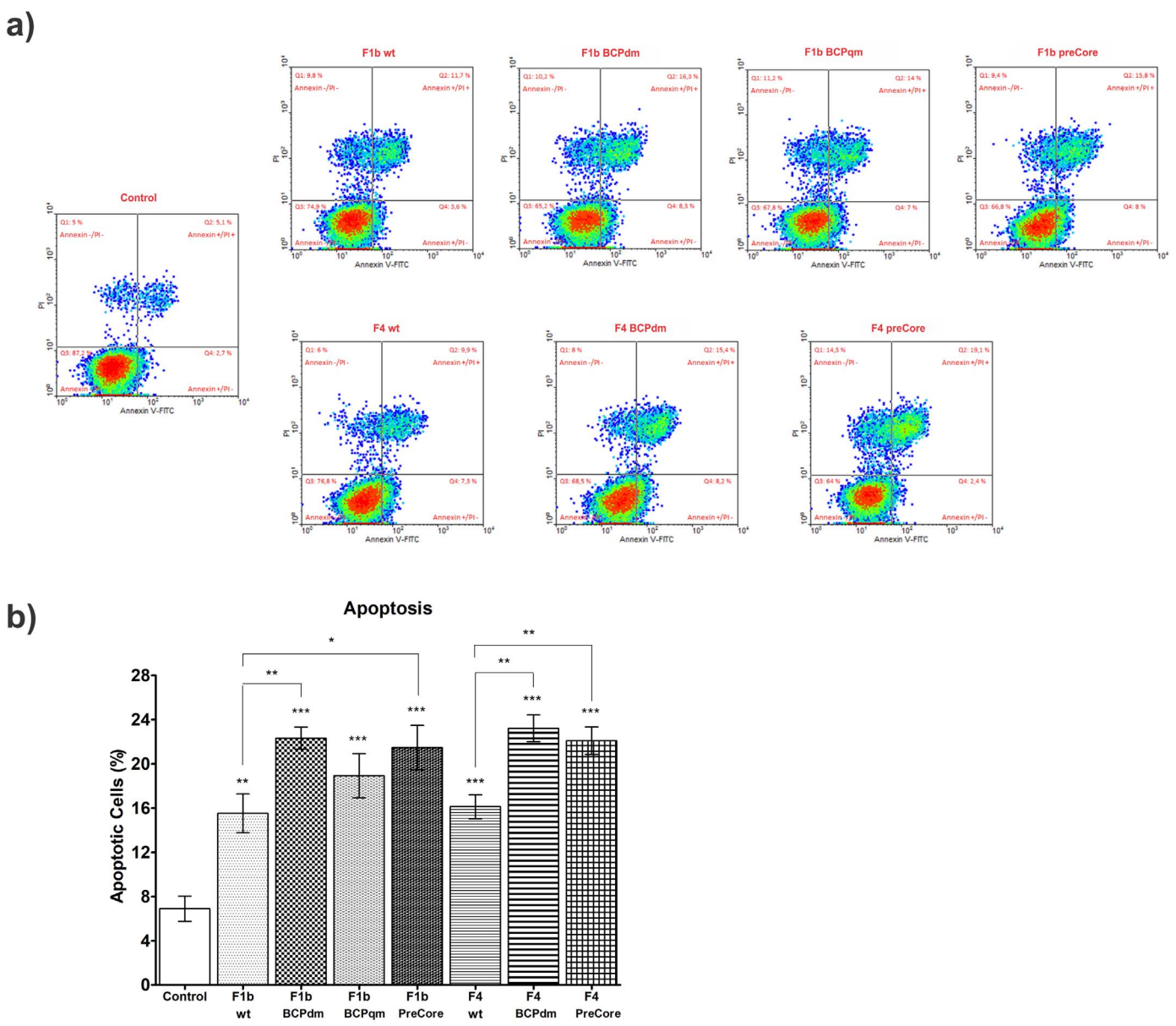

Fig. 2. Apoptosis detection in Huh-7 cells transfected with full-length HBV genome by flow cytometry. a) Huh-7 cells were transfected with pUC 19 empty vector (control), F1b wt, F1b BCPdm, F1b BCPqm, F1b preCore, F4 wt, F4 BCPdm and F4 preCore variants. Seventy-two hours post-transfection, cells were stained with annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Q1: necrotic cells; Q2: late apoptotic cells; Q3: viable cells; Q4: early apoptotic cells. Graphics show 20,000 events. b) Quantification of early and late apoptotic cells. Shown values represent the mean ± standard deviation of three independent experiments. Statistically significant changes compared to control cells are indicated with asterisks above the bars and statistically significant changes between the viral variants are indicated with asterisks above the brackets. * $p < 0.05$; ** $p < 0.005$ and *** $p < 0.0001$.

intracellular pathways. The BCP mutations, were described to alter viral replication and the expression of HBeAg, but due to the overlapping of the ORFs, they also affect the X gene, and consequently, HBV-X. The mutants 1762T/1764A result in L130M and V131I, respectively (Kidd-Ljunggren et al., 1997). HBV-X is a multifunctional regulatory protein with transcriptional transactivator activity on a number of cellular and viral promoters. By interacting with various transcription factors or components of signal transduction pathways, HBV-X regulates a wide variety of cellular processes, including apoptosis (Murakami, 2001; Rawat et al., 2012). Hence, the increase in apoptosis observed in BCP mutants may be in part due to the mutations in HBV-X.

In the present study, no significant differences were observed between sgts F1b and F4 in their capability to induce apoptosis. These results are in agreement with a previous study reporting an increase in apoptosis in transfected Huh-7 cells with HBV-X from genotypes A, B, C and D, however, no significant differences in apoptosis induction was detected between different genotypes (Kanda et al., 2004). On the contrary, a study conducted by Lu and Chen (2005) demonstrated that in HepG2 cells, genotype B exhibits stronger pro-apoptotic capability than genotypes A and C. The authors suggested that this difference might be associated to viral heterogeneity in a HBV-X region with homology to the BH3 domain of the Bcl-2 family proteins (aa 116–130). Therefore, based on these reports, the implication of genotype in the induction of apoptosis still remains controversial.

HBeAg is a protein that promotes the establishment of persistent infections by the immunomodulation of innate immune signal transduction pathways, via interaction and targeting of toll-like receptor (TLR) mediated signaling pathways (Lang et al., 2011). Furthermore, there is evidence that HBeAg may play an important role in the regulation of cytokine production in human hepatocytes. In a recent study, HBeAg has been reported to inhibit IL-18 signaling and IFN-γ expression (Jegaskanda et al., 2014). In addition, Wu et al. (2010)

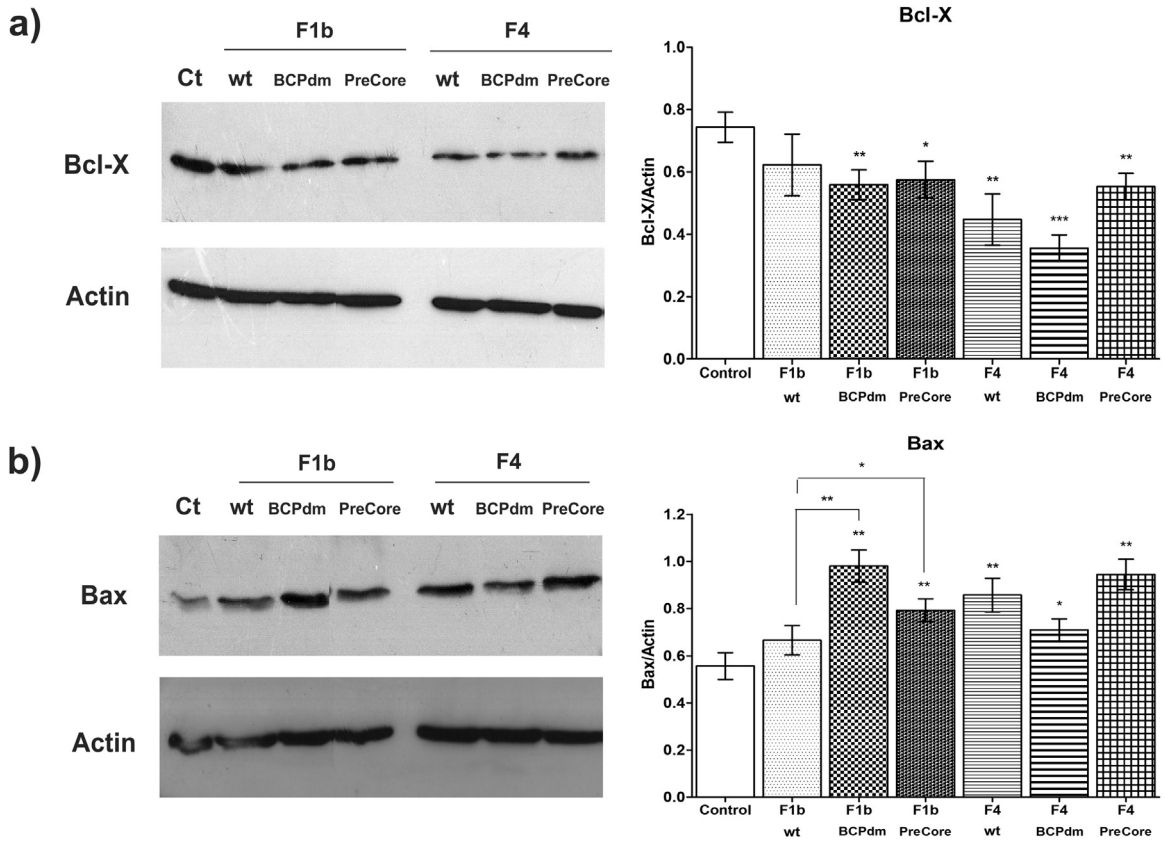

Fig. 3. Expression of Bcl-2 family proteins in Huh-7 cells transfected with full-length HBV genomes. Huh-7 cells were transfected with pUC 19 empty vector (control), F1b wt, F1b BCPdm, F1b preCore, F4 wt, F4 BCPdm and F4 preCore variants. Seventy-two hours post-transfection, total cell proteins were extracted and expression levels of Bcl-X (a) and Bax (b) were determined by Western Blot. Relative intensity of the bands was quantified by normalization to beta-actin, using ImageJ software. Shown values represent the mean ± standard deviation of three independent experiments. * $p < 0.05$; ** $p < 0.005$ and *** $p < 0.0001$.

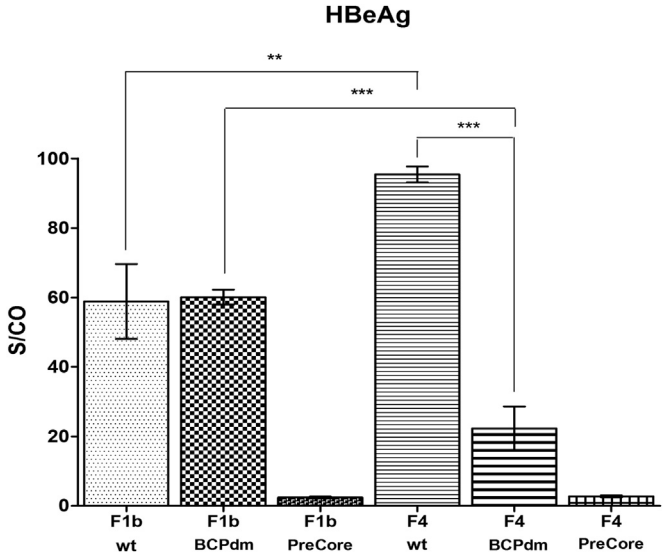

Fig. 4. Expression of e antigen (HBeAg) in Huh-7 cells transfected with full-length HBV genomes. Huh-7 cells were transfected with F1b wt, F1b BCPdm, F1b preCore, F4 wt, F4 BCPdm and F4 preCore variants. Seventy-two hours post-transfection supernatants were collected and HBeAg was quantified using an ARCHITECT analyzer. S/CO: Sample/Cut off value. Shown values represent the mean ± standard deviation of three independent experiments. ** $p < 0.005$ and *** $p < 0.0001$.

demonstrated that inflammatory cytokine production (TNF, IL-6, IL-8, IL-12A, IFN-α1, and IFN-β) is inhibited by HBeAg. These pro-inflammatory cytokines activate a number of intracellular mechanisms including apoptosis of infected cells (Adams, 2003). Therefore, HBeAg abrogation might result in higher levels of inflammatory cytokines, leading to an increase in apoptosis induction.

On the other hand, it has been shown that HBeAg can inhibit p53-mediated apoptosis, implying that intracellular HBeAg can exert an anti-apoptotic effect in hepatoma cells (Liu et al., 2016). The authors have proposed that HBeAg can suppress p53-dependent apoptosis by interacting with NUMB and promoting HDM2-mediated ubiquitination and degradation of p53. The observation that transfection with the preCore mutants, lacking HBeAg expression, significantly increased apoptosis supports the anti-apoptotic activity of HBeAg, proposing a novel role for this viral protein.

It has been reported that the mitochondria-dependent apoptotic pathway is involved in the development of liver diseases (Chen et al., 2001; Ehrmann et al., 2000). The Bcl-2 family of proteins is defined as the key regulator of apoptosis in the mitochondrial pathway, consisting of both suppressors and promoters of apoptosis. The interplay between Bcl-2 family members plays a critical role in deciding whether a cell undergoes apoptosis or not (Chipuk et al., 2010; Siddiqui et al., 2015).

The results obtained in this study showed that full-length HBV transfection increased the expression of the pro-apoptotic protein Bax and decreased the expression of the anti-apoptotic Bcl-X protein in Huh-7 cells. These data indicate the modulation of the Bcl-2 family members, that promotes HBV-induced apoptosis. Even though other studies have demonstrated that HBV infection activates apoptosis (Bhoola and Kramvis, 2016; Lu et al., 2007; Yeganeh et al., 2015), this is the first report which suggests the modulation of the mitochondrial pathway in HBV-induced apoptosis.

The secretion of HBeAg antigen in transfected cells showed variations in the expression of the antigen between viral variants and, also, between sgts. The expression of HBeAg was higher for sgt F4 wt than for

sgt F1b wt. Furthermore, sgt F4 BCPdm showed a significant reduction compared to sgt F4 wt, which was not observed for the sgt F1b. These results suggest that both sgts might induce apoptosis using alternatives pathways.

Overall in the present study we have demonstrated that HBV genome transfections with sgts F1b and F4 induce apoptosis in the human hepatocyte cell line Huh-7. HBV-mediated apoptosis was independent of the viral subgenotype, and associated to the modulation of the Bcl-2 family proteins. The BCP and preCore naturally occurring mutations increased apoptosis induction. However, the increase in apoptosis was not associated with enhanced viral replication, but may be related to mutations at the X protein. Finally, the increase of apoptosis observed in the preCore mutants supports the anti-apoptotic activity of HBeAg.

The results of this work help to describe the molecular mechanisms by which different HBV variants contribute to the pathogenesis of chronic HBV infections. Furthermore, this work provides new pieces of knowledge that contribute to the biological and molecular characterization of genotype F, native of Latin American population, and responsible for most HBV chronic infections in Argentina.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2017.10.016.

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