Hepatitis B e Antigen Expression by Hepatitis B Virus Subgenotype A1 Relative to Subgenotypes A2 and D3 in Cultured Hepatocellular Carcinoma (Huh7) Cells

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Key Words

Apoptosis · Endoplasmic reticulum stress · Secretory pathway · Hepatitis B e antigen · Hepatitis B virus · Unfolded protein response

Abstract

Background: Hepatitis B virus (HBV) is hyperendemic in southern Africa, with subgenotype A1 prevailing. The pre-core/core (preC/C) region of A1, encoding for hepatitis B e antigen (HBeAg), has unique sequence characteristics, differentiating it from subgenotypes A2 and D3. Our aim was to follow the expression of HBeAg in vitro by the three subgenotypes. **Methods:** Huh7 cells were transfected with plasmids belonging to subgenotypes A1, A2, and D3. Using indirect immunofluorescence, the expression of HBeAg was followed, as was the activation of the unfolded protein response (UPR) and subsequent activation of apoptosis. **Results and Conclusions:** Following transfection with D3, HBeAg passed through the secretory pathway earlier than cells transfected with genotype A. Cells transfected with A1 showed a lower expression of the preC/C precursor in the secretory pathway and a higher co-localization in the nucleus. Cells transfected with A1 showed greater endoplasmic reticulum (ER) stress and an earlier, prolonged activation of the UPR seen by the higher activity of three ER-localized

transmembrane transducers (double-stranded RNA-dependent protein kinase-like ER kinase, activating transcription factor 6, and inositol-requiring enzyme 1), on day 3 compared to day 5. Moreover, our study also found that cells transfected with A1 had increased apoptosis.

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Introduction

In southern Africa, infection with the hepatitis B virus (HBV), the prototype member of the family *Hepadnaviridae* [1, 2], is prevalent in the Black population [3]. Genotype A is the dominant genotype, with subgenotype A1 prevailing [4, 5]. This subgenotype has been associated with a high hepatocarcinogenic potential [6, 7]. Unique sequence modifications, within the regulatory *cis*-acting elements and the polymerase of subgenotype A1, may be responsible for the reduced HBV DNA levels associated with infection with this subgenotype when compared to subgenotype A2 and genotype D carriers [4, 8–12]. Moreover, compared to subgenotype A2 or genotype D, subgenotype A1 is associated with early seroconversion of hepatitis B e antigen (HBeAg) to antibodies against HBeAg [6, 8, 9, 13], such that ~5% of subgenotype A1 carriers infected are HBeAg positive in adulthood, com-

pared to $\geq 40\%$ seen for carriers infected with genotypes B or C in other hyperendemic areas of the world [14–16]. This high rate of HBeAg negativity in subgenotype A1 is attributable to the presence of mutations in the basic core promoter/precore (preC) region that affects the synthesis of HBeAg at the transcriptional, translational, and post-translational level [10, 11, 17, 18].

The precore/core (preC/C) region of HBV encodes for the preC/C fusion protein that is the precursor of HBeAg [19]. This precursor preC/C fusion protein is post-translationally modified by cleavage at a fixed site on the amino end in the endoplasmic reticulum (ER) and a variable site on the carboxyl end, in the Golgi apparatus, giving rise to the mature heterogeneous non-particulate soluble HBeAg protein [19–21]. HBeAg is secreted into the serum or expressed on the surface of hepatocytes [22].

The ER quality control mechanism ensures that only correctly folded polypeptide chains traverse along the secretory pathway [23]. This ER quality control mechanism can be compromised when unfolded or misfolded proteins accumulate and aggregate in the lumen of the ER resulting in ER stress [24–26]. In order to overcome this ER stress, all eukaryotic cells have evolved a specific response mechanism known as the unfolded protein response (UPR) or ER stress response [26–28]. In mammalian cells, initiation of the UPR occurs through activation of three signalling cascades in a sequential order by three ER-localized transmembrane transducers, which include: (1) double-stranded RNA-dependent protein kinase-like ER kinase (PERK), (2) activating transcription factor (ATF) 6, and (3) inositol-requiring enzyme (IRE) 1 [27, 28]. Activation of these signalling cascades alters the cell's transcriptional and translational programmes to cope with the stressful conditions and resolve protein-folding defects, thus ensuring cell survival [26–28]. If the UPR fails, cells initiate lethal programmes such as autophagy or cell death by either apoptosis or necrosis [27, 29, 30].

The objective of the present study was to compare expression of HBeAg in the secretory pathway, activation of the UPR, and subsequent activation of apoptosis in Huh7 cells transfected with replication-competent plasmids belonging to one of three subgenotypes: A1, A2, and D3.

Materials and Methods

Construction of Replication-Competent Plasmids

1.28-mer replication-competent plasmids for wild-type subgenotypes A1 (KM519453), A2 (KM519454), and D3 (KM519455) were constructed and were able to replicate and express proteins

in vitro [31]. Ethics approval to perform this study was obtained from the University of the Witwatersrand Research Ethics Committee (Medical), Johannesburg, South Africa.

Cell Culture and Transfection

Huh7 cells were transfected as described previously [31]. Twenty-four hours prior to transfection, 6×10^5 Huh7 cells were plated into each of the 6-cm culture dishes containing either 6 coverslips for indirect immunofluorescence or no coverslips for immunoblotting or reverse transcriptase (RT) polymerase chain reaction (PCR) (or 6×10^3 cells plated into 96-well tissue culture plates for ELISA) and incubated overnight at 37°C in a humidified incubator containing 5% (w/v) CO_2 . For each transfection experiment, an eGFP control was included in triplicate, in order to measure transfection efficiency 1 day after transfection, by determining the number of cells that had been successfully transfected. This was measured using an Olympus IX71 fluorescence microscope system (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

For the determination of the activation of the UPR, stress-inducing compounds were included as controls and added to Huh7 cells 24 h prior to cell harvest: (1) 5 $\mu\text{g}/\text{ml}$ tunicamycin, a nucleoside antibiotic, which functions in the inhibition of N-linked glycosylation resulting in the accumulation of unfolded proteins in the ER [32, 33], (2) 2 $\mu\text{g}/\text{ml}$ thapsigargin, which functions in Ca^{2+} depletion from the ER and consequently results in ER stress [33], and (3) 5 $\mu\text{g}/\text{ml}$ ionomycin, a Ca^{2+} ionophore, which functions in Ca^{2+} depletion from the ER and consequently results in ER stress [32].

For the determination of the activation of cell death, the following controls were included and added to Huh7 cells 24 h prior to cell harvest: (1) 7 $\mu\text{g}/\text{ml}$ cycloheximide, a protein synthesis inhibitor that induces the activation of the JNK pathway and subsequent apoptosis [34], and (2) 1:1,000 H_2O_2 , which functions in the dose-dependent activation of the necrosis through the activation of the activator protein 1 [35].

Subcellular Localization of HBeAg in the Secretory Pathway

On days 3 and 5, Huh7 cells grown on coverslips and transfected with the various 1.28-mer replication-competent plasmids were prepared for indirect immunofluorescence as previously described [36], with the exception that cells were blocked with 3% (w/v) bovine serum albumin incubated at 4°C until used. Cells were then incubated overnight at 4°C with primary antibodies: 1:50 mouse monoclonal antibody against HBeAg (7e9), which does not react with HBcAg [37] [kindly donated by Dr. Peter Revill, Victorian Infectious Diseases Reference Laboratory, Melbourne, Vic., Australia; pers. commun.] in combination with either 1:500 rabbit monoclonal antibody against protein disulphide isomerase (C_8H_{16}) (Cell Signalling Technology, Danvers, Mass., USA) or 1:1,000 rabbit polyclonal antibody against ER-Golgi intermediate compartment (ERGIC)-53/p58 (Sigma-Aldrich Inc., St. Louis, Mo., USA), which binds to their respective protein, located in the ER and ERGIC, respectively. The following day, cells were washed three times, 10 min per wash, with PBS and incubated for 1 h with secondary antibodies: 1:1,000 donkey anti-mouse (IgG) (H + L) antibody labelled with Alexa Fluor 488 or 546, and 1:1,000 goat anti-rabbit (IgG) (H + L) antibody labelled with Alexa Fluor 546 or 488 (all purchased from Invitrogen). Cells were then washed three times, 10 min per wash, with PBS after which the coverslips were mounted on tissue slides using ProLong[®] Gold

Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen), cured overnight, and sealed the following day. The cells were viewed and the images captured and analysed using the LSM 780 confocal laser scanning microscope and camera and the ZEN 2010, release version 6.0 software (Carl Zeiss Microimaging GmbH by Carl Zeiss AG, Oberkochen, Germany).

Immunoblotting Analysis for the Activation of the PERK and ATF6 Pathways of the UPR

The protocol for immunoblotting analysis was done as described previously by Bhoola et al. [31]. However, when detecting for phosphorylated protein, cells were lysed with SDS total cell lysis buffer supplemented with Complete, EDTA-free, Protease Inhibitor Cocktail Tablets and PhosSTOP (Roche Diagnostic GmbH by Roche Applied Science, Mannheim, Germany). The nitrocellulose membrane was incubated overnight at 4°C with one of the following primary antibodies: 1:50,000 mouse monoclonal antibody against α -tubulin (clone B-5-1-2) (Sigma-Aldrich Inc.), 1:25,000 rabbit polyclonal antibody against GFP (ab6556), 1:2,000 rabbit polyclonal antibody against ATF6 α (ab37149) (both from Abcam Inc., Cambridge, Mass., USA), and 1:1,000 eukaryotic initiation factor (eIF) 2 α [serine (Ser) 51 (9721)] (Cell Signalling Technology Inc.). The following day, the nitrocellulose membrane was incubated with either 1:3,000 goat anti-mouse IgG (H + L)-HRP conjugate or 1:3,000 goat anti-rabbit IgG (H + L)-HRP conjugate containing 1:10,000 Precision Protein™ Strep-Tactin-HRP conjugate (all from Bio-Rad Laboratories, Hercules, Calif., USA) depending on the species of the primary antibody. Thereafter, the protein signal of interest was developed with the SuperSignal® West Dura Extended Duration Substrate (Pierce Biotechnology by Thermo Fisher Scientific Inc., Waltham, Mass., USA).

RT-PCR and Restriction Digestion Analysis for the Activation of the IRE1/XBP1 Pathway

The protocol to determine the activation of the IRE1/XBP1 pathway was adapted from Fribley et al. [38]. On days 3 and 5, RNA was extracted from Huh7 cells transfected with replication-competent plasmids using the Trizol® Plus RNA Purification Kit (Invitrogen by Life Technologies, Carlsbad, Calif., USA) following the manufacturer's protocol. Thereafter, an aliquot of 500 ng of RNA was reverse transcribed to synthesize cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories) following the manufacturer's protocol. Subsequently, the cDNA was used as a template to amplify part of the spliced and unspliced XBP1 mRNA using primers specific for the XBP1 gene (primers were designed using the following sequences as template: accession Nos. NM_005080.3 for the unspliced form and NM_001079539.1 for the spliced form [39]; XBP1 F-5'-GAACCAGGAGTTAAGACAGC-3' and XBP1 R-5'-AGTCAATACCGCCAGAATCC-3' were used to amplify the 198- and 174-bp DNA fragments for unspliced and spliced XBP1 mRNA, respectively). PCR was performed in a MyCycler Thermocycler (Bio-Rad Laboratories) using the following reaction conditions: 5 μ l ammonium persulphate, 1.5 μ l 50 mM MgCl₂, 4.0 μ l 10 mM dNTP solution, 1.5 μ l 10 μ M XBP1 F primer, 1.5 μ l 10 μ M XBP1 R primer, 26.0 μ l SABAX water for injections, 0.5 μ l 5 U/ μ l BIOTAQ™ DNA Polymerase (Bioline Reagent Ltd., London, UK), and 10 μ l 1:10 diluted cDNA; thermal cycling conditions were as follows: initial denaturation at 95°C for 2 min followed by 25 cycles of denaturation at 95°C for 30 s, an-

nealing at 55°C for 30 s, elongation at 72°C for 1 min, and a final elongation at 72°C for 10 min. Confirmation of the unspliced form of XBP1 PCR product was done using PstI, which is only found in the unspliced form of XBP1 mRNA, resulting in the generation of two fragments of 114 and 84 bp. The restriction digestion conditions were as follows: 7.0 μ l nuclease-free water, 2.0 μ l 10 $\times$ Buffer O, 1.0 μ l 10 U/ μ l PstI (Fermentas Molecular Biology Tools by Thermo Fisher Scientific), and 10.0 μ l XBP1 PCR product, which was incubated at 37°C for 4 h. Results were analysed by agarose gel electrophoresis using a 3% (w/v) agarose gel.

Measurement of Apoptosis and Total Cell Death

Extracellular cleaved cytokeratin (CK) 18 fragments, which are generated through the cleavage of CK18 by active cysteinyl aspartate-specific protease (caspase)-9 that acts as a marker of apoptosis in epithelial cells, including hepatocytes, were quantified using the M30-Apoptosense® ELISA kit (Peviva AB, Bromma, Sweden) [40, 41]. Total cell death was determined extracellularly by the quantitative measurement of soluble total (cleaved and uncleaved) CK18, which is expressed at high levels in most cells and in serum when released from epithelial cells during cell death, and was detected using the M65® ELISA kit [41, 42].

Statistical Analysis

Quantification of extracellular cleaved and total CK18 was statistically analysed using STATA® 12.0: Data Analysis and Statistical Software (StataCorp LP, College Station, Tex., USA). The significance of differences observed between samples was determined using the one-way ANOVA test and Scheffé's comparison method. $p < 0.05$ was regarded as statistically significant. Analysis was obtained from three independent transfection experiments performed in triplicate.

Fig. 1. Subcellular localization of HBV HBeAg in the early secretory pathway. HBeAg expression was followed by transfecting Huh7 cells with wild-type replication-competent plasmids containing authentic HBV promoters belonging to subgenotypes A1, A2, and D3 wild-type replication-competent plasmids and containing a CMV promoter for subgenotype D3. Mock-transfected cells served as the negative control. These cells were fixed on days 3 and 5 after transfection, after which the cells were subjected to indirect immunofluorescence. HBeAg was detected by the binding of primary mouse monoclonal antibody against HBeAg (7e9) and detected with secondary donkey anti-mouse (IgG) (H + L) antibody, labelled with Alexa Fluor 488 in the case of negative control (mock), subgenotypes D3 (Exo), D3 and A2, and Alexa Fluor 546 in the case of subgenotype A1, shown in green. The ER and ERGIC were detected by the binding of primary rabbit monoclonal antibody against protein disulphide isomerase (PDI; C₈H₁₆) (**a**) and primary rabbit polyclonal antibody against ERGIC-53/p58 (**b**), respectively, and detected with secondary goat anti-rabbit (IgG) (H + L) antibody, labelled with Alexa Fluor 546 in the case of negative control (mock), subgenotypes D3 (Exo), D3, and A2, and Alexa Fluor 488 in the case of subgenotype A1, shown in red. The nuclei were counterstained with DAPI, shown in blue (scale bars = 10 μ m and magnification 1,000 $\times$).

(For figure see next page.)

Results

Subcellular Localization of HBeAg in the Secretory Pathway

The expression of HBeAg was followed by transfecting Huh7 cells with wild-type replication-competent clones belonging to subgenotypes A1, A2, and D3 containing an authentic promoter and subgenotype D3 containing a CMV promoter. No HBeAg expression was detected when cells were mock transfected (fig. 1a, b: 1–8). In order to differentiate between the nucleus and the cytoplasm, cells were counterstained with DAPI (fig. 1a, b: 1, 5, 9, 13, 17, 21, 25, 29, 33, and 37). In order to follow the expression of HBeAg, intracellular organelle markers were used. Protein disulphide isomerase, which locates the ER, showed a fine reticular staining pattern that extended throughout the cytoplasm (fig. 1a: 2, 6, 10, 14, 18, 22, 26, 30, 34, and 38), whereas ERGIC-53, which locates the ERGIC, showed a more punctate and juxtanuclear staining pattern (fig. 1b: 2, 6, 10, 14, 18, 22, 26, 30, 34, and 38). The mouse monoclonal antibody against HBeAg binds to the preC region and thus did not differentiate between the precursor and mature HBeAg.

Subgenotype D3 plasmids containing either the exogenous CMV or the authentic HBV promoter expressed HBeAg, with the former showing a more punctate and granular appearance in the nucleus (fig. 1a, b: 11 and 15), and the latter a more diffuse and finely reticular appearance (fig. 1a, b: 19 and 23). Cytoplasmic HBeAg expression in cells transfected with plasmid containing a CMV promoter showed a more punctate and granular appearance on day 3 that progressed to a fine reticular and diffuse appearance on day 5 (fig. 1a, b: 11 and 15), whereas cells transfected with plasmid containing authentic HBV promoters showed a more reticular and diffuse appearance on day 5 (fig. 1a, b: 19 and 23). By following the expression on both days 3 and 5, our results suggest that transfection with the CMV promoter-driven plasmid led to an earlier expression compared to transfection with the HBV promoter-driven plasmid (fig. 1a, b: 9–24).

Subgenotype A1, A2, and D3 plasmids containing an authentic HBV promoter expressed HBeAg. Nuclear expression of HBeAg in cells transfected with either subgenotype A1 or A2 plasmids showed a diffuse and granular expression (fig. 1a, b: 27, 31, 35, and 39) compared to the fine reticular expression in cells transfected with subgenotype D3 plasmid (fig. 1a, b: 19 and 23). The frequency of nuclear localization of HBeAg was higher for subgenotype A1 than cells transfected with either subgenotypes A2 or D3, which had a similar frequency of nuclear

localization (fig. 1a, b: 19, 23, 27, 31, 37, and 39). Cytoplasmic expression of HBeAg in cells transfected with subgenotype A1 plasmid showed a diffuse and finely reticular expression (fig. 1a, b: 37 and 39) compared to the more reticular and distinctly granular appearance observed for cells transfected with either subgenotypes A2 or D3 (fig. 1a, b: 19, 23, 27, and 31). The expression of HBeAg occurred earlier in cells transfected with subgenotype D3 (fig. 1a, b: 17–24). Further, the magnitude of expression of HBeAg through the secretory pathway as visualized by co-localization of HBeAg with the ER and ERGIC was the lowest for subgenotype A1, consecutively increasing for subgenotypes A2 and D3 (fig. 1a, b: 17–40). Moreover, highest nuclear expression of HBeAg was seen for subgenotype A1, consecutively decreasing for subgenotypes A2 and D3 (fig. 1a, b: 17–40).

Activation of the PERK Pathway by the Various Subgenotypes of HBV

The activation of the PERK pathway is the first and least stringent of the UPR pathways that are initiated in cells in response to ER stress [26–28]. Activation of this pathway was determined by immunoblotting analysis with rabbit polyclonal antibody against phosphorylated eIF2 α (Ser51) (No. 9721), which detects endogenous levels of phosphorylated Ser51 in eIF2 α within the cells. eIF2 α is phosphorylated to the dissociation of immunoglobulin heavy chain binding protein from PERK, resulting in its activation, and is thus a good indicator for the activation of the PERK pathway [26–28]. Activation of the PERK pathway was seen in all samples, with the activity of the PERK pathway being higher on day 3 relative to day 5 (fig. 2). The activation of the PERK pathway occurred earlier for cells transfected with subgenotype D3 containing a CMV promoter compared to cells transfected with subgenotype D3 containing an authentic HBV promoter (fig. 2). On day 3, the activation of the PERK pathway occurred earliest for cells transfected with either subgenotypes A2 or D3 when compared to cells transfected with subgenotype A1 (fig. 2). There was no difference in the activation of the PERK pathway between subgenotypes A1 and A2 on day 5.

Activation of the ATF6 Pathway by the Various Subgenotypes of HBV

The activation of the ATF6 pathway is the second and intermediate stringent adaptive response pathway of the UPR that is activated in cells in response to ER stress, which is not overcome by the PERK pathway [26–28]. Activation of this pathway was determined by immuno-

Fig. 2. Activation of the PERK pathway of the UPR by subgenotypes A1, A2, and D3 of HBV. The activation of the PERK pathway was determined from Huh7 cells that were transfected with subgenotype A1, A2, or D3 1.28-mer replication-competent plasmids and the positive control replication-competent plasmid and harvested on days 3 and 5 after transfection. The immunoblots show the expression of control proteins α -tubulin and GFP and experimental protein phosphorylated eIF2 α . α -Tubulin was detected by the binding of primary mouse monoclonal antibody against

α -tubulin. GFP was detected by the binding of primary rabbit polyclonal antibody against GFP. Phosphorylated eIF2 α was detected by the binding of primary rabbit polyclonal antibody against eIF2 α . All primary antibodies were detected using either secondary goat anti-mouse IgG (H + L)-HRP antibody or goat anti-rabbit IgG (H + L)-HRP antibody. The Precision Plus Protein WesternC™ Standards were detected with the use of Precision Protein™ Strep-Tactin-HRP conjugate. The expression of the protein of interest was determined relative to that of α -tubulin.

blotting analysis using polyclonal antibody against ATF6 α , which detects the active 50-kDa form of ATF6 α . Activation of the ATF6 pathway was seen in all samples with activity being higher on day 3 than on day 5 (fig. 3). This pathway activation occurred earlier for cells transfected with subgenotype D3 containing an authentic

HBV promoter compared to cells transfected with subgenotype D3 containing a CMV promoter (fig. 3). The activation occurred sooner for cells transfected with subgenotype A2 compared to cells transfected with subgenotypes D3 and A1 (fig. 3).

Fig. 3. Activation of the ATF6 pathway of the UPR by subgenotypes A1, A2, and D3 of HBV. The activation of the ATF6 pathway was determined from Huh7 cells that were transfected with subgenotype A1, A2, or D3 1.28-mer replication-competent plasmids and the positive control replication-competent plasmid and harvested on days 3 and 5 after transfection. The immunoblots show the expression of control proteins α -tubulin and GFP (**a**) and experimental protein cleaved ATF6 α (**b**). α -Tubulin was detected by the binding of primary mouse monoclonal antibody against α -tubulin. GFP was detected by the binding of primary rabbit poly-

clonal antibody against GFP. Cleaved ATF6 α was detected by the binding of primary rabbit polyclonal antibody against ATF6 α . All primary antibodies were detected using either secondary goat anti-mouse IgG (H + L)-HRP antibody or goat anti-rabbit IgG (H + L)-HRP antibody. The Precision Plus Protein WesternC™ Standards were detected with the use of Precision Protein™ Strep-Tactin-HRP conjugate. The expression of the protein of interest was determined relative to that of α -tubulin. To facilitate analysis, results from one blot have been shown as two different blots.

Activation of the IRE1/XBP1 Pathway by the Various Subgenotypes of HBV

The activation of the IRE1/XBP1 pathway is the third and most stringent adaptive response pathway of the UPR, which is activated in cells in response to ER stress, which is not overcome by the PERK and ATF6 pathways [26–28]. Activation of this pathway was determined by RT-PCR analysis through the amplification of the spliced and unspliced forms of XBP1 mRNA, which represent the active and inactive forms of XBP1, respectively. Activation of the IRE1/XBP1 pathway occurred in all transfections, with the activity of the IRE1/XBP1 pathway being increased on day 5 relative to day 3 for cells transfected with subgenotype A1, decreased for cells transfected with subgenotype D3 containing either

a CMV or authentic HBV promoter, and showed no difference for cells transfected with subgenotype A2 (fig. 4). As a result of harvesting cells on days 3 and 5, we can only infer that the activation of the IRE1/XBP1 pathway occurred earlier than day 3 for cells transfected with subgenotype D3 plasmids containing either a CMV or authentic HBV promoter (fig. 4). Thus, activation occurred earlier for cells transfected with either subgenotype A2 or D3 plasmids than with subgenotype A1 plasmids (fig. 4).

Activation of Apoptosis by the Various Subgenotypes of HBV

Expression of cleaved soluble CK18 fragments served as the marker of apoptosis, whereas expression of total

Fig. 4. Activation of the IRE1/XBP1 pathway of the UPR by subgenotypes A1, A2, and D3 of HBV. The activation of the IRE1/XBP1 pathway was determined from Huh7 cells that were transfected with subgenotype A1, A2, or D3 1.28-mer replication-competent plasmids and the positive control replication-competent plasmid and harvested on days 3 and 5 after transfection. Activation of this pathway was determined by RT-PCR and restriction

digestion and semi-quantification determined by using 500 ng of total RNA for each sample. All lanes labelled 'A' show the amplification of total (spliced and unspliced form) XBP1 mRNA after PCR. All lanes labelled 'B' show the digestion of the total PCR product. Digestion with *Pst*I results in the digestion of the unspliced form of XBP1.

CK18 served as the marker of total cell death, as occurs as a result of apoptosis and necrosis [40–42]. Following transfection with subgenotype D3, there was no statistical difference in either the cleaved CK18 fragment or total CK18 between the construct with the authentic promoter compared to the one with the CMV promoter (fig. 5a, b). On day 5, a statistically significantly higher expression of cleaved CK18 fragments was observed when cells transfected with subgenotype A1 plasmid were compared to cells transfected with the subgenotype A2 plasmid ($p < 0.05$; fig. 5a). On day 3, a statistically significantly higher expression of total CK18 was observed when cells trans-

fectected with subgenotype A1 plasmid were compared to cells transfected with subgenotype D3 plasmid ($p < 0.05$; fig. 5b).

Discussion

Infection by viruses that utilize the secretory pathway for the generation of viral proteins can induce chronic ER stress [43]. In order to tolerate this ER stress, the activation of the UPR on a timescale that ranges from days to years is required [44]. To date, no study has looked at the

Fig. 5. Quantitative measurements for the activation of apoptosis and total cell death by subgenotypes A1, A2, and D3 of HBV. The expression of cleaved CK18 fragments (**a**) and total soluble CK18 (**b**) was determined from medium collected on days 3 and 5 of Huh7 cells that were transfected with subgenotype A1, A2, or D3 1.28-mer replication-competent plasmids and the positive control replication-competent plasmid. Apoptosis was detected by the extracellular quantitative measurement of cleaved CK18 fragments, which are generated through the cleavage of CK18 by caspase-9. Total cell death was determined by the extracellular quantitative

measurement of soluble total (cleaved and uncleaved) CK18, which is expressed in serum when released from hepatocytes during cell death. **c** The resultant figure of the ratio of cleaved soluble CK18 fragments to total soluble CK18. **d** The resultant figure of the ratio of cleaved CK18 fragments to total soluble CK18 subsequent to subtraction of the mean ratio of the negative controls. Statistically significant changes ($p < 0.05$) between samples for cleaved CK18 fragments and total CK18 have been shown by the different symbols. Error bars represent a 5% difference from the absolute value.

activation of the UPR in cells transfected with HBV replication-competent plasmids. In addition to following the expression of HBeAg in the early secretory pathway, we studied the activation the three UPR pathways as well as subsequent apoptosis following transfection of Huh7 cells with HBV 1.28-mer replication-competent plasmids. Furthermore, we determined whether transcriptional promoters and the subgenotype of HBV affected the activation of these pathways.

Cells transfected with replication-competent plasmids belonging to subgenotypes A1, A2, and D3 showed dis-

tinct differences in the expression pattern and distribution of HBeAg. In our study, the magnitude of expression of HBeAg through the early secretory pathway was consistently lower for subgenotype A1 and consecutively increased for subgenotypes A2 and D3 on days 3 and 5. This is the result of variations in the Kozak region (positions 1809 and 1812 from the *EcoRI* site [45]) of subgenotype A1 compared to subgenotypes A2 and D3 [18]. In subgenotype A1, the ¹⁸⁰⁹GCAC¹⁸¹² to ¹⁸⁰⁹TCAT¹⁸¹² variation coverts the Kozak region from an optimal to suboptimal translational context [4] and results in the decreased

translation of HBeAg by a leaky scanning mechanism [46]. Moreover, the expression of HBeAg by subgenotype D3 occurred earlier when compared to subgenotypes A1 and A2.

The activation of all three pathways of the UPR in transfected Huh7 cells occurred at different time points (fig. 2–4), and the trends were similar to those seen in chemically stressed cells. The activity of the PERK and ATF6 pathways was inferred to be highest on day 3 compared to day 5, whereas the activity of the IRE1/XBP1 pathway was the lowest on day 3 compared to day 5. The PERK pathway had the lowest stringency, the ATF6 pathway had an intermediate stringency, and the IRE1/XBP1 pathway had the highest stringency [26–28]. In agreement with other studies [26–28], this difference in stringency was demonstrated by the fact that the IRE1/XBP1 pathway showed no activation in the negative controls, whereas the PERK and ATF6 pathways were active. These results concur with the suggestion that adaptation to chronic or severe ER stress, in order to avoid apoptosis, occurs via complete or partial suppression of one or more of the UPR pathways [44]. It has been suggested that the PERK pathway would be the most likely candidate for suppression as it is rapidly downregulated by downstream negative feedback loops within the UPR, and its long-term activation would be deleterious to adaptation because it activates proapoptotic CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) and its targets [44]. Furthermore, following expression of the PERK pathway, the next likely candidate would be the IRE1/XBP1 pathway because prolonged activation of this pathway leads to the activation of the proapoptotic c-jun amino-terminal kinase (JNK) pathway and possibly to the activation of caspase-12 [44]. On the other hand, the ATF6 pathway is the most likely candidate to remain activated during chronic ER stress because activation of this pathway conserves the quality control function of the ER [44]. It does so by increasing the expression of molecular chaperones and the components of the ER-associated degradation pathway, ensuring that only correctly folded polypeptide chains traverse along the secretory pathway as well as increasing the protein capacity of the ER [23].

In the presence of an exogenous CMV promoter, subgenotype D3 HBeAg was expressed earlier and accumulated in the ER (fig. 1). The presence of the exogenous CMV promoter led to the earlier activation of the PERK pathway, but not to that of the ATF6 and IRE1/XBP1 pathways, and led to an increased level of total cell death, predominantly as a consequence of necrosis (fig. 2–5).

Therefore, the presence of the exogenous CMV promoter leads to greater cellular toxicity, when compared to that of the authentic HBV promoters. This cellular toxicity occurred independently of the UPR. Previously, it has been shown that the CMV promoter induces the activation of tumour necrosis factor- α , a proinflammatory cytokine, which subsequently leads to the activation of necrosis in an established tumour [47]. Although the CMV promoter is the most commonly used promoter in mammalian expression plasmid vectors because it is a highly robust and efficient transcriptional regulatory element that is activated in a broad range of cell types [48], its use may not always reflect what happens in the presence of authentic HBV promoters.

The cells transfected with subgenotype A1 had higher PERK, ATF6, and IRE1/XBP1 activity than cells transfected with subgenotypes A2 or D3 (fig. 2–4). The persistent activation of the UPR when cells were transfected with subgenotype A1 may be the result of the difference in HBeAg expression described in the present study, as well as the increased replication and expression of HBcAg shown previously using the same constructs [31]. Furthermore, cells transfected with subgenotype A1 seemed to have the highest level of apoptosis (fig. 5). There was a temporal increase in the level of apoptosis for cells transfected with either subgenotype A1 or D3, whereas there was a decrease in cells transfected with subgenotype A2. The level of apoptosis observed for the different subgenotypes is in agreement with a previous study, which showed that in the clinical setting, patients infected with subgenotype A1 had significantly higher levels of apoptosis compared to those infected with genotype D [49].

In conclusion, transfection of Huh7 cells with 1.28-mer HBV replication-competent plasmids supported the expression of HBeAg and led to the activation of all three arms of the UPR, namely the PERK, ATF6, and the IRE1/XBP1 pathways. The stronger CMV promoter led to an increased expression of HBeAg and more necrosis than apoptosis when compared to authentic HBV promoters. The different subgenotypes of HBV can affect the expression of HBeAg and activation of all three UPR pathways. It will be informative to further investigate the induction of the different branches of the UPR following transfection with HBV replication-competent plasmids at earlier and more frequent time points as well as over an extended timescale.

Acknowledgements

We would like to thank the National Research Foundation (GUN No. 65530), the Medical Research Council, the University of the Witwatersrand, and the Poliomyelitis Research Foundation for funding. Mouse monoclonal antibody against HBeAg (7e9) was

kindly donated by Dr. Peter Revill, Victorian Infectious Disease Reference Laboratory, Melbourne, Vic., Australia, and the Huh7 cell line (Japan Health Science Research Resources Bank, catalogue No. JCRB0403) was kindly donated by Prof. Hans Will, Heinrich Pette Institute, Leibniz Institute for Experimental Virology and Immunology, University of Hamburg, Hamburg, Germany.

References

- Marion PL, Robinson WS: Hepadna viruses: hepatitis B and related viruses. *Curr Top Microbiol Immunol* 1983;105:99–121.
- Summers J, Mason WS: Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* 1982;29:403–415.
- Mphahlele M, Francois G, Kew M, Van Damme P, Hoosen A, Meheus A: Epidemiology and control of hepatitis B: implications for eastern and southern Africa. *South Afr J Epidemiol Infect* 2002;17:12–17.
- Kimbi GC, Kramvis A, Kew MC: Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* 2004;85:1211–1220.
- Kramvis A, Weitzmann L, Owiredu WK, Kew MC: Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. *J Gen Virol* 2002;83:835–839.
- Kew MC: Hepatitis viruses and hepatocellular carcinoma. *S Afr Med J* 1994;84:550–556.
- Gopalakrishnan D, Keyter M, Shenoy KT, Leena KB, Thayumanavan L, Thomas V, Vinayakumar K, Panackel C, Korah AT, Nair R, Kramvis A: Hepatitis B virus subgenotype A1 predominates in liver disease patients from Kerala, India. *World J Gastroenterol* 2013;19:9294–9306.
- Kramvis A, Kew MC, Bukofzer S: Hepatitis B virus precore mutants in serum and liver of Southern African Blacks with hepatocellular carcinoma. *J Hepatol* 1998;28:132–141.
- Kramvis A, Bukofzer S, Kew MC, Song E: Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of southern African black adult carriers of the virus. *Hepatology* 1997;25:235–240.
- Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, Gish RG, Kramvis A, Kew MC, Yoshihara N, Shrestha SM, Khan M, Miyakawa Y, Mizokami M: A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 2004;40:747–755.
- Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, Gish RG, Tanaka Y, Kato T, Orito E, Ueda R, Miyakawa Y, Mizokami M: Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol* 2004;85:811–820.
- Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, Acharya SK, Gish RG, Kramvis A, Shimada T, Izumi N, Kaito M, Miyakawa Y, Mizokami M: Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* 2006;44:915–924.
- Song E, Dusheiko GM, Bowyer S, Kew MC: Hepatitis B virus replication in southern Africa blacks with HBsAg-positive hepatocellular carcinoma. *Hepatology* 1984;4:608–610.
- Dusheiko GM, Bowyer SM, Sjogren MH, Ritchie MJ, Santos AP, Kew MC: Replication of hepatitis B virus in adult carriers in an endemic area. *J Infect Dis* 1985;152:566–571.
- Botha JF, Ritchie MJ, Dusheiko GM, Mouton HW, Kew MC: Hepatitis B virus carrier state in black children in Ovamboland: role of perinatal and horizontal infection. *Lancet* 1984;1:1210–1212.
- Kew MC, Kassianides C, Berger EL, Song E, Dusheiko GM: Prevalence of chronic hepatitis B virus infection in pregnant black women living in Soweto. *J Med Virol* 1987;22:263–268.
- Kramvis A, Kew M, Francois G: Hepatitis B virus genotypes. *Vaccine* 2005;23:2409–2423.
- Kramvis A, Kew MC: Molecular characterization of subgenotype A1 (subgroup Aa) of hepatitis B virus. *Hepatol Res* 2007;37:S27–S32.
- Messageot F, Salhi S, Eon P, Rossignol JM: Proteolytic processing of the hepatitis B virus e antigen precursor. Cleavage at two furin consensus sequences. *J Biol Chem* 2003;278:891–895.
- Salhi S, Messageot F, Carlier D, Jean-Jean O, Rossignol JM: Identification of a cellular protein specifically interacting with the precursor of the hepatitis B e antigen. *J Viral Hepat* 2001;8:169–173.
- Laine S, Thouard A, Derancourt J, Kress M, Sitterlin D, Rossignol JM: In vitro and in vivo interactions between the hepatitis B virus protein P22 and the cellular protein gC1qR. *J Virol* 2003;77:12875–12880.
- Milich DR, Chen MK, Hughes JL, Jones JE: The secreted hepatitis B precore antigen can modulate the immune response to the nucleocapsid: a mechanism for persistence. *J Immunol* 1998;160:2013–2021.
- Bukau B, Weissman J, Horwich A: Molecular chaperones and protein quality control. *Cell* 2006;125:443–451.
- Ma Y, Hendershot LM: Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. *J Biol Chem* 2003;278:34864–34873.
- Malhotra JD, Kaufman RJ: The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol* 2007;18:716–731.
- Rutkowski DT, Kaufman RJ: A trip to the ER: coping with stress. *Trends Cell Biol* 2004;14:20–28.
- Ron D, Walter P: Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 2007;8:519–529.
- Schröder M, Kaufman RJ: The mammalian unfolded protein response. *Annu Rev Biochem* 2005;74:739–789.
- Kaufman RJ: Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 2002;110:1389–1398.
- Shore GC, Papa FR, Oakes SA: Signaling cell death from the endoplasmic reticulum stress response. *Curr Opin Cell Biol* 2011;23:143–149.
- Bhoola NH, Reumann K, Kew MC, Will H, Kramvis A: Construction of replication competent plasmids of hepatitis B virus subgenotypes A1, A2 and D3 with authentic endogenous promoters. *J Virol Methods* 2014;203:54–64.
- Cybulsky AV, Takano T, Papillon J, Khadir A, Liu J, Peng H: Complement C5b-9 membrane attack complex increases expression of endoplasmic reticulum stress proteins in glomerular epithelial cells. *J Biol Chem* 2002;277:41342–41351.
- Chan JY, Kwong M: Impaired expression of glutathione synthetase genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. *Biochim Biophys Acta* 2000;1517:19–26.
- Guo YL, Baysal K, Kang B, Yang LJ, Williamson JR: Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor- α in rat mesangial cells. *J Biol Chem* 1998;273:4027–4034.
- Xu Y, Bradham C, Brenner DA, Czaja MJ: Hydrogen peroxide-induced liver cell necrosis is dependent on AP-1 activation. *Am J Physiol* 1997;273:G795–G803.

- 36 Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P: Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature* 1979;281:646–650.
- 37 Walsh R, Nuttall S, Revill P, Colledge D, Cabuang L, Soppe S, Dolezal O, Griffiths K, Bartholomeusz A, Locarnini S: Targeting the hepatitis B virus precore antigen with a novel IgNAR single variable domain intrabody. *Virology* 2011;411:132–141.
- 38 Fribley A, Zhang K, Kaufman RJ: Regulation of apoptosis by the unfolded protein response. *Methods Mol Biol* 2009;559:191–204.
- 39 Reinhardt S, Schuck F, Grosgen S, Riemschneider M, Hartmann T, Postina R, Grimm M, Endres K: Unfolded protein response signaling by transcription factor XBP-1 regulates ADAM10 and is affected in Alzheimer's disease. *FASEB J* 2014;28:978–997.
- 40 Leers MP, Kolgen W, Bjorklund V, Bergman T, Tribbick G, Persson B, Bjorklund P, Ramaekers FC, Bjorklund B, Nap M, Jornvall H, Schutte B: Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* 1999;187:567–572.
- 41 Hagg M, Biven K, Ueno T, Rydlander L, Bjorklund P, Wiman KG, Shoshan M, Linder S: A novel high-through-put assay for screening of pro-apoptotic drugs. *Invest New Drugs* 2002;20:253–259.
- 42 Biven K, Erdal H, Hagg M, Ueno T, Zhou R, Lynch M, Rowley B, Wood J, Zhang C, Toi M, Shoshan MC, Linder S: A novel assay for discovery and characterization of pro-apoptotic drugs and for monitoring apoptosis in patient sera. *Apoptosis* 2003;8:263–268.
- 43 van Anken E, Braakman I: Versatility of the endoplasmic reticulum protein folding factory. *Crit Rev Biochem Mol Biol* 2005;40:191–228.
- 44 Rutkowski DT, Kaufman RJ: That which does not kill me makes me stronger: adapting to chronic ER stress. *Trends Biochem Sci* 2007;32:469–476.
- 45 Gunther S, Fischer L, Pult I, Sterneck M, Will H: Naturally occurring variants of hepatitis B virus. *Adv Virus Res* 1999;52:25–137.
- 46 Ahn SH, Kramvis A, Kawai S, Spangenberg HC, Li J, Kimbi G, Kew M, Wands J, Tong S: Sequence variation upstream of precore translation initiation codon reduces hepatitis B virus e antigen production. *Gastroenterology* 2003;125:1370–1378.
- 47 Old LJ: Tumor necrosis factor (TNF). *Science* 1985;230:630–632.
- 48 Foecking MK, Hofstetter H: Powerful and versatile enhancer-promoter unit for mammalian expression vectors. *Gene* 1986;45:101–105.
- 49 Beutler B, Cerami A: Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature* 1986;320:584–588.