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Apoptosis of Hepatitis B Virus-Infected Hepatocytes Prevents Release of Infectious Virus▽

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Apoptosis of infected cells is critically involved in antiviral defense. Apoptosis, however, may also support the release and spread of viruses. Although the elimination of infected hepatocytes is required to combat hepatitis B virus (HBV) infection, it is still unknown which consequences hepatocyte apoptosis has for the virus and whether or not it is advantageous to the virus. To study this, we designed a cell culture model consisting of both HBV-producing cell lines and primary human hepatocytes serving as an infection model. We showed that the release of mature, enveloped virions was 80% to 90% reduced 24 h after the induction of apoptosis in HBV-replicating hepatoma cells or HBV-infected hepatocytes. Importantly, HBV particles released from apoptotic hepatocytes were immature and nonenveloped and proved not to be infectious. We found an inverse correlation between the strength of an apoptotic stimulus and the infectivity of the virus particles released: the more potent the apoptotic stimulus, the higher the ratio of nonenveloped capsids to virions and the lower their infectivity. Furthermore, we demonstrated that HBV replication and, particularly, the expression of the HBx protein transcribed from the viral genome during replication do not sensitize cells to apoptosis. Our data clearly reject the hypothesis that the apoptosis of infected hepatocytes facilitates the propagation of HBV. Rather, these data indicate that HBV needs to prevent the apoptosis of its host hepatocyte to ensure the release of infectious progeny and, thus, virus spread in the liver.

Human hepatitis B virus (HBV) is a small DNA virus characterized by a pronounced liver tropism. HBV replicates and assembles exclusively in hepatocytes without the need for cell disruption. Progeny viral particles are released through the secretory pathway. The "noncytopathic" behavior of HBV has been demonstrated with stably transfected hepatoma cell lines (32, 36) and with HBV-infected primary human hepatocytes (PHH) (35). The noncytopathic replication strategy explains why HBV infection *per se* does not cause liver damage in HBV-transgenic mice (14) or HBV carriers infected around birth and why it elicits little innate immune response (51). When the immune system becomes activated, however, inflammatory liver disease called hepatitis B becomes evident, and the infection may be cleared. Although HBV obviously does not need cell destruction to release infectious progeny, it is still debated whether HBV might sensitize the host hepatocyte to apoptosis to enhance its spread in the liver (41, 45).

The viral genome (3.2 kb), consisting of a partially double-stranded, relaxed circular DNA (rcDNA), shows an extremely compact organization, with overlapping open reading frames and regulatory elements. Upon viral uptake into hepatocytes, the HBV capsid is transported to the nuclear pore complex, where the rcDNA genome is released into the nucleus. Inside the nucleus the rcDNA is converted to a covalently closed circular DNA (cccDNA) by cellular enzymes, which serves as a transcription template for the 3.5-kb pregenomic/precore RNA and three subgenomic RNAs. The pregenomic RNA is bifunctional. On the one hand, it is reverse transcribed into a new rcDNA within the viral capsid forming in the cytoplasm; on the other hand, it serves as mRNA for the viral capsid and polymerase proteins. The precore RNA encodes a nonstructural protein, which is processed and secreted as HBV e antigen (HBeAg). The two subgenomic RNAs encode three viral envelope proteins, the large (L) protein, the middle (M) protein, and, predominantly, the small (S) protein, which are densely packed into the lipid bilayer of the viral envelope. Infected cells secrete, in large excess to virions, subviral particles, which are empty envelopes of a spherical and filamentous shape that can be detected in the serum of infected individuals as hepatitis B surface antigen (HBsAg) (10).

A third subgenomic RNA encodes a regulatory protein called HBx, which is thought to be required to establish *in vivo* infection (53) and displays pleiotropic effects when studied in cell culture-based assays. It was shown previously that HBx prevents apoptosis by interfering with cellular proteins involved in CD95- and transforming growth factor β (TGF-β)-mediated apoptosis pathways (7, 28, 37) or by directly interacting with p53 (8, 17, 49) or caspase-3 (12, 22, 24).

In contrast to the proposed antiapoptotic functions of HBx, diverse reports described that the overexpression of HBx sensitizes liver cells to apoptosis in a dose-dependent manner (18, 26, 29, 39, 45). It has been reported that HBx induces apoptosis in a both p53-dependent (3, 48) and p53-independent (38, 45) manner. It may also harm the integrity of mitochondrial membranes (26, 44). However, Su et al. indicated that HBx-dependent apoptosis may depend on additional triggers (41).

To explain the paradox between the pro- and antiapop-

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totic functions of HBx, it was proposed that HBx might boost viral replication early after hepatocyte infection and induces apoptosis at later stages to facilitate efficient HBV particle release and to minimize antiviral inflammatory responses (26, 29, 38, 45).

None of these assumptions, however, has yet been proven in an infection model, and apoptosis induction by HBV infection has not been documented *in vivo*. Other persistent viruses such as HIV, human T-cell leukemia virus type 1 (HTLV-1), or human papillomavirus (HPV) (5, 50, 52) may induce the apoptosis of their host cells, but the physiological relevance is not entirely clear. Therefore, this study focuses on the biological significance of apoptosis for HBV propagation. In addition, this study investigates whether the expression of HBx transcribed from the viral genome during HBV replication alters the sensitivity of cells to apoptosis.

MATERIALS AND METHODS

Cells and cell culture conditions. The human hepatoma cell lines HepG2, HepG2.2.15, and HepG2 H1.3±x were grown on collagen-coated culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, and 1% nonessential amino acids. HepAD38 cells were cultured in DMEM-F12 medium with 10% FCS in the presence of 0.3 μg/ml tetracycline and 400 μg/ml neomycin (G418). Cell culture media and ingredients were obtained from Invitrogen (Karlsruhe, Germany). Cells were maintained at 37°C in a 5% CO_2 atmosphere. To boost HBV production, confluent HBV-replicating hepatoma cell lines were cultured without splitting in Williams E medium supplemented with 5% FCS and the ingredients described above plus 2.4 μg/ml hydrocortisone, 0.5 μg/ml inosine, and 0.75% dimethyl sulfoxide. To induce HBV production in HepAD38 cells, cells were cultured in DMEM-F12 medium with 1% FCS in the absence of tetracycline for at least 10 days.

PHH were isolated from surgical human liver biopsy specimens by collagenase type IV (Worthington, Lakewood, NJ) perfusion and subsequent differential centrifugation. A total of 1×10^6 cells/ml were seeded onto collagen-coated 6- or 12-well plates and cultured in Williams E medium supplemented as described previously (35). Surgical human liver biopsy specimens were obtained with informed consent of the donor as approved by the local ethics committee.

Induction of apoptosis. In order to induce apoptosis in hepatoma cell lines, cell culture medium was removed, and adherent cells were exposed to UV-C (20 mJ/cm^2) by using a UV chamber (GS Gene Linker; Bio-Rad). Cells were subsequently covered with medium and cultured for 24 h as described above. Anti-CD95 antibody (Ab) treatment was applied to induce apoptosis in PHH. For this, we used the monoclonal anti-Apo1 antibody (kindly provided by H. P. Krammer, DKFZ, Heidelberg, Germany) at concentrations of 100 ng/ml in the presence of 50 ng/ml protein A (Fluka, Germany) as described previously (35).

Apoptosis assays. (i) Caspase-3 assay. The activity of caspase-3/7 was determined as described previously (6). Briefly, cytosolic protein extracts from 10^6 cells were prepared in 200 μl lysis buffer (20 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). Five microliters of cytosolic protein extracts was added to 100 μl caspase buffer {20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and 10% sucrose} and incubated with 100 μM caspase-3 substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC; Alexis, Lausen, Switzerland). The releases of fluorogenic AFC from cleaved caspase-3 substrate were compared after 30 min at 30°C by using the Genios Pro fluorometric plate reader (Tecan, Männedorf, Switzerland).

(ii) DNA fragmentation analysis. A total of 10^6 cells were harvested and resuspended in lysis buffer (100 mM Tris [pH 8.5], 5 mM EDTA, 200 mM NaCl, 0.2% SDS) containing 3 μg/ml proteinase K (Roche, Karlsruhe, Germany) for 16 h at 37°C. Afterwards, RNase A (Macherey-Nagel, Düren, Germany) digestion was performed for 30 min at 37°C, and low-molecular-weight DNA was extracted with phenol-chloroform. The DNA was subjected to 1.5% agarose gel electrophoresis, and laddering was visualized by ethidium bromide staining.

(iii) Cell viability assays. Live cells were stained for intracellular esterase activity by using 2 μM calcein AM. Dead cells were visualized by fluorescence microscopy using 2 μM ethidium homodimer 1 (EthD-1) (Live/Dead viability/cytotoxicity kit; Molecular Probes Europe BV). In addition, cell viability was determined by tetrazolium XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) colorimetric assay (Roche Diagnostics, Penzberg, Germany) and quantified by photometric measurements.

HBV infection of PHH. At 4 days postseeding, PHH were infected with HBV in the presence of 5% polyethylene glycol 8000 (PEG) (Merck, München, Germany) overnight. For infection, we used either frozen HBV aliquots (−80°C) with a defined virus titer or we transferred directly the supernatant of HBV-producing HepG2.2.15 cells or supernatant from previously HBV-infected PHH with or without apoptosis treatment. The frozen HBV aliquots consisted of the concentrated supernatant of HepG2.2.15 cells using Centricon Plus-70 (Biomax 100; Millipore Corp., Billerica, MA). If apoptosis has been induced by anti-CD95 antibodies, the recipient PHH cultures were pretreated with two caspase inhibitors for 1 h to prevent remaining anti-CD95 antibody activity: 2 μM DEVD-aldehyde (Biomol, Plymouth, PA) and 100 μM z-Val-Ala-Asp-fluoromethylketone (z-VAD; Alexis, Lausen, Switzerland).

Assays for detecting HBV infection. The establishment of HBV infection was assayed by determining nuclear HBV cccDNA, progeny release, and HBeAg secretion from infected cells.

The amount of progeny HBV was measured by HBV DNA dot blotting. Cell culture media (0.4 ml) of HBV-infected PHH (corresponding to 5×10^5 cells) were dotted onto a positively charged nylon membrane, hybridized with a ^{32}P-labed HBV DNA probe, and quantified with a PhosphorImager instrument (Molecular Dynamics, Sunnyvale, CA) relative to a dilution series of HBV DNA (8 to 500 pg). HBeAg was determined by an Axsym assay in cell culture medium (HBe 2.0; Abbot, Wiesbaden, Germany).

Total DNA was extracted from 5×10^5 PHH by using microspin columns (NucleoSpinTissue; Macherey-Nagel, Dueren, Germany). Real-time PCR was performed to quantify HBV cccDNA by using LightCycler FastStart DNA Master^{Plus} SYBR green I mix (Roche Diagnostics, Mannheim, Germany) and cccDNA-specific primers (46). HBV cccDNA was normalized to mitochondrial DNA (51). The cutoff for detection was 50 copies of cccDNA per 10^4 cells.

Characterization of released HBV particles. HBV particles contained in 2 ml of cell culture medium (from 1.5×10^6 cells) were sedimented into a 1.5-ml 1.15-g/ml to 1.44-g/ml CsCl step gradient covered with 0.5 ml of 20% sucrose by centrifugation at 55,000 rpm for 16 h at 10°C in an SW 61 swing-out rotor (XL-70 ultracentrifuge; Beckman). Twelve fractions were collected from bottom to top and subjected to HBV DNA dot blot analysis.

For further analysis, CsCl fractions of identical densities from three different runs were pooled, and 200 μl was dialyzed by using Slide-A-Lyzer minidialysis units (10,000-molecular-weight cutoff [MWCO]; Pierce, Rockford, IL) for 1 h at room temperature (dialysis buffer [137 mM NaCl, 5 mM KCl, 10 mM Tris, and 1 mM $MgCl_2 \cdot H_2O$]). Total nucleic acids were extracted from the respective fractions with a QIAamp MinElute virus spin kit (Qiagen, Hilden, Germany) and analyzed by quantitative PCR (qPCR) (one-step reverse transcription [RT]-PCR LightCycler RNA Master SYBR green I kit; Roche Diagnostics, Mannheim, Germany) using HBV-specific primers (46). To quantify HBV RNA, DNA digestion (DNase I; Roche, Mannheim, Germany) was performed for 1 h at room temperature before nucleic acids were quantified as described above. Double treatment with DNase I and RNase H (Roche, Mannheim, Germany) confirmed the complete removal of HBV DNA.

Immunoprecipitation (IP) using protein G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HBsAg (polyclonal goat antiserum), and anti-capsid antibodies (Dianova, Hamburg, Germany) was performed at 4°C for 16 h to characterize the HBV particle structure within the indicated density fractions. Total nucleic acid was extracted from the pulldown as well as from the supernatant, followed by a relative quantification of nucleic acid content by using HBV-specific primers (44). HBV envelopment was verified by an HBsAg enzyme-linked immunosorbent assay (ELISA) (Murex; Abbott, Wiesbaden, Germany). As an external standard, a dilution series of recombinant HBsAg (2 to 100 ng/ml) (Engerix-B; GlaxoSmithKline, London, Great Britain) was used.

Southern blot analysis was performed to characterize HBV genomes within the particles. For this, particles were concentrated from cell culture medium from about 2×10^8 cells and separated by CsCl gradient centrifugation. DNA was extracted from defined fractions, separated by 1.2% agarose gel electrophoresis, and blotted onto a positively charged nylon membrane. HBV DNA was detected by hybridization with a ^{32}P-labeled HBV DNA probe.

RESULTS

HBV-producing hepatoma cell lines are not sensitized to undergo apoptosis. We compared the vitality of HBV-replicating cell lines to that of parental cells upon UV-C irradiation to investigate whether HBV replication or the expression of HBx in the context of an HBV genome alters the sensitivity of cells toward apoptosis. HepG2-H1.3 (31) and HepG2-H1.3x− (L. Lucifora, S. Arzberger, et al., submitted for publication) cells replicating wild-type or x-deficient HBV, respectively, were both established in our laboratory recently. The expression level of HBx when it is transcribed from the viral genome is extremely low and cannot be confirmed by traditional biochemical methods (4, 42). Therefore, the production of wild-type and x-deficient HBV was confirmed by the sequencing of intracellular cccDNA and progeny virus (Lucifora et al., submitted). In addition, we used HepAD38 cells, which replicate HBV in a tetracycline-regulated fashion (19).

Prior to apoptosis induction by UV-C light, we ensured that all cell lines efficiently replicated HBV and secreted $\geq 5 \times 10^6$ enveloped, DNA-containing particles per ml cell culture medium per day. Therefore, it was necessary to culture confluent cell layers for 7 days under conditions inducing cell differentiation (19, 32). We determined cell viability before and 24 h after UV-C exposure by fluorescence microscopy (Fig. 1a and b) and by an XTT test measuring metabolic activity (Fig. 1c). The viability of HBV-replicating cells after treatment was not significantly different from that of the corresponding control cells (Fig. 1c), indicating that HBV replication, including HBx expression in the context of the viral genome, does not sensitize host cells to apoptosis.

To confirm apoptosis induction, we measured the activation of execution caspase-3/7 and analyzed the fragmentation of genomic DNA in HepG2.2.15 cells upon apoptosis induction by UV-C irradiation, anti-CD95 treatment, or both (Fig. 1d and e). HepG2.2.15 (Fig. 1d) but also HepG2 H1.3 and HepG2 H1.3x− (Fig. 1c) cells were partially resistant to a single treatment. However, a combination of UV-C irradiation plus anti-CD95 antibody treatment acted synergistically and significantly increased caspase-3/7 activity (Fig. 1d).

Induction of apoptosis in HepG2.2.15 cells alters the structure of progeny HBV. In order to assess the biological significance of apoptosis for the HBV life cycle, we first analyzed the release of HBV particles after apoptosis induction. Therefore, we induced apoptosis in HBV-producing HepG2.2.15 cells using UV-C light, anti-CD95 antibodies, or a combination of both and characterized virus particles released using CsCl density gradient centrifugation to separate HBV DNA, naked capsids, and enveloped virions due to their sedimentation properties (Fig. 2a). Depending on the strength of the apoptotic stimulus applied (Fig. 1d and e), we found an inverse correlation with the ratio of enveloped HBV virions to HBV DNA-containing naked capsids released. After a strong apoptotic stimulus (combination of UV-C irradiation and anti-CD95 antibody treatment), more than 90% of the released particles consisted of nonenveloped capsids predominantly sedimenting at a buoyant density of 1.36 to 1.37 g/ml (Fig. 2a), whereas the total amount of HBV particles released did not change significantly during the first 24 h following apoptosis induction.

HBV particles sedimenting at low-density (buoyant density, 1.24 to 1.28 g/ml; fraction 8 [F8] and F9) and high-density (buoyant density, 1.36 to 1.37 g/ml; F3 and F4) (Fig. 2b) CsCl fractions were further characterized by immunoprecipitation using polyclonal anti-HBsAg and anti-capsid antibodies, respectively, followed by analysis of the HBV DNA content. Without apoptosis treatment, about 80% of the released viral genomes were found in enveloped HBV particles in the low-density fractions, whereas after apoptosis treatment, naked

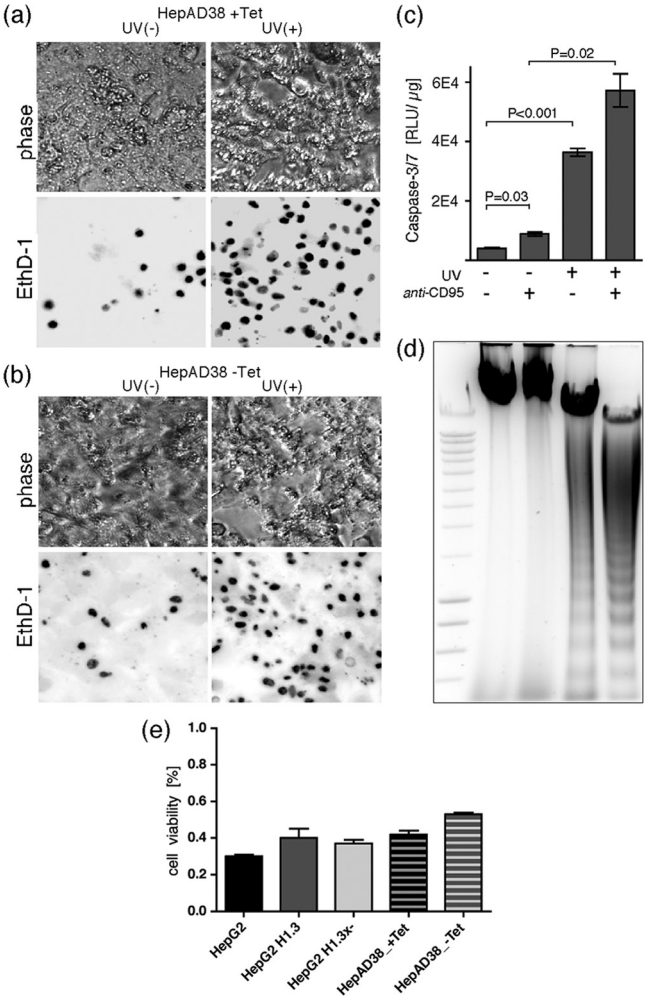

FIG. 1. Sensitivity of HBV-producing HepG2 cells to apoptosis. HepG2, HepG2-H1.3, HepG2-H1.3x−, and HepAD38 cells were treated with UV-C light (UV) or anti-CD95 monoclonal Ab (MAb). (a and b) Phase-contrast microscopy (top) showed morphological changes of HepAD38 cells without (+Tet) (a) and with (−Tet) (b) HBV replication 24 h after UV-C irradiation (20 mJ/cm^2). Dead cells were visualized by ethidium homodimer (EthD-1) staining (bottom). (c) Cell viabilities of the indicated cell lines after UV-C irradiation were determined by an XTT assay. Data are expressed as percentages of untreated cells (means ± standard deviations [SD]; $n = 3$). (d) Detection of caspase-3/7 activity by cleavage of Ac-DEVD-AFC fluorogenic substrates. Cell lysates of HepG2.2.15 cells were prepared 4 h after treatment with anti-CD95 Ab (1 μg/ml), UV-C irradiation, or a combination of both. Unexposed cells cultured in parallel served as a control. Data show the relative light units (RLU) per μg of protein (mean ± SD; $n = 3$). (e) Analysis of DNA fragmentation of HepG2.2.15 cells 30 h after treatment. Low-molecular-weight DNA was separated on a 1.4% agarose gel and visualized by ethidium bromide staining.

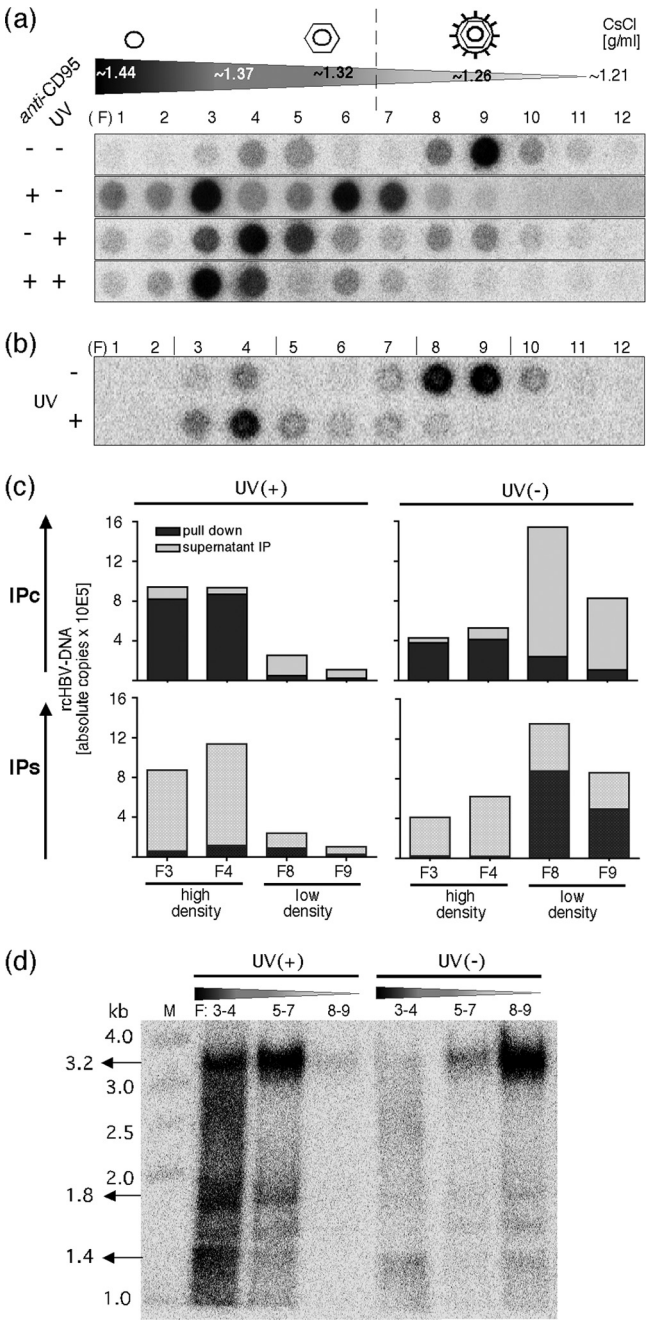

FIG. 2. Physical properties of HBV particles released from HepG2.2.15 cells after apoptosis induction. (a and b) Characterization of HBV particles released due to their density profiles. (a) Twenty-four hours after UV-C irradiation (20 mJ/cm^2), anti-CD95 MAb treatment (1 μg/ml), or a combination of both, cell culture media were subjected to CsCl gradient centrifugation, and collected fractions (F) were analyzed by dot blotting using a ^{32}P-labeled HBV DNA probe. The pictogram illustrates CsCl densities and expected distributions of naked HBV DNA, naked capsids, and virions. (b) Density profile of concentrated culture medium 24 h after UV-C irradiation. (c) Immunoprecipitation by HBc (IPc) or HBs (IPs) antigen of the indicated density fractions followed by absolute quantification of the isolated nucleic acid contents using HBV DNA-specific primers. (d) HBV DNA Southern blot analysis of the indicated density fractions. To differentiate between circular and linear HBV DNA genomes, XhoI digestion was performed. The obtained fragments are marked by arrows: the 3.2-kb fragment was derived from circular DNA, and the 1.8- and 1.4-kb fragments were derived from linear DNA. M, molecular weight marker.

HBV DNA-containing capsids accumulated in the high-density fractions (Fig. 2c). Additionally, Southern blot analysis was performed to characterize the DNA structure of the HBV genomes obtained by density profiling (Fig. 2d). Linear and circular HBV DNA forms were distinguished by XhoI digestion, resulting in one 3.2-kb fragment and two fragments of 1.4 and 1.8 kb, respectively. Without any treatment, circular HBV DNA was detected exclusively in the low-density fractions (F8 and F9) containing enveloped virions. After apoptosis induction, in contrast, the majority of HBV particles was detected in higher-density fractions (F3 and F4), with naked capsids containing a mixture of circular and linear HBV DNA genomes as well as further less-well-defined HBV DNA structures (Fig. 2d). On the basis of these results, we concluded that apoptosis interrupted HBV replication and resulted in the release of immature HBV particles.

HBV particles released from apoptotic cells are not infectious. We used PHH, which can be infected with HBV (35), to dissect whether released viral particles are infectious or not. We infected freshly isolated PHH with HBV obtained from HepG2.2.15 cells at a multiplicity of infection (MOI) of 200 enveloped, DNA-containing HBV particles/cell. At day 1 postinfection (p.i.), we still detected HBV DNA and HBeAg contained in the inoculum. Productive infection started after day 3 p.i., with secreted HBV DNA and HBeAg levels increasing ≥10-fold until day 10 p.i. (Fig. 3a, left). When neutralizing anti-HBsAg antibodies were added to the virus inoculum, we detected neither the establishment of a productive HBV infection (Fig. 3a, right) nor the formation of cccDNA (Fig. 3b), demonstrating that *in vitro* infection of PHH relies on an intact viral envelope.

In a second approach, we examined the infectivity of the HBV particles released by transferring the cell culture medium of HepG2.2.15 cells 24 h after apoptosis treatment onto PHH. Depending on the strength of the apoptotic stimuli, the infectivity of HBV particles was markedly reduced. When particles from apoptotic cells were used as an inoculum, 75% to 84% fewer HBV progeny were produced (Fig. 3c). In addition, the proportion of HBV-infected PHH was significantly lower, as indicated by the amount of HBV cccDNA (Fig. 3d). Thus, the HBV particles released from apoptotic HepG2.2.15 cells were noninfectious. The small number of remaining enveloped particles explains the low level of infectivity still observed.

Apoptosis in HBV-infected PHH abrogates virus spread. Since apoptosis pathways may be altered in hepatoma cell lines, we confirmed our findings after the induction of apoptosis in primary HBV-infected hepatocytes. We allowed the establishment of HBV infection for 8 days before apoptosis induction by anti-CD95 antibody treatment, which is a potent proapoptotic stimulus for primary hepatocytes (35). By comparing the sedimentation characteristics of the HBV particles released, we found a pattern similar to that observed for hepatoma cell lines. HBV DNA-containing particles released from untreated HBV-infected PHH consisted of >85% of enveloped virions (buoyant density, 1.24 to 1.28 g/ml), whereas 90% of particles released after anti-CD95 antibody treatment were naked capsids sedimenting in two populations at 1.32 or 1.36 to 1.37 g/ml, respectively (Fig. 4a). We assumed that they were attributed to different maturation levels of the capsids.

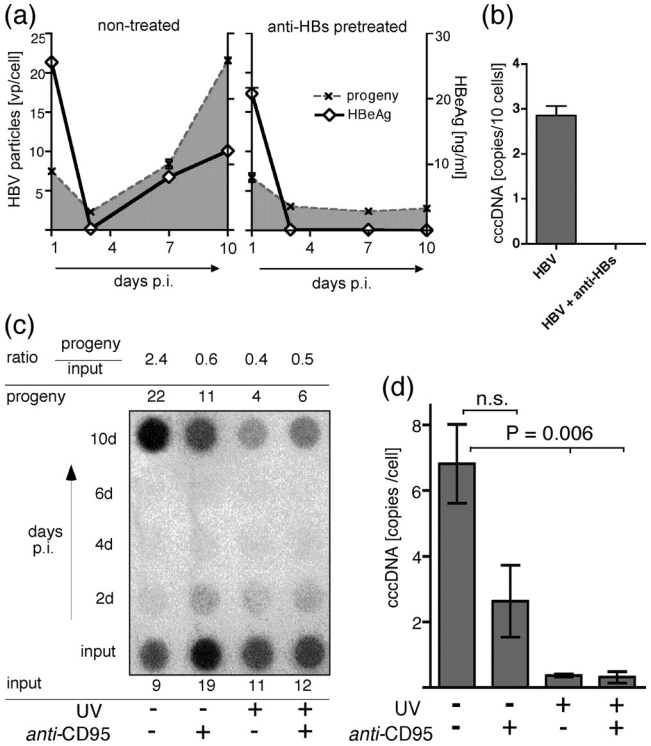

FIG. 3. Analysis of the infectivity of HBV released from HepG2.2.15 cells. (a) PHH cultures were infected with HBV particles (left) or particles preincubated with neutralizing anti-HBs antibodies (right). Productive infection was monitored by progeny HBV DNA dot blot analysis (filled area) and HBeAg secretion (continuous line) up to 10 days p.i. HBV DNA-containing particles (viral particles [vp]) per cell were quantified by using a PhosphorImager apparatus (mean ± SD; $n = 3$). (b) Quantification of HBV cccDNA at 10 days p.i. using qPCR relative to the mitochondrial DNA content. Data are expressed as the number of copies of cccDNA per 10 cells (mean ± SD; $n = 3$). (c) Culture media of HepG2.2.15 cells collected 24 h after apoptosis induction with anti-CD95 MAb (1 μg/ml), UV-C irradiation (20 mJ/cm^2), or combined treatments were transferred onto PHH. At the indicated time points, the establishment of infection was monitored by HBV progeny release using HBV DNA dot blot analysis of PHH culture media. The input was calculated from the amount of HBV DNA-containing particles per cell used for infection. In addition, the number of HBV progeny was calculated from the amount of HBV DNA-containing particles per cell released at day 10 p.i. (d) At day 10 p.i., with media from apoptosis-treated or mock-treated cells, PHH were lysed, and HBV cccDNA was quantified by qPCR. The values obtained were normalized to mitochondrial DNA content. Numbers of HBV cccDNA copies per cell are given (mean ± SD; $n = 3$). *P* values were analyzed by a Student's *t* test.

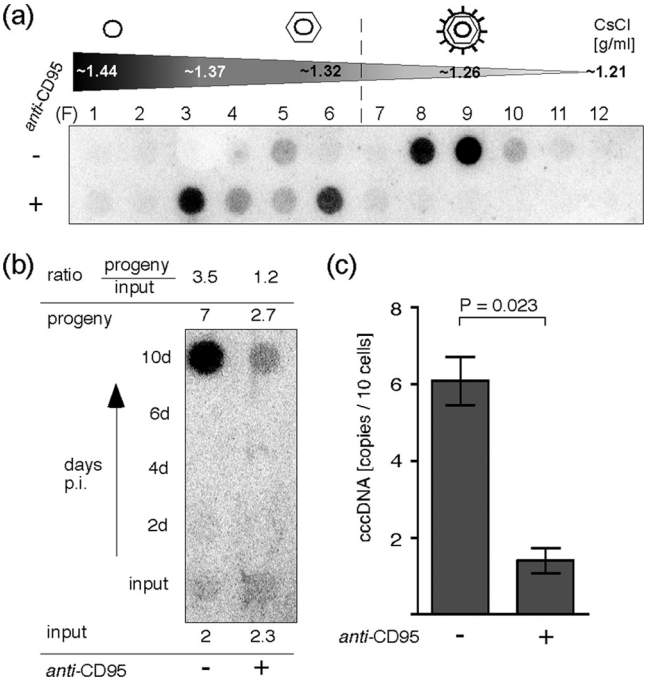

FIG. 4. Structural characterization and infectivity of HBV particles released from apoptotic PHH. Thirty hours after anti-CD95 MAb (130 ng/ml) treatment and mock treatment of HBV-infected PHH, cell culture media were collected. (a) HBV particles contained were sedimented into a CsCl gradient. Shown are data from analyses of fractions (F) collected by dot blotting using a ^{32}P-labeled HBV DNA probe. (b) Culture media of HBV-infected PHH undergoing anti-CD95 or mock treatment were transferred to PHH of a different donor. At the indicated time points, the establishment of a productive HBV infection was analyzed by progeny HBV DNA dot blot analysis. From the signal detected, the input MOI (HBV DNA-containing particles per cell used for infection) was calculated relative to an external standard. In addition, the number of progeny HBV was calculated from the amount of HBV DNA-containing particles released per cell at day 10 p.i. (c) Quantification of HBV cccDNA in PHH at day 10 p.i. using a specific qPCR. Results were normalized to mitochondrial DNA content. HBV cccDNA copies per cell are given (mean ± SD; $n = 3$). *P* values were determined by a Student's *t* test.

In order to analyze whether the HBV particles released were able to spread infection to other hepatocytes, we transferred the culture media from anti-CD95- and mock-treated HBV-infected PHH to freshly isolated PHH obtained from a different donor. Prior to infection, we incubated recipient PHH with the caspase inhibitors z-VAD and DEVD for 2 h to block the residual activity of the anti-CD95 antibodies. The establishment of a secondary infection was followed by the measurement of HBV progeny release (Fig. 4b) and the formation of cccDNA (Fig. 4c). Although the amounts of HBV DNA-containing particles used for infection were comparable in both experiments, the establishment of infection determined by the quantification of cccDNA was significantly ($P = 0.023$) less effective when the culture medium from apoptotic PHH was used. These results further support the finding that the induction of apoptosis inhibits the propagation of HBV.

Immature HBV particles are released from PHH after apoptosis induction. Distinct types of HBV particles were detected in the cell culture medium of mock-treated and apoptotic hepatocytes. In order to characterize those particles in more detail, we separated HBV particles released from mock- or anti-CD95-treated PHH by CsCl gradient centrifugation and analyzed envelopment by an HBsAg ELISA (Murex; Abbott) (Fig. 5a). Real-time PCR was used to determine the maturation of the viral capsids by quantifying the HBV RNA and HBV DNA contents following DNase and RNase digestion, respectively (Fig. 5b and c).

When cells were mock treated, HBsAg was detected only in F8 to F10 (buoyant density, 1.24 to 1.28 g/ml) (Fig. 5a), confirming the separation of naked and enveloped HBV particles. In F8 and F9 we detected the largest amount of total HBV

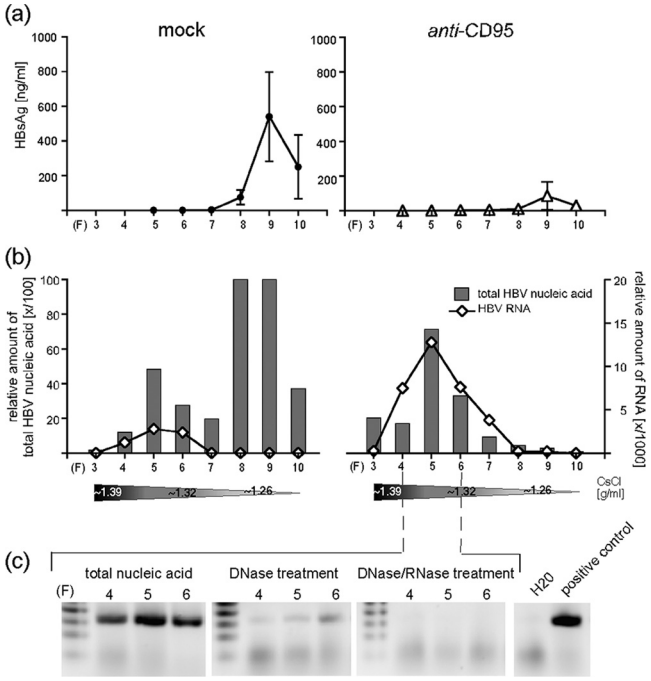

FIG. 5. Characterization of HBV particles released from apoptotic PHH. Supernatants of HBV-infected PHH cultures were collected 24 h after anti-CD95 MAb (130 ng/ml) or mock treatment and subjected to CsCl gradient centrifugation. Twelve fractions (F) with decreasing densities (F1 to F12) were collected; F3 to F10 were used for further characterization. (a) HBsAg ELISA indicating enveloped HBV particles. Average values from three experiments ± standard errors of the means are given. (b) Qualitative and quantitative analysis of encapsidated viral nucleic acids using one-step RT-PCR. To discriminate between DNA and RNA, DNase digestion was performed. (Left) Mock treatment. (Right) Anti-CD95 treatment. The total amount of HBV nucleic acids (DNA plus RNA) (gray bars) detected in F8 of mock-treated cells was set to 100%. HBV RNA levels (continued line) of all samples are reported as parts per thousand. (c) Amplification products of qPCR runs of F4 to F6 with the indicated treatments were separated on a 2% agarose gel and visualized by ethidium bromide staining.

nucleic acids but no RNA (Fig. 5b), confirming that they contained mature, enveloped virions. A second, small peak of total HBV nucleic acids was found around F5 (buoyant density, 1.34 g/ml) corresponding to nonenveloped capsids.

After the induction of apoptosis, we found a markedly reduced amount of HBsAg and no nucleic acids in F8 to F10, confirming that no more enveloped DNA-containing particles were released. Since HBsAg ELISA also detects subviral particles, we conclude that apoptosis induction blocks the secretion of subviral particles as well as virions. In F4 to F6, the amount of HBV RNA was 6-fold higher than that detected after mock treatment (Fig. 5c).

Taken together with results shown in Fig. 2d, we assume that apoptosis blocks HBV replication and capsid maturation and prevents the release of enveloped virions as well as subviral particles but induces the release of nonenveloped capsids. Although we cannot pinpoint the pathway by which this release occurs, our data clearly demonstrate that the particles are not mature and are noninfectious.

DISCUSSION

This study was designed to investigate whether HBV influences the sensitivity of hepatocytes to apoptosis and if apoptosis induction in HBV-infected hepatocytes may support virus spread within the liver. This is of importance since it has been debated whether or not HBV replication actively induces the apoptosis of its host cell to enhance progeny virus release or to modify the immune response in its host (39, 41, 43, 45).

We show for the first time in a biological context that apoptosis of HBV-infected hepatocytes does not support virus propagation. We demonstrate that apoptosis induction severely interrupts the HBV life cycle, resulting in the release of noninfectious HBV capsids. Importantly, we found an inverse correlation between the strength of the apoptotic stimulus, the structure of the HBV particles released, and their infectivity: the more potent an apoptotic stimulus, the higher the ratio of nonenveloped capsids to mature HBV virions and the lower the viral infectivity. Our data provide compelling evidence that apoptosis induction of infected hepatocytes reduces the spread of HBV progeny in the host and, therefore, is deleterious to the virus.

Although we and others have shown that neither HBV replication nor the expression of HBV proteins in the context of a replicating HBV genome spontaneously induces the apoptosis of the host cell (1, 35), some studies indicated that the overexpression of the viral HBx protein sensitizes host cells to apoptosis (18, 26, 29, 39, 45). Pan and coworkers (28) provided an explanation for this, pointing out that the ability of HBx to promote or inhibit apoptosis depends on hepatocyte differentiation and whether cells are quiescent or dividing. In our experiments, we needed to differentiate the cells to support HBV replication (13, 32). By comparing differentiated, HBV-replicating HepG2-H1.3±x cells with parental HepG2 cells, we confirmed that HBV does not sensitize its host cell to apoptosis. In accordance with these functional data, gene expression profiling of HepG2-H1.3, HepG2-H1.3x−, and HepG2.2.15 in comparison to parental HepG2 cells using Illumina human WG-6 microarray gene chips revealed that HBV replication in the presence and absence of the viral HBx protein does not alter the expression profile of proteins with proapoptotic potential (S. Arzberger, J. L. Schultze, and U. Protzer, unpublished observation).

It has been argued that HBV might induce the apoptosis of its host cell, especially at the onset of an acute HBV infection, to facilitate HBV particle release. This was described previously for cytopathic DNA viruses such as adenovirus or simian virus 40 (SV40) (9, 15, 16) but may also apply to HBV contained in secretory vesicles. Thus, hepatocyte apoptosis may enhance the spread of progeny virus in the host but may also modify cellular immune responses because apoptosis induction allows the cross-presentation of viral proteins by dendritic cells in the context of apoptotic bodies (25, 47). To challenge the first hypothesis, we artificially induced apoptosis in either HBV-producing hepatoma cell lines or HBV-infected PHH, analyzed virus particles released into the cell culture medium, and additionally transferred them to new PHH to imitate a surrounding of liver cells still susceptible for a second round of HBV infection.

Irrespective of whether CD95 was triggered or cells were

subjected to UV-C light to initiate apoptosis, we demonstrated that apoptosis induction interrupted the release of infectious HBV particles. We observed that HBV-replicating hepatoma cell lines as well as primary cells release huge amounts of capsids after apoptosis induction in a dose-dependent manner for hitherto unknown reasons.

Interestingly, after CD95-induced apoptosis, which was a weak apoptotic stimulus in hepatoma cell lines but a strong stimulus in PHH, cells released two distinct populations of capsids sedimenting at different densities. We hypothesized that these capsid populations corresponded to different maturation levels of capsids within the HBV replication cycle, and we therefore analyzed their RNA and DNA contents, respectively. HBV DNA as well as RNA-containing nonenveloped capsids were released by apoptotic hepatocytes. However, we could not assign the two distinct populations of viral capsids to different nucleic acid contents. Therefore, we speculate that the different biochemical characteristics are caused by an association with cellular membranes in apoptotic bodies or by structural changes within the HBV capsids during maturation (11, 34).

The release of nonenveloped HBV capsids upon apoptosis was somewhat unexpected, but it has also been observed for hepatocytes in which intracellular budding and virion secretion have been blocked due to the absence of the viral L glycoprotein (40). In addition, it has been shown that HBV capsids survive T-cell-induced apoptosis of murine hepatocytes (28a). However, nonenveloped capsids are hardly found in the blood of infected patients and chimpanzees (30). Either this particle release pathway is active only during hepatocyte apoptosis or nonenveloped particles are rapidly phagocytosed and degraded. The latter option is supported by a series of data obtained by Milich and colleagues showing that B cells very efficiently bind capsids via the constant region of the B-cell receptor triggering rapid uptake (2, 20, 21, 27).

How exactly HBV capsids are released remains elusive. It is open regarding why the capsids are secreted and not degraded directly by activated caspases. However, the majority of the potential aspartyl cutting sides are probably hidden in the tridimensional structure of the HBV capsids, and the relatively large "protein aggregate" may be hard to dissolve within the cell. We assume that the release of capsids is linked to cell-autonomous defense mechanisms purging out foreign protein aggregates. An alternative interpretation is a partial loss of cell membrane integrity upon apoptosis induction. Although this is contradictory to data showing that intrinsic and CD95 receptor-mediated apoptosis leave membrane structures intact (23), it explains why apoptotic hepatocytes release alanine aminotransferase (ALT) upon apoptosis induction (33).

Second-round infection experiments documented that the HBV capsids released were not infectious, since they established no HBV cccDNA in the inoculated PHH, a prerequisite to initiate the HBV replication cycle in a newly infected cell (Fig. 3d and 4d). The remaining low-level infection observed was most likely caused by HBV particles which had been enveloped before apoptosis interrupted the viral replication cycle. The assumption that an intact envelope is essential for viral entry was supported by the observation that the coating of the HBV envelope by antibodies abolished infectivity (Fig. 3a and b).

Our data clearly showed that the apoptosis of HBV-infected hepatocytes blocked the further spread of HBV infection from these cells. Although we cannot rule out the possibility that surrounding hepatocytes may engulf apoptotic bodies containing HBV capsids *in vivo*, this uptake mechanism probably directs the capsids to the wrong cellular compartment. Thus, our study clearly refutes the assumption that the apoptosis of infected hepatocytes may facilitate HBV particle spread. Furthermore, it supports the notion that it is more advantageous for HBV to inhibit than to support apoptosis.

In conclusion, we demonstrated for the first time that the induction of apoptosis in HBV-infected hepatocytes interrupted HBV propagation. We support the concept that apoptosis is a powerful antiviral defense mechanism erasing HBV infection. We demonstrated that it would be detrimental to the virus to actively sensitize its host cell to apoptosis. In keeping with this, the virus profits from reducing the sensitivity of hepatocytes to apoptosis.

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