Hepatitis delta virus epigenetically enhances clusterin expression via histone acetylation in human hepatocellular carcinoma cells

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INTRODUCTION

Superinfection and co-infection of hepatitis delta virus (HDV) and hepatitis B virus (HBV) are associated with fulminant hepatic failure (Smedile et al., 1982) and chronic hepatitis (Buti et al., 1987; Colombo et al., 1983; Rizzetto et al., 1979). Carriers of HDV may develop liver cirrhosis (Fattovich et al., 1987) and hepatocellular carcinoma (HCC) (Oliveri et al., 1991; Verme et al., 1991). For these reasons, HDV is a major cause of severe liver disease. HDV produces only one known protein, the HDV antigen (HDAg). HDAg consists of two distinct isoforms, the small form (S-HDAg; 195 aa, 24 kDa) and the large form (L-HDAg; 214 aa, 27 kDa) (Bonino et al., 1986). The large form contains an extra 19 aa at the C terminus that consists of a stretch of variable but genotype-specific membrane-attaching sequence and serves as the virion assembly signal (Chang et al., 1991). L-HDAg is synthesized only late in the virus replication cycle, as a result of an RNA editing event that alters the termination codon of the open reading frame for S-HDAg (Polson et al., 1996). The function of these two antigens in HDV pathogenesis is less defined than in HDV replication. Recently, modulation of transforming growth factor-β signalling cascades by L-HDAg has been reported and linked to HDV-associated liver fibrosis (Choi et al., 2007).

There is evidence suggesting that both HDAg isoforms are transcriptional inducers. Wei & Ganem (1998) reported that L-HDAg, but not S-HDAg, had the capacity to activate the expression of co-transfected genes driven by a variety of promoters, including the pre-S, S and C promoters of HBV. In addition, Goto et al. (2000) reported that L-HDAg activates serum response factor-dependent and serum response element-dependent pathways. Huang et al. (2001) also found that expression of B23, a nucleolar phosphoprotein, is upregulated by these two HDAs. Thus, S-HDAg and L-HDAg are both capable of regulating cellular gene expression, but L-HDAg seems to activate more heterologous promoters than S-HDAg.

In order to understand better the cellular genes regulated by HDV infection, we screened transcriptionally induced genes from the human HCC cell line Huh7 expressing either isofrom of HDAg. The clusterin gene was identified as an upregulated gene in response of both HDAg and HDV replication. Moreover, we demonstrated that this upregulation was mediated by histone acetylation within the clusterin promoter. To evaluate the effect of clusterin expression in Huh7 cells, we treated HDAg-expressing Huh7 cells with a DNA-damaging agent, adriamycin (ADR), to measure their chemosensitivity. Our results showed that enhanced expression of clusterin...
protein in HDAg-expressing cells was associated with a decrease in chemosensitivity to ADR.

**METHODS**

**Selection of Huh7 cell lines that stably express HDAg.** Human hepatoma Huh7 cells and human lung cancer H1299 cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 IU penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Transfection of the plasmids pcDNA/GFP, pcDNA/L-HDAg and pcDNA/S-HDAg into Huh7 cells was carried out using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Huh7 cells stably expressing HDAg were selected by G418 (1.5 mg ml⁻¹; Sigma). The cells were then isolated, amplified and examined by Western blot analysis.

**GeneFishing PCR.** GeneFishing PCR (Seegene) is similar to subtracted hybridization of differential display genes (Kim et al., 2004). RNA from Huh7 cells expressing HDAg was prepared for reverse transcription of cDNA according to the manufacturer’s instructions. This was followed by reamplification with differential display gene primers. An agarose gel was used to compare the intensity of bands after ethidium bromide staining. The PCR-amplified band of interest was extracted and ligated into a TA vector followed by transformation into XL-10 competent cells. The DNA of the TA vector was purified and sequenced.

**Northern blot analysis.** Total RNA was extracted from Huh7 cells using TRizol reagent (Gibco-BRL) and 30 μg cellular RNA was electrophoresed in a 1.2 % agarose gel containing formaldehyde. The RNA was blotted onto a nitrocellulose membrane (Hybond-C Extra; Amersham) and probed with in vitro [³²P]cCMP-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH), clusterin and S-HDAg DNA probes (1 x 10⁶ c.p.m. ml⁻¹). After 24 h hybridization at 42 °C, the membrane was washed and detection was carried out by autoradiography.

**Quantitative real-time PCR (qPCR).** qPCR was used to quantify the levels of the clusterin in the samples. Before generating the cDNA, 2 μg purified RNA (Ultraspec RNA Isolation System BL-10200) was reverse-transcribed with a high-capacity cDNA archive kit (Applied Biosystems). The reactions were subsequently performed in triplicate using TaqMan Gene Expression Assays (Applied Biosystems), which included pre-optimized probe and primer sets specific for the genes being validated. The assay ID numbers of the validated genes were Hs00156548_m1 for clusterin and Hs99999905_m1 for GAPDH, used as the internal standard reference. Data were collected using an ABI PRISM 7000 sequence detection system.

**Western blot analysis.** Cells were washed with PBS and harvested for sonication in the presence of Protease Inhibitor Cocktail (Sigma). The protein concentration was determined by a Bradford assay and 20 μg cell lysate was analysed by SDS-PAGE. After transfer to PVDF membrane, the proteins were reacted with polyclonal anti-HDAg, anti-clusterin (Santa Cruz Biotechnology), anti-acetylated H3 (Upstate), anti-H3 (Abcam) or anti-β-actin (Sigma) antibody, followed by anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Calbiochem). A chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech) was applied to determine the levels of protein expression. The Image J software used for analysis was downloaded from the National Institutes of Health (http://rsb.info.nih.gov/ij/).

**Construction of the clusterin promoter for a luciferase reporter gene assay.** To subclone the clusterin upstream promoter region, genomic DNA from Huh7 cells was extracted for PCR amplification. The forward primer clu-F(−988), 5’-GATTTCTAACTGGGA-GGCCT-3’, and reverse primer clu-R(−133), 5’-GGTCAGCCGGC-ACCTGTGCC-3’, were used to isolate the clusterin 5′ regulatory region from −133 to −988 (pGL−988), inserted into the KpnI and BglII restriction sites of the pGL3-Basic plasmid (Promega). The same insertion was digested with KpnI and NotI to obtain the pGL−410 construct. To delete the upstream sequence of the clusterin promoter, the forward primer clu-F(−307-Kpn), 5’-GATAGGTACCCTT-CCAGAAGCTCCCT-3’ (KpnI site underlined), and reverse primer clu-R(−133-Bgl), 5’-TCTGAGATCGTACGGCGCAACC-TGT-3’ (BglII site underlined), were used to amplify the pGL−307 construct containing the heat-shock response element (HSE), AP-1 and AP-2 sequences. To delete these sequences, the forward primer clu-F(−295-Kpn), 5’-GATAGGTACCCTT-CCAGAAGCTCCCT-3’, and clu-R(−133-Bgl) primer were used to construct pGL−259. All of the constructs underwent DNA sequencing analysis.

**Luciferase and β-galactosidase reporter gene assays.** To evaluate the role of L-HDAg in clusterin promoter activity, cells were transiently co-transfected with the constructed clusterin reporter plasmid (1 μg) or the control vector (pGL3-Basic) with pSV-βGal (0.5 μg; Promega) and the expression plasmid of L-HDAg (0.3 μg) in a 35 mm dish in duplicate. Transfected cells were harvested after 48 h and lysed with cell culture lysis reagent (Promega). The efficiency of transfection was determined by assaying for β-galactosidase activity using o-nitrophenyl-β-d-galactopyranoside substrate (Promega) and spectrophotometrically measuring the absorbance at 420 nm. Luciferase activity was measured with a Microplate Luminometer (TR717; Applied Biosystems) and calibrated against β-galactosidase activity.

**Chromatin immunoprecipitation (Chip).** Cells (3 x 10⁶) were fixed with DMEM containing 1 % formaldehyde for 10 min at 37 °C followed by the addition of glycine (125 mM) for 5 min at 37 °C to quench the fixative. The cells were then washed with PBS/0.5 mM EDTA and scraped with fresh PBS/EDTA solution. The cell pellet was centrifuged at 4200 g for 4 h and resuspended in 200 mM NaCl, 1 % NP-40, 0.25 % sodium deoxycholate, 2 mM EDTA, 1 mM PMSF and Protease Inhibitor Cocktail and transferred to a microfuge tube for sonication. After sonication, cell debris was removed by centrifugation at 12000 g for 10 min at 4 °C. The supernatant was pre-cleared with protein A-Sepharose and BSA (0.5 mg ml⁻¹) for 1 h at 4 °C. An aliquot (50 μl) of pre-cleared chromatin was stored at −20 °C as the ‘input DNA control’. The supernatant was divided into equal aliquots. Pre-immune serum was added to one aliquot (pre-immune control) whilst anti-acetylated H3 antibody (1 μg) was added to the other, and they were incubated overnight at 4 °C on a rotating wheel. Salmon sperm DNA (1 mg ml⁻¹) was added to reduce non-specific binding of histone and DNA, followed by the addition of protein A-Sepharose (45 μl) for another 2 h of rotation. The immune complex was collected by centrifugation in a microcentrifuge followed by a low-salt wash [0.1 % SDS, 1 % Triton X-100, 3 mM EDTA, 150 mM NaCl and 20 mM Tris/HCl (pH 8.0)], a high-salt wash (low-salt wash with 500 mM NaCl) and a LiCl salt wash [0.25 M LiCl, 1 % sodium deoxycholate, 1 mM EDTA and 20 mM Tris/HCl (pH 8.0)] at 4 °C before elution from the Sepharose beads. The immune complex was dissociated with elution buffer (0.1 M NaHCO₃, and 1 % SDS) and separated from the beads by centrifugation. The eluted complex was adjusted to 200 μM with NaCl and incubated at 65 °C for 4 h. The DNA was then precipitated with absolute alcohol and resuspended in 20 μl H₂O. The clusterin response element sequence was amplified by PCR using the forward primer clu-F(−307-Kpn) and the reverse primer clu-R(−133-Bgl).

**HDV replication using HDV cDNA or RNAs.** Huh7 cells were seeded onto a 60 mm diameter dish and incubated for 24 h.

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Lipofectamine 2000 (12 μl) was diluted with 0.5 ml opti-MEM (Gibco-BRL Life Technologies) and incubated at room temperature for 5 min. Plasmid containing a dimer of HDV cDNA (HDV-D2G, 4 μg) was added to another aliquot of opti-MEM (0.5 ml). The diluted DNA was then combined with diluted Lipofectamine 2000 and further incubated at room temperature for 20 min. Cells were washed twice with 1 × PBS, which was then replaced with 1 ml opti-MEM, and the DNA–liposome complexes were added to the Huh7 cells. After 3 h incubation, the medium was removed and replaced with fresh medium. Genomic HDV RNA (HDV-D2G) was transcribed from HinIII-linearized HDV D2G plasmid using an AmpliScribe T7, T3 and SP6 High Yield Transcription kit (Ambion). Capped mRNAs for S-HDAg were prepared by in vitro transcription using the T7 mMESSAGE mMACHINE (Ambion) transcription kit from plasmid PX9 linearized by HinIII digestion (Sheu, 2002). HDV RNA co-transfection was also performed using Lipofectamine 2000. The DNA was replaced with PX9 cap mRNA (2 μg) and HDV-D2G genomic RNA (2 μg).

Chemosensitivity (MTT) assay. Chemosensitivity to ADR (Sigma) was determined using an MTT colorimetric assay. Approximately 6 × 10^4 cells per well were seeded onto 48-well plates. After 24 h incubation, the cells were exposed to various concentrations of ADR with fresh medium for 48 h. At the end of the exposure period, the supernatant was removed and cells were washed with PBS. Then, 150 μl MTT (1 mg ml⁻¹; Sigma) was added to each well and incubated at 37 °C for 3 h. After the supernatant had been removed and the cells washed with PBS, 200 μl 2-propanol solution was added per well to dissolve the water-insoluble formazan salt. The plates were shaken at 50 r.p.m. at room temperature for 10 min. Finally, the absorbance was measured at 570 nm using an ELISA plate reader (Molecular Devices SPECTRA max 340 PC). The assays were repeated three times and the chemosensitivity was reported as mean values.

RESULTS

Clusterin expression is induced in Huh7 cells stably expressing HDAg and is associated with HDV replication

To isolate potential cellular genes activated by L-HDAg or S-HDAg, we used a PCR screening method (GeneFishing). We found that one primer set generated an L-HDAg-specific induced band (Fig. 1a), which was identified as the human clusterin gene. To confirm this result, the mRNA level of clusterin was further examined by Northern blot analysis (Fig. 1b). We found that clusterin was upregulated up to 2.5-fold in the presence of L-HDAg. Clusterin (Blaschuk et al., 1983) is a secreted glycoprotein that is translated from a single mRNA as a pre-protein. This pre-protein is an intracellular 449 aa polypeptide chain with an apparent molecular mass of 60 kDa (c-CLU). Proteolytical cleavage of glycosylated c-CLU into an alpha- and beta-chain linked by five disulfide bridges (Choi-Miura et al., 1992) results in the mature form of the secreted clusterin protein (s-CLU). The processed clusterin protein appears as a smear of about 40 kDa as detected by Western blot (Leskov et al., 2003). Clusterin is present in most animal tissues and body fluids, and a broad range of functions has been reported (reviewed by Trougakos & Gonos, 2002). Among the various properties attributed to clusterin, the most frequently investigated are its small heat-shock protein-like chaperone activity and its involvement in cell death regulation (Trougakos & Gonos, 2006). Interestingly, overexpression of clusterin inhibits apoptosis and confers chemoresistance to prostate cancer (Miyake et al., 2006).

To determine whether induction of clusterin was associated only with L-HDAg expression, total cell lysates from two clones each of individually selected S-HDAg- and L-HDAg-expressing cells were further analysed by Western blotting with anti-clusterin, anti-beta-actin and anti-HDAg antibodies. The levels of c-CLU were higher in L-HDAg- and S-HDAg-expressing cells compared with the parental Huh7 cells (Fig. 1c). Interestingly, the levels of s-CLU were also substantially higher in L-HDAg- and S-HDAg-expressing cells. Therefore, we examined the medium of HDAg stably expressing cells for s-CLU by Western blot analysis. As expected, the levels of s-CLU increased in L-HDAg- and S-HDAg-expressing cells (Fig. 1c). The induction of clusterin in response to HDAg was shown to be specific, as transfection of a plasmid containing green fluorescent protein (GFP) into Huh7 cells exerted no effect on clusterin expression. To demonstrate further that clusterin expression was regulated at the mRNA level, we carried out qPCR (Fig. 1d). Our results showed that both HDAgs induced a modest increase in clusterin expression in Huh7 cells but that S-HDAg seemed to have the greatest effect.

As upregulation of clusterin in HDAg-expressing Huh7 cells might not necessarily be associated with the true HDV life cycle, we next investigated whether expression of clusterin could be regulated in cells synthesizing HDV RNA by transfecting the replication-competent HDV dimeric cDNA plasmid HDV-D2G into Huh7 cells. This resulted in replication of HDV RNA (Fig. 2b) and detectable HDAg protein expression (Fig. 2a). As the editing of mRNA of S-HDAg to L-HDAg is associated with the HDV replication processes (Polson et al., 1996), the presence of L-HDAg after 4 days of transfection provided further evidence of successful HDV RNA replication using the HDV-D2G plasmid. The expression of clusterin was affected in the early stages of viral RNA replication, when cells only express S-HDAg (2 days after transfection). Expression of clusterin increased further when L-HDAg appeared (4 days after transfection). The clusterin mRNA level was also measured (Fig. 2c). We found that HDV replication led to up to a 1.7-fold increase in clusterin transcription. The level of clusterin mRNA in the replication system was not altered as much as in the Huh-HDAg-expressing system, which might be due to the transfection efficiency of HDV-D2G. Thus, regulation of clusterin expression was associated with the HDV life cycle and both HDAgs induced clusterin expression.

Induction of clusterin is not directly mediated by the clusterin response element (CRE) in Huh7 cells

To elucidate the molecular mechanism of clusterin induction mediated by HDAg, we cloned the genomic 5′
regulatory region (−998 to −133) of human clusterin from Huh7 cells for a luciferase reporter gene assay. This 5′ upstream region contains a recognized HSE and transcription factor AP-1 and AP-2 binding sites that are part of the CRE (Fig. 3a). A series of luciferase reporter constructs containing the full-length clusterin 5′ regulatory region and various deletions (pGL −988, pGL −410, pGL −307 and pGL −259; Fig. 3a) were constructed to study whether L-HDAg activates clusterin promoter activity. Surprisingly, L-HDAg had no effect on any of the reporter constructs in Huh7 cells (results not shown).

Activation of clusterin transcription has been reported to be regulated by the CRE located in the 5′ promoter region...
upon treatment by heat shock (Michel et al., 1997) or with a proteasome inhibitor (MG132) (Loison et al., 2006). Therefore, we treated Huh7 cells with MG132 to determine whether endogenous clusterin was induced via the CRE. As it has been demonstrated that p53 can suppress s-CLU induction responses (Criswell et al., 2003) and Huh7 cells carry mutant p53 (Aden et al., 1979), we used p53-null H1299 cells (Chang et al., 2006) with similar p53 status to Huh7 cells as the positive control for MG132 treatment. No induction of clusterin in response to MG132 was found in Huh7 cells, whereas a dose-dependent enhancement of clusterin expression was observed in H1299 cells (Fig. 3b). Thus, it appeared that induction of clusterin in Huh7 cells is most likely not mediated by the CRE located in the 5′ promoter region, and so L-HDAg may induce clusterin via another mechanism.

Expression of clusterin is induced by histone deacetylase (HDAC) inhibitor in Huh7 cells

Interestingly, other reports have demonstrated that clusterin expression is regulated by the epigenetic mechanisms of promoter methylation (Lund et al., 2006) and histone acetylation (Lund et al., 2006; Nuutinen et al., 2005). Therefore, we examined whether this mechanism was responsible for HDAg-induced clusterin expression. As induction of clusterin by the HDAC inhibitor trichostatin A (TSA) has been observed in human neuroblastoma cells (Nuutinen et al., 2005), we started with an examination of the effect of TSA on endogenous clusterin expression in Huh7 cells. Various amounts of TSA were added to Huh7 cells and clusterin protein was detected by Western blot analysis. The levels of histone subunit H3 acetylation were detected as the results of HDAC inhibition. The expression levels of clusterin protein (Fig. 4a) and mRNA (Fig. 4b) positively correlated with the concentrations of TSA in Huh7 cells. Thus, increasing histone acetylation induced clusterin expression in Huh7 cells.

Histone H3 is heavily acetylated in Huh7 cells stably expressing L-HDAg or S-HDAg

We next tested whether a chromatin modification such as acetylation of histones is responsible for HDAg-induced clusterin expression. Examination of the levels of acetylation of histone subunit H3 in HDAg-expressing Huh7 cells revealed that L-HDAg- and S-HDAg-expressing cells displayed higher levels of H3 acetylation than their parental Huh7 cells (Fig. 5a). Stable expression of GFP enhanced neither clusterin expression (Fig. 1c) nor H3 acetylation (Fig. 5a). To find out whether hyperacetylated H3 increased in the 5′ regulatory region of clusterin, we
performed a ChIP assay using anti-acetylated histone H3 antibody to immunoprecipitate the 5' regulatory region of clusterin. TSA-treated (100 ng ml\(^{-1}\)) Huh7 cells were also examined and served as the hyperacetylation control for the ChIP assay. Our results showed that cells expressing L-HDAg had up to 2-fold more histone H3 hyperacetylation in the CRE region than the parental cells (Fig. 5b). The enhanced H3 hyperacetylation of the CRE region correlated with the increased levels of mRNA detected by Northern blotting (Fig. 1b) and qPCR (Fig. 1d). Our data thus suggested that induction of clusterin by HDAgs is via an epigenetic mechanism in Huh7 cells.

**Fig. 3.** HDAg induction of clusterin is not directly mediated by the CRE in Huh7 cells. (a) Schematic diagram of the 5' regulatory region of the clusterin promoter. To investigate the induction of clusterin transcription by L-HDAg, the individual luciferase reporter constructs shown and an L-HDAg-expressing plasmid were co-transfected into Huh7 cells and the cells were analysed as described in Methods. (b) Expression of endogenous clusterin in Huh7 cells is not upregulated by MG132. Total protein from Huh7 and H1299 cells (20 \(\mu\)g) was analysed by Western blotting following MG132 treatment to detect levels of endogenous clusterin expression.

**Fig. 4.** Endogenous clusterin expression is regulated epigenetically by histone acetylation. (a) Endogenous clusterin expression induced by various amounts of TSA was examined in Huh7 cells. Cellular lysates (20 \(\mu\)g) were analysed by 10% SDS-PAGE and detected with the indicated antibodies. The degree of clusterin stimulation was calculated as fold stimulation by normalization to the intensity of Huh7 cells without TSA. (b) qPCR measurement of clusterin mRNA levels in TSA-treated cells.

**Fig. 5.** Hyperacetylation of histone H3 is associated with HDAg expression in Huh7 cells. (a) Huh7 cells stably expressing HDAg contain more acetylated histone H3. The histone H3 protein was detected with anti-acetylated H3 and anti-H3 antibodies in Huh7 cells stably expressing either isoform of HDAg. (b) Recruitment of acetylated histone H3 to the CRE in Huh7 cells expressing L-HDAg. Cell lysates were used for the ChIP assay. The lysate of TSA-treated (100 ng ml\(^{-1}\) for 24 h) Huh7 cells was the positive control for acetylated histone H3 and treatment with pre-immune serum was the negative control for ChIP of acetylated histone H3.

HDV epigenetically regulates clusterin expression
Transient transfection of HDAs is associated with histone H3 hyperacetylation and clusterin upregulation

To demonstrate that induction of clusterin in Huh7 cells stably expressing HDAs was not an artefact of the response to G418 selection, transient transfection of constructs containing HDAs into Huh7 cells was conducted. Various amounts of HDAg-expressing plasmids were transfected with Huh7 cells and HDAs levels were examined on day 3 post-transfection. Clusterin protein and histone H3 acetylation were also detected by Western blot analysis (Fig. 6a). In addition, we measured clusterin mRNA level from the corresponding samples by qPCR (Fig. 6b). The data showed that levels of expressed HDAs correlated with clusterin expression and H3 hyperacetylation. To examine further the transient association of HDAs with clusterin induction, HDAs and clusterin protein were analysed over a 5-day period (Fig. 6c). When the amount of HDAs decreased on day 5 post-transfection, clusterin and acetylated histone H3 showed corresponding reductions. These data provided additional evidence to support the association of HDAs with histone acetylation and induction of clusterin. We also transiently transfected a GFP expression vector (pEGFP-C1) into Huh7 cells to exclude the possibility that induction of H3 acetylation is non-specific to HDAs. No obvious H3 acetylation was observed in response to the expression of GFP (Fig. 6a, b).

Fig. 6. Hyperacetylated H3 is detected in Huh7 cells transiently transfected with L-HDAg and S-HDAg expression plasmids. (a) Correlation of HDAg expression levels with clusterin expression and H3 hyperacetylation by Western blot analysis. (b) qPCR measurement of clusterin mRNA levels in S-HDAg-, L-HDAg- and pEGFP-C1-transfected cells. The amount of plasmid was supplemented to 4 μg with pcDNA3.1 control plasmid and transfected into Huh7 cells in a 60 mm dish. (c) Transient expression of HDAs correlates with transient clusterin expression and H3 hyperacetylation. Cellular lysates (20 μg) were harvested for Western blot analysis. The acetylated levels of H3 protein correlated well with the expression levels of HDAg.
HDV replication enhances histone acetylation and clusterin expression

We further tested whether histone acetylation is truly associated with HDV replication using a cDNA-free HDV replication system (Sheu & Lai, 2000). The in vitro-synthesized HDV dimeric RNA from the cDNA plasmid HDV-D2G and capped mRNA of S-HDAg were co-transfected into Huh7 cells and cellular lysates were prepared for Western blot analysis (Fig. 7a). On day 1 post-transfection, S-HDAg was expressed and a low level of histone H3 acetylation was observed. On day 3 post-transfection, L-HDAg was expressed and histone H3 showed significant acetylation. The hyperacetylated H3 proteins were maintained steadily throughout the assay period. In addition, the levels of clusterin closely correlated with the levels of histone H3 acetylation and reached the highest level on day 7 post-transfection. The levels of clusterin mRNA increased simultaneously with HDV replication under the same conditions (Fig. 7b). To test whether H3 acetylation was induced by RNA transfection, we transfected only the genomic HDV RNA into Huh7 cells as a mock control. The mock control showed neither H3 hyperacetylation nor clusterin induction. Therefore, we concluded that HDV replication specifically induces clusterin expression via histone hyperacetylation, which may explain why clusterin transcription was only moderately induced by HDAgs.

Huh7 cells stably expressing HDAgs have decreased sensitivity to ADR treatment

Clusterin is a highly conserved protein and has many cellular functions. Interestingly, clusterin has been found to be elevated in the serum of HCC and HBV-positive patients (Feng et al., 2005). As clusterin has chaperone activity in response to cell damage (Trougakos & Gonos, 2006), we used the topoisomerase II inhibitor ADR, a chemotherapeutic drug, to induce cell death and analysed its cytotoxic effect in an MTT assay. At up to 900 ng ADR ml⁻¹ (1.55 μM), S-HDAg-expressing cells showed the least sensitivity and the parental Huh7 cells showed the most sensitivity to ADR treatment (Fig. 8a). Kinetic analysis also revealed that S-HDAg- and L-HDAg-expressing cells were less sensitive to ADR treatment than the parental Huh7 cells (Fig. 8b). Therefore, HDAgs may provide a benefit to Huh7 cells, allowing them to survive ADR treatment.

To characterize the role of secreted clusterin in the cytoprotective effect, we collected the medium from S-HDAg- and L-HDAg-expressing cells after 48 h incubation and used it as the source of s-CLU. The medium used for cell treatment was a mixture of one part fresh and one part collected medium to avoid metabolic toxicity from the collected medium. As shown in Fig. 8(c), S-HDAg-expressing cells supplemented with conditioned medium survived better than cells with fresh medium. Similar results were obtained for L-HDAg-expressing cells (Fig. 8d). Thus, s-CLU, present in the collected medium, appeared to have a protective effect against ADR treatment.

DISCUSSION

Our data demonstrate that clusterin is an authentic HDV-regulated gene that is specifically and transcriptionally induced by L-HDAg and S-HDAg. We attempted to define the L-HDAg response element of the 5′ region of the human clusterin promoter. Unfortunately, L-HDAg was unable to induce activity of the clusterin 5′ region above existing levels (Fig. 3a). In addition, we were surprised to observe that histone acetylation is associated with HDV replication and that hyperacetylation of histones resulted in clusterin induction in Huh7 cells.
Clusterin expression is apparently regulated by two pathways. One is through activation of the transcription factors HSE, AP-1 and AP-2, which induce promoter activity (Loison et al., 2006). The other pathway is mediated by chromatin remodelling through hyperacetylation of histones, followed by transcriptional activation (Nuutinen et al., 2005). Our data showed that clusterin expression is differentially regulated with regard to cell-type specificity. We also demonstrated the novel finding that HDAgs activate clusterin expression epigenetically through histone acetylation in Huh7 cells.

In order to complete the virus life cycle, HDV has to at least partially alter the cellular machinery for its own proliferation. We showed that once HDV replication is initiated, as evidenced by production of S-HDAg, acetylation of histone H3 increases and synthesis of L-HDAg enhances this modification further. The ability of HDAs to mediate histone acetylation may facilitate the completion of virus replication. Histone acetyltransferases (HATs) and HDACs are the key enzymes that alter the acetylation status of chromatin. Reversible acetylation of viral genes by many viruses has been reported and the interaction of viral proteins with HAT or HDAC has also been demonstrated (Gray & Teh, 2001). For example, adenovirus protein E1A alters CBP/p300 HAT activity (Chakravarti et al., 1999; Hamamori et al., 1999) and simian virus 40 T antigen modulates CBP HAT activity (Valls et al., 2003). Human papilloma virus type 16 also regulates HDAC activity via the E7 oncoprotein to stimulate cell growth (Brehm et al., 1999). Apparently, many oncogenic viruses regulate histone-modifying enzymes to induce tumorigenesis. Currently, we do not know how HDAg regulates histone acetylation. It would be interesting to investigate whether histone acetylation affects HDV replication. We speculate that HDAg interacts directly with either HATs to enhance HAT activity or HDACs to repress HDAC activity. Recently, the acetyltransferases CBP and p300 have been reported to interact with HDAs and to modulate HDV RNA replication (Huang et al., 2008), and linker histone H1e also binds S-HDAg (Lee & Sheu, 2008), providing extra evidence for the potential of HDAs to regulate histone acetylation.

Clusterin has been reported to play a significant role in tumorigenesis (reviewed by Trougakos & Gonos, 2002), but the role of clusterin in liver diseases has not been well established. Overexpression of clusterin (89%) in HCC has been reported from tissue array data and the expression
pattern suggests that clusterin is secreted from tumour cells (Kang et al., 2004). Surprisingly, the frequency of clusterin overexpression increases substantially in metastatic HCC when compared with primary tumours (Lau et al., 2006). Our data also suggest that HDAg elevates the level of secreted clusterin. Therefore, the ability to upregulate the expression of clusterin by HDV may contribute to the long-term, cumulative and detrimental effects of liver pathogenesis. In this report, HDAg-expressing cells had increased resistance to ADR treatment, demonstrating the protective effect of clusterin in HCC cells.

Our study indicates that HDV is able to activate the expression of clusterin, a protein with many potential cellular functions that may also participate in liver pathogenesis. Furthermore, patients with intrahepatic delta antigen have a more severe liver disease than those without it (Colombo et al., 1983). As HDV is often found together with HBV in viral hepatitis patients (Colombo et al., 1983), HDAgS may collaborate with the HBV X protein, a viral transactivator (Waris & Siddiqui, 2003), to further increase the oncogenic potential in the liver of HBV/HDV carriers. Therefore, HBV and HDV together may accelerate the development of severe liver disease.

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