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Innate immune evasion by hepatitis B virus-mediated downregulation of TRIF

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ABSTRACT

Hepatocytes are the target host cells during hepatitis B virus (HBV) infection. Recent studies indicate that the innate immune response of hepatocytes plays an important role in inhibiting HBV replication. TIR-domain-containing adaptor inducing interferon-beta (TRIF) is a key component in innate immune signaling pathways. In this study, we found that the TRIF protein was downregulated in human hepatoma cell lines and liver tissue samples harboring HBV. Hepatitis B virus X protein (HBX) reduced TRIF protein expression in a dose-dependent manner via the proteasomal pathway. HBX appeared to not directly interact with TRIF as these proteins failed to co-immunoprecipitate when overexpressed in hepatoma cells. TRIF upregulation in hepatoma cell lines dramatically inhibited HBV transcription and expression of its viral proteins. Cellular apoptosis induced by TRIF was also confirmed in hepatoma cell lines. Taken together, these findings reveal a new mechanism for HBV evasion of the cellular innate immunity by HBX-mediated degradation of TRIF protein in hepatocytes.

1. Introduction

Hepatitis B virus (HBV) infection is among the global top ten causes of death [1]. Chronic HBV infection significantly increases the risk of liver cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC) [2]. Upon entry of HBV in the body, interactions between the virus and host dictate whether the outcome of the infection eventually clears or persists. Innate immunity is the first line of defence of the body against infection. Innate immune cells depend mainly on the identification of viral pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) present at the cell surface and in specific intracellular compartments to detect the invading virus. Toll-like receptors (TLRs) are currently the most extensively studied class of PRRs. Once PAMPs are recognized by TLRs, downstream signaling pathways initiate the antiviral effects by recruitment of adaptor proteins and consequent positive regulation of the expression of type I interferons (IFNs) and various inflammatory cytokines [3].

TLR3 signaling is an important pathway in innate immunity, and is impaired by many viruses [4–8]. Previous studies have indicated that TLR3 signaling plays an important role against HBV and is in turn suppressed by HBV [9]. As an adaptor protein in the TLR3 signaling, TIR-domain-containing adaptor inducing interferon-beta (TRIF) is often targeted. Hepatitis A virus, hepatitis C virus and coxsackievirus B3 have been shown to induce the cleavage of TRIF [4–6]. Hepatocytes are not classical innate immune cells; however, they harbor PRRs and corresponding signaling pathways [7,8,10–14]. A growing body of evidence suggests that activation of PRR-mediated innate immunity in hepatocytes inhibits the replication and protein expression of HBV [10,14,15]. From an evolutionary point of view, the virus needs to develop a strategy to escape the host immune responses for its survival. Although disruption of TLR3 signaling in hepatocytes by HBV has been reported [7,8], it remains unclear whether there is a regulating role of HBV on TRIF expression.

In the present study, we detected the expression of TRIF in hepatoma cell lines HepG2 and Huh7 and in liver tissues with and without HBV expression at the transcriptional and protein levels. We found that TRIF protein expression was reduced by HBV mediated by the HBX protein in a dose-dependent and proteasome-dependent manner. The experiments presented here also showed that overexpression of TRIF dramatically suppressed

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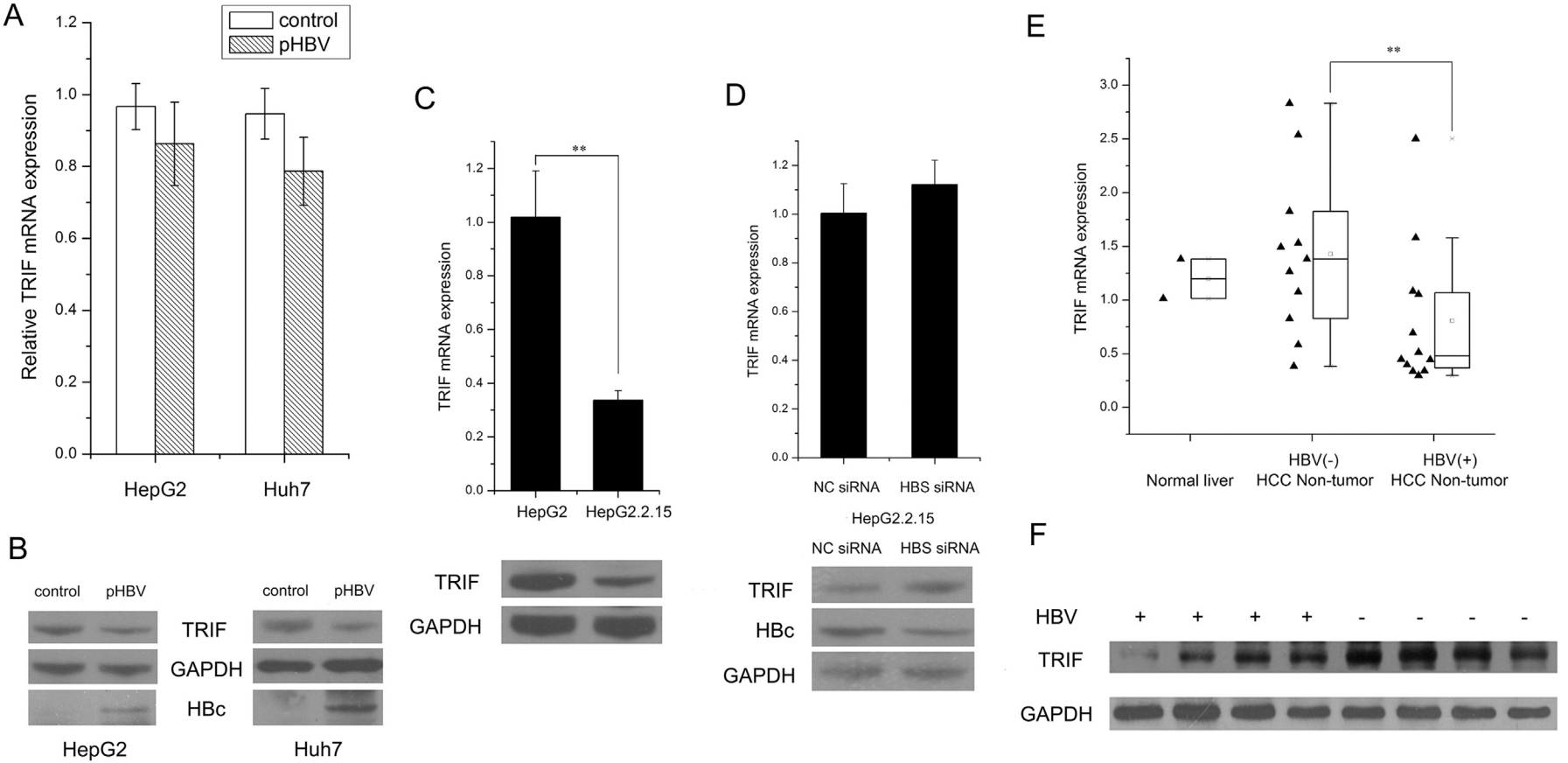

Fig. 1. Reduced TRIF expression in HBV-related hepatoma cells and liver tissues. HepG2 or Huh7 cells were transiently transfected with pHBV or control vector. Gene transcription (A) and protein expression (B) of TRIF were determined 48 h after transfection. (C) TRIF transcript (upper panel) and protein (lower panel) expression in HepG2 and HepG2.2.15 cells. (D) HepG2.2.15 cells were transfected with NC siRNA or HBS siRNA for 48 h, and then TRIF transcript and protein expression were detected. (E) Box plot of TRIF gene transcription levels in normal liver samples from 2 HBV-negative healthy donors (normal liver), non-tumorous liver tissues from 11 HBV-related (HBV− HCC non-tumor) and 11 HBV-unrelated HCC patients (HBV + HCC non-tumor). (F) Representative Western blots were shown to illustrate the TRIF protein expression between the HBV-related and HBV-unrelated HCC patients. $^{**}P < 0.01$.

HBV expression and replication as well as induced cell apoptosis in hepatoma cell lines. Our current data highlight the repressive function of TRIF on HBV and describe a new strategy utilized by HBV to impair the host innate immunity.

2. Materials and methods

2.1. Reagents, cell lines and transfection

The proteasome inhibitor MG-132 (Sigma–Aldrich, USA) was dissolved in dimethyl sulfoxide and diluted to the working concentration with fresh media before use. Human hepatoma cell lines HepG2 and Huh7 were cultured in high glucose Dulbecco's Modified Eagle Medium (Gibco, USA) with 10% fetal bovine serum (Gibco). HepG2.2.15 cells were also cultured in the same conditions except with G418 (Sigma–Aldrich) supplemented at 400 μg/ml. Transient transfections with plasmids or siRNA were performed using Lipofectamine 2000 Reagent (Invitrogen, USA) following the manufacturer's protocol.

2.2. Patient samples

Liver tissues were obtained from the First Affiliated Hospital of Zhejiang University in China, including 2 normal samples from HBV-negative healthy donors, 11 non-tumorous samples from HBV-related HCC patients and 11 samples from non-HBV-related HCC patients. All tissues were obtained with informed consent and stored at −80 °C before use.

2.3. Plasmids and siRNAs

To construct a TRIF expression vector, we amplified the coding sequence of TRIF from HepG2 cDNA and then cloned it into a pcDNA3.1(−) vector (Invitrogen). The primer pair that included restriction enzyme cut sites and terminal protective bases was designed as follows: 5′-AACTCGAGACCATGGCCTGCACAGGCCCATCAC-3′ (XhoI) and 5′-CGCAAGCTTTCATTCTGCCTCCTGCGTCTTGTCC-3′ (HindIII). To construct an HBX expression plasmid with the pcDNA3.1(−) vector, we amplified the coding sequence of HBX from a wild-type HBV expression plasmid, and the primer pair was designed as follows: 5′-AACTCGAGACCATGGCTGCTAGGCTGTGCTGCCAAC-3′ (XhoI) and 5′-CGCAAGCTTTTAGGCAGAGGTGAAAAAGTTGC-3′ (HindIII). A plasmid carrying 129% length of HBV genome (subtype ayw) and an HBX-deficient HBV plasmid were obtained from James Ou (Keck School of Medicine, University of Southern California, Los Angeles, CA, USA) and designated as pHBV and pHBVX-in this study. The *Renilla* luciferase reporter plasmid pRL-TK was purchased from Promega (USA). Empty vector pUC19 (Takara Biotechnology, China) and pcDNA3.1(−) were used as controls. HBS siRNA targeting the HBV S gene was used to silence HBV expression. An siRNA duplex with scramble sequences was used as a negative control (NC). Sequences of siRNA duplexes in this study were designed as follows: HBS siRNA (sense: 5′-GUCUGUACAACAUCUUGAGTT-3′; antisense: 5′-CUCAAGAUGUUGUACAGACTT-3′) and NC siRNA (sense: 5′-CGCGCUUUGUAGGAUUCGTT-3′; antisense: 5′-CGAAUCCUACAAAGCGCGCTT-3′). All siRNA duplexes were synthesized and purified by Sangon Biotech (China).

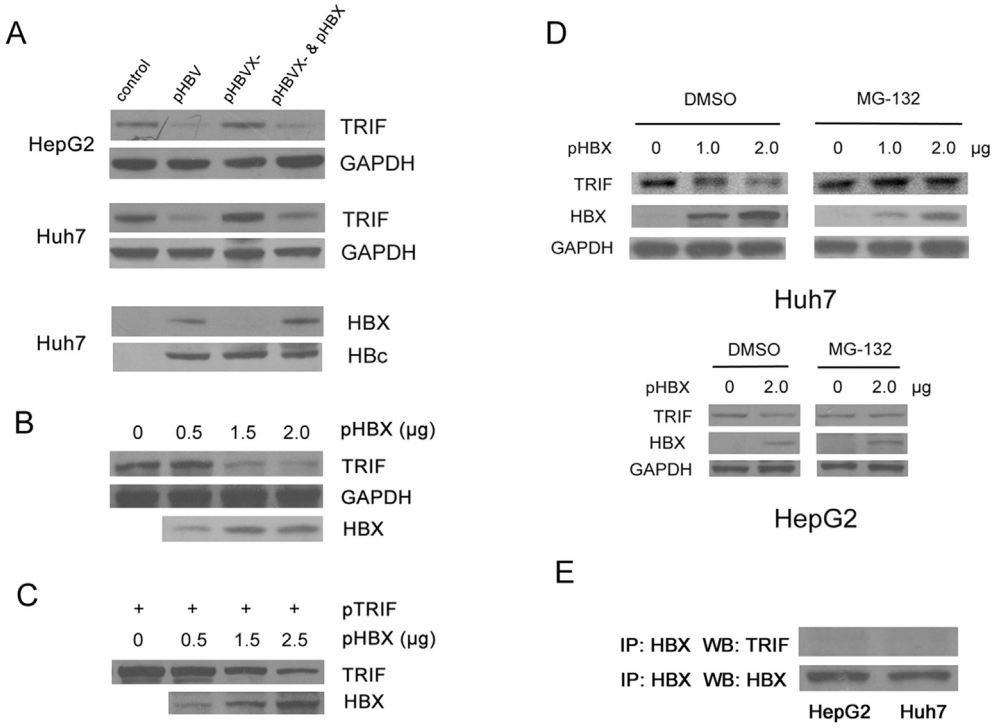

Fig. 2. HBX induced proteasome-dependent TRIF degradation. (A) HepG2 or Huh7 cells were transiently transfected with a control vector, pHBV, pHBVX-, or pHBVX-plus pHBX. Cells were harvested at 72 h post-transfection, and TRIF protein was detected by Western blot. (B) Huh7 cells were transfected with 0, 0.5, 1.5 or 2.0 μg of pHBX plasmid, and an empty vector was used to balance the total DNA input. Cells were harvested 72 h later, and TRIF or HBX protein was analyzed. (C) Huh7 cells were transfected with a fixed amount of pTRIF plasmid together with the indicated amount of pHBX plasmid, balanced with empty vector. At 72 h post-transfection, TRIF and HBX protein were detected. To ensure equal amounts of the pTRIF plasmid among different groups, pRL-TK was co-transfected as an internal control plasmid, and each lysate was normalized to the *Renilla* luciferase activity. (D) Huh7 or HepG2 cells were transfected with the indicated amount of pHBX. Twelve hours after transfection, cells were treated with or without 2.5 μM MG-132 for 36 h. Cells were then lysed and assessed for TRIF expression. (E) HepG2 or Huh7 cells were co-transfected with pHBX and pTRIF plasmids. In these cells harvested at 48 h post-transfection, the binding of HBX to TRIF were detected by co-immunoprecipitation analysis.

2.4. Quantitative real-time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For tissue RNA extraction, frozen tissues were ground to fine powder in liquid nitrogen using a mortar and then processed in the same manner as with the cells. One microgram of total RNA was reverse transcribed to cDNA using the M-MLV reverse transcriptase (Promega). Real-time PCR was carried out using SYBR Premix DimerEraser (Takara Biotechnology) with the 7500 Real-Time PCR System (Applied Biosystems, USA). HBV core antigen (HBc) gene-specific primers were used for the detection of HBV. Sequences of primers used were as follows: HBc, 5′-AGTGTGGATTCGCACTCC-3′ (sense) and 5′-GAGTTCTTCTTCTAGGGGACC-3′ (antisense); GAPDH, 5′-ATGACATC AAGAAGGTGGTG-3′ (sense) and 5′-CATACCAGGAAATGAGCTTG-3′ (antisense); TRIF, 5′-GGCCCATCACTTCCTAGCG-3′ (sense) and 5′-GAGAGATCCTGGCCTCAGTTT-3′ (antisense).

2.5. Western blot and co-immunoprecipitation assays

Cells were homogenized in lysis buffer. Proteins were prepared from frozen tissues by grinding to fine powder in liquid nitrogen and then treated with lysis buffer. For Western blot analysis, the sample lysates were separated by 12% SDS-PAGE, and proteins were transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk in 0.05% Tween-TBS, followed by sequential incubation with the desired primary and secondary antibodies. The target proteins were visualized using an ECL detection system (GE Healthcare, USA). GAPDH was detected as a loading control. The primary antibodies were anti-GAPDH, anti-HBc (Santa Cruz Biotechnology, USA), anti-TRIF (Cell Signaling Technology, USA) and anti-HBX (Abcam, UK). For co-immunoprecipitation, the sample lysates were incubated with the primary antibody and protein A/G-plus agarose beads (Santa Cruz Biotechnology) overnight at 4 °C. After washing the immunocomplexes four times with cold lysis buffer, the immunoprecipitated proteins were dissociated from the beads by boiling in sample loading buffer and then separated by SDS-PAGE for Western blot analysis as described above.

2.6. Luciferase assay

Cells were seeded in a 6-well plate one day before co-transfection with pRL-TK and other constructs. The cells were then lysed at indicated time points after transfection, and the lysates were cleared by centrifugation. The *Renilla* luciferase activity was detected using the *Renilla* Luciferase Assay System (Promega).

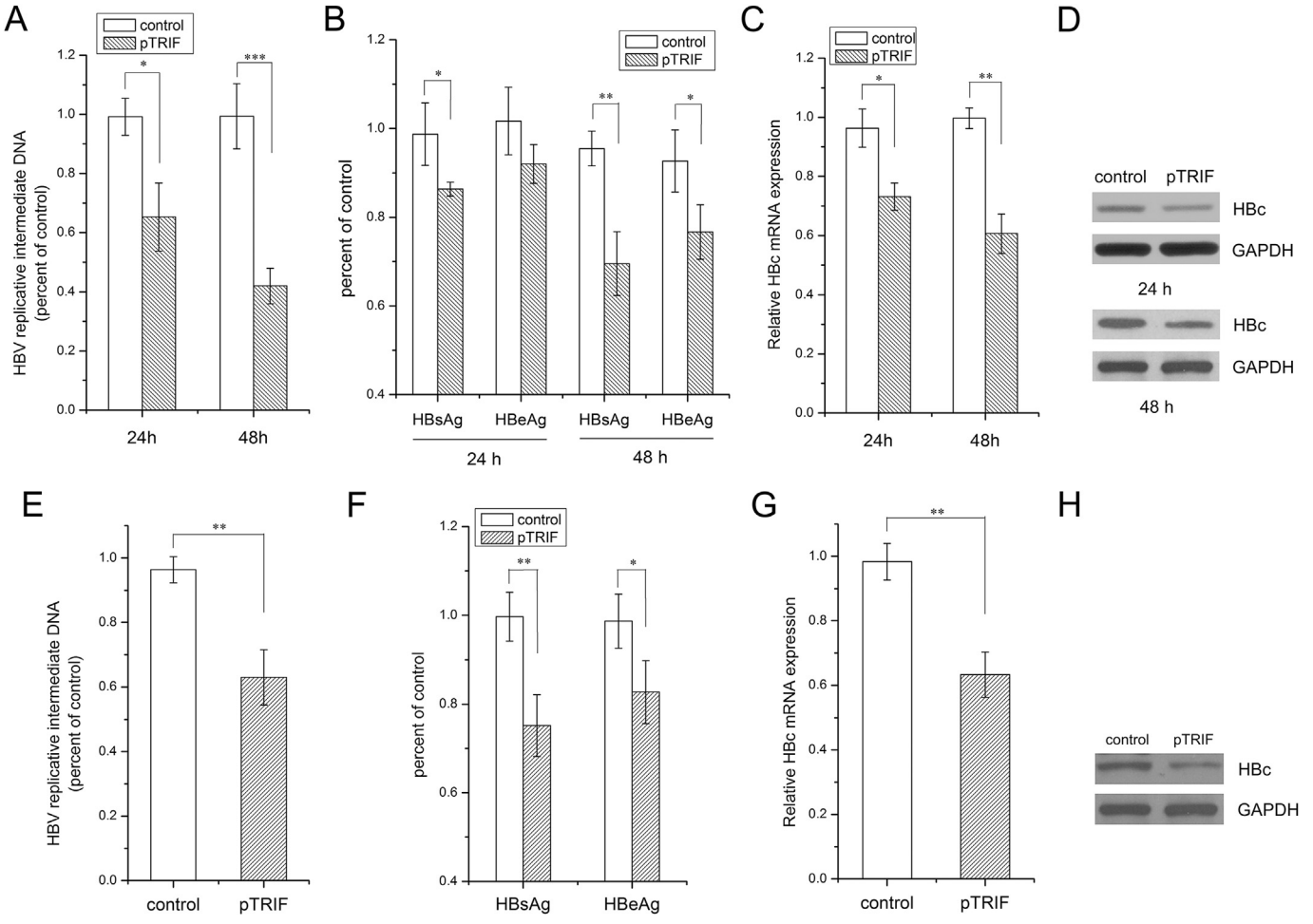

Fig. 3. TRIF inhibited HBV replication and protein expression in both transient and stable HBV-transfected cells. Huh7 cells were transfected with pTRIF or control plasmid, together with pHBX plasmid. At 24 h or 48 h post-transfection, the cellular HBV replicative intermediate DNA (A), HBsAg and HBeAg levels in culture supernatants (B), HBc gene transcription (C) and protein expression (D) were measured. HepG2.2.15 cells were transfected with pTRIF or control plasmid. The cellular HBV replicative intermediate DNA (E), HBsAg and HBeAg levels in culture supernatants (F), HBc gene transcription (G) and protein expression (H) were assessed 48 h post-transfection. Data were normalized to *Renilla* luciferase activity (from co-transfection of pRL-TK) for analysis of HBV DNA replicative intermediates. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

2.7. ELISA

Levels of HBV surface antigen (HBsAg) and e antigen (HBeAg) in culture supernatants were determined by using ELISA kits (Kehua Bio-engineering, China) following the manufacturer's protocol.

2.8. Analysis of intracellular HBV DNA replicative intermediates

Cells were harvested by trypsinization and divided into two aliquots. One half was used for detection of *Renilla* luciferase activity, and the other half was used for analysis of HBV DNA replicative intermediates. Briefly, cells were lysed in 0.5 ml lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA and 1% NP-40 at 37 °C for 15 min. Cell debris and nuclei were removed by centrifugation, and the supernatant was mixed with 130 μl of 35% PEG 8000 containing 1.5 M NaCl. After 1 h of incubation in ice, the core particles were collected by centrifugation at 12,000 × *g* for 10 min at 4 °C. The precipitates were then incubated for 30 min at 37 °C with 100 μl of digestion buffer (15 μg RNase A and 10 units of DNase I), followed by treatment at 55 °C for 1 h with 400 μl of protein digestion buffer (200 μg/ml proteinase K, 10 mM EDTA, 1% SDS and 100 mM NaCl). The samples were extracted by the phenol–-chloroform method, and DNA was precipitated with ethanol and then dissolved in TE buffer. HBc primers were used for PCR to analyze viral replicative intermediates. Transfection efficiency was normalized to *Renilla* luciferase activity.

2.9. Apoptosis assay

DAPI (Sigma–Aldrich) was added to the culture medium at a final concentration of 2 μg/ml. After 30 min of incubation, cells were observed under an *inver*ted fluorescence microscope. Several morphological features of cells indicated apoptosis, including cytoplasmic shrinkage, membrane blebbing, detachment from the plate, loss of membrane integrity, chromatin condensation and nuclear fragmentation. Caspase-3/7 activity assays (Promega) were carried out according to the manufacturer's protocol.

2.10. Statistical analysis

Results were represented as mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using the Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. TRIF is downregulated in HBV-related hepatoma cells and liver tissues

To determine if HBV deregulates TRIF expression, the gene transcription and protein expression of TRIF in hepatoma cells with or without HBV expression were examined. Decreased TRIF protein levels were observed in HepG2 and Huh7 cells transiently transfected with the pHBV plasmid, while no significant change was seen at the transcriptional level (Fig. 1A and B). We therefore speculated that a post-translational event caused the downregulation of TRIF protein. Next, we examined TRIF expression in HBV stably transfected HepG2.2.15 cells compared with the parental HepG2 cells. Unlike the previous results, a downregulated TRIF transcript level was observed along with the reduction in its protein level (Fig. 1C). After suppression of HBV expression in HepG2.2.15 cells using HBS siRNA, the TRIF gene transcription was not increased, while TRIF protein was upregulated (Fig. 1D). These findings indicated the occurrence of post-translational modification(s) of TRIF in HBV stably transfected cells. In order to further verify in vivo whether TRIF downregulation correlated with HBV expression, TRIF expression was also examined in human liver samples. Both gene transcription and protein expression of TRIF were lower in non-tumorous liver tissues from the 11 HBV-related HCC patients (HBV + HCC non-tumor) compared with those in tissues from the 11 non-HBV-related HCC patients (HBV− HCC non-tumor) (Fig. 1E and F). TRIF expression in the two normal liver tissues seemed comparable to that of the HBV- HCC non-tumor group, although statistical analysis was not performed due to the small number of cases. Base on our data, TRIF protein expression is

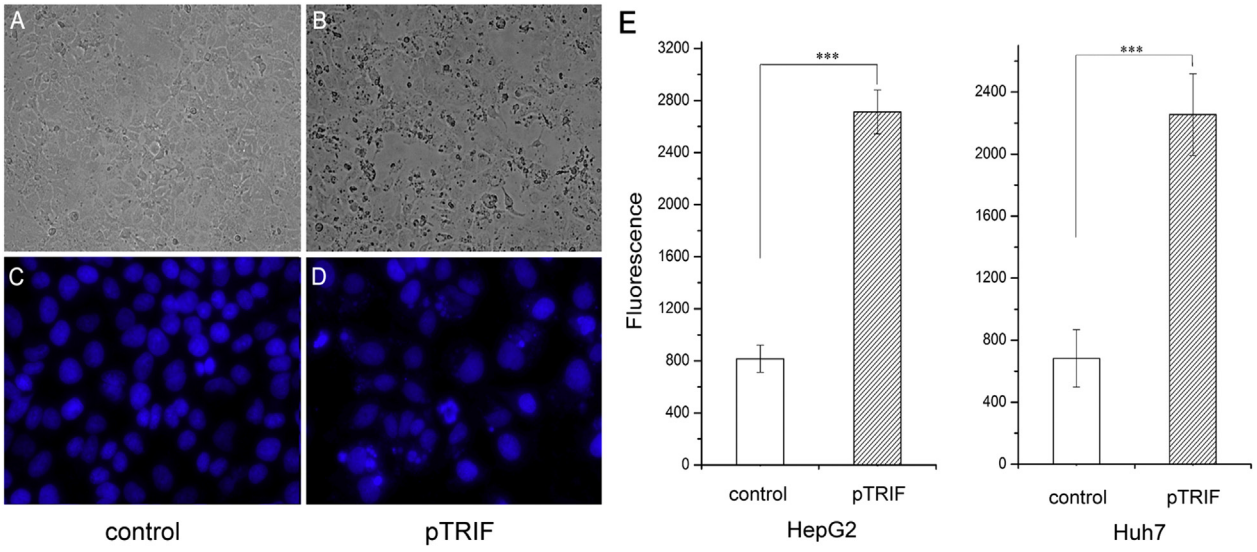

Fig. 4. TRIF protein promoted apoptosis of hepatoma cells. (A–D) Huh7 cells were transfected with pTRIF or control plasmid. DAPI was added to the culture medium at a final concentration of 2 μg/ml 48 h later. The cellular morphological features were observed with an inverted fluorescence microscope after 30 min of incubation (A–B, magnification 200 × ; C–D, magnification 400 ×). (E) HepG2 or Huh7 cells were transfected with pTRIF or control plasmid. Caspase-3/7 activities were assessed 48 h post-transfection. ***$P < 0.001$.

decreased in HBV-related hepatoma cells and liver tissues in spite of the discrepancy in TRIF transcription level between stable and transient HBV-transfected cells.

3.2. HBX reduces TRIF protein expression

HBX, a viral protein encoded by the HBV genome, has been shown to affect protein ubiquitination [11,16,17] and interact with the TRIF protein [11]. To test the possibility that HBX was responsible for the TRIF downregulation, we transfected hepatoma cells with the HBV expression plasmid (pHBV), X-deficient HBV expression plasmid (pHBVX-) or HBX expression plasmid (pHBX) in combination with pHBVX-. The TRIF protein expression was reduced in pHBV transfectants but not in HBVX-transfectants, and it was restored to the wild-type level by introduction of pHBX (Fig. 2A). The expression of HBV core and X protein in Huh7 cells was detected to confirm successful transfection of these plasmids (Fig. 2A, bottom panel). Transfection of pHBX led to a dose-dependent decrease in expression of endogenous TRIF in Huh7 cells (Fig. 2B). To reconfirm the effect of HBX on TRIF protein levels, we transfected Huh7 cells with pTRIF plasmid together with increasing amounts of pHBX plasmid. The exogenous TRIF protein expression was also negatively modulated by HBX in a dose-dependent manner (Fig. 2C). In order to define whether the TRIF reduction occurred through proteasome-mediated degradation, we treated cells with the proteasome inhibitor MG-132 after transfection of pHBX. Treatment with MG-132 abrogated the decrease of TRIF (Fig. 2D), indicating that the HBX promoted TRIF degradation via the proteasomal pathway. Furthermore, the interaction between HBX and TRIF protein was not detected by co-immunoprecipitation in Huh7 and HepG2 cells co-transfected with the TRIF and HBX expression plasmids (Fig. 2E). Together, these results suggest that HBX indirectly induces proteasome-dependent TRIF degradation in hepatoma cells.

3.3. HBV replication and protein expression are inhibited by TRIF

To investigate the effects of TRIF on HBV replication and expression of its proteins, levels of HBsAg and HBeAg in the culture supernatants, cellular HBV replicative intermediate DNA, viral transcription and HBV core protein were measured in Huh7 cells after co-transfection with pTRIF and pHBV plasmids. Overexpression of TRIF dramatically inhibited HBV transcription and replication and reduced viral protein levels (Fig. 3A–D). These results were also confirmed in stable HBV-transfected HepG2.2.15 cells (Fig. 3E–H).

3.4. TRIF promotes apoptosis of hepatoma cells

To determine the effect of TRIF overexpression on cell apoptosis, we transfected Huh7 cells with pTRIF or control plasmid for 48 h, and then morphological features of the cells were examined under an inverted fluorescence microscope. In contrast to the controls, cells overexpressing TRIF appeared shrunken with broken membranes and showed detachment from the culture substrate, karyopyknosis, chromatin condensation and nuclear fragmentation, indicating that they were undergoing apoptosis (Fig. 4A–D). We further analyzed the caspase activity in HepG2 and Huh7 cells transfected with pTRIF or control plasmid. Caspase-3/7 activities were significantly increased in both cell lines when TRIF protein was upregulated (Fig. 4E). Hence, these results revealed that TRIF can induce apoptosis in hepatoma cells.

4. Discussion

The innate host defence establishes a state against viral survival within infected cells and neighboring uninfected cells [18]. As the target cells is the site of replication and the main location of virus–host interactions, intracellular host defences play important roles in limiting viral spread. TLR3 is a widely studied PRR which can recognize dsRNA generated during viral infection and consequently initiate a series of intracellular events that ultimately induce the production of antiviral effector genes [19,20]. As the adaptor of TLR3 signaling, TRIF is often deregulated as part of an evasion strategy by viruses. A recent study reported that expression of TRIF in peripheral blood mononuclear cells was decreased in chronic HBV infected patients when compared with healthy controls [21]. However, the impact of HBV expression on TRIF protein in hepatocytes has not been reported. To our knowledge, the present study is the first to report the decreased protein expression of TRIF in HBV-related hepatoma cells and liver tissues and to describe the HBX-mediated degradation of TRIF protein in hepatocytes, which presents a new mechanism for HBV evasion of the cellular innate immunity.

We demonstrated that HBX induced TRIF degradation through a proteasomal pathway in hepatoma cells. This was not surprising since the majority of intracellular proteins are degraded via the ubiquitin–proteasome pathway [22]. HBX has been reported to actively influence protein ubiquitination; for instance, HBX can cleave Lys63-linked polyubiquitin chains from many proteins [11] and induce ubiquitin-mediated protein degradation [16,17]. However, no interaction between the HBX and TRIF proteins was detected in our co-immunoprecipitation experiment, which conflicted with a previous result [11]. Thus, the underlying mechanism responsible for the downregulation of TRIF by HBX needs further investigation.

Activation of TLR3 signaling in hepatocytes by an agonist inhibits HBV replication [15]. However, whether the overexpression of the adaptor protein TRIF in hepatocytes elicits antiviral effects on HBV remains to be investigated. For example, although STAT-1 is the key protein in the signaling pathway for type I and type II IFNs, its overexpression is not sufficient to augment IFN signaling and inhibit HBV replication [23]. A previous study demonstrated that upregulation of a truncated TRIF protein (a mutant lacking the sequence responsible for apoptosis induction) in hepatoma cells potently inhibited HBV replication [10]. In this study, the inhibition of HBV replication and protein expression was also observed when the full-length TRIF protein was overexpressed. TRIF induces apoptosis [24,25]. We reconfirmed it in hepatoma cells. As a non-cytopathic virus, HBV uses the host cellular machinery to reproduce. In other words, host cell survival is a prerequisite for HBV persistence. From this perspective, the TRIF-induced apoptosis of infected cells effectively limits the spread of the virus, and we thus believe that downregulation of TRIF by HBV facilitate its survival in hepatocytes.

In conclusion, our results reveal that TRIF overexpression in hepatocytes inhibits the HBV replication and expression of viral proteins. HBV can mitigate this inhibition by degrading the TRIF protein, which may be a new strategy for evading host cellular innate immune responses. These findings enhance our understanding of the mechanisms of persistent viral infection and may provide guidance for new therapeutic strategies in the treatment of HBV.

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