Transforming Growth Factor-β1 Suppresses Hepatitis B Virus Replication Primarily Through Transcriptional Inhibition of Pregenomic RNA

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Transforming growth factor–beta1 (TGF-β1) is a pleiotropic cytokine with pivotal roles in the regulation of cellular functions and immune responses. In this study, we found that TGF-β1 was able to effectively suppress hepatitis B virus (HBV) replication. In the presence of TGF-β1, the level of viral replicative intermediates was dramatically decreased, both in actively dividing cells and in confluent cells. At the same time, the levels of viral transcripts, core protein, and nucleocapsid were significantly diminished by TGF-β1 treatment. Interestingly, the inhibitory activity of TGF-β1 was associated with preferential reduction of the level of pregenomic RNA compared with pre-C mRNA. Further analysis indicated that TGF-β1 might exert its antiviral effect primarily through reducing expression of the HBV core protein by transcriptional regulation instead of posttranscriptional modification. ***Conclusion:*** **TGF-β1 may play a dual role in HBV infection, in the suppression of immune responses against viral infection and in the direct inhibition of viral replication, resulting in minimization of liver damage in patients with chronic hepatitis.** (HEPATOLOGY 2007;46: 672-681.)

Hepatitis B virus (HBV) infection remains one of the most serious viral infections in humans. More than 350 million people worldwide suffer from chronic hepatitis B, many of whom develop cirrhosis and hepatocellular carcinoma.[1] The pathogenesis of HBV-induced liver diseases involves complicated mechanisms revolving around viral replication and immune responses against HBV infection. The molecular mechanisms of HBV replication have been studied extensively and are well documented,[2] whereas the immunological mechanisms against HBV infection are less understood. The distinct cell populations of the liver, namely, hepatocytes, Kupffer cells, sinusoidal endothelial cells, and stellate cells, are arranged in a highly organized manner. Hepatocytes and their surrounding nonparenchymal cells are known to secret various cytokines, including interferons, interleukin (IL)-1, IL-6, IL-10, IL-12, IL-18, TNF-α, and transforming growth factor–beta1 (TGF-β1).[3-5] It has been demonstrated that during HBV infection viral clearance occurs before destruction of infected hepatocytes in both the chimpanzee and the transgenic mouse model.[6,7] Some experimental results have implied that cytokines are involved in the suppression of HBV replication noncytopathically. Clinical observations indeed have revealed several cytokines to be elevated during the progression of hepatitis.[8] These cytokines are likely to be involved in the regulation of immune responses against viral infection and may directly inhibit viral replication. Recently, several cytokines including IL-18, IFNs, and TNF-α have been reported to be effective in the suppression of HBV replication both in the livers of HBV transgenic mice and in a cell culture system.[9-12] However, the effect of TGF-β1, which is the major cytokine secreted by hepatocytes and nonparenchymal cells, on HBV replication has not been studied.

Abbreviations: cccDNA, covalently closed circular DNA; HBcAg, HBV core protein; HBeAg, HBV e antigen; pgRNA, pregenomic RNA; TGF-β1, transforming growth factor–beta1.

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TGF-β1 is a pleiotropic cytokine that has biological effects on a variety of cellular responses, including cellular growth inhibition, cell differentiation, apoptosis, and extracellular matrix (ECM) formation.[13-16] In addition, a pivotal function of TGF-β1 is to modulate the immune response through the regulation of immune cells. It has been shown that TGF-β1 has an inhibitory effect on T-cell proliferation through the inhibition of IL-2 production[17] and the differentiation of T cells.[18] The addition of exogenous TGF-β1 to cultures of stimulated B cells inhibits subsequent proliferation and immunoglobulin (Ig) secretion.[19] Under clinical investigation, TGF-β1 is one of the cytokines that is elevated in patients with chronic hepatitis B (CHB).[20,21] Studies have revealed that patients with chronic hepatitis and cirrhosis have significantly higher levels of plasma TGF-β1 (3.0–3.7 ng/mL) than do normal subjects. In some patients with hepatocellular carcinoma (HCC), the concentration of plasma TGF-β1 may be as high as 19.3 ng/mL.[22] It has also been shown that the addition of TGF-β1 causes significant suppression of the antigen-specific and the antigen-nonspecific cellular and humoral immune responses of peripheral-blood mononuclear cells (PBMCs) in patients with CHB.[23] Furthermore, TGF-β1 has been reported to promote the development of liver fibrosis and cirrhosis,[24] and high cytokine levels are associated with the severity of liver fibrosis in patients with chronic hepatitis C.[25] Taken together, the results of these studies suggest that TGF-β1 may play an important role in regulating the progression of viral chronic hepatitis, cirrhosis, and carcinoma.

Recently, we established an HBV-producing cell line, 1.3ES2, that is capable of replicating virus efficiently and is able to maintain a steady pool of HBV covalently closed circular DNA (cccDNA).[26] We have demonstrated that TGF-β1 can effectively suppress HBV replication in 1.3ES2 cells. In the presence of TGF-β1, the levels of viral replicative intermediates, core protein, and viral transcripts decreased simultaneously. Further investigation indicated that TGF-β1 exerted its antiviral effect through preferential reduction of the synthesis of HBV pregenomic RNA (pgRNA) and core protein. Based on the observation that the concentration of TGF-β1 is significantly elevated in CHB patients and TGF-β1 can effectively suppress HBV replication in cultured liver cells, our results raise the possibility that TGF-β1 may play an important role in the course of HBV infection.

Materials and Methods

Cell Culture and Transfection. HepG2 and 1.3ES2 cells were maintained as previously described.[26] To assess the antiviral effect of TGF-β1, the cells were treated with 10 ng/mL of TGF-β1 (R&D System Inc., Minneapolis, MN). The culture medium was refreshed every 2 days during the experiments. For transfection, cells were grown on 60-mm dishes and transfected with 7 μg of plasmid DNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in serum-free medium. Transfection efficiency was monitored via cotransfection with pEGFP-N1 (Clontech, Mountain View, CA), and the experimental results were then normalized against the transfection efficiency.

Southern Blot Analysis. Twenty micrograms of total DNA was digested with *Hin*dIII and separated on a 1.2% agarose gel. The gels were treated as described,[26] and the DNA samples were transferred onto nylon membranes (Amersham, Freiburg, Germany). After ultraviolet crosslinking and prehybridization, the membranes were hybridized with [^{32}P]labeled DNA probe generated by a random-primed labeling kit (Amersham, Freiburg, Germany) using full-length HBV DNA.

Western Blot Analysis and Immunoprecipitation. One hundred micrograms of total protein was separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA). The membranes were then blocked with nonfat milk and incubated with anti-HBV core antibody (DakoCytomation, Glostrup, Denmark) or antiactin antibody (Sigma, St. Louis, MO). The immunoblot signals were examined using enhanced chemiluminescence reagent (PerkinElmer Life Sciences, Melbourne, Australia). HBV core protein (HBcAg) and HBV e antigen (HBeAg) were immunoprecipitated from an equal amount of culture medium or cell lysate using anticore antibody. The samples were precleared with normal serum (Sigma, St. Louis, MO)/protein-A–Sepharose beads (Pharmacia, Germany) and then incubated with anticore antibody/protein-A–Sepharose beads. After incubation, the immunoprecipitates were washed 3 times with NET buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40). The immune complexes were separated by 15% SDS-PAGE, and western blot analysis was carried out. The signals were quantified by densitometry analysis (AlphaEaseFC Image Analysis Software, Alpha Innotech, San Leandro, CA) to calculate the inhibitory effect of TGF-β1.

Particle Blotting Analysis. Analysis of intracellular HBV core particles was performed as previously described by Schneider et al.[27] Briefly, cell lysates were separated using a 1.2% native agarose gel and transferred onto polyvinylidene fluoride membranes. Core particles were examined by immunoblot analysis using anticore antibody.

To detect capsid-associated nucleic acids, the samples were transferred onto nylon membranes. Capsid-associated nucleic acids were released from the core particles in situ by denaturing the membranes with 0.2 N NaOH/1.5 M NaCl, and neutralizing with 0.2 N Tris-HCl/1.5 M NaCl. Finally, the membranes were hybridized with HBV-specific probe.

Northern Blot Analysis. Total RNA was isolated using TRIzol solution (Invitrogen, Carlsbad, CA), which was followed by phenol/chloroform extraction and isopropanol precipitation. Fifteen micrograms of total RNA was separated using a 1.2% formaldehyde-agarose gel and transferred onto nylon membranes. The membranes were then hybridized with ^{32}P-labeled DNA probes as already described.

Hirt Extraction. HBV cccDNA was isolated as previously described.[26] After complete lysis of the cells with 3 mL of Hirt solution, 750 μL of 5N NaCl was added to the cell lysate, and the mixture was incubated on ice overnight. The cell lysates were centrifuged to collect the supernatant and extracted with phenol/chloroform. HBV cccDNA was then precipitated by ethanol and examined by Southern blot analysis.

Detection of HBsAg. HBV surface antigen (HBsAg) in the culture medium was determined based on the manufacturer's protocol for the enzyme-linked immunosorbent assay (ELISA) kit (Evernew Biotech Inc., Taipei, Taiwan). Each experiment was performed in triplicate and repeated 3 times independently.

Ribonuclease (RNase) Protection Assay. The HBV-specific RNA probe, which was designed according to the previous study by Moriyama et al.,[28] was synthesized *in vitro* using T7 RNA polymerase in the presence of [α-^{32}P]deoxycytidine triphosphate (dCTP). The RNase protection assay was performed as described by Konishi et al.[29] Briefly, 100 μg of total RNA was hybridized with the RNA probe in molar excess. After digestion with RNase A and RNase T1 (Roche, Mannheim, Germany), the protected RNA was separated using an 8% polyacrylamide/7M urea gel and visualized by X-ray film exposure. Dephosphorylated ϕX174 *Hin*fI DNA marker was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and used as the size marker. The signals were quantified by densitometry analysis to calculate the inhibitory effect of TGF-β1. The RPA experiment was repeated 3 times independently.

Results

TGF-β1 Treatment Suppressed HBV Replication in Both Actively Dividing Cells and Confluent Cells. To evaluate the antiviral effects of TGF-β1 on the cell culture system, the HBV-producing cell line 1.3ES2 was established by stably transfecting HepG2 cells with a 1.3-fold HBV genome.[26] The 1.3ES2 cells were chosen because of the production of viral replicative intermediates and its capacity to support cccDNA formation. To identify the effective dose that interfered with HBV replication, various concentrations of TGF-β1 were tested in the culture medium. After prolonged treatment for 6 days, a reduction in the HBV replicative intermediates could be observed with TGF-β1 at concentration as low as 1 ng/mL, and the effect was maximal at 5–10 ng/mL of TGF-β1 (Fig. 1A). Interestingly, it has been reported that patients with chronic hepatitis have significantly higher levels of plasma TGF-β1 (3.0–3.7 ng/mL) than do healthy subjects.[22] Our data suggest that elevated TGF-β1 might play a role or roles in controlling the replication of HBV in a physiological environment. To investigate the kinetics of the TGF-β1 effect on reducing viral replicative intermediates, 10 ng/mL of TGF-β1 was supplemented 2 days after plating (day 2), and total cellular DNA was extracted from the cultured dishes every 2 days until day 8. The amounts of viral replicative intermediates increased continuously with time in the control cells, but no such increase was observed in the TGF-β1-treated cells (Fig. 1B). These results indicated that synthesis of the viral replicative intermediates was significantly suppressed by TGF-β1. Our data also revealed less HBV replication in actively dividing cells (before day 6) and then significantly increased HBV replication when the cells reached confluence (after day 6). Of significance was that the accumulation of the HBV replicative intermediates continued in the confluent control cells even after up to 30 days of culture (data not shown). The effect of TGF-β1 on cell proliferation was also detectable in the growth curves (Fig. 1C). Growth was inhibited by about 30% after 4 days of treatment with TGF-β1, and the magnitude of inhibition remained constant until day 8. There was no obvious apoptotic cell death detected by trypan blue staining and by DNA fragmentation analysis (data not shown) of both the TGF-β1-treated and the control groups. These results are similar to those of previous studies that showed HepG2 cells were apoptosis resistant when treated with TGF-β1.[30,31]

As shown in the previous experiment, optimal HBV replication occurred after cells reached confluence as compared to actively dividing cells. We next examined whether TGF-β1 treatment could also inhibit the synthesis of HBV replicative intermediates in the confluent cells. TGF-β1 was added after the cells had reached confluence on day 6 after plating, and total cellular DNA was extracted every 2 days until day 12. Similar to its effect on actively dividing cells, TGF-β1 also had an antiviral effect

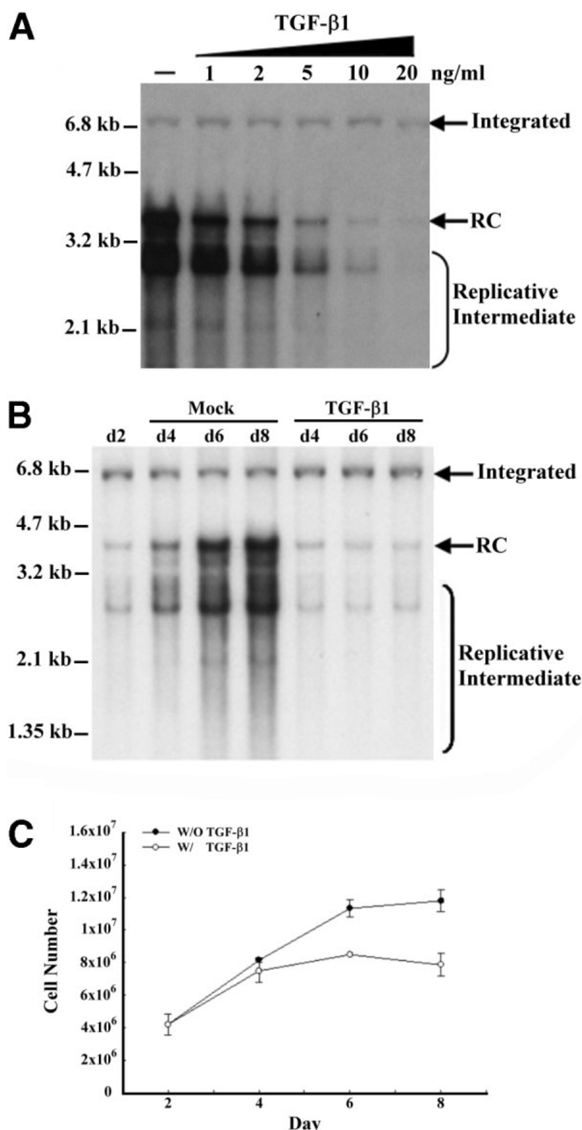

Fig. 1. Suppression of HBV replication by TGF-β1 in actively dividing cells. (A) Cells were treated with various doses (0, 1, 2, 5, 10, 20 ng/mL) of TGF-β1 2 days after plating to monitor the inhibitory effect of TGF-β1 on HBV replication. After prolonged treatment with TGF-β1 for 6 days, total DNA was extracted and subjected to *Hin*dIII digestion. The integrated viral genome and viral replicative intermediates were examined by Southern blot analysis using an HBV-specific probe. The intensity of the integrated HBV genome of each lane was used as internal control for an equal amount of sample loading. Bands corresponding to the integrated genome (integrated), the relaxed circular double-stranded DNA (RC), the replicative intermediates, and the size markers are indicated individually. (B) Cells were treated with or without 10 ng/mL of TGF-β1 2 days after plating (day 2) and kept in culture for 6 days (day 8). Total DNA was extracted at the indicated times, and *Hin*dIII digestion was carried out. The integrated HBV genome and viral replicative intermediates were examined as described above. (C) Curve of control and TGF-β1-treated cells. The cells were treated with or without 10 ng/mL of TGF-β1 2 days after plating and kept in culture for 6 days. Cell number was determined every 2 days by the trypan blue exclusive method.

in confluent cells. The amount of viral replicative intermediates decreased gradually with TGF-β1 treatment, unlike the continuous increase that occurred in the control cells (Fig. 2A). This result confirmed that TGF-β1

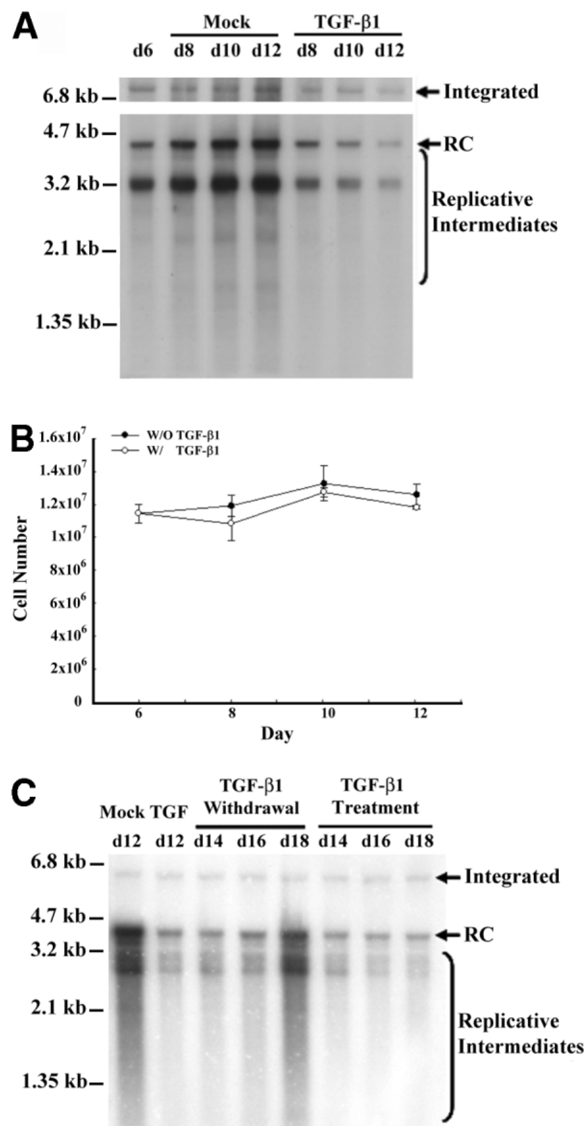

Fig. 2. Suppression of HBV replication by TGF-β1 in confluent cells. (A) Cells were treated with or without 10 ng/mL of TGF-β1 6 days after plating (day 6), when the cells had reached confluence, and were kept in culture for 6 days (day 12). Total DNA was extracted at the indicated times, and *Hin*dIII digestion was carried out. The integrated viral genome and viral replicative intermediates were then examined by Southern blot analysis using an HBV-specific probe. The intensity of the integrated HBV genome of each lane was used as internal control for an equal amount of sample loading. (B) Curve of control and TGF-β1-treated cells. The cells were treated with or without 10 ng/mL of TGF-β1 as mentioned above. Cell number was determined every 2 days by the trypan blue exclusive method. (C) After prolonged treatment with TGF-β1 for 6 days (day 12), TGF-β1 was removed from the culture medium, and then the culture was incubated in the absence (TGF-β1 withdrawal panel) or the presence (TGF-β1 treatment panel) of TGF-β1 for another 6 days (day 18). Total DNA was extracted at the indicated times, *Hin*dIII digestion was carried out, and subjected to Southern blot analysis to reveal the level of viral replicative intermediates.

could indeed suppress HBV replication and that this inhibitory effect was not limited to the growth stage of the cells. The effect of TGF-β1 on cell proliferation is shown in Fig. 2B. The growth inhibitory effect of TGF-β1 was not obvious for confluent cells because most of the cell population was growth-arrested in the G_1 phase (data not shown). To examine whether the TGF-β1 effect was irreversible, we subsequently washed out the cytokine on day 12 and maintained cells in the presence or the absence of TGF-β1 for 6 more days, from day 14 to day 18. The level of HBV replicative intermediates increased in the culture after TGF-β1 was withdrawn from the medium, whereas continued treatment with TGF-β1 maintained the decreased level of HBV replicative intermediates (Fig. 2C). These results indicated that the antiviral effect of TGF-β1 is reversible and not likely to be a result of TGF-β1-induced apoptosis.

TGF-β1 Treatment Suppressed HBV Replication through Reduction in Core Protein Synthesis. To investigate whether TGF-β1 could inhibit core particle formation, expression of intracellular core particles was measured with or without TGF-β1 treatment in actively dividing cells and in confluent cells (as specified in Figs. 1 and 2). Cell lysates were separated using a 1.2% agarose gel, and intracellular core particles were analyzed by the particle blotting method. Particle-associated core protein and embedded HBV nucleic acids were then detected by a specific anticore antibody and an HBV-specific probe, respectively. Following TGF-β1 treatment, the formation of core particles was significantly suppressed, as shown by the reduced levels of both core particles and encapsidated nucleic acids in the growing and confluent cells (Figs. 3A and 4A, upper panels). To explore the mechanism by which TGF-β1 reduced the synthesis of intracellular core particles, we further analyzed the expression of core protein in the cell lysates by Western blot. Core protein was also significantly decreased by TGF-β1 (Figs. 3A and 4A, lower panels). These results indicated that TGF-β1 treatment reduced the expression of core protein, which subsequently decreased expression of intracellular core particles in cells.

To address whether this TGF-β1-mediated core protein was mediated through interference with the expression of viral transcripts, total RNA was extracted and examined by Northern blot analysis. In contrast to the continuous accumulation of viral transcripts in the mock control, the amount of viral transcripts was reduced after TGF-β1 treatment in both dividing and confluent cells (Figs. 3B and 4B). However, the magnitude of the reduction was not as dramatic as the inhibition of HBV replicative intermediates, core particles, and encapsidated nucleic acids already described. Furthermore, in a similar

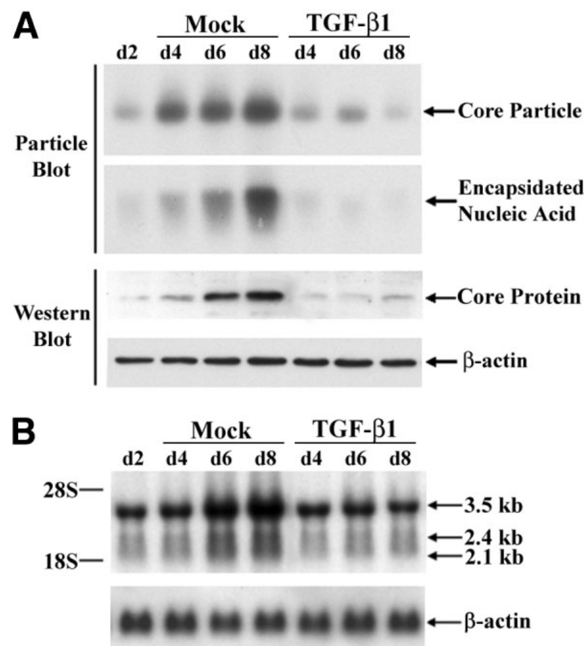

Fig. 3. TGF-β1 affects formation of intracellular nucleocapsid and synthesis of core protein and viral transcripts in actively dividing cells. (A) Cells were treated with or without 10 ng/mL of TGF-β1 2 days after plating (day 2) and kept in culture for 6 days (day 8). For particle blot analysis (upper panel), cell lysates were harvested at the indicated time points, and equal amounts of samples were assayed for core particles by native agarose gel electrophoresis and immunoblot analysis with antibodies against core protein. Core particle–associated viral nucleic acids were detected by Southern blot analysis of the disrupted core particles after transfer to a nitrocellulose membrane using an HBV-specific probe. For Western blot analysis (lower panel), cell lysates were subsequently separated by SDS-PAGE, and the core protein was detected by immunoblot analysis with specific antibodies. Expression of β-actin was used as an internal control for sample loading. (B) Total RNA was extracted at the same time points and subjected to Northern blot analysis to reveal the expression profile of the HBV transcripts, namely, the 3.5-, 2.4-, and 2.1-kb transcripts. The loading amount of total RNA was normalized using a β-actin-specific probe.

manner, the amount of viral transcripts increased gradually after withdrawal of TGF-β1 (Fig. 4C).

TGF-β1 Treatment Did Not Affect Existing Pool of HBV cccDNA. The observation that TGF-β1 effectively reduced the level of viral replicative intermediates raised the possibility that TGF-β1 might also affect the formation of HBV cccDNA. To examine the effect of cytokines on the amount of cccDNA produced, viral nucleic acid was prepared by Hirt extraction and subjected to heat denaturation and restriction enzyme digestion. Following melting at 85°C for 5 minutes, replicative intermediates including relax-circular and duplex-linear form DNA, but not cccDNA, were denatured into single-stranded DNA. Next *Eco*RI digestion was carried out to linearize the intact cccDNA, and mobility was shifted from the cccDNA position to the position of the duplex-linear form DNA (3.2 kilobases [kb]). Southern blot analysis

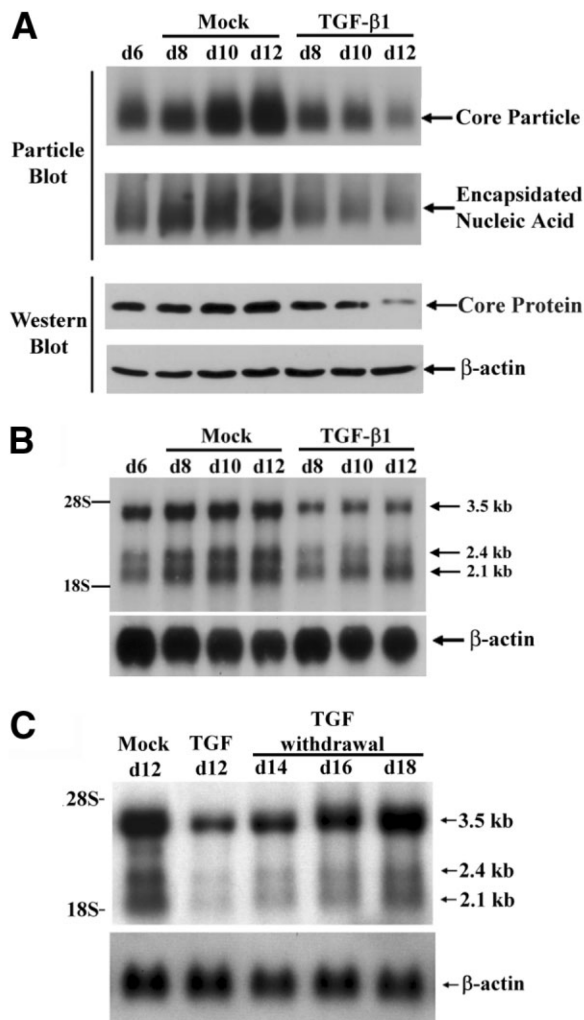

Fig. 4. TGF-β1 affects formation of intracellular nucleocapsid and synthesis of core protein and viral transcripts in growth-confluent cells. (A) Cells were treated with or without 10 ng/mL of TGF-β1 6 days after plating (day 6) and kept in culture for 6 days (day 12). For particle blot analysis (upper panel), cell lysates were harvested at the indicated times, and equal amount of samples were assayed for core particles by native agarose gel electrophoresis and immunoblot analysis using antibodies against the core protein. Core particle–associated viral nucleic acids were detected by Southern blot analysis of the disrupted core particles after transfer to a nitrocellulose membrane using an HBV-specific probe. For Western blot analysis (lower panel), cell lysates were subsequently separated by SDS-PAGE, and the core protein was detected by immunoblot analysis with specific antibodies. Expression of β-actin was used as an internal control for sample loading. (B) Total RNA was extracted at the same time points and subjected to Northern blot analysis to reveal the expression profile of the HBV transcripts. The loading amount of total RNA was normalized using a β-actin-specific probe. (C) After prolonged treatment with TGF-β1 for 6 days (day 12), TGF-β1 was withdrawn from the culture medium and the culture incubated in the absence of TGF-β1 for another 6 days (day 18). Total RNA was extracted at the indicated times and subjected to Northern blot analysis to reveal the expression profile of the HBV transcripts.

demonstrated that unlike the significant decline in the level of viral replicative intermediates in the presence of TGF-β1, the amount of HBV cccDNA was not affected by cytokine treatment (Fig. 5A,B). Evidently, TGF-β1 treatment was able to effectively suppress the production of viral replicative intermediates but failed to reduce the pool of cccDNA in our experiment.

TGF-β1 Treatment Differentially Suppressed Synthesis of HBcAg and HBeAg. Our results demonstrated that TGF-β1 treatment could efficiently decrease the expression of core protein (Figs. 3A and 4A). In contrast, the amount of HBsAg in the culture medium was not significantly altered according to the ELISA analysis. A representative result is shown in Fig. 6A. We used Western blot analysis to further examine whether TGF-β1 could exert a similar inhibitory effect on the expression of HBeAg. After treatment with TGF-β1 for 6 days, culture medium and cell lysates were prepared to determine the levels of secreted HBeAg, extracellular HBcAg (capsid-associated core protein), and intracellular HBcAg by the immunoprecipitation method. Previous studies have indicated

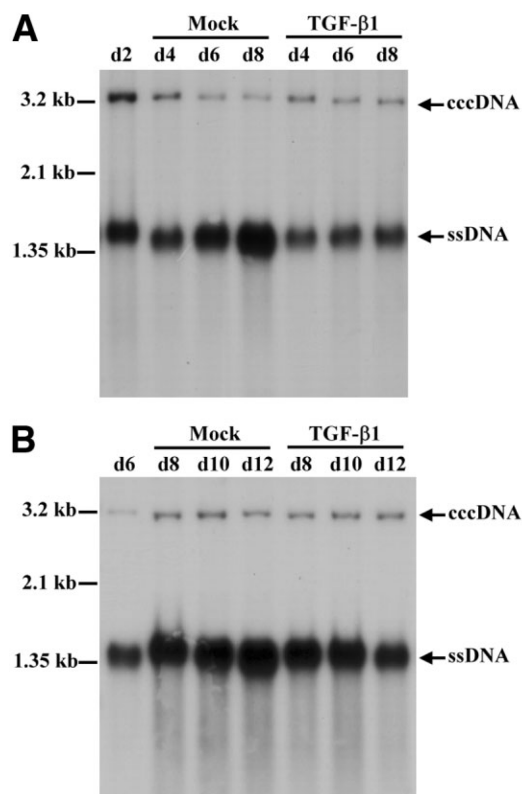

Fig. 5. Effect of TGF-β1 on the amount of HBV cccDNA. (A) Actively dividing cells (day 2) were treated with or without 10 ng/mL of TGF-β1 for 2, 4, and 6 days (day 4, day 6, and day 8) and subjected to Hirt extraction to prepare HBV cccDNA. The Hirt extract was thermally denatured at 85°C for 5 minutes, digested with *Eco*RI, and analyzed by Southern blotting using an HBV-specific probe. Bands corresponding to HBV cccDNA and HBV ssDNA are indicated. (B) Confluent cells (day 6) were treated with or without 10 ng/mL of TGF-β1 for 2, 4, and 6 days (day 8, day 10, and day 12) and subjected to Hirt extraction to prepare HBV cccDNA. The Hirt extract was thermally denatured at 85° for 5 minutes, digested with *Eco*RI, and analyzed by Southern blotting using an HBV-specific probe.

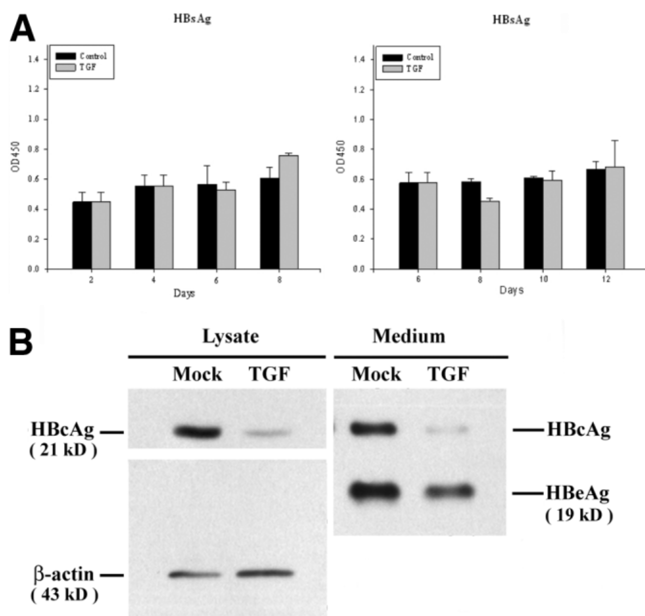

Fig. 6. TGF-β1 treatment differentially regulates expression of the HBV core protein (HBcAg) and HBeAg. (A) Actively dividing cells (day 2) or growth-confluent cells (day 6) were treated with or without TGF-β1 for 6 days (days 8 and 12, respectively). The levels of secreted HBsAg in the culture medium were measured at the indicated time points by ELISA. (B) Growth confluent cells were treated with or without TGF-β1 for 6 days. The culture medium and cell lysates were then harvested for the following experiments. An equal amount of cell lysate was directly subjected to SDS-PAGE, and core protein was detected by immunoblot analysis using specific antibodies. Expression of β-actin was used as an internal control for sample loading. Similarly, an equal amount of culture medium was immunoprecipitated with antibodies against the core protein. Samples were then subjected to Western blot analysis to examine HBcAg and HBeAg simultaneously. The signals were quantified by densitometry analysis to measure the percentage reduction in HBcAg and HBeAg caused by the TGF-β1 treatment.

that both enveloped and nonenveloped viral particles can be secreted in the tissue culture system.[32] We found that HBcAg and HBeAg had different sensitivities to TGF-β1 treatment. Densitometry analysis showed that the amount of HBcAg in the cell lysate and culture medium was dramatically reduced, by 75%, compared with that in the mock control, but HBeAg was only moderately reduced, by 24%, after a similar treatment (Fig. 6B). These results suggested that the expression of HBcAg and HBeAg is differentially regulated by TGF-β1.

TGF-β1 Treatment Induced Preferential Inhibition of HBcAg Synthesis Through Reduction in HBV pgRNA. The 3.5-kb transcripts contained 2 types of RNA, pre-C mRNA and pgRNA. These can be regulated coordinately or differentially. The pre-C mRNA encodes the precore protein, which is then processed into secreted HBeAg. In contrast, pgRNA encodes the core protein and polymerase protein, which are packaged into viral capsids.[33] Because expression of HBcAg, but not HBeAg, was dramatically decreased by TGF-β1, we postulated that pgRNA was probably more profoundly inhibited than was pre-C mRNA after TGF-β1 treatment. To investigate this idea, we performed an RNase protection assay (RPA) that uses a protection probe that can specifically distinguish between the expression of pgRNA and the expression of pre-C mRNA. A representative experiment is shown in Fig. 7A, demonstrating that the RPA probe was able to protect the 5′ end of pgRNA, the pre-C mRNA, and the 3′ ends of all the viral transcripts. These generated 3 sizes of protected fragments: a 66-nucleotide pgRNA, a 97-nucleotide pre-C mRNA, and a 121-nucle-

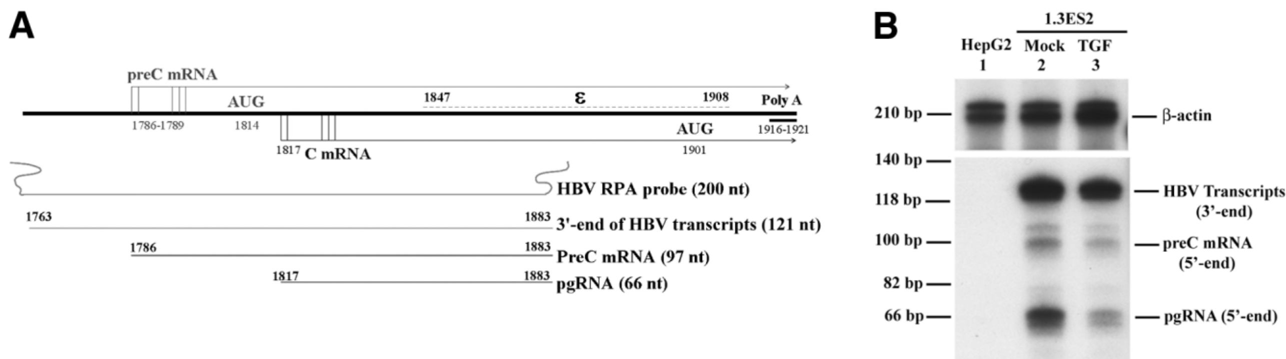

Fig. 7. TGF-β1 treatment differentially regulates the expression of pre-C mRNA and pregenomic RNA. (A) Illustration of the specific probe designed to distinguish pre-C mRNA from pgRNA by RNase protection analysis (RPA). The predicted size of the protected fragment was 66 nucleotides for pgRNA and 97 nucleotides for pre-C mRNA. The protected size of the 3′ ends of all viral transcripts was 121 nucleotides. (B) Growth-confluent cells were treated with or without 10 ng/mL of TGF-β1 for 6 days and subjected to RNA extraction. One hundred micrograms of total RNA was incubated with 3×10^5 cpm of the ^{32}P-labeled RPA probe, which was followed by RNaseA/RNaseT1 digestion. The protected RNA fragments were then separated by 8% polyacrylamide/7M urea gel electrophoresis and analyzed by exposure to X-ray film. HepG2 cells served as a negative control for HBV transcripts, and a β-actin-protected fragment was used as an internal control for sample loading. Fragments corresponding to the 3′ ends of all viral transcripts, the 5′ end of pre-C mRNA, the 5′ end of pgRNA, β-actin, and the size markers were indicated. The signals were quantified by densitometry analysis to measure the percentage reduction in the various RNA species induced by TGF-β1 treatment.

otide 3′ ended of all HBV transcripts. By densitometry analysis, our data demonstrated that despite TGF-β1 treatment resulting in a mere 20% reduction in the total amount of viral transcripts (Fig. 7B, the band indicating the 3′ end of the HBV transcripts), the expression of pgRNA was inhibited very significantly (75%) as compared to only a moderate reduction (30%) of pre-C mRNA (Fig. 7B). Therefore, the differential reduction of pgRNA and pre-C mRNA correlated closely with the differential expression of HBcAg and HBeAg in cells after TGF-β1 treatment. Taken together, this provided that the failure to form HBV nucleocapsids after TGF-β1 treatment may have primarily been a result of a specific reduction in both HBcAg and pgRNA.

To further investigate whether the TGF-β1-mediated reduction of HBV pgRNA was a result of transcriptional regulation or posttranscriptional modification, 2 HBV-related plasmids were constructed and transfected into HepG2 cells. The plasmid p1.3XHBV contained a 1.3-fold HBV genome, in which the transcription of pgRNA was controlled by the HBV core promoter. The other plasmid, p1XHBV, contained a 1-fold HBV genome in which the transcription of pgRNA was regulated by the cytomegalovirus (CMV) promoter. After transfection and treatment with or without TGF-β1 for 3 days, the capsid-associated viral genome and core protein were detected to evaluate the antiviral effect of TGF-β1. As shown in Fig. 8, our results demonstrated that TGF-β1 was able to suppress HBcAg synthesis and HBV replication when the cells were transfected with p1.3XHBV. However, TGF-β1 did not affect HBcAg synthesis and viral replication when cells were transfected with p1XHBV. These results indicated that TGF-β1 inhibited HBV replication primarily through transcriptional suppression of the pgRNA rather than by posttranscriptional modification. The molecular mechanisms involved in this TGF-β1-induced transcriptional suppression of pgRNA are now under investigation.

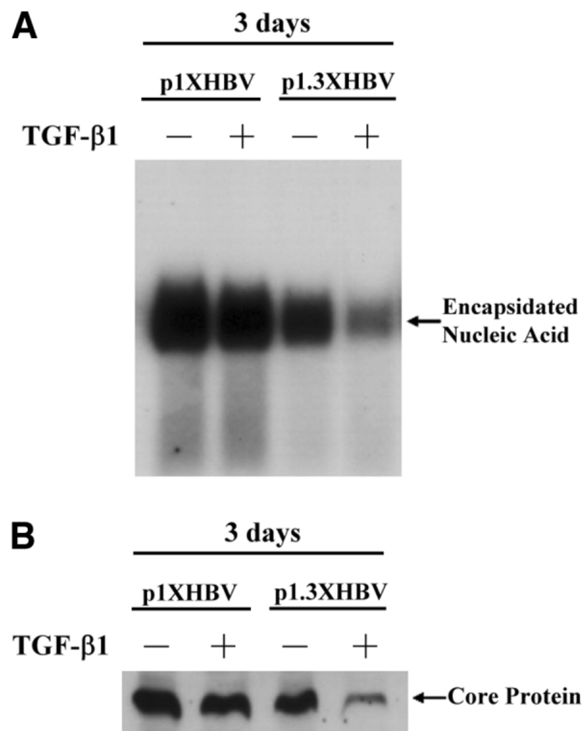

Fig. 8. Suppression of HBV replication by TGF-β1 was specifically mediated through interference with the HBV promoter. HepG2 cells were transfected with HBV genome–containing plasmids (p1.3XHBV contains a 1.3-fold HBV genome in which the transcription of pgRNA is controlled by the HBV core promoter and enhancer elements; p1XHBV contains 1-fold HBV genome in which the transcription of pgRNA is regulated by CMV promoter). These cells were treated with or without 10 ng/mL of TGF-β1 for 3 days to evaluate the inhibitory effect of TGF-β1 on HBV replication. (A) For particle blot analysis, cell lysates were harvested and separated by native agarose gel electrophoresis. Core particle–associated viral nucleic acids were then detected by Southern blot analysis of the disrupted core particles using an HBV-specific probe. (B) For Western blot analysis, cell lysates were separated by SDS-PAGE, and the core protein was detected by immunoblot analysis using a specific antibody against the HBV core protein.

Discussion

TGF-β1 Is Able to Effectively Suppress HBV Replication. In this study, we found that TGF-β1 could suppress HBV replication in an HBV-producing cell line. Our current results demonstrated first that TGF-β1 suppressed synthesis of viral replicative intermediates both in actively dividing cells and in growth-confluent cells and, second, that TGF-β1 exerted its antiviral effect primarily through suppressing expression of pgRNA, which encodes the core protein. Several pieces of evidence supported the hypothesis that this antiviral effect was not a result of TGF-β1-induced apoptosis. These include the lack of obvious apoptotic cell death as detected by trypan blue staining as well as the absence of DNA fragmentation in both the TGF-β1-treated cells and the control cells. Furthermore, the inhibitory effect on HBV replication could be reversed after removal of TGF-β1. This conclusion is also supported by earlier findings showing that HepG2 cells are resistant to TGF-β1-induced apoptosis.[30,31]

The present results also illustrated that TGF-β1 could be effective at a dose as low as 1 ng/mL and in this study was most effective at doses between 5 and 10 ng/mL. These effective doses are consistent with a range within the increased TGF-β1 levels found in the plasma of patients with chronic hepatitis, cirrhosis, and HCC.[22] Recently, TGF-β1 was found to suppress viral RNA replication and protein expression of the hepatitis C virus (HCV) replicon.[34] This result coincides with our finding and fully supports our observations that TGF-β1 could

be an antiviral cytokine and could be cooperating with other cytokines to restrict virus replication during chronic hepatitis. Furthermore, TGF-β1 can exert a potent immunosuppressive effect via its effects on lymphocyte proliferation, differentiation, and survival.[35] The presence of TGF-β1 cause significant dose-dependent inhibition of HBV-specific immune responses such as IFN-γ expression, antibody production, and proliferation of PBMCs in chronic hepatitis B patients.[23] These effects could minimize the inflammatory responses and consequently reduce the immunopathological effect of virus infection. Taken together, we speculated that TGF-β1 may play a dual role during HBV infection. TGF-β1 could possibly reduce the cytotoxic effect against HBV-infected hepatocytes, which is induced by immune responses against virus infection. On the other hand, TGF-β1 could directly inhibit HBV replication to restrict the outbreak of virus that results from the immunosuppressive effect. In this way, although the HBV replication cannot be completely eliminated, hepatocyte damage is restricted and the survival and functioning of the liver is prolonged. Therefore, TGF-β1 may be important to the progression and chronic character of HBV infection. However, whether this hypothesis is true *in vivo* and the possible applications of TGF-β1 in the therapy of HBV patients need to be investigated further.

Antiviral Effect of TGF-β1 Is Mediated Through Transcriptional Suppression of HBV pgRNA. We have demonstrated that there is a dramatic decrease in HBV core protein in the presence of TGF-β1 and that this process is mediated through a specific reduction in pgRNA. This raised the possibility that the reduction in pgRNA might be a result of a decrease in pgRNA synthesis at the transcription level or a degradation of pgRNA at the posttranscription level. We found that the promoter activity of the HBV core promoter elements was inhibited by TGF-β1 but that this inhibitory effect disappeared when the HBV core promoter elements were replaced by other transcriptional regulatory elements, namely, the CMV promoter. These results indicated that TGF-β1 induced a decrease in the synthesis of pgRNA primarily through transcriptional regulation rather than through posttranscriptional modification.

In contrast, TGF-β1 appeared to inhibit the synthesis of pre-C mRNA to a lesser extent than pgRNA. It is known that pgRNA encodes the core/polymerase proteins and is the substrate for viral encapsidation. Therefore, expression of pgRNA directly affects the level of viral replication. Previous studies have established that both ubiquitous and liver-specific transcriptional factors are able to contribute to the differential transcription of pre-C and pregenomic mRNA.[36] For example, overexpression of transcription factor SP1, hepatocyte nuclear factor 4 (HNF-4), peroxisome proliferators activated receptor α (PPARα), and retinoid X receptor α (RXRα) lead to increased synthesis of pgRNA but have little effect on the synthesis of pre-C mRNA.[37-39] On the other hand, a range of inhibitory effects have been reported for ectopically expressed hepatocyte nuclear factor-3β (HNF-3β), one of which is suppression of HBV replication associated with preferential reduction in the level of pgRNA relative to pre-C mRNA.[40] Apparently, our observation of the differential regulation of pgRNA and pre-C mRNA by TGF-β1 is not without precedent. Thus, it is possible that suppression of HBV replication occurs primarily through reduction in various hepatocyte nuclear factors that are specific to regulation of pgRNA transcription, leading to a decrease in the transcriptional activity of pgRNA.

Antiviral Mechanism of TGF-β1 Is Different from That of IFNs or TNF-α. It has been well documented that IFNs and TNF-α are able to suppress HBV replication in both the HBV-transgenic mice model and cell culture system. The antiviral effect of these molecules could act either on the regulation of assembly or on the stability of the pgRNA-containing HBV capsids; specifically, the expression of viral transcripts is not profoundly affected.[12] Furthermore, the antiviral activity of IFN-γ has been reported to be dependent on proteasome activation and Jak signaling,[41,42] whereas suppression of HBV replication by TNF-α would seem to be mediated through the NF-κB signaling pathway.[27] In this study, TGF-β1 was demonstrated to exert its inhibitory effect on HBV replication primarily through transcriptional repression of pgRNA. We also found that the expression of *MxA* (target gene for type I IFN) and *GBP1* (guanylate-binding protein 1, target gene for IFN-γ) were only slightly increased by TGF-β1 according to RT-PCR analysis. Our results clearly indicate that the regulatory mechanism by which TGF-β1 affects viral replication is distinctly different from those of IFN-γ and TNF-α. However, we cannot completely rule out the possibility of crosstalk between the TGF-β1 and IFN target genes and that this might play a minor role in the antiviral effect of TGF-β1. Because the cellular effects of TGF-β1 are transduced through activation of both receptor-regulated Smads and members of the mitogen-activated protein kinase (MAPK) family,[43] further study is needed of the signaling pathways crucial for the suppression of HBV replication or its possible cooperation with other cytokines.

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