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Antiviral effect of *Curcuma longa* Linn extract against hepatitis B virus replication

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A B S T R A C T

Ethnopharmacological relevance: A medicinal herb *Curcuma longa* Linn has been used for treating various liver diseases caused by hepatitis B virus (HBV) in Asia.
Aim of the study: The study was performed in order to investigate the antiviral activity of *Curcuma longa* Linn against HBV replication in liver cells.
Materials and methods: Aqueous extract of *Curcuma longa* Linn (CLL) was prepared and used to analyze its antiviral activity against HBV replication in HepG 2.2.15 cells, which contain HBV genomes. The inhibitory effect of CLL on HBV replication was examined by testing the levels of secreted HBV surface antigens (HBsAg), HBV DNAs, and HBV RNAs in HepG 2.2.15 cells using ELISA, Southern blot, and Northern blot analyses. Cytotoxic activities of CLL extract on various liver cells were analyzed by MTT assay. To dissect the inhibitory mechanism of CLL extract on HBV replication, the levels of p53 protein and p53 mRNAs were analyzed by Western blot and RT-PCR in HepG 2.2.15 cells. The repression of CLL extract on HBV transcription was analyzed by RT-PCR and CAT assay.
Results: : CLL extract repressed the secretion of HBsAg from HepG 2.2.15 cells. CLL extract also suppressed the production of HBV particles and the level of intracellular HBV RNAs in HepG 2.2.15 cells, suggesting that CLL extract inhibits HBV replication. We found that the anti-HBV activity of CLL extract is mediated through enhancing the cellular accumulation of p53 protein by transactivating the transcription of p53 gene as well as increasing the stability of p53 protein. It turned out that CLL extract repressed the transcription of HBx gene by suppressing HBV enhancer I and X promoter through p53 protein. In addition, CLL extract did not have any cytotoxic effects on liver cells.
Conclusion: These data showed that CLL extract represses HBV replication through enhancing the level of p53 protein. CLL extract can be used as a safe and specific drug for patients with liver diseases caused by HBV infection.

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1. Introduction

Liver diseases are major pandemics. In Africa and Asia, the main causes of liver diseases are viral infections. In addition, viral hepatitis has increased in Europe and America recently. Hepatitis B virus (HBV) infection can cause acute and chronic hepatitis, liver cirrhosis, and liver carcinoma in human (Ganem and Prince, 2004). Globally, two billion people are serologically positive in HBV infection. More than 360 million people chronically infected by HBV have higher risk for liver cirrhosis or hepatocellular carcinoma (HCC). HBV infection increases the risk for the development of HCC some 100-fold and is responsible for over 500,000 deaths each year from liver cirrhosis and HCC (Parkin et al., 2001, 2005).

In clinical practice, the therapy of HBV associated liver diseases consist of two general medicine categories, which are immunomodulators such as interferon-alpha (IFN-α) and direct antiviral nucleoside analogs such as lamivudine (Ganem and Prince, 2004; Farrell and Teoh, 2006). However, IFN-α is suitable for only a minority of patients and has severe side effects. Furthermore, it is expensive (Farrell and Teoh, 2006; Leemans et al., 2007). Nucleoside analogs also have disadvantages, such as long-term therapy and high drug resistance rate (Zoulim, 2004; Lok et al., 2007). The ideal treatment for patients infected with HBV should be effective and cheap with finite duration and little side effects. Although various treatments are implemented, the availability of anti-HBV drugs is currently far away from fulfill-

Abbreviations: HBV, hepatitis B virus; CLL, *Curcuma longa* Linn; HBsAg, HBV surface antigens; HCC, hepatocellular carcinoma; IFN-α, interferon-alpha; ELISA, enzyme-linked immunosorbent assay; HBx, HBV X protein.

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ing these conditions. Thus, new drugs are developed to be used alone or in combination with existing treatments. Recently, it has been reported that small interfering RNA effectively inhibited HBV replication (McCaffrey et al., 2003; Ren et al., 2006), yet it will take a while to use siRNA in clinical treatment. Therefore, alternative strategies are needed to combat HBV associated liver diseases.

Several surveys from Western countries have demonstrated that the use of herbal medicine is rapidly increasing (Kessler et al., 2001; De Smet, 2002). Herbal medicines are promising complementary ways of supporting patients, who are receiving conventional treatments, which include radiotherapy and chemotherapy (Boil, 1996). Recently, herbal medicines have been accepted as one of the main sources of drug development. Herbal medicines such as *Silymarin, Glycyrrhizin*, and *Phyllanthus amarus* show promising activities for liver diseases treatment, however, scientific proof of their efficacy in liver diseases is insufficient (Muriel and Rivera-Espinoza, 2007; Stickel and Schuppan, 2007).

CLL has been used as a traditional Asian medicine to treat gastrointestinal upset, arthritic pain, urinary tract infection, and liver ailments for a long time (Dixit et al., 1988; Luper, 1999). However, the precise pharmacological mechanism of CLL is mostly unknown. The main component curcumin also has been shown to have several pharmacological activities such as anti-inflammatory, anti-oxidant, and anti-microbial effects (Maheshwari et al., 2006). It also has been reported that curcumin inhibited the development of liver cirrhosis (Bruck et al., 2007).

Here, we report that CLL extract has antiviral activity against HBV by decreasing the transcription of HBV X (HBx) gene and does not have cytotoxicity on liver cells. We also found that CLL extract efficiently enhanced the cellular accumulation of p53 protein through transcriptional activation of p53 gene and inhibition of p53 protein degradation. These results indicated that the antiviral activity of CLL extract against HBV is due to, partly at least, the inhibition of HBV gene expression through p53-mediated pathway. Hence, CLL extract can be used as an efficient herbal medicine for viral liver diseases.

2. Materials and methods

2.1. Cell culture and transfection

Chang cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. HepG2 cells and HepG 2.2.15 (HBV producing HepG2 cells, which contain HBV genomic DNAs) were maintained in MEM-alpha. Transfection was performed with ExGen 500 transfection reagent (MBI Fermentas) according to the manufacture's direction.

2.2. Chemical compounds and antibodies

Curcumin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), cycloheximide, and anti-β-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-p53 antibody was purchased from Oncogene Science (Manhasset, NY). *Curcuma longa* Linn was obtained from a local market in Geumsan, Korea. The rhizoma of this plant was identified and authenticated by Prof. HS YOO. Voucher specimens (#CL-2005-03-Rh) have been deposited at the Institute of Traditional Medicine and Bioscience in Daejeon University. The dried rhizoma of *Curcuma longa* was boiled with distilled water for 3 h. Total extract was centrifuged at 5000 rpm for 30 min and the supernatant of the extract was lyophilized. The lyophilized extract was dissolved in distilled water to produce 100 g/L of CLL extract.

2.3. Plasmids

Wild-type p53 and dominant-negative mutant p53 (p53 DN) expression plasmids were provided by Dr. Baker (Johns Hopkins Univ., USA). The pHBVENHI-CAT plasmid containing HBV enhancer I and X promoter and the pHBVENHII-CAT plasmid containing HBV enhancer II were provided by Dr. Lee (Indiana Univ., USA).

2.4. Cell viability assay

HepG 2.2.15 cells (5×10^2 cells/96 well plate) were cultured with 200 mg/L and 500 mg/L of CLL extract for 0, 3, 6, and 9 days. After cultivation, cell viability was determined by MTT assay. Briefly, MTT (2 μg) was added to each well and incubated for 3 h. Then, culture medium was removed from each well by aspiration and DMSO was added to dissolve the purple formazan of MTT. The absorbance was read by microplate reader at a wavelength of 550 nm. For microscopic analysis, HepG 2.2.15 cells (3×10^5 cells/plate) were cultured with either 200 mg/L or 500 mg/L of CLL extract for 9 days.

2.5. Determination of the secreted HBV surface proteins

The secreted HBV surface proteins in culture media from HepG 2.2.15 cells treated with CLL extract at final concentrations of 200 mg/L and 500 mg/L for 9 days were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacture's protocol (Green Cross, Korea).

2.6. Preparation of viral particles and Southern blot analysis

HBV virus particles secreted in culture medium from HepG 2.2.15 were collected according to the method of Sells et al. (1987). HepG 2.2.15 cells (3×10^6 cells/plate) were cultured in the presence of CLL extract for 9 days, while the medium was changed every 3 days. Then, the culture medium was centrifuged at 2000 × *g* for 15 min and polyethylene glycol was added to the supernatant to a final concentration of 10% (v/v). The virus pellets were collected by centrifuging at 10,000 × *g* for 15 min and resuspended in TNE buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). The virus suspension was adjusted to 1% sodium dodecylsulfate (SDS) and 0.5 mg/ml proteinase K and incubated at 55 °C for 3 h. The digest was extracted with phenol:chloroform and the viral DNAs were precipitated with ethanol. The DNA pellet was dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and electrophoresed in 1% agarose gel followed by blotting onto Hybond-N$^+$ membrane (Amersham). The blot was hybridized with a ^{32}P-labeled HBV DNA probe in Church hybridization buffer (Church and Gilbert, 1984), washed with 2× standard saline citrate (SSC) and 0.2% SDS at room temperature for 1 h, and autoradiographed.

2.7. Northern blot analysis and RT-PCR analysis

Total RNAs were isolated from cells using easy-Blue RNA extraction kit (iNtRON Biotech). RNAs were subjected to electrophoresis on a 1% formaldehyde–agarose gel and transferred onto Hybond-N$^+$ membrane. HBV RNAs were detected with ^{32}P-labeled HBV DNA probes in Church hybridization buffer. The blots were then stripped and rehybridized with a ^{32}P-labeled GAPDH DNA probe for normalization.

For RT-PCR analysis, RNA samples were prepared from cells and were reverse-transcribed at 37 °C for 90 min using cDNA synthesis kit (Amersham). PCR was performed with the specific primers for the genes of HBV, p53, and β-actin; polymerase (P) gene (5′CCCTCCTTTCCTCACATTCA3′ and 5′TCCTTGTTGGGGTTGAAGTC3′), core (C) gene (5′TTCGAGATCTCCTCGACACC3′ and 5′GGCGAGGGAGTTCTTCTTCT3′), surface antigen (S) gene (5′GGAAGCTTCTACAGCA-

TGGGAGGT′3 and 5′GGGGGCCCGTTTGGTTTTATTA3′), X gene (5′GGAATTCGCACCTCATGTTA3′ and 5′GCTCTAGACTACTCCCCCAACTCC3′), p53 gene (5′CGTCTGGGGATCCTG CATTC3′ and 5′GTTCCGTCCCTCGAGATTACC3′), and β-actin gene (5′CAAGAGATGGCCACGGCTGCT3′ and 5′TCCTTCTGCATCCTGTCGGCA3′). PCR products were electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide.

2.8. CAT assay

HepG2 cells were transfected with plasmids by calcium phosphate method (Choi et al., 1997). At 24 h after transfection, cells were treated with CLL extract for 24 h. CAT assay was carried out as previously reported (Kim et al., 2005). CAT activity was quantified by measuring the conversion of [^{14}C] chloramphenicol to its acetylated form using an isotope scanner.

2.9. Western blot analysis

Cell lysates were prepared in lysis buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 1 mM orthovanadate, 0.5 mM dithiothreitol, and 20 μM leupeptin. Proteins were separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to Immobilon-P membranes (Millipore), and analyzed by Western blot using specific antibodies. To detect the stability of p53 protein, HepG2 cells were treated with 500 mg/L of CLL extract for 4 h and cultured in the presence of cycloheximide (3 μg/ml) to inhibit new protein synthesis.

3. Results and discussion

3.1. CLL extract has antiviral activity against HBV replication

Although CLL has been used for a long time as a traditional Asian medicine to treat liver ailments caused by HBV infection (Dixit et al., 1988; Luper, 1999), it is unclear whether CLL has antiviral activity against HBV replication. Hepatitis B surface antigen (HBsAg) and HBV DNA are general markers for HBV replication. The elimination of HBsAg is generally accepted as an indication of a complete cure state of HBV epidemiology along with the eradication of HBV DNA. *In vitro* antiviral activity of CLL extract was analyzed in HepG 2.2.15 cells, which secret HBV Dane particles and HBsAg in culture media. First, the level of HBsAg secretion was analyzed by ELISA in HepG 2.2.15 cells treated with 200 mg/L and 500 mg/L of CLL extract for 9 days (Fig. 1A). In HepG 2.2.15 cells, the level of HBsAg steadily increased in culture media depending on the culture periods. However, treatment of HepG 2.2.15 cells with CLL extract resulted in about an 80% inhibition of HBsAg secretion compared with untreated controls.

Although a decrease in HBsAg production indicates decrease in HBV replication, we further confirmed the anti-HBV activity of CLL extract by measuring the amount of HBV particles released in the culture media from HepG 2.2.15 cells. HBV particles were collected from the culture media of cells treated with 200 mg/L and 500 mg/L of CLL extract every 3 days for 9 days and HBV DNAs were prepared from HBV particles. The level of HBV DNA was analyzed by SDS-PAGE and Southern blot assay using ^{32}P-labeled HBV DNA probe. The treatment of CLL extract on HepG 2.2.15 cells reduced the level of HBV viral DNAs suggesting that CLL extract strongly inhibited the extracellular release of HBV particles from HepG 2.2.15 cells (Fig. 1B). Now, it is evident that CLL extract strongly suppresses HBV replication.

HBV transcription is the first step for viral replication after virus infection. The RNA transcript products of HBV are pregenomic RNA and various RNAs encoding for four different viral proteins. Pregenomic RNA (3.5 kb) encodes the viral core and polymerase, which is a reverse transcriptase that is required for HBV viral DNA replication. Viral RNAs of 2.4/2.1 kb encode the viral surface proteins. To confirm the effect of CLL extract on viral transcription, HepG 2.2.15 cells were cultured in the presence of CLL extract and HBV RNAs were prepared from HepG 2.2.15 cells. Northern blot analysis was performed to measure the levels of various HBV RNAs. It turned out that CLL extract suppressed the levels of HBV RNAs suggesting that CLL extract might repress HBV transcription (Fig. 1C). Therefore, the suppression of HBV replication by CLL extract is most likely the result from repression of HBV RNA transcription.

To test whether the suppression of HBV replication by CLL extract might be due to its cytotoxicity, cytotoxic effects of CLL extract were examined in HepG 2.2.15 cells by MTT assay and microscopic analysis. No apparent cytotoxicity of CLL extract was detected in HepG 2.2.15 cells up to 9 days suggesting that the suppression of HBV replication by CLL extract was not caused by its cytotoxicity (Fig. 1D). We also investigated the possible cytotoxicity of CLL extract in Chang cells derived from normal liver and no cytotoxicity of CLL extract was detected (Fig. 1D). In addition, there were no morphological changes induced by CLL extract in either HepG 2.2.15 cells or Chang cells (Fig. 1E). These data exhibited that CLL extract can be practically used as a safe herbal medicine to treat patients with liver diseases induced by HBV infection.

3.2. CLL extract elevates the level of p53 protein and its inhibitory effect on HBV replication is mediated by p53 protein

p53 protein is closely related to liver diseases including HCC (Martin and Dufour, 2008). Furthermore, mutation and deletion of p53 gene frequently occur in HCC (Wang et al., 2000). p53 protein is well known as a tumor suppressor associated with many cellular regulations such as cell cycle control, apoptosis, DNA replication, transcription, and DNA repair (Prives and Hall, 1999). p53 protein also has been reported to interfere with the replication of some viruses including HBV (Lee et al., 1995; Puisieux et al., 1995). p53 protein binds to HBV enhancer and represses the transcription from the HBV enhancer (Ori et al., 1998). It has been reported that some transcriptional inhibitors of HBV act through elevation of p53 protein (Chi et al., 1998). Since CLL extract inhibits the transcription of HBV, it is possible that the inhibitory effect of CLL extract on HBV transcription may be related to p53 protein.

To test whether CLL extract affects the transcription of p53 gene, HepG 2.2.15 cells were treated with CLL extract for 9 days and p53 RNA was analyzed by RT-PCR. The levels of p53 mRNA in HepG 2.2.15 cells were significantly increased by CLL extract (Fig. 2A). Next, we investigated whether the increase of p53 RNA by CLL extract in HepG 2.2.15 cells is dependent on the presence of HBV genome, since HepG 2.2.15 cells contain HBV genome. HepG2 cells, which are HCC cells deficient of HBV genomes, were treated with CLL extract and p53 RNA was analyzed by RT-PCR. As shown in Fig. 2B, CLL extract remarkably increased the levels of p53 mRNA in HepG2 cells suggesting that the increase of p53 RNA by CLL extract in HCC cells is independent of the presence of HBV genome.

It is also possible that CLL extract may regulate the stability of p53 protein. To test the effect of CLL extract on the stability of p53 protein, HepG2 cells were treated with CLL extract and were exposed to cycloheximide to prohibit new protein synthesis. The cell extracts were prepared and analyzed by Western blot with an anti-p53 antibody. While the level of p53 protein in HepG2 cells decreased rapidly after inhibition of protein synthesis, the stability of p53 protein in HepG2 cells exposed to CLL extract was

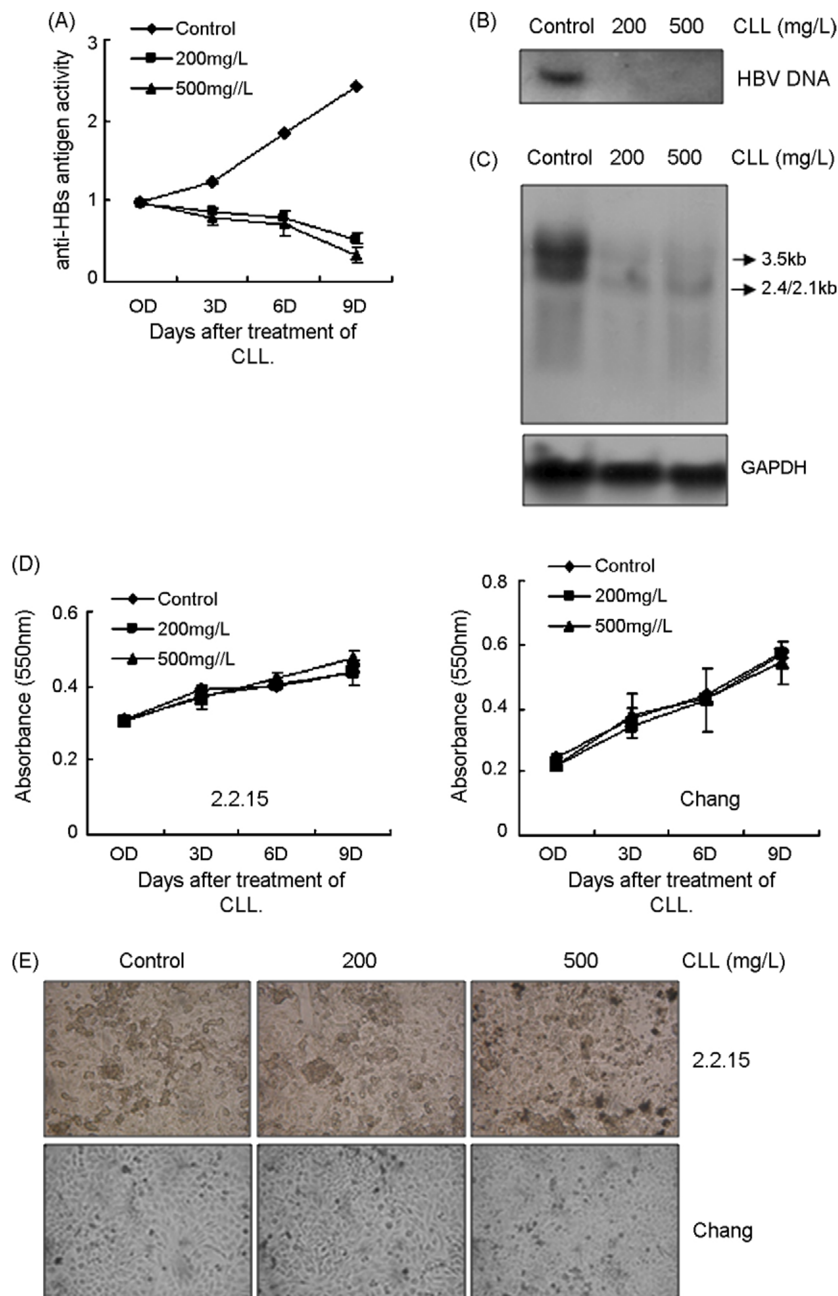

Fig. 1. Effects of CLL extract on HBV replication in HepG 2.2.15 cells. (A) CLL extract reduces the levels of secreted HBsAg in culture media from HepG 2.2.15. The levels of secreted HBsAg in culture media of HepG 2.2.15 cells untreated or treated with CLL extract for 9 days were analyzed by ELISA. (B) CLL extract reduces the levels of virus particles secreted in culture medium from HepG 2.2.15 cells. The secreted HBV particles in culture media of HepG 2.2.15 treated with CLL extract for 9 days were collected and HBV genomic DNAs were prepared. The level of HBV genomic DNAs were analyzed by Southern blotting with ^{32}P-labeled HBV specific DNA probe. (C) CLL extract represses HBV transcription. Total viral RNAs were prepared from HepG 2.2.15 cells and analyzed by Northern blotting with ^{32}P-labeled HBV specific DNA probe. (D) Cytotoxic activities of CLL extract on HepG 2.2.15 cells and Chang cells were analyzed by MTT assay. Data represent the mean ± S.D. of the three independent experiments. (E) The morphology of liver cells treated with CLL extract was analyzed with light microscopy.

prolonged, suggesting that CLL extract enhances the stability of p53 protein (Fig. 2C). It is clear that CLL extract enhances not only the transcription of p53 gene but also the stability of p53 protein.

To confirm whether the antiviral activity of CLL extract was due to the elevation of p53 protein, we investigated the role of p53 protein in regulating HBV replication by transient transfection of plasmids expressing wild-type and dominant-negative mutant of p53 protein into HepG 2.2.15 cells. We analyzed the effect of p53 protein on the production of HBV particles in HepG 2.2.15 cells by Southern blot analysis (Fig. 2D). The over-expression of p53 protein and the treatment of CLL extract abolished the production of HBV particles in HepG 2.2.15 cells. On the other hand, the expression of dominant-negative mutant p53 protein derepressed the HBV replication even in the presence of CLL extract in HepG 2.2.15 cells. We also analyzed the HBV transcription by Northern blot analysis (Fig. 2E). The expression of dominant-negative mutant p53 protein restored the HBV transcription suppressed by CLL extract in HepG 2.2.15 cells. In addition, the possible p53-mediated alteration in the viability of the cells was ruled out, because p53-transfected cells exhibited no difference in morphology and viability compared with the control cells (data not

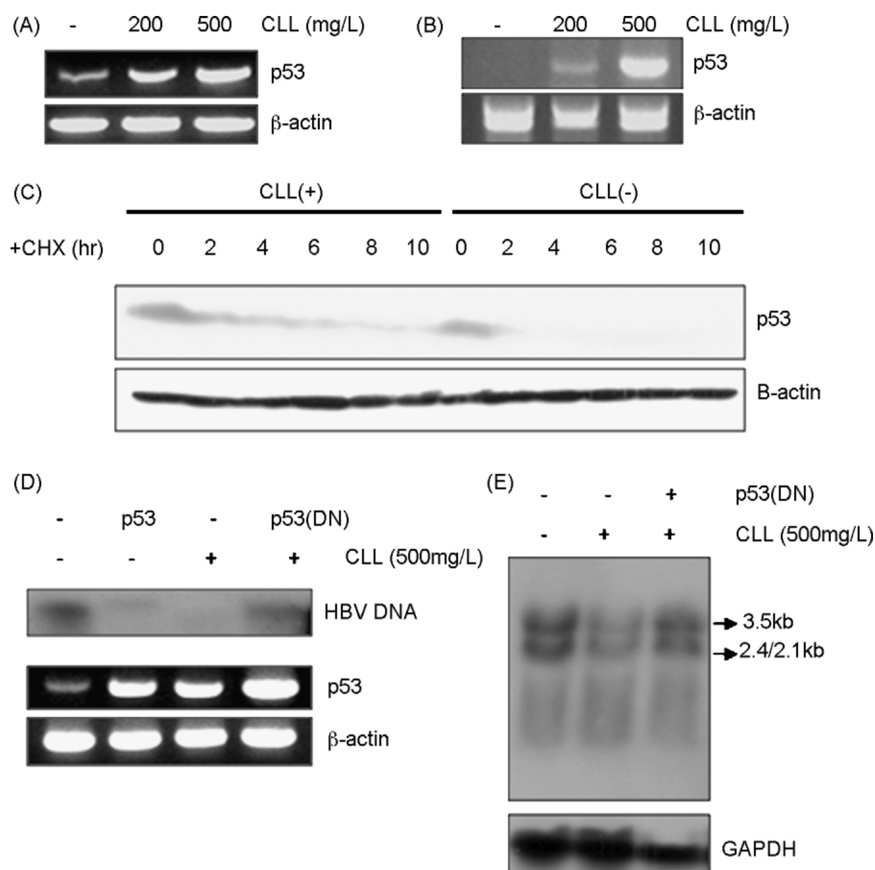

Fig. 2. The elevated level of p53 protein induced by CLL extract and p53-mediated repression of CLL extract on HBV replication. (A) CLL extract enhances the mRNA level of p53 gene in HepG 2.2.15 cells. Viral RNAs were prepared from HepG 2.2.15 cells treated with CLL extract and RT-PCR was performed with specific primers for p53 gene. (B) CLL extract enhances the mRNA level of p53 gene in HepG2 cells. Viral RNAs were prepared form HepG2 cells treated with CLL extract and RT-PCR was performed with specific primers for p53 gene. (C) CLL extract enhances the stability of p53 protein. HepG2 cells were treated with 500 mg/L of CLL extract for 4 h and were further incubated with cycloheximide (3 μg/ml) for 0, 2, 4, 6, 8, and 10 h. Cell lysates were prepared and the stability of p53 protein was determined by Western blot analysis. β-Actin is an internal control. (D) The HBV replication is repressed by p53 protein. HepG 2.2.15 cells were transfected with plasmids expressing either p53 protein or p53 dominant-negative mutant (DN) protein. At 48 h after transfection, cells were treated with 500 mg/L of CLL extract for 9 days. The culture medium was harvested and virus particles were precipitated with PEG. To quantify the level of virus particles, viral DNAs were prepared from virus particles and analyzed by Southern blotting with ^{32}P-labeled HBV specific DNA probe. (E) CLL extract represses the transcription of HBV genome through p53 protein. HepG 2.2.15 cells were cultured with CLL extract and transfected with plasmid expressing p53 DN protein. The viral RNAs were prepared from cells and subjected to Northern blot analysis.

shown). Taken together, we confirmed that p53 protein is an important regulatory mediator involved in the anti-HBV activity of CLL extract.

3.3. *CLL extract represses HBV enhancer and X promoter through regulation of p53 protein*

Since p53 protein plays an important role in the anti-HBV activity of CLL extract, we further analyzed the possible mechanism of how p53 protein regulates HBV replication in liver cells treated with CLL extract. HepG 2.2.15 cells were transfected with plasmids expressing either wild-type p53 protein or dominant-negative mutant p53 protein along with the treatment with 500 mg/L of CLL extract for 9 days. The levels of viral RNAs of HepG 2.2.15 cells treated with CLL extract were analyzed by RT-PCR using the primers of four HBV genes. Especially, CLL extract significantly decreased the level of HBx mRNA in HepG 2.2.15 cells. The mRNA level of viral polymerase gene was also slightly decreased (Fig. 3A). In addition, the repressed transcription of HBx gene was remarkably restored by dominant-negative mutant p53 protein. It has been reported that the transcription of HBx gene is derived by HBV enhancer I and X promoter, which is controlled by p53 protein that binds to a specific region within the HBV enhancer (Ori et al., 1998). To clarify the striking effect of CLL extract on the inhibition of HBx gene transcription, we analyzed the inhibitory effect of CLL extract on HBV enhancer I and X promoter. Since we confirmed that p53 protein is an important regulatory mediator involved in the anti-HBV activity of CLL extract, first, we tested the possible role of p53 protein in the repression of HBV enhancer I and X promoter by CAT assay. HepG2 cells were transfected with a CAT-reporter plasmid pHBVenhI-CAT containing HBV enhancer I and X promoter. As previously reported, p53 protein strongly repressed HBV enhancer I and X promoter in HepG2 cells (Fig. 3B-1). In addition, CLL extract suppressed HBV enhancer I and X promoter in a dose-dependent manner suggesting that CLL extract enhances the level of p53 protein and subsequently the elevated p53 protein suppresses HBV enhancer I and X promoter (Fig. 3B-2). About an 87% inhibition of transcription from HBV enhancer I and X promoter was observed in HepG2 cells treated with 500 mg/L of CLL extract (Fig. 3B-2). In addition, the suppression of HBV enhancer I and X promoter by CLL extract was partially restored by dominant-negative mutant p53 protein confirming that CLL extract inhibits the transcription of HBx gene through p53 protein-mediated pathway (Fig. 3B-2). Since HBx protein plays an essential role in HBV replication, the transcriptional repression of HBx gene by CLL extract through suppression of enhancer I and X promoter via p53-mediated pathway explain how CLL extract inhibits HBV replication.

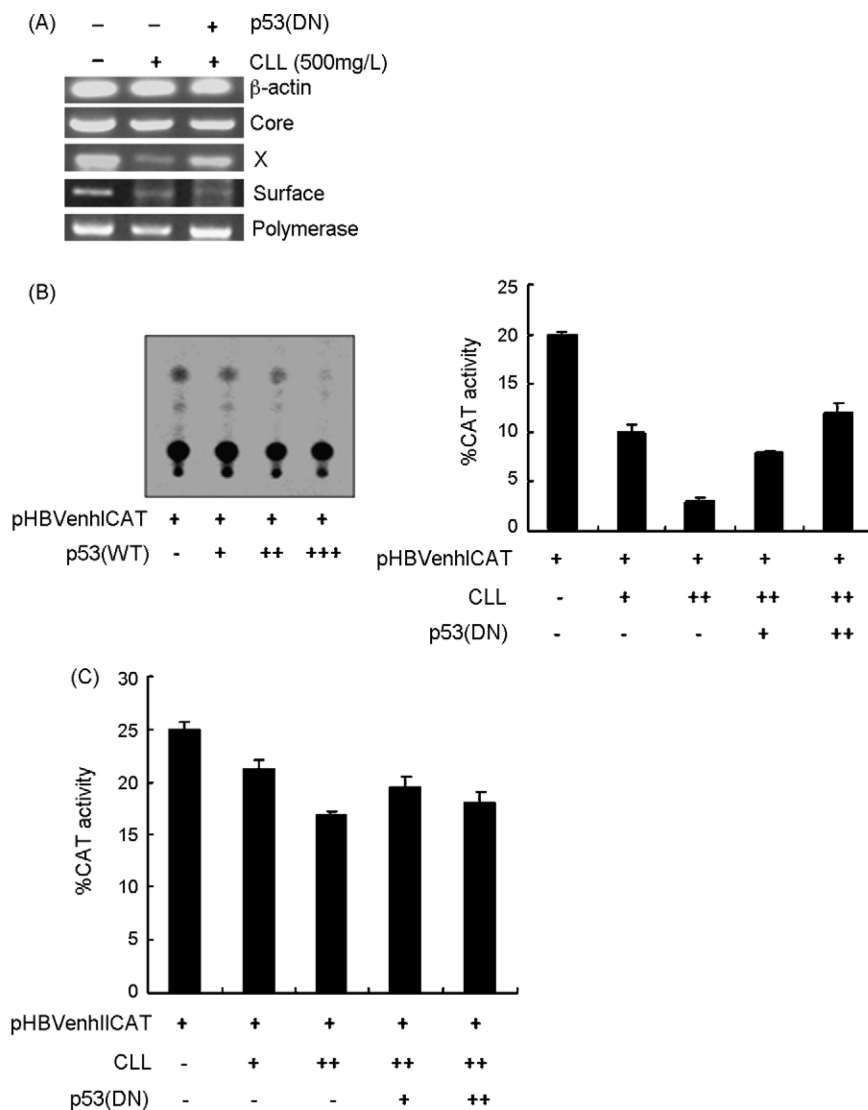

Fig. 3. The effect of CLL extract on the expression of HBV genes. (A) CLL extract represses the mRNA level of HBx gene in HepG 2.2.15 cells. HepG 2.2.15 cells were transfected with plasmids expressing p53 DN protein. At 48 h after transfection, cells were untreated or treated with 500 mg/L of CLL extract for 9 days. Viral RNAs were prepared from cells and RT-PCR was performed with specific primers for HBV genes. (B-1) To test whether p53 protein represses the HBV enhancer I/HBx promoter, various amounts of p53 expression plasmid (0, 0.25, 0.5, and 1 μg) were transfected into HepG2 cells along with 0.5 μg of pHBVenhI-CAT plasmid containing the HBV enhancer I/HBx promoter in front of CAT gene. At 48 h after transfection, cell lysates were prepared and CAT assay was performed. (B-2) HepG2 cells were transfected with reporter CAT plasmid, pHBVenhI-CAT, along with plasmid expressing p53 DN protein. At 48 h after transfection, cells were treated with 200 mg/L (+) and 500 mg/L (++) of CLL extract. Cell lysates were prepared and subjected to CAT assay. CAT activity was quantified by measuring the conversion of [^{14}C] chloramphenicol to its acetylated form using an isotope scanner. Data are mean values obtained from three independent experiments and bars represent standard deviation. (C) HepG2 cells were transfected with reporter CAT plasmid, pHBVenhII-CAT, containing the HBV enhancer II in front of CAT gene along with plasmid expressing p53 DN protein. At 48 h after transfection, cells were treated with 200 mg/L (+) and 500 mg/L (++) of CLL extract and CAT assay was carried out.

While HBV enhancer I is located upstream of the X promoter, enhancer II is located upstream of the core promoter and the polymerase promoter. Since CLL extract did not suppress the transcription of the core gene, though it suppressed the transcription of the polymerase gene slightly, we tested the effect of CLL extract on HBV enhancer II. HepG2 cells treated with CLL extract were transfected with pHBVenhII-CAT expressing CAT gene under the control of HBV enhancer II and CAT analysis was performed. We observed about an 28% inhibition of CAT activity in cells treated with 500 mg/L of CLL extract and CAT activity was partially restored by dominant-negative mutant p53 protein suggesting that p53 protein did not repress HBV enhancer II as much as enhancer I (Fig. 3C). Since CLL extract slightly represses HBV enhancer II while HBV enhancer II affects core promoter and polymerase promoter, it may explain why mRNA levels of core gene and viral polymerase gene were slightly regulated by CLL extract (Fig. 3A).

3.4. *Curcumin, a component of CLL extract plays a role in suppression of HBV replication*

We analyzed components of CLL extract by liquid chromatography. Among several components of CLL extract, there was a yellow pigment, curcumin (data not shown). Previous studies have demonstrated that curcumin is a compound derived from CLL and can be used for cancer prevention due to its anti-tumor, anti-atherogenic, and anti-inflammatory properties (Li et al., 2007). *In vivo*, curcumin suppresses carcinogenesis of the skin and the liver in mice (Chuang et al., 2000) and *in vitro*, it has been shown to inhibit the growth of a wide variety of tumor cell lines including colon, ovar-

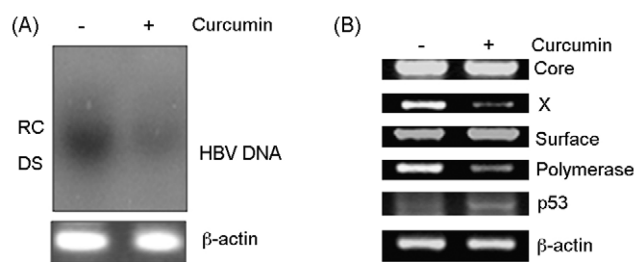

Fig. 4. Anti-HBV activity of curcumin in HepG 2.2.15 cells. (A) Virus particles were precipitated with PEG from the culture medium of HepG 2.2.15 cells treated with 100 μM curcumin for 9 days. Viral DNAs were prepared from precipitated virus particles and analyzed by Southern blotting with ^{32}P-labeled HBV specific probe. (B) Viral RNAs were prepared from HepG 2.2.15 cells and RT-PCR was performed using specific primers of HBV genes.

ian, breast, bladder, and prostate cancers (Mehta et al., 1997; Collett and Campbell, 2004; Choudhuri et al., 2005; Shi et al., 2006; Tong et al., 2006). To investigate whether curcumin plays a role in anti-HBV activity of CLL extract, HepG 2.2.15 cells were treated with 100 μM curcumin for 9 days and HBV particles in media were collected. HBV DNAs were isolated from HBV particles and analyzed by Southern blot assay. Curcumin reduced the level of HBV particles secreted from HepG 2.2.15 cells (Fig. 4A). We also tested whether curcumin could regulate the mRNA levels of endogenous p53 gene and HBV genes in HepG 2.2.15 cells. HepG 2.2.15 cells were treated with 100 μM curcumin for 6 days and total RNAs were analyzed by RT-PCR. Curcumin increased mRNA level of endogenous p53 gene and decreased mRNA levels of HBx gene and P gene, while it had no effect on the transcription of C gene and S gene (Fig. 4D). These data coincided with those of CLL extract. Manzan et al. reported that the highest yield of curcuminoid containing curcumin and its derivatives in CLL extract was approximately 0.16% of the extract (Manzan et al., 2003). Accordingly, in our experiments, 500 mg/L of CLL extract was used to test the anti-HBV activity which might maximally contain 0.8 mg/L of curcumin. We used 100 μM of curcumin which is 36.8 mg/L to test the anti-HBV activity of curcumin. Interestingly, 5 mg/L of curcumin had no effect on anti-HBV activity (data not shown). These results suggest that while curcumin plays a role in anti-HBV activity of CLL extracts, CLL extract possesses higher anti-HBV activity when compared with curcumin alone (compare Fig. 1B with Fig. 4A). Now it is evident that CLL extract contains curcumin and possibly other soluble components which act synergistically with curcumin for anti-HBV activity.

In this study, we demonstrated that CLL extract suppressed the HBV replication and the transcription of HBV genes in HepG 2.2.15 cells which produce HBV particles. We also found that CLL extract specifically decreased the transcription of HBx gene. Interestingly, CLL extract elevated the transcription level of endogenous p53 gene in a dose-dependent manner and inhibited the degradation of p53 protein in HepG2 cells. In addition, it turned out that the elevated p53 protein induced by CLL extract represses HBV enhancer I and X promoter and subsequently represses the HBV replication. Moreover, CLL extract does not have any cytotoxic effect on human liver cells. Taken together, our results indicate that CLL extract containing anti-HBV activity could possibly be used for the treatment of patients with liver diseases caused by HBV infection.

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