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Green tea extract and its major component epigallocatechin gallate inhibits hepatitis B virus in vitro

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Abstract

Hepatitis B virus (HBV) infection is endemic in Asia and causes major public health problems worldwide. Present treatment strategies for HBV infections are not satisfactory and the clinical limitation of current antiviral drugs for HBV, such as lamivudine, is causing rapid emergence of drug-resistant viral strains during the prolonged therapeutic treatment. In this research, the efficacy of a natural green tea extract (GTE) against HBV in a stably expressed HBV cell line HepG2-N10 is examined. The expression of viral antigens, HBsAg and HBeAg, were determined by using enzyme linked immuno-absorbent assay (ELISA). Quantitative real-time-PCR (Q-PCR) was used for the determination of extracellular HBV DNA and intracellular replicative intermediates and nuclear covalent closed circular DNA (cccDNA). HBV mRNAs were also analyzed by reverse transcription PCR (RT-PCR). Results showed that the 50% effective concentration (EC_{50}) of GTE on HBsAg, HBeAg, extracellular HBV DNA and intracellular HBV DNA were 5.02, 5.681, 19.81, and 10.76 μg/ml, respectively. While the concentration of GTE with the inhibition percentage of 50% on proliferating cells (CC_{50}) was 171.8 μg/ml. Similar analysis of the principal component of GTE, epigallocatechin gallate (EGCG), revealed it has relative weaker efficacy compared to GTE.

Keywords: Hepatitis B virus; Green tea extract; Epigallocatechin gallate; HBeAg; HBsAg HBV DNA; Repliction intermediates; cccDNA

1. Introduction

Hepatitis B virus (HBV) infection is one of the major public health problems worldwide. The World Health Organization (WHO) estimates that one third of the world's population has been infected by HBV and 5% of them are chronically infected (Lee, 1997). Additionally, previous results have shown that HBV plays a major role in occurrence and development of liver fibrosis (Kelleher and Afdhal, 2006; Sprengers et al., 2002) and hepatocelluar carcinoma (Cha et al., 2004; Koike, 1995; Tang et al., 2006). Interferon and nucleotide analogs such as lamivudine (3TC), adefovir and entecavir are approved drugs used for the treatment of HBV infection. However, relative lower cure rate, side effects and quick accumulation of drug-resistant mutants have limited their application (Dienstag et al., 1999; Fattovich et al., 1988; Lai et al., 1998). The unsatisfactory therapeutic application of interferon (IFN) and nucleotide analogs have strengthened the need for novel anti-HBV agents.

Green tea and its major constituent polyphenols are best known for their various biological and pharmacological properties including anti-oxidative (Song et al., 2005), antibacterial (Stapleton et al., 2004), antitumor (Mukhtar and Ahmad, 2000; Yang et al., 2002) and antiviral activity (Chang et al., 2003; Song et al., 2005). Studies have shown that green tea extracts (GTE) and its major component, epigallocatechin gallate (EGCG) can inhibit the invasion of human umbilical vein endothelial cells (Yamakawa et al., 2004), prevent tumor blood vessel growth (Pfeffer et al., 2003) and offer protection against mutagenesis (Lee et al., 2003). The anti-diabetic application of GTE has been validated in animal models with insulin resistance (Wu et al., 2004). GTE comprises other biological activities such as anti-inflammatory (Dona et al., 2003) and anti-aging activity as well (Hsu, 2005). EGCG has also been found to be capable of strongly inhibiting the replication of HIV in cultured peripheral blood cells (Fassina et al., 2002) by binding directly to CD4 and interfering with the binding of gp120 to the host receptor (Kawai et al., 2003). Antiviral activities of EGCG have also been

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reported against the infection of influenza virus (Imanishi et al., 2002; Nakayama et al., 1993; Song et al., 2005) and inhibiting the expression of antigenic proteins from Epstein–Barr virus (Chang et al., 2003), as well as influencing the expression of host regulation factors essential for viral replication (Weber et al., 2003).

In this study, the anti-HBV activity of GTE and its major component EGCG were investigated in a stably expressed HBV cell line, HepG2-N10. HBV antigens, the extracellular HBV DNA, intracellular replicative intermediates, nuclear covalent closed circular DNA (cccDNA) as well as HBV mRNA were determined after the treatment with GTE and EGCG. Our results indicated that both GTE and EGCG are efficient HBV inhibitors, but EGCG alone was not as efficient as GTE, indicating there might be other active components of GTE.

2. Materials and methods

2.1. GTE, EGCG and 3TC

The GTE used in this study was a product of the Xasino-herb Corporation, China and the standard sample of EGCG (99%) was a product of Hangzhou Gosun Biotechnologies Corporation, China. 3TC was supplied by the National Center for Drug Screening of China. Except for the HPLC experiment, the GTE, EGCG and 3TC were dissolved in DMSO and stored at −20 °C. Stocks were diluted with culture medium to the desired working concentration and with DMSO for concentrations lower than 0.2% (v/v).

2.2. HPLC analysis of the components of the GTE

The components of GTE were determined by HPLC using a Gilson supeclo column (5 μm, 4.6 mm × 250 mm, Gilson analysis system). The EGCG and GTE were dissolved individually in methanol to working concentrations of 1.2 and 0.8 mg/ml, respectively. Dissolved samples were filtered through a 0.45 μm filter before injection into the column. The mobile phase was doubly distilled water/methanol/phosphoric acid with a volume ratio of 65:35:0.1. The mobile solution was degassed with sonication. The isocratic elution flow rate was 1.0 ml/min and the measurement wavelength was 280 nm.

2.3. Cell line

The human hepatoblastoma cell line HepG2-N10 used in this analysis was kindly provided by Dr. Ningshao Xia of Xiamen University. The cell line was generated by transfecting HepG2 cells with a transfer plasmid which contains a 1.3 unit length of genotype A HBV genome (subtype adw2). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 380 μg/ml G418 (Sigma, St. louis, MO, USA). The cells were incubated in humidified incubators at 37 °C with 5% CO_2 (Innaimo et al., 1997).

2.4. MTT assay

The cytotoxicity of the drugs to HepG2-N10 cells was detected by determining the survival rate of the cells using the MTT method. The stock solution of 3-(4, 5-dimethylthiozol-2-yl)-3,5-dipheryl tetrazolium bromide (MTT, Sigma) was prepared using sterilized phosphate-buffered saline to 5 mg/ml. For each well of 96-well plates (Corning, USA), 200 μl of 2×10^5/ml HepG2-N10 cells was added and cultured at 37 °C for 24 h. The medium was removed and replaced with fresh medium containing GTE or EGCG of various concentrations at an interval of every 3 days. The control wells contained an equivalent amount of solvent. After 6 days, the cells were treated with the MTT assay as described elsewhere (Liu et al., 2003). The culture medium was removed and washed twice with PBS. MTT reagents were then added to the monolayer of cells. After incubation at 37 °C for 4 h, the formanzan particles were solubilized by adding 150 μl DMSO and the absorbance at wavelength of 490 nm was measured. The inhibition rate of the cells (%) was calculated as [average value of the control wells (A490-A630) − average value of the study wells (A490-A630)]/average value of the control wells (A490-A630) × 100%. The concentration of the drugs with an inhibition percentage of 50% on proliferating cells (CC_{50}) were calculated according to Berkson logit method (Berkson, 1968). Concentrations below CC_{50} were selected for the following antiviral experiments.

2.5. Treatment of GTE, EGCG and 3TC on HepG2-N10 cells

One milliliter of 1×10^5/ml HepG2-N10 cells was added into each well of the 24-wells plastic plate (Corning, USA). After incubation at 37 °C for 24 h, fresh culture media with a variety of concentrations of GTE, EGCG or 3TC were added and replaced every 2 days. After the treatment with the drugs for 6 days, the media and the cells were collected and used for further experiments.

2.6. Detection of the expression of antigens of HBV in vitro

The expression levels of HBsAg and HBeAg were detected by commercial ELISA kit (HRP) (Shanghai Kehua Bio-engineering Corporation, China) according to the manufacture's instructions. The media samples were collected after 6 days of treatments, centrifuged at 5000 r/min for 10 min to drop cellular debris, and then immediately used for ELISA. The samples were diluted to appropriate concentration with PBST buffer (pH7.4, tween, 0.5%) before measurement according to the manufacturer's instructions. Inhibition rates (%) were calculated as [average value of the control wells without drug − average value of the study wells (A450-A630)]/[average value of the control wells without drug (A450-A630) − average value of negative control wells (A450-A630)] × 100%. 3TC was used in these assays as a positive control.

2.7. Quantitative real-time PCR

To determine the quantity of extracellular HBV DNA in the medium and intracellular replicative intermediates within the treated cells, quantitative real-time PCR (Q-PCR) was performed with Eva green fluorescence dye. The primers used for Q-PCR were HBV-F: 5′-ACTTCGCTTCACCTCTGCACG-3′ and HBV-R: 5′-GGCAACATACCTTGA TAATCCAGA-3′. The target PCR fragment was located at the surface antigen S gene. A plasmid containing the cloned HBV genome was used as a standard. Viral DNA was extracted using an adapted salt-extraction method (Aljanabi and Martinez, 1997). To analyze the intracellular replicative intermediates, the cells treated with various drugs were lyzed with 500 μl lysis buffer (0.5% NP-40, 20 mM Tris, PH 7.5, 150 mM NaCl, and 1 mg/ml bovine serum albumin) (Hirsch et al., 1988). The lysate was centrifuged at 2000 rpm for 5 min and the virons in the supernatant were separated by addition of equal volume of 20% PEG-8000 and centrifuged at 12,000 rpm for 20 min after being cooled at 4 °C for 2 h. The intermediates DNAs in pellet were then extracted using the adapted salt-extraction method. The real-time PCR program consists of an initial denaturing step at 95 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 15 s at 58 °C and 30 s at 72 °C.

To determine the quantity of cccDNA, a pair of specific PCR primers across two gaps of relaxed circular HBV genome was synthesized. The objective fragment was expected to be amplified from the cccDNA template but not from the relaxed circular HBV DNA (rcDNA). The forward primer was HBV-ccc-F: 5′-CGGTCTGGGGCAAAGCTCAT-3′ and the reverse one was HBV-ccc-R: 5′-GCACAGCTTGGAGGCTTGA-3′. The cccDNA presented in the nuclear of the harvested cells were extracted using a plasmid rapid isolation kit (BioDev.) according to manufacturer's instructions. To further eliminate the rcDNA, the isolated cccDNA was treated with Plasmid-Safe ATP-depended DNase (EPICENTRE) for 2 h at 37 °C. This ATP-depended DNase degrades linear single-stranded and double-stranded DNA, but has little activity on closed circular double-stranded DNA. The PCR was initiated by 2 min at 95 °C followed by 15 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C for 38 cycles.

2.8. RT-PCR analysis of HBV mRNA

The total mRNA of HBV of GTE treated cells were detected by RT-PCR using a one-step RT-PCR kit (Qiagen, CA) with a pair of primers located at the X gene of the HBV genome The forward primer was HBV-X-F: 5′-CCTTCTTACTCTACCGTTCC-3′ and the reverse one was HBV-X-R: 5′-GACCAATTTATGCCTACAGCC-3′. Total HepG2-N10 RNA was extracted from the collected cell samples with trizol reagent (Invitrogen, USA). The extracted RNA was digested with RNase-free DNaseI (Promega, USA) for 30 min according to the manufacturer's instructions. One microlitre of total RNA was used for RT-PCR. As an internal reference, an β-actin gene segment was amplified with primers β-actin-F: 5′-CACCAACTGGGACGACAT-3′ and β-actin-R: 5′-ACAGCCTGGATAGCAACG-3′. The program of RT-PCR was 50 °C (30 min), 95 °C (15 min), followed by 35 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (30 s). The RT-PCR products were analyzed by electrophoresis.

2.9. Statistical analysis

Statistical analysis was performed by using the SPSS software package (SPSS for windows release 13.0. Inc., Chicago, IL, USA). Results were shown as mean value ± S.D. The Tukey test of significant difference was used to evaluate the difference between the test samples and control. A P value < 0.05 was considered statistically significant. The one-way ANOVA test was used to evaluate the difference between the test samples. EC50 and TI50 were calculated with Berkson logit method (Berkson, 1968).

3. Results

3.1. HPLC analysis of GTE and EGCG

The GTE and EGCG were determined by HPLC according to the procedure described in Section 2. Results are shown in Fig. 1. The retention time of standard EGCG was detected at 5.792 min (Fig. 1A). At least nine components were determined in GTE sample by using HPLC. The highest peak was the fifth one, which had a same retention time as the standard sample of EGCG. This indicated that the main component of GTE was EGCG (Fig. 1B). Apart from EGCG, the peaks corresponded to unknown products, among which peak 6, 7, and 9 had larger peak areas in comparison to the rest.

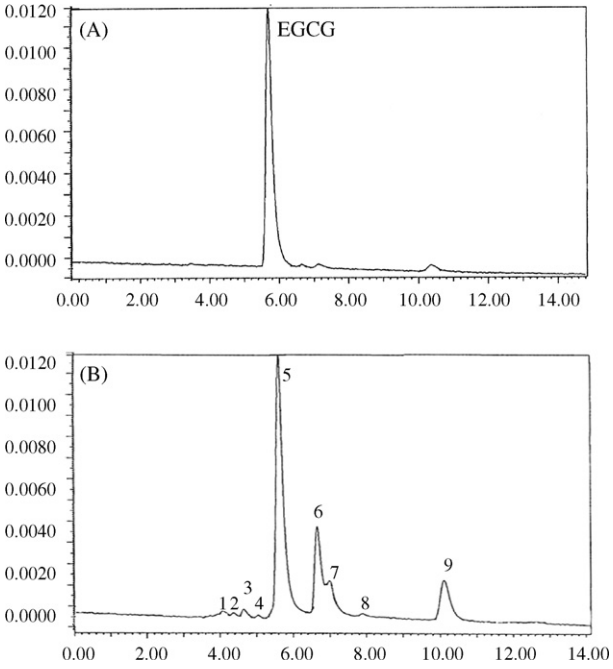

Fig. 1. Reverse-phase HPLC analysis of EGCG (A) and GTE (B). The detection wavelength was 280 nm.

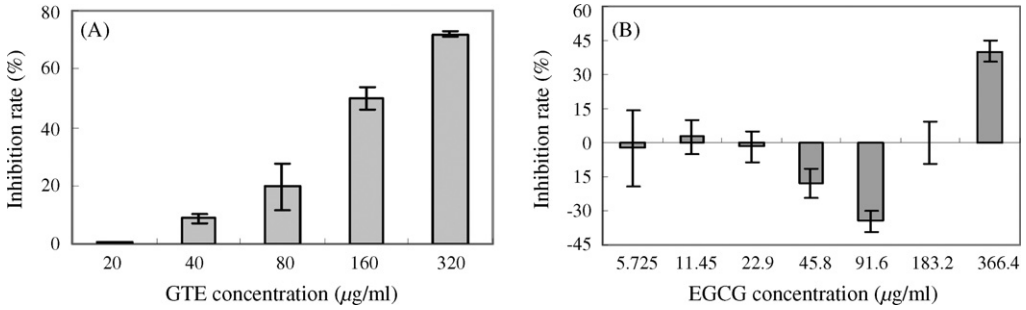

Fig. 2. Evaluation of GTE (A) and EGCG (B) cytotoxicity to HepG2-N10 with MTT assay. Results were expressed as inhibition rates of control (mean ± S.D.) from six independent experiments.

3.2. Cellular toxicity of GTE and EGCG

The cytotoxicity of GTE and EGCG were evaluated by using the MTT assay. As shown in Fig. 2, GTE had low toxicity to HepG2-N10 cells. The CC_{50} of GTE was 171.8 μg/ml. In our study, at concentrations higher than 91.6 μg/ml, EGCG showed significant cytotoxicity to HepG2-N10 cells, visible under an optical ordinary microscope (data not shown). The OD value of the cells treated with EGCG, however, varied and the inhibition rate were sometimes negative (Fig. 2). Therefore, the CC_{50} of EGCG could not be determined by MTT. Concentrations no more than 91.6 μg/ml EGCG were therefore chosen for the following experiments.

3.3. GTE and EGCG inhibited the expression of HBV antigens

The HBsAg and HBeAg in the medium were determined after treatment with different concentrations of GTE, EGCG and 3TC using the ELISA method. Results indicated that the treatment of GTE and EGCG inhibited both HBsAg and HBeAg of HBV simultaneously, dose-dependently and efficiently ($P < 0.01$; Fig. 3). The CC_{50} of GTE was 171.8 μg/ml. The EC_{50} of HBsAg and HBeAg were 5.02 and 5.681 μg/ml in HepG2-N10 cells, respectively. After treatment for 6 days with GTE at a concentration of 40 μg/ml, the HBsAg of HBV was almost entirely inhibited with an inhibition rate of 99%. At the same concentration of GTE, the inhibition rate of HBeAg was 93%. EGCG could also down regulate the expression of antigens effectively, but the effect was weaker than GTE when a corresponding concentration was used. The inhibition rate on HBsAg and HBeAg of 3TC was 15 and 19%, respectively at 2 μM, much weaker compared to GTE and EGCG. The results indicated that 3TC has little effect on the trafficking and the secretion of HBsAg and HBeAg of HepG2-N10.

3.4. Treatment of GTE dramatically inhibited the production of the extracellular HBV DNA by HepG2-N10 cells

Q-PCR revealed that GTE could inhibit the production of HBV DNA in a dose-dependent manner ($P < 0.01$) (Fig. 4A).

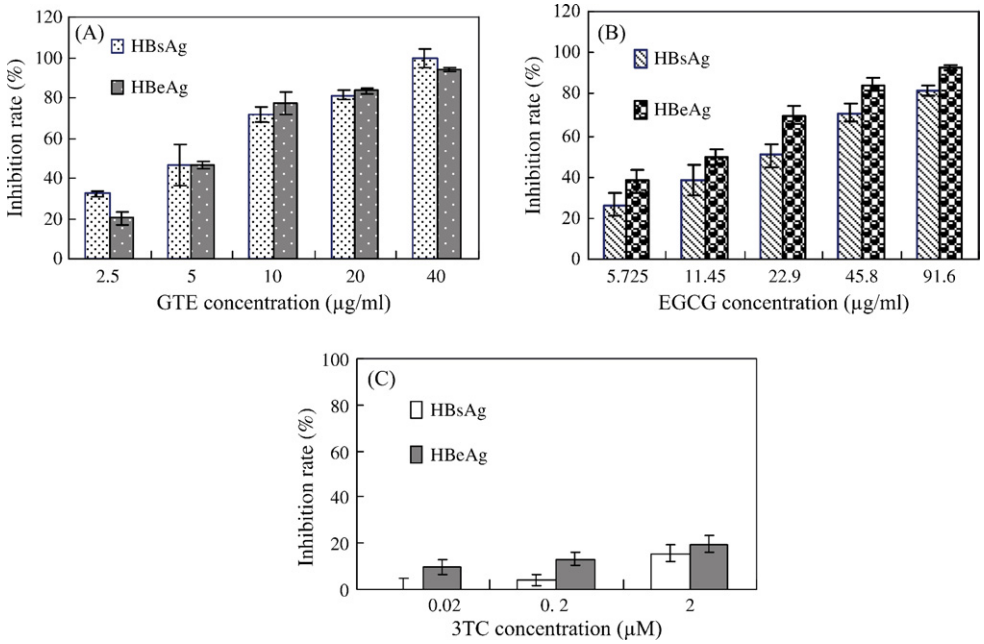

Fig. 3. Analysis of HBsAg and HBeAg secreted by HepG2-N10 cells treated with different concentrations of GTE (A), EGCG (B) and 3TC (C). Results are expressed as inhibition rate of control (mean ± S.D.) from six independent experiments.

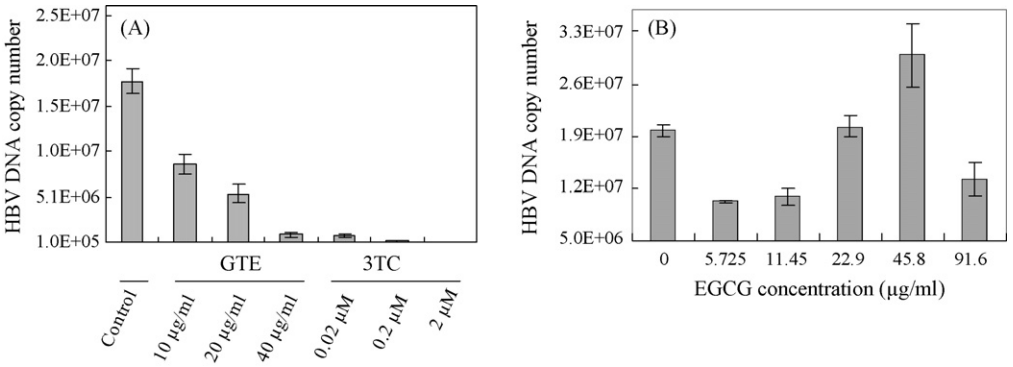

Fig. 4. Real-time PCR analysis of the effect of GTE, EGCG and 3TC on HBV DNA in media from HepG2-N10 cells after treatments for 6 days. Results were expressed as copies per milliliter culture medium (mean ± S.D.) from three independent experiments. (A) GTE and 3TC and (B) EGCG.

The concentrations of GTE from 10 to 40 μg/ml could efficiently decrease the production of HBV DNA. At 40 μg/ml, about 95% of HBV DNA was inhibited effectively after treatment of 6 days and the copy number of HBV DNA decreased to 10^5 copies/ml. The EC_{50} of extra-cellular HBV DNA was 19.81 μg/ml when treatment with GTE. After incubation with 5.725 μg/ml EGCG, the copy number of HBV DNA detected was 1.05×10^7 copies/ml, representing about 50% reduction of HBV DNA in the medium of HepG2-N10 compared to that of the control (2.8×10^7 copies/mL) (Fig. 4B). However, when the concentration of EGCG was increased, there was not a corresponding increase in inhibition.

Treatment of 3TC with concentrations of 0.02, 0.2, and 2 μM, decreased the amount of HBV DNA significantly (Fig. 4A). The HBV DNA copies were reduced to 10^5 copies/ml with three different concentrations of 3TC. The data suggested that the inhibition effect of GTE at 40 μg/ml was comparable to that of 3TC at 0.02 μM.

3.5. GTE and EGCG down-regulated intracellular replicative intermediates in the HepG2-N10 cells

Our results indicated that there was a dramatic reduction of replicative intermediates after the treatment with GTE and the reduction was dose dependent (Fig. 5A). An inhibition of around 70% of replicative intermediates was observed after 6 days with 40 μg/ml GTE treatment. The EC_{50} of replicative intermediates was 10.76 μg/ml when treatment with GTE. A dramatic decrease of the replicative intermediates of more than 50% was observed with the treatment of EGCG at different concentrations (Fig. 5B). The HBV replicative intermediates copies were reduced significantly to less than 10^7 copy/ml with three different concentrations of 3TC.

3.6. GTE and EGCG inhibited the production of HBV cccDNA

The effects of GTE, EGCG and 3TC on the levels of cccDNA of the HepG2-N10 cells were investigated by real-time PCR (Fig. 6). The cccDNA of HBV in karyons decreased after treatment with GTE, EGCG and 3TC. Increasing doses of the three agents resulted in the progressive inhibition in the accumulation of cccDNA present within cells (Fig. 6). At a concentration of 40 μg/ml of GTE, the cccDNA were decreased by more than 90%. After treatment with high concentration EGCG (22.9 μg/ml), the level of cccDNA was down regulated by nearly 60%. Treatment with 0.02, 0.2 and 2.0 μM 3TC resulted in a greater than 90% inhibition of accumulation of HBV cccDNA after 6 days of treatment.

3.7. GTE inhibited the level of HBV mRNA

Results of RT-PCR showed that the HBV mRNA of the cells treated with GTE was decreased markedly in comparison to that

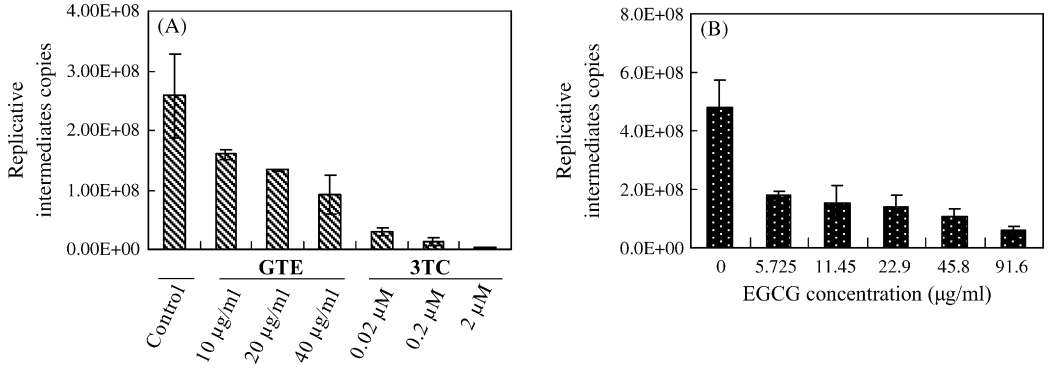

Fig. 5. Real-time PCR analysis of the effect of GTE, EGCG and 3TC on intracellular replicative intermediates from HepG2-N10 cells after treatments for 6 days. Results are expressed as copies per milliliter cell lysates (mean ± S.D.) from three independent experiments. (A) GTE and 3TC and (B) EGCG.

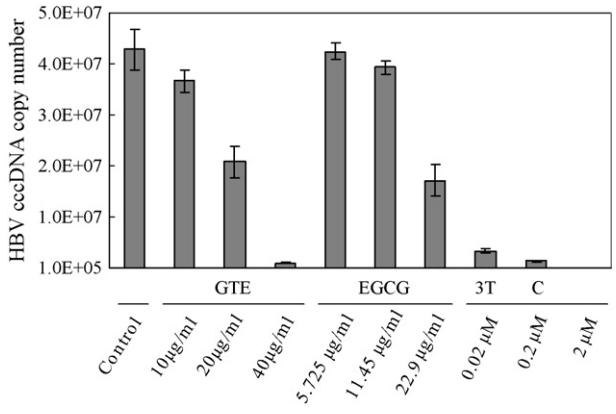

Fig. 6. Real-time PCR results of intracellular HBV cccDNA after GTE, EGCG or 3TC treatment of HepG2-N10 cells for 6 days. Results were expressed as copy numbers per well (mean ± S.D.) from three independent experiments.

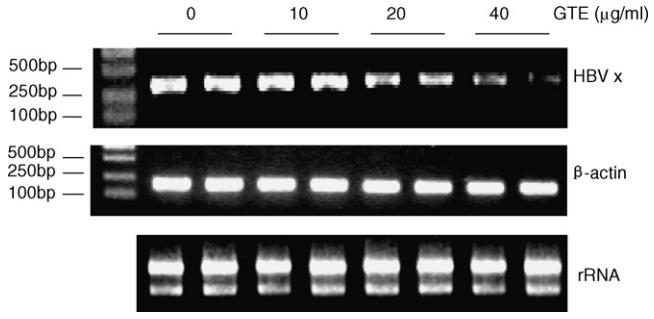

Fig. 7. RT-PCR results of HBV mRNAs and β-actin mRNA after treatment of GTE with different concentrations for 6 days. The 18S and 28S rRNA of the RNA samples are shown.

of the control (Fig. 7) and the inhibitory effect increased with the increase of GTE concentration. GTE concentration, consistent with an effect at the HBV transcription level, which indicated that GTE functions at the HBV transcriptional level.

4. Discussion

In the past few decades, there has been great interest regarding the anti-inflamminatory, antioxidant and antiviral properties of polyphenols found in green tea. By analyzing HBsAg, HBeAg and HBV DNA, we examined the effects of GTE and its major component EGCG on HBV in vitro in the HepG2-N10 cell line.

Results of this research suggest that both GTE and EGCG could inhibit production of HBV. EGCG, the major component of GTE, had weaker effects than GTE. This result suggests that other components of GTE could also increase inhibition effects against HBV. HPLC results confirmed that there were additional components in GTE (Fig. 1). This is consistent with many previous data regarding other diseases that report the effects of a single component were often much lower than those for compounds (Heaton et al., 2002; Miyata, 2007; Munk, 1997).

In this study, GTE exhibited a powerful inhibitory effect on the expression of HBsAg and HBeAg. Studies show that HBeAg plays a role in viral persistence. It has been suggested that the HBeAg may promote HBV chronicity by functioning as an immuno-regulatory protein and is involved in immune tolerance of HBV-infected individuals (Chen et al., 2005; Milich et al., 1990). Our finding suggests that GTE has potential as a therapeutic compound to overcome host immune tolerance induced by HBeAg during HBV infection.

In our study, 3TC can significantly reduce the HBV DNA level in supernatant of HepG2-N10. This result was similar to the 3TC effect on HBV DNA of HepG2 2.2.15 reported by Doong et al. (Doong et al., 1991). In their experiments, 0.5 μM 3TC resulted in nearly complete cessation of viral DNA replication by southern blot. In our experiments, 3TC was found to have no obvious inhibitory effects on HBsAg and HBeAg secretion. Similar results have been reported by other investigators (Shuangsuo et al., 2006). In clinical research, it has also been shown that most patients do not clear either HBeAg or HBsAg after long-term treatment with 3TC even though their serum HBV DNA was markedly reduced (Nevens, 1997).

In this study quantitative PCR was used to examine the effects of GTE, EGCG and 3TC on cccDNA. The results showed that cccDNA levels were decreased effectively by GTE, EGCG and 3TC (Fig. 6). Our results are consistent with previous reports that showed 3TC could cause a greater than 90% inhibition of non-protein-associated with RC and cccDNA by HepG2 cells (Delaney et al., 1999).

Green tea polyphenols have been shown to have efficacy in various models of inflammatory liver injury and are widely used in treatment of human liver diseases such as hepatitis C and alcoholic cirrhosis (Gloro et al., 2005). Recent studies have also shown that green tea extracts have an inhibitory effect on interstinal a-glucosidase (Zhong et al., 2006); Glycosidases including glucosidases are essential for the processing of glycoproteins and glycolipids in viruses. It is reported that glucosidase inhibitors are potent inhibitors of secretion of HBV particles (Mehta et al., 1997). Antiviral studies have shown that green tea polyphenol has an inhibitory effect on influenza virus (Imanishi et al., 2002; Nakayama et al., 1993; Nishikawa et al., 2006; Song et al., 2005), Epstein–Barr virus (Chang et al., 2003), adenovirus (Weber et al., 2003) and human immunodeficiency virus (Fassina et al., 2002). It can inhibit the expression of specific antigens (EBV), interfering with the binding of the envelope protein to its receptor (influenza virus and HIV), inhibiting the viral enzyme activity (influenza virus) and influencing some host regulative factors. These studies indicate that GTE exhibited its antiviral effects with multiple mechanisms. In our study, GTE significantly inhibited the expression of specific HBV antigens, reducing the replication of viral DNA, and down-regulating the production of replicative intermediates and cccDNA. Our RT-PCR analysis of HBV mRNAs showed GTE could interfere with the transcription of HBV. As the antigen expression, extracellular DNA, intermediates DNA, cccDNA are all related to the transcription level of HBV mRNA, reduction of HBV mRNA might play a key role in the GTE anti-HBV function. Nevertheless, the precise mechanism for GTE anti-HBV is still unknown and further investigation is necessary.

In summary, the present studies indicate that GTE reduced the production of HBV specific antigens, the level of extracellular HBV DNA, intracellular replicative intermediates and cccDNA in a dose-dependent manner, and reduction of HBV mRNA was

observed. Although further studies are required to elucidate the molecular mechanisms, these results suggest that GTE could be a candidate agent for the therapy of hepatitis B virus infection.

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