RESEARCH ARTICLE

Preventive Effects of a Major Component of Green Tea, Epigallocathechin-3-Gallate, on Hepatitis-B Virus DNA Replication

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Abstract

Background: Hepatitis B virus infection is one of the major world health problems. Epigallocatechin-3 gallate is the major component of the polyphenolic fraction of green tea and it has an anti-viral, anti-mutagenic, anti-tumorigenic, anti-angiogenic, anti-proliferative, and/or pro-apoptotic effects on mammalian cells. In this study, our aim was to investigate the inhibition of HBV replication by epigallocatechin-3 gallate in the Hep3B2.1-7 hepatocellular carcinoma cell line. Materials and Methods: HBV-replicating Hep3B2.1-7 cells were used to investigate the preventive effects of epigallocatechin-3 gallate on HBV DNA replication. The expression levels of HBsAg and HBeAg were determined using ELISA. Quantitative real-time-PCR was applied for the determination of the expression level of HBV DNA. Results: Cytotoxicity of epigallocathechin-3-gallate was not observed in the hepatic carcinoma cell line when the dose was lower than 100 μM. The ELISA method demonstrated that epigallocatechin-3 gallate have strong effects on HBsAg and HBeAg levels. Also it was detected by real-time PCR that epigallocatechin-3 gallate could prevent HBV DNA replication. Conclusions: The obtained data pointed out that although the exact mechanism of HBV DNA replication and related diseases remains unclear, epigallocatechin-3 gallate has a potential as an effective anti-HBV agent with low toxicity.

Keywords: Hepatitis B - Hepatocellular carcinoma - EGCG - green tea - *in-vitro*

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Introduction

Hepatitis B virus (HBV) infection is one of the major world health problems; although the availability of effective vaccines and several antiviral drugs (Baumert, 2007; He, 2011) One third of the world's population has been infected by HBV and 5% of them are chronically infected according to the World Health Organization (WHO) estimation (Xu, 2008). Some studies show that long-term HBV infection may lead some diseases such as hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma. Increasing evidence shows that persistent HBV infection in the liver is associated with heavy liver disease, such as cirrhosis and hepatocellular carcinoma (Park, 2006; Pang, 2014).

HBV is a DNA virus consisting of a nucleocapsid which contains the 3.2 kb circular partially double-stranded DNA genome. The HBV nucleocapsid is surrounded by an envelope. Entry of HBV into healthy hepatocytes has long been recommended as a potential target for antiviral intervention (He, 2011; Huang, 2014). Although several pharmacological treatment strategies have been applied to treat Hepatitis B, a final and exact antiviral therapy against HBV infection has not been identified. The therapeutic effects of interferon-α and nucleoside analogues, such as entecavir, lamivudine and adefovir, are not fully convincing because such treatments may be accompanied by side effects, high costs and drug resistance (Feld, 2002; Perrillo, 2005; Tillmann, 2009). Several studies reported that some natural products have ability to inhibit HBV replication using various mechanisms. For example, oxymatrine was shown to prevent HBV DNA synthesis (Xu, 2010). A helioxanthin derivative was demonstrated to diminish HBV promoter activities and thus suppress HBV gene expression and DNA synthesis (Ying, 2007; Tseng, 2008). Therefore, there is an urgent and strict necessity to develop highly effective anti-HBV drugs for the long-term treatment of HBV infection with fewer side effects and at a lower cost.

Epigallocatechin-3 gallate (EGCG) is the major component of the polyphenolic fraction of green tea. Along with other tea catechins, and polyphenols in general, it is an antioxidant that is thought to prevent tumorigenesis by protecting cellular components from

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oxidative damage via free radical scavenging. Indeed, a number of studies have demonstrated the free radical scavenging activities of EGCG, as well as its anti-viral, anti-mutagenic, anti-tumorigenic, anti-angiogenic, anti-proliferative, and/or pro-apoptotic effects on mammalian cells both *in vitro* and in vivo (Xu, 2008; Mukhtar, 2000; Hsieh, 2009; Bettuzzi, 2006; Qiao, 2009; Philips, 2009). However, the mechanism by which EGCG inhibits HBV replication is unclear.

In this study, our aim was to investigate the inhibition of HBV replication by EGCG in Hep3B2.1-7 hepatocellular carcinoma cell line. At that point, the results showed that EGCG treatment has a role that reduces the HBV DNA production and HBV antigens such as HBsAg and HBeAg in a dose-dependent manner.

Materials and Methods

Cell line, cell culture and treatment

The human hepatocellular carcinoma cell line Hep3B2.1-7 (ATCC: HB-8064) which can stably produce complete virion particles and high levels of HBV proteins, was purchased from ATCC. It is an inducible HBV replicating cell line and contains a slight over-length HBV genome. In the present study, it was used as a model of HBV-infected hepatocytes. The Hep3B2.1-7 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, USA), 100 U/ml penicillin and 100 μM streptomycin (Sigma) at 37°C in a humidified incubator at 5% CO_2.

Hep3B2.1-7 cells were plated at a density of 1×10^5 cells/ml into 24-well plates and incubated for 24 h. Different concentrations (0, 50, 100, 200 and 400 μM) of EGCG (Sigma, for cell culture) were added to the medium. Cells were grown in the presence of EGCG for 9 d, and the supernatant was collected every other day. After incubation with EGCG for 9 d, the concentrations of HBsAg, HBeAg, and HBV DNA in the supernatant were determined.

MTT Assay

The cytotoxicity of EGCG to Hep3B2.1-7 cells was detected by determining the survival rate of the cells using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Liu, 2003). 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. Hep3B2.1-7 cells were cultivated at a density of 1×10^5 cells/well in 96-well plates in 100 µl and treated with EGCG (0, 50, 100, 200 and 400 μM) for 9 d. The culture medium containing the drugs was changed every other day. On the last day, the culture medium was removed and washed twice with PBS. 10 µl of MTT (5 g/ml) was added to each well and further incubated in a CO_2 incubator at 37°C for 4 h. The optical density (OD) at 490 nm was obtained. Each experiment was performed in triplicate. 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 (CC50) was calculated according to Berkson logit method (Berkson, 1968).

HBsAg and HBeAg detection by ELISA

After incubation with EGCG for 9 d, the cell supernatant was collected and subjected to 10000×g centrifugation to remove cellular debris. Secreted HBsAg and HBeAg were quantified by enzyme-linked Immunosorbent assay (ELISA) kits (DRG, Germany) following the manufacturer's instructions. The data were calculated by the following formula: inhibition of control (%)=(1−ODT/ODC)×100%, where ODT and ODC represent the cell number-adjusted OD of the test drugs and the control, respectively.

Real-time Quantitative PCR (qPCR) detection of HBV DNA

The quantity of HBV DNA in the culture supernatant and cells was determined with a real-time system (Applied Biosystems, USA) by using. The supernatant of the cells cultured for 6 and 9 d was collected, and the DNA was extracted from 200 µl of medium using the DNA Extraction Kit (QIAGEN) according to the manufacturer's recommendations by an automated system (QIAcube, QIAGEN). The HBV DNA sequence was amplified with a forward primer HBV-DNA-F: 5'-CCTTCTTACTCTACCGTTCC-3', a reverse primer: HBV-DNA-R: 5'-GACCAATTTATGCCTACAGCC-3' and reference gene (Actin): β-actin-F: 5'-CACCAACTGGGACGACAT-3'. The PCR was initiated by 50°C for 2 min followed by 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 10 s. Relative gene expression were calculated by $2^{(-\Delta\Delta Ct)}$ method.

Statistical Analysis

Statistical analysis was performed by using the SPSS software package (SPSS for windows release 17.0). The one-way ANOVA test was used to evaluate the difference between the test samples.

Results

The cytotoxicity of EGCG was evaluated by using the MTT assay. Our results showed that EGCG showed significant cytotoxicity to Hep3B2.1-7 cells, above 100 μM. As shown in Figure 1, the cytotoxic effects of EGCG was quite slight (10% inhibition of cell viability) when 100 µM of EGCG were used. However, this effect was detected as significantly high (48% inhibition of cell viability) and (60% inhibition of cell viability) when 200 and 400 µM of EGCG were used, respectively. So that, the maximum non-toxic concentration was 100 µM, and this was used as the highest concentration of EGCG in the HBV inhibition assay.

The levels of HBeAg and HBsAg in the supernatant of EGCG-treated Hep3B2.1-7 cells were measured by ELISA. The obtained data by ELISA method showed that EGCG inhibit the expression of HBsAg and HBeAg levels in Hep3B2.1-7 cells in a dose-dependent manner. After

treatment of 9 d, 100 μM EGCG significantly inhibited the secretion of HBsAg and HBeAg levels by %58,6 and %48,5 respectively ($p<0.05$). These results indicated that EGCG could down-regulate HBsAg and HBeAg levels (Figure 2).

According to the data that were obtained from qPCR showed us that EGCG could inhibit the production of HBV DNA in a dose-dependent manner. However, after treatment with 50 and 100 μM EGCG, a decrease, but not so significant, in the HBV DNA level in the supernatant was observed. The last two concentrations (200 and 400 μM) of EGCG could efficiently reduce the extracellular HBV DNA level. Fold changes of HBV DNA expression levels were shown in Figure 3. At that point the data pointed out that at 200 and 400 μM concentrations; respectively about 65% and 80% of HBV DNA was inhibited effectively after treatment with EGCG ($p<0.05$).

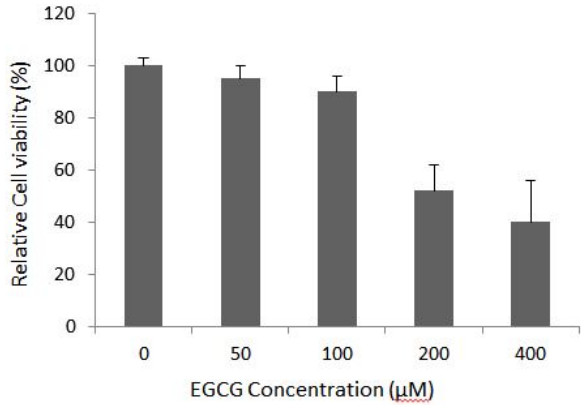

Figure 1. The Cytotoxic Effects of EGCG on Hep3B2.1-7 Cells

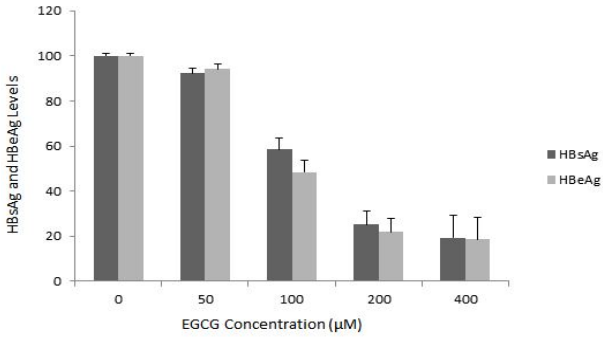

Figure 2. The Effects of EGCG on HBsAg and HBeAg Antigen Expression Levels

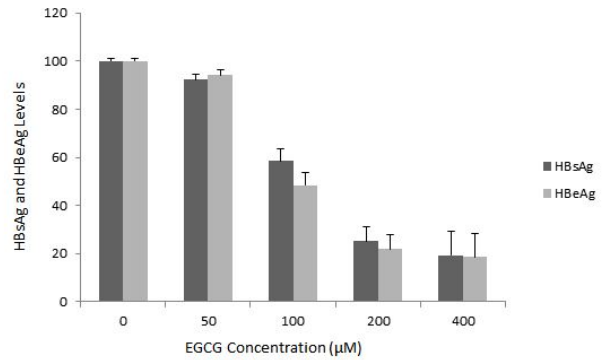

Figure 3. The Preventive Effect of EGCG on HBV DNA Expression Level

Discussion

Green tea, Camellia sinensis, contains significant amounts of polyphenol catechins such as C, EGC, ECG and EC. EGCG is also one of the catechins and the major compound of green tea (Huang, 2014). Recently, there has been great interest regarding the anti-inflammatory, anti-oxidant, anti-cancer, anti-apoptotic and antiviral properties of polyphenols found in green tea (Liu, 2003; Miyata, 2007; Fassina, 2002; Doong, 1991). Some studies reported that green tea has an activity against some viruses such as HIV, HTLV-1, HCV, Influenza and HBV (Li, 2011; Roh, 2011; Harakeh, 2014; Kim, 2013). EGCG, the major component of green tea, has been demonstrated to show numerous effects, including antiangiogenic, anti-proliferative, and apoptotic effects in various tumors, and could inhibit several downstream signaling molecules in multiple signaling pathways (Hastak, 2003). Additionally, EGCG stops the progression of hepatic fibrosis by inhibiting hepatic stellate cell (HSC) activation and matrix metalloproteinase-2 activity (Zhen, 2007).

In this study, we wanted to evaluate the potential anti-HBV activity of EGCG *in vitro* in the Hep3B2.1-7 cell line by analyzing HBV antigens (HBsAg, HBeAg) and HBV DNA. Our results showed that EGCG could significantly reduce the expression level of HBV DNA. The secretion level of HBeAg and HBsAg compared with control group (0 μM) and the results worked in both a dose-dependent manner. Similar data about have been reported with some studies (He, 2011; Xu, 2008).

Green tea polyphenols, especially EGCG, have been demonstrated to have serious 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, 2005). As mentioned above, these polyphenols have an inhibitory effect on some viruses. It can inhibit the expression level of some antigens, receptors or proteins of these viruses such as Epstein-Barr virus, influenza virus, adenovirus and Human Immunodeficiency Virus (Fassina, 2002; Imanishi, 2002; Chang, 2003; Weber, 2003).

Researchers have recently focused on the potential effects of some natural extracts to treat cancers. EGCG is one of them and known to prevent each step of carcinogenesis (Tachibana, 2009; Chen, 2009). Based on this information, we tried to show the preventive effect of EGCG on hepatocellular carcinoma by reducing HBV DNA copies. It is known that detailed replication mechanism of HBV remains unclear. However, it is estimated that various cellular factors are included in HBV genome replication and EGCG may interact with certain cellular proteins and affect the DNA replication mechanism.

As a result, our data showed that EGCG has strong effects on Hepatitis B virus DNA and antigens. Anti-HBV activity of EGCG through decreasing the secretion levels of HBeAg and HBsAg and expression level of HBV DNA was shown, parallel with the current literature data. However, to determine whether EGCG treatment could reduce the rates of some diseases -especially liver damage resulting from HBV infection- and to confirm these in-

vitro studies results, further in-vivo studies are needed. On the other hand, the treatment of HBV all over the world is quite difficult and expensive. This economic situation may lead an extra burden to the countries budget. So that, identification of economic drugs from natural medicines can be a good perspective to solve this problem after antiviral activities of them are exactly proved both invivo and invitro methods. Finally, we can say that although the exact mechanism of HBV DNA replication and related diseases remains unclear, EGCG has a potential as an effective anti-HBV agent with low toxicity.

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