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Suppression of cytotoxin-induced cell death in isolated hepatocytes by tea catechins

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Abstract

To elucidate the hepatoprotective effects of green tea catechins, the following experiments were conducted utilizing (−)-epigallocatechin-3-gallate (EGCG), the major component of green tea catechin, together with other catechins. The protective effects of catechins against hepatotoxins, bromobenzene or rubratoxin B, were examined in primary cultures of rat hepatocytes. Bromobenzene and rubratoxin B are known to induce necrosis and apoptosis of cells, respectively. After 24-h treatment with toxin, EGCG and (−)-epigallocatechin-3-(3″-*O*-methyl)gallate (EGCg-3″-OMe) suppressed the bromobenzene-induced morphological change and dose-dependently prevented bromobenzene-induced cell death. Both catechins also prevented apoptotic cell death caused by rubratoxin B. In rubratoxin B-treated cells, both catechins were found to suppress the activation of caspase-3 by rubratoxin B. The results in the present study suggest that EGCG and EGCg-3″-OMe are potent hepatoprotective agents. This report is the first to show that catechins suppress cytotoxin-induced cell death.

Keywords: EGCG ((−)-epigallocatechin-3-gallate); Hepatoprotection; Cell death cytotoxin-induced; Caspase

1. Introduction

The liver is the primary site of detoxification in the body and acts as the first line of defense against infectious, toxic, or carcinogenic agents coming from the gut (Bertolino et al., 2000). Therefore, disruption of the integrity of liver function leads to fatal cases or irreversible organ death. Various factors that cause liver injury have been described. In liver injury, hepatocytes die via necrosis or apoptosis. From the viewpoint of the clinical treatment of liver failure, it is important to protect normal hepatocytes from injury by using hepatoprotective agents. Therefore, potent hepatoprotective and nontoxic compounds are required to treat liver failure. Hepatoprotective agents are also required to protect cells from necrosis and apoptosis. Chemicals such as bromobenzene damage the liver cells via free radical reactions, resulting in the induction of cell necrosis (Lau and Monks, 1988; Thor and Orrenius, 1980). Rubratoxin B-induced liver failure produces fulminant hepatitis and induces cell apoptosis (Siraj and Hayes, 1979; Natori et al., 1970; Nagashima and Goto, 2000). The rubratoxin B model is the more promising experimental basis for understanding the mechanism of clinical liver complaints and for evaluating the efficacy of hepatoprotective agents. In the present study, we used bromobenzene and rubratoxin B as cytotoxins for screening effective hepatoprotective agents against necrosis and apoptosis.

Green tea catechins are the major constituents of tea leaves and are consumed by Japanese and Chinese people mainly in the form of beverages. The effects of green tea catechins have been widely studied and antioxidative (Kawase et al., 2000; Yoshino et al., 1994; Ho et al., 1992), antiallergic (Ohmori et al., 1995; Sano et al., 1999), antimutagenic/anticarcinogenic (Jankun et al., 1997; Shiraki et al., 1994; Yamane et al., 1991), and antibacterial effects (Fukai et al., 1991) have been docu-

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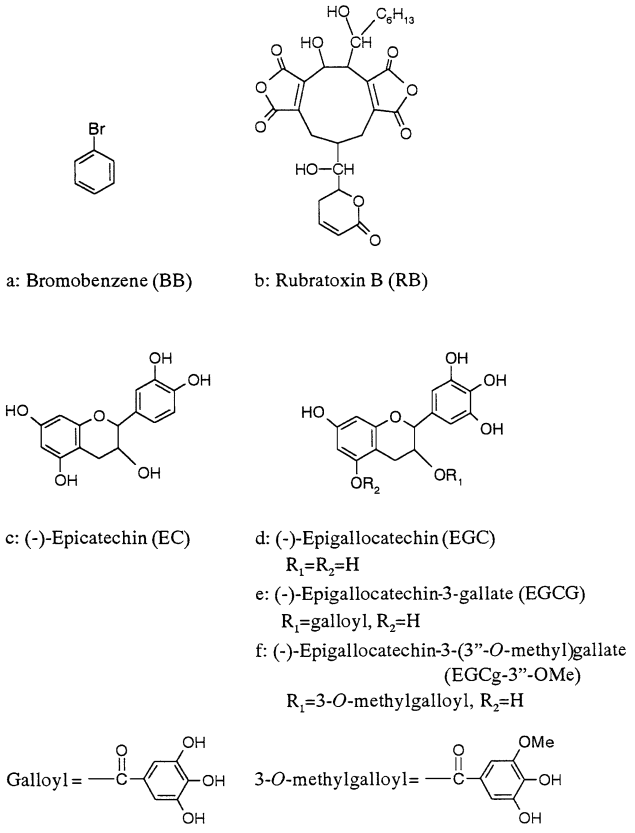

Fig. 1. Structural formulas of hepatotoxins and catechins used in this study.

mented. Among the green tea catechins, (−)-epigallocatechin-3-gallate (EGCG) is present in the greatest abundance (54%) and is found to be the most effective for scavenging reactive oxygen species. As for the antibacterial effects of catechins, (−)-epigallocatechin (EGC) shows the most potent activity compared to the other constituents of green tea catechins. Although catechins have been attracting much attention because of their functions, only few studies concerning the hepatoprotective activity of this material are available.

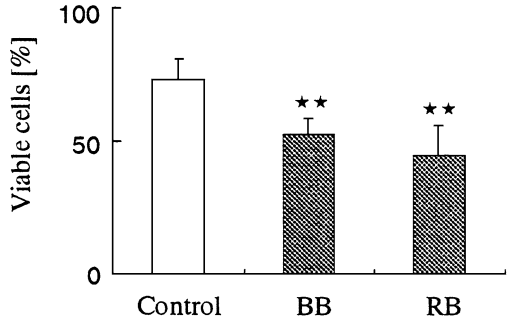

Fig. 2. Effects of hepatotoxins on the viability of cultured rat hepatocytes. Hepatocytes were cultured in medium containing 1 mM bromobenzene or 77.1 μM rubratoxin B for 24 h. Data represent the results of three independent measurements performed in duplicate and the results are expressed as mean percentages of viable cells ± S.D. $^{\star\star}P < 0.01$ compared with the control.

The hepatoprotective effect of catechins against cytotoxin (bromobenzene or rubratoxin B (mycotoxin of *Penicillium* sp.))-induced cell death was now examined. The results showed that EGCG and epigallocatechin-3-(3″-*O*-methyl)gallate (EGCg-3″-OMe) protected the hepatocytes from bromobenzene and rubratoxin B in a dose-dependent manner and could be potent hepatoprotective agents. This is the first report showing that catechins suppress cytotoxin-induced cell death of hepatocytes.

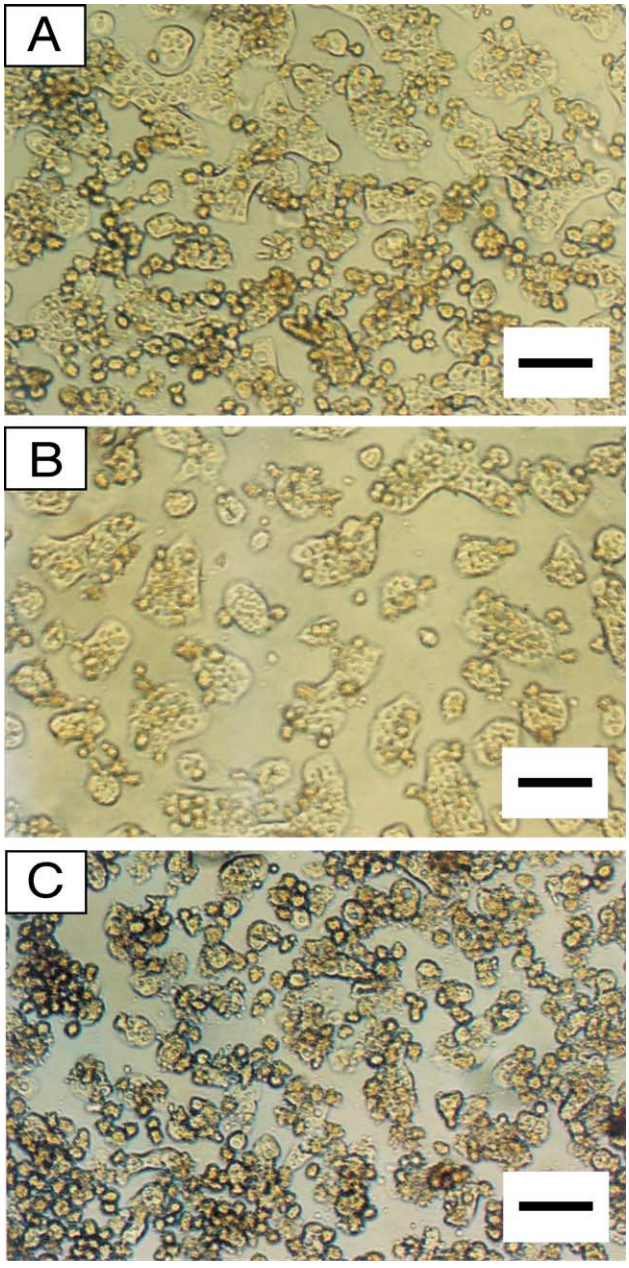

Fig. 3. Morphological study showing the protective effects of EGCG in bromobenzene-treated rat hepatocytes. (A) Control, (B) treated with 1 mM bromobenzene, (C) treated with 1 mM bromobenzene and 0.2 mM EGCG concomitantly for 24 h (magnification × 100). Bars show 100 μm.

2. Materials and methods

2.1. Chemicals

Bromobenzene and rubratoxin B were purchased from Wako (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Catechins, (−)-epicatechin, (−)-epigallocatechin, (−)-epigallocatechin-3-gallate (EGCG) and epigallocatechin-3-(3″-O-methyl)gallate (EGCg-3″-OMe) were isolated from green tea leaf (Saijo, 1982). According to high performance liquid chromatography (HPLC) analysis, the purity of the catechins is over 99% for epicatechin, epigallocatechin and EGCG. The purity of EGCg-3″-OMe is over 95%. The chemical structures of these materials are shown in Fig. 1. Other chemicals were obtained from local commercial sources, and used without further purification.

2.2. Media

The basal medium A consisted of William's E medium (WE, ICN Biochemical, Costa Mesa, CA), 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 ng/ml amphotericin B, 100 ng/ml aprotinin (Nacalai Tesque, Kyoto, Japan), and 10% (v/v) fetal bovine serum (ICN Biochemicals). Medium B consisted of medium A supplemented with 1 nM insulin and 1 nM dexamethasone.

2.3. Isolation of hepatocytes

Hepatocytes were isolated from male Sprague–Dawley rats weighing 150–200 g by perfusing the liver with collagenase (from *Clostridium histolyticum*; Sigma-Aldrich) using the method of Seglen (Seglen, 1976). A hepatocyte preparation of more than 90% viability at the time of isolation was used for the experiments. Cells were seeded at a density of 1×10^5 cells/cm^2 onto polystyrene culture plates (Nippon Becton and Dickinson, Tokyo, Japan), and were incubated for the first 6 h in Medium B under conditions with humidified air with 5% CO_2 at 37 °C. After 6 h, Medium B was replaced by Medium A.

2.4. Treatment of cells with hepatotoxins in the presence of catechins

One day after the isolation of hepatocytes, the medium was replaced by fresh Medium A containing 0.8% (v/v) dimethyl sulfoxide (DMSO) and hepatotoxins (bromobenzene, rubratoxin B). The final concentrations of bromobenzene and rubratoxin B were adjusted to 1 mM and 77.1 μM (Nagashima and Goto, 2000), respectively. In the experiments investigating the hepatoprotective effects of catechins, various doses of catechins were added to Medium A, and the final concentration of each catechin was adjusted to 0.01, 0.02 or 0.2 mM. Cells were incubated under each set of conditions for another 24 h, and the number of living cells was determined by the trypan blue exclusion assay. Briefly, 0.2% (w/v) trypan blue solution was added to trypsinized cell suspension and viable cells which were not stained were counted. A control run was performed in medium containing only 0.8% (v/v) DMSO.

2.5. Morphological observation

Confluent cells in 1 ml of medium with chemicals were cultured in each well of a 12-well culture plate for 24 h, followed by microscopic examination using a phase contrast microscope (OLYMPUS LH20A).

2.6. Assay for caspase-3 activity

Cells were harvested and washed once with ice-cold phosphate buffered saline(−) and then resuspended in hypotonic cell lysis buffer at a concentration of 10^8 cells/ml. The cells were lysed by subjecting them to four cycles of freezing and thawing. The cell lysates were then centrifuged at 15,000 × *g* for 20 min at 4 °C and the supernatant fraction was collected. Caspase-3 activity was measured using a CaspACE Assay System Colorimetric Kit (Promega) (substrate: Acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD-pNA)), optimized for assay of caspase-3 activity, following the manufacturer's instructions. The assay kit used ensures that caspase-3 activity is measured specifically.

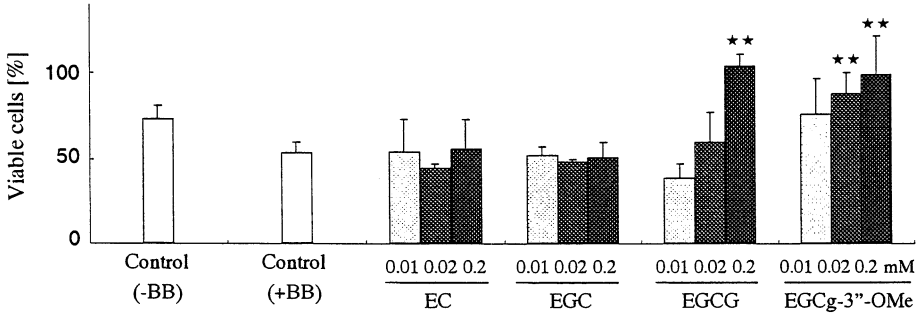

Fig. 4. Protective effects of catechins on bromobenzene-induced acute damage to isolated rat hepatocytes. Results shown are means ± S.D. of three independent measurements. ★$P<0.05$, ★★$P<0.01$ compared with the control. Final concentration of bromobenzene was 1 mM.

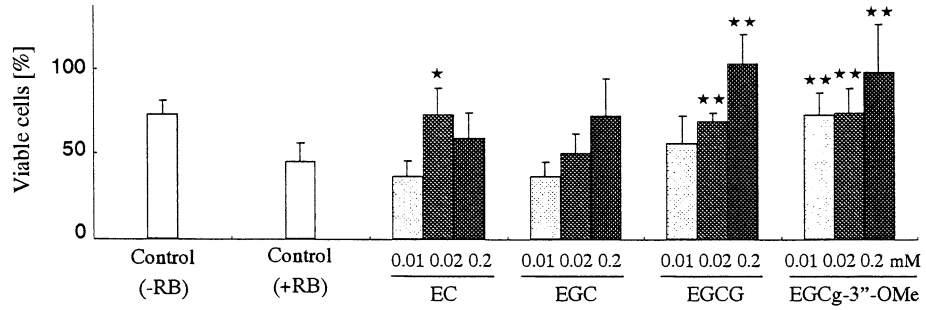

Fig. 5. Protective effects of catechins on rubratoxin B-induced acute damage to isolated rat hepatocytes. Results shown are means ± S.D. of three independent measurements. $^{\star}P<0.05$, $^{\star\star}P<0.01$ compared with the control. Final concentration of rubratoxin B is 77.1 μM.

2.7. Statistics

All assays and quantitative measurements were performed in triplicate each time. Hepatocytes were prepared from one rat to avoid effects of differences in hepatocyte origin. The data were analyzed for statistical significance using Student's *t*-test.

3. Results

3.1. Effects of hepatotoxins on the viability of cultured rat hepatocytes

The effects of bromobenzene and rubratoxin B on the viability of hepatocytes are shown in Fig. 2. Cell viability was significantly reduced by treatment with bromobenzene and rubratoxin B compared to that of the control run. The solvent (0.8% DMSO) itself had no effect on cell viability (data not shown). All experiments in this study were repeated twice to ensure reproducibility.

3.2. Protective effects of catechins against bromobenzene

Bromobenzene is known to induce cell necrosis (Lau and Monks, 1988; Thor and Orrenius, 1980). Treatment with bromobenzene caused marked morphological changes to the cells compared with the control run, as shown in Fig. 3A,B. In contrast, hepatocytes treated with bromobenzene in the presence of EGCG showed no morphological changes, as seen in the control run (Fig. 3C). This indicates that EGCG impaired the toxicity of bromobenzene.

To represent quantitatively the protective effects of catechins against bromobenzene, different concentrations of epicatechin, epigallocatechin, EGCG and EGCg-3″-OMe were added to bromobenzene-treated cells. Cell viability was examined by means of the trypan blue exclusion assay and the results are shown in Fig. 4. In the control run, only bromobenzene was added to the cultured cells. Epicatechin and epigallocatechin showed no protective effects while EGCG and EGCg-3″-OMe showed dose-dependent protective effects on the cytotoxicity of bromobenzene. At the highest final concentration (0.2 mM), EGCG and EGCg-3″-OMe significantly ($P<0.01$) protected the cells and maintained the initial cell viability.

3.3. Protective effects of catechins against rubratoxin B

Different concentrations of epicatechin, epigallocatechin, EGCG and EGCg-3″-OMe were added to rubratoxin B-treated cells (Fig. 5). In the control run, only rubratoxin B was added to the cells. Similar to the results for bromobenzene-treated cells, no protective effects of epicatechin or epigallocatechin could be observed; conversely, EGCG and EGCg-3″-OMe showed protective effects on the cytotoxicity of rubratoxin B. At the highest final concentration (0.2 mM) used, EGCG and EGCg-3″-OMe maintained initial cell viability as in the case of bromobenzene.

3.4. Suppression of induction of caspase-3 by catechins

Rubratoxin B is known to induce apoptosis (Nagashima and Goto, 2000), but the mechanism had remained unclear. The activity of caspase-3 was measured in cells treated with EGCG (0.2 mM) or EGCg-3″-OMe (0.2 mM) (Fig. 6). The results show that rubratoxin B induced caspase-3 activity significantly. EGCG and EGCg-3″-

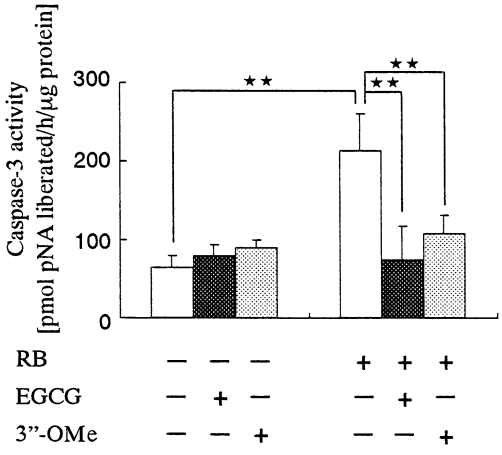

Fig. 6. Suppression of caspase-3 activity by EGCG and EGCg-3″-OMe (0.2 mM) in rubratoxin B-treated rat hepatocytes. Results shown are means ± S.D. of three independent measurements. $^{\star\star}P<0.01$ compared with the control.

OMe suppressed the activity of caspase-3 down to the control level. DMSO (solvent) did not affect on caspase-3 activity.

4. Discussion

Various authors have reported anticancer effects of green tea catechins (Okabe et al., 1997; Yang et al., 1998; Ahmad et al., 1997; Zhao et al., 1997; Spencer et al., 2001; Islam et al., 2000). In those studies, catechins showed anticancer effects by inducing apoptosis in cancer cells, thereby decreasing cell viability and increasing caspase-3 activity in such cells (Ahmad et al., 1997; Zhao et al., 1997; Spencer et al., 2001; Islam et al., 2000). Similar effects of catechins were observed in our previous study using the rat hepatoma cell line H4-II-E-C3, where the viability of cells treated with 0.02 mM EGCG decreased to 43.9% of the untreated control, and decreased to 16.5% on treatment with 0.2 mM EGCG. However, the effects of catechins on isolated hepatocytes have been little studied, especially from the point of view of hepatoprotection.

In the present study, we examined the protective effect of catechin on the liver as used for liver-failure treatment. Our data showed clearly that EGCG protects hepatocytes against cell death caused by two types of toxicity, one induced by bromobenzene and the other by rubratoxin B. EGCG is a potential hepatoprotective agent. It has been reported that bromobenzene is activated metabolically via cytochrome *P*450s (CYPs) and the resulting reactive intermediates induce covalent binding, enzyme inactivation and lipid peroxidation, then finally lead to the death of cells by necrosis (Lau and Monks, 1988; Thor and Orrenius, 1980; Lauriault et al., 1992). It was considered that the protective effect of catechins against bromobenzene was caused by the suppression of CYPs-induction or by scavenging of highly reactive intermediates. In the present study, the expression of CYPs (1A1, 2B1/2, 2E1, 3A2) was also examined (data not shown). The expression levels of CYPs appear to be almost the same under EGCG-present and EGCG-free conditions in bromobenzene-treated cells. Therefore, catechins did not affect the expression of CYPs, but were considered to exert hepatoprotective effects by scavenging reactive intermediates. The fact that catechins scavenge reactive intermediates is well known. We demonstrated that EGCG scavenges a stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). EGCG, 0.02 mM, scavenged 63.7% of DHHP in a 20-min reaction.

Rubratoxin B is well known to induce apoptosis in various kinds of cells (Nagashima and Goto, 2000). The mechanism of apoptosis induction has not been clarified yet. The activity of caspase-3 was shown to be activated in rubratoxin B-treated hepatocytes. The results suggested that caspase-3 activation is related to rubratoxin B-induced cell death. EGCG lowered the activity of caspase-3, the executioner caspase. Such suppression of caspase-3 activation is thought to be one of the mechanisms for EGCG protection against RB toxicity. In contrast to this, EGCG activated caspase-3 in cancer cells, as mentioned above. The differences in the behavior of EGCG have not been clarified, and should be investigated in future studies. We also found that EGCG did not protect HL-60 human promyelotic leukemia cells treated with rubratoxin B. Since EGCG protected normal hepatocytes and induced the apoptosis of cancer cells, it could be a safe antitumor drug.

Among antitumor substances, bacterial toxins have been shown to interfere with tumor growth and are therefore considered to be potential antitumor drugs. Rubratoxin B, both in vitro and in vivo, shows strong antitumor activity against Yoshida ascites sarcoma of the rat (Fimiani and Richetti, 1993). However, the toxicity or danger associated with the use of mycotoxins cannot be completely ignored. We suggest that rubratoxin B can be used as an antitumor agent in the presence of green tea catechins that regulate the undesirable aspects of this mycotoxin.

EGCg-3″-OMe, a rare constituent of green tea catechins (0.07 ~ 0.52%), was isolated from tea leaf in 1982 (Saijo, 1982) and Sano et al. (1999) reported that EGCg-3″-OMe had strong antiallergic activity. The antioxidative property of EGCg-3″-OMe has been previously reported by us (Kawase et al., 2000). However, other biological and pharmacological properties of EGCg-3″-OMe have never been reported because this compound is difficult to isolate due to its very small amount in tea leaf. The present study also concerned the hepatoprotective properties of EGCg-3″-OMe against the chemical toxicity of bromobenzene and rubratoxin B. EGCg-3″-OMe showed the strongest hepatoprotective activity, equaling that of EGCG. The hepatoprotective effect of catechins will also be examined in vivo and the results will be reported later.

In conclusion, we found that EGCG and EGCg-3″-OMe are potent hepatoprotective agents. As catechins are from tea leaf, they may be very safe; this is very important in clinical use. This report is the first to show the hepatoprotective effect of catechins against cell death induced by both toxins.

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