Enhancing Effect of Zinc on Hepatoprotectivity of Epigallocatechin Gallate in Isolated Rat Hepatocytes

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The influence of metal ions (Fe^{2+}, Cu^{2+}, Zn^{2+}) on the hepatoprotective activity of epigallocatechin gallate (EGCG) against hepatotoxin-induced cell injury was investigated. Primary cultures of rat hepatocytes were treated with a well-known hepatotoxin, bromobenzene (BB), in the presence of EGCG only or EGCG plus each metal ion. After 24 h, 0.02 mM EGCG did not show protective activity on the cultured hepatocytes. In contrast, the hepatocytes were protected against BB in the presence of 0.02 mM EGCG and 0.02 mM zinc. The addition of only zinc could not protect hepatocytes against BB. These results suggest that the formation of the zinc–EGCG complex is very important in the enhancement of the hepatoprotective activity of EGCG. The complexation of EGCG with zinc was confirmed by UV–VIS absorption spectroscopy.

Key words epigallocatechin gallate (EGCG); zinc; hepatoprotection; radical scavenge

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.[1)] Therefore, the disruption of the integrity of liver function leads to fatal cases or even irreversible organic death. In liver injury, it is supposed that the intervention of free radicals in normal metabolic processses is responsible for the pathological changes. Free radicals attack biomolecules and induce lipid peroxidation, enzyme inactivation, and finally cell necrosis.[2)]

Green tea catechins are the major constituents of tea leaves and have attracted significant interest in terms of their clinical and curative effects on many diseases. These effects of catechins are due to the radical scavenging and antioxidant activities inherent in polyphenolic compounds.[3—5)] To date, many attempts not only to take advantage of their desirable bioactivities, but also to enhance those activities, have been made.[6—11)] We found that epigallocatechin gallate (EGCG) showed hepatoprotective activity against hepatotoxins, bromobenzene (BB) and rubratoxin B (RB) (to be reported elsewhere). From the viewpoint of the clinical treatment of liver failure, it is important to protect normal hepatocytes from injury by using hepatoprotective agents with high activity and in small doses. Thus, the hepatoprotective effect of EGCG must be enhanced for its clinical use.

Investigation of pharmaceutical activities of metal-flavonoid complexes has been attracting interest recently. Satoh *et al.* reported that the cytotoxic activity of gallic acid for cancer cells was modified by metals.[11)] In a study using the oxygen electrode method, Kumamoto *et al.* found that copper strongly increased the antioxidant activity of EGCG.[6)] Thus, the different desirable effects of flavonoids, such as anticancer and antioxidant effects, are found to be enhanced by metals.

In this study, to enhance the hepatoprotective activity of EGCG, the effects of adding metal ions (Fe^{2+}, Cu^{2+}, Zn^{2+}) were examined by using BB as the hepatotoxin. It was found that zinc enhanced the hepatoprotective activity of EGCG. Also, the oxidation potential and the free radical scavenging activity of Zn–EGCG were evaluated using cyclic voltammetry and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) stable radical, respectively. The results are reported herein.

MATERIALS AND METHODS

Chemicals BB was purchased from Wako Pure Chemical Industries (Osaka, Japan). (−)-Epigallocatechin-3-gallate (EGCG) was isolated from green tea leaf.[12)] According to HPLC analysis, the purity of EGCG is higher than 95%. $FeSO_4 \cdot 7H_2O$ was obtained from Wako Pure Chemical Industries (Osaka, Japan). $CuCl_2 \cdot 2H_2O$ and $ZnCl_2$ were from Nacalai Tesque (Kyoto, Japan). DPPH was from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were obtained from local commercial sources, and used without further purification.

Media The basal medium A consisted of William's E medium (WE, ICN Biochemical, Costa Mesa, CA, U.S.A.), 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 the medium A supplemented with 1 nM insulin and 1 nM dexamethasone.

Isolation of Hepatocytes Hepatocytes were isolated from male Sprague-Dawley rats weighting 150—200 g by perfusing the liver with collagenase (from *Clostridium histolyticum*; Sigma-Aldrich, St. Louis, MO, U.S.A.) using the method of Seglen.[13)] Hepatocyte preparation with 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 on polystyrene culture plates (Nippon Becton and Dickinson, Tokyo, Japan), and incubated for 6 h in Medium B under the conditions of humidified air with 5% CO_2 at 37 °C. After 6 h, Medium B was exchanged for Medium A.

Preparation of Metal–EGCG Complexes The metal–EGCG complexes were prepared by mixing EGCG with metal salts in water. The formation of the complex was characterized by UV–VIS absorption spectroscopy using a

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BioSpec-1600 spectrophotometer (Shimadzu, Kyoto, Japan).

Treatment of Cells with Hepatotoxin in the Presence of EGCG and Metals One day after the isolation of hepatocytes, the medium was replaced with fresh Medium A containing 0.8% (v/v) dimethyl sulfoxide (DMSO) and BB. The final concentration of BB was adjusted to 1 mM. In the experiments investigating the hepatoprotective activities of EGCG, metals and metal–EGCG mixtures, different concentrations of each reagent were added to Medium A. Cells were incubated under each condition for another 24 h, and then the number of viable 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 that were not stained were counted. A control run was performed in the medium containing only 0.8% (v/v) DMSO.

Cyclic Voltammetry Cyclic voltammetry was used to observe the redox behaviors of EGCG and metal–EGCG complexes.[14—17] Cyclic voltammograms were recorded with a 315A potentiostat (Huso Electrochemical System, Kawasaki, Japan), in methanol : 0.1 M phosphate buffer (pH 7.5), (1 : 1, v/v). A glassy carbon (GC) electrode served as the working electrode, an Ag/AgCl electrode was used as the reference electrode, and a platinum electrode was used as a counter electrode. Before each measurement, the GC working electrode was freshly polished with 0.25 μm diamond slurry and rinsed with distilled water. The electrolytic cell was thermostatted at 25.0±0.1 °C.

Antioxidant Determination EGCG and metal–EGCG complex were dissolved in 0.1 M acetate buffer (pH 6.0), and then 1 ml of each solution was added to the same volume of 500 μM DPPH in ethanol (final concentration of 250 μM). The mixture was vortexed and left to stand for 20 min at room temperature in the dark. The absorbance at 517 nm by DPPH was measured using a BioSpec-1600 spectrophotometer (Shimadzu, Kyoto, Japan).[18,19]

Statistics All assays and quantitative measurements were performed in triplicate and the data were analyzed for statistical significance by the Student's *t*-test.

RESULTS

Protective Effects of EGCG against BB Treatment of the hepatocytes with BB reduced cell viability significantly compared to that of the control run (without BB). To examine the effective concentration for realizing the protective activity of EGCG against BB toxicity, different concentrations of EGCG were added to BB-treated cells. Cell viability was examined by means of the trypan blue exclusion assay and the results are shown in Fig. 1. EGCG at 0.2 mM significantly ($p<0.01$) protected the cells from BB toxicity. At this condition, viable cell number was kept at inocubated cell number, although in control (-BB), viable cells decreased by cell death associated with monolayer culture. EGCG protected hepatocytes from such cell death associated with cultivation (Fig. 2). The effect of EGCG (0.2 mM) is considered to be derived from simultaneous protection from both BB and cell death associated with monolayer culture. EGCG at 0.02 mM showed no protective activity against BB toxicity. According to these results, 0.02 mM EGCG was used to examine the effects of metals on the BB toxicity in the subsequent study.

Effects of Metals on the Hepatoprotective Activity of EGCG against BB To elucidate the effects of metal ions (Fe^{2+}, Cu^{2+}, Zn^{2+}) on the hepatoprotective activity of EGCG, each metal was mixed with EGCG at 0.02 mM. The complex formation with copper was characterized by the shift of the EGCG absorbance to the long-wave length region. In the cases of iron and zinc, the absorbance of EGCG increased and the spectra of both became broader (Fig. 3). Mixtures of 0.02 mM EGCG and 0.02 mM metal were added to BB-treated cells and the results are shown in Fig. 4. EGCG at 0.02 mM showed no protective activity against BB toxicity. However, the protective activity was observed by the addition of 0.02 mM EGCG and 0.02 mM zinc. In the case of iron or copper, cell viabilities were almost of the same level as that in

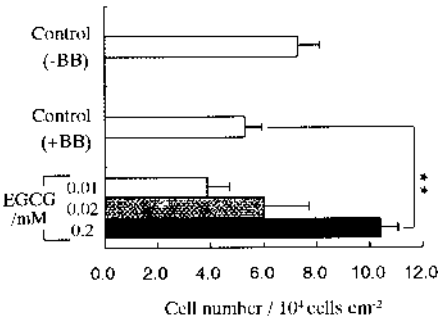

Fig. 1. Protective Activity of EGCG at Different Concentrations against Bromobenzene (BB)-Induced Acute Damage to Isolated Rat Hepatocytes

Hepatocytes were cultured in the medium containing 1 mM BB for 24 h. Data shown represent the analysis of three independent measurements and results are expressed as mean number of viable cells±S.D. $**p<0.01$ compared with the control.

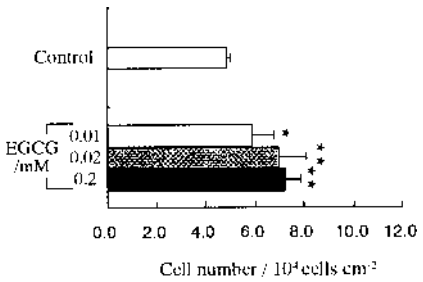

Fig. 2. Effect of EGCG at Different Concentrations against Cells Death Associated with Monolayer Culture

Hepatocytes were cultured in the medium for 24 h. Data shown represent the analysis of three independent measurements and results are expressed as mean number of viable cells±S.D. $*p<0.05$, $**p<0.01$ compared with the control.

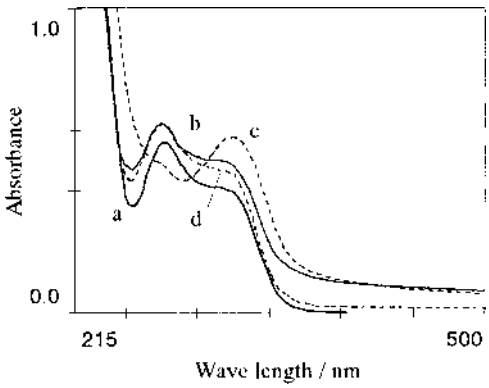

Fig. 3. UV–VIS Absorption Spectra of Metal–EGCG Complexes

a: EGCG, b: Fe–EGCG, c: Cu–EGCG, d: Zn–EGCG.

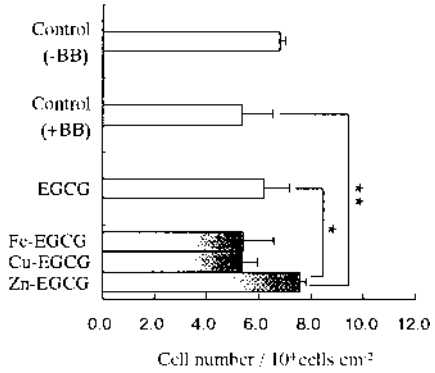

Fig. 4. Effects of Metals on the Hepatoprotective Activity of EGCG against BB Toxicity

Each of $FeSO_4$, $CuCl_2$ and $ZnCl_2$ solutions was mixed with equimolar EGCG solution at the final concentration of 0.02 mM. Results shown are means±S.D. of three independent measurements. $*p<0.05$, $**p<0.01$ compared with the control. Final concentration of BB is 1 mM.

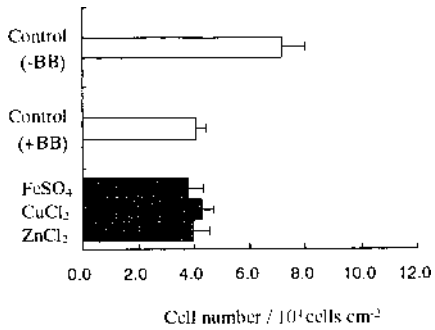

Fig. 5. Effects of the Addition of Only Metals on Cultured Rat Hepatocytes Treated with 1 mM BB

Each of $FeSO_4$, $CuCl_2$ and $ZnCl_2$ solutions at the final concentration of 0.02 mM was added to the medium. Results shown are means±S.D. of three independent measurements.

the case of EGCG.

To confirm the above effect arised from complex formation between EGCG and metal, protection by metals against BB was examined. Addition of only metals did not protect cells against BB toxicity as shown in Fig. 5.

Cytotoxic Effects of Metals The cytotoxic effect of each metal was also examined to confirm the safety of dosing metals to living cells. Each metal at 0.02 mM was added to normal cells and cell viability was measured (Fig. 6). Of the metals examined, only copper reduced cell viability and exhibited an undesirable cytotoxic effect. Copper is known to produce oxygen radicals and act as a pro-oxidant. The reduced viability of cells is considered to be due to this effect. Iron showed no cytotoxic effect, although it has the ability to produce oxygen radicals. Zinc had no toxic effect and was proven safe for administration.

Optimal Ratio of Zinc to EGCG Then, to determine the optimum ratio of zinc to EGCG for protecting liver cells from BB toxicity, Zn–EGCG complexes were prepared by mixing zinc and EGCG at different ratios and the hepatoprotective activities were examined (Fig. 7). The hepatoprotective activity of Zn–EGCG was increased with increasing ratio of $[ZnCl_2]$/[EGCG] in the complex. A linear response curve was obtained up to a $[ZnCl_2]$/[EGCG] ratio of 0.66. At ratios larger than 0.66, hepatoprotective activity became almost constant. Therefore, we applied two types of regression

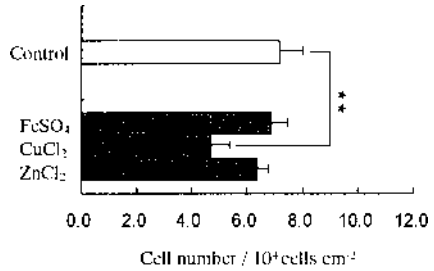

Fig. 6. Cytotoxic Effects of Metal Ions (0.02 mM) on Cultured Rat Hepatocytes

Results shown are means±S.D. of three independent measurements. $**p<0.01$ compared with the control.

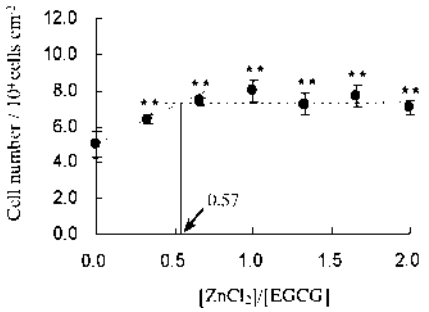

Fig. 7. Effect of $[ZnCl_2]$/[EGCG] Ratio on the Hepatoprotective Activity of Zn–EGCG Complex against BB Toxicity

([EGCG]=0.02 mM) $**p<0.01$ compared with the value at $[ZnCl_2]$/[EGCG] ratio of zero.

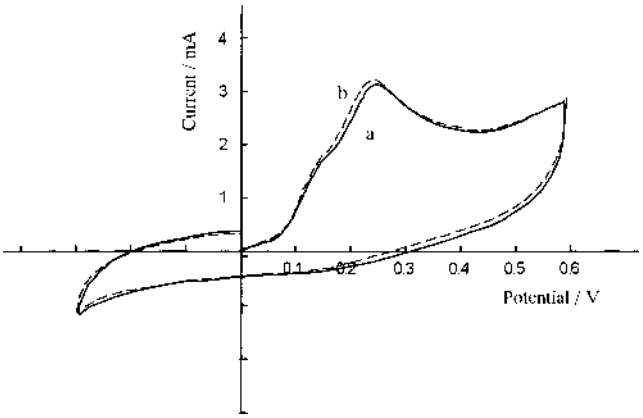

Fig. 8. Cyclic Voltammogram of Metal–EGCG Complex

a: EGCG, b: Zn–EGCG.

lines indicated below to fit to the experimental data points. Here, a, b and c are constants.

$$0<\text{ratio}<0.66: \quad (\text{cell number}\times 10^{-4}/\text{cm}^2)=a+b\times(\text{ratio}) \tag{1}$$

$$0.66<\text{ratio}: \quad (\text{cell number}\times 10^{-4}/\text{cm}^2)=c \tag{2}$$

The regression lines are represented by dotted lines and fitted well to the experimental data. The unknown constants obtained from the regression analyses were $a=5.00$, $b=4.24$, and $c=7.32$. The ratio of $[ZnCl_2]$/[EGCG] at the intersection of two lines was 0.57.

Cyclic Voltammetry To understand the effects of metal ions on the redox properties of EGCG, we measured the cyclic voltammogram of each material, and the results are shown in Fig. 8. The oxidation potential of EGCG did not change in the absence or presence of zinc.

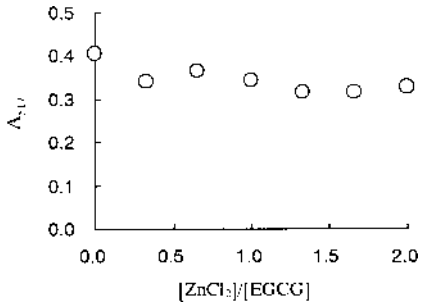

Fig. 9. Effect of $[ZnCl_2]$/[EGCG] Ratio on the Radical Scavenging Activity of Zn–EGCG Complex

[EGCG] = 0.02 mM.

Measurement of Radical Scavenging Activity The radical scavenging activities of EGCG and Zn–EGCG were examined using DPPH. This method is based on the reduction of DPPH, a stable free radical. This reaction has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidative activity of food and plant extracts. Figure 9 shows the radical scavenging activity of Zn–EGCG at various ratios of $[ZnCl_2]$/[EGCG], evaluated by the decrease of the absorbance at 517 nm by DPPH. The activity was slightly increased with increasing ratio of zinc. The results suggest that the formation of Zn–EGCG has no marked effect on the radical scavenging activity of EGCG.

DISCUSSION

Zinc is an essential trace element and is capable of protecting against hepatotoxicity produced by a variety of chemicals. Zinc pretreatment had been shown to protect the liver in many mammal models.[20—24] The mechanism of the zinc-mediated liver protection is related to the increase in hepatic metallothionein concentration. Metallothionein is easily inducible by metal ions and can scavenge reactive oxygen species that cause serious cellular injuries. Zinc acetate was approved by the FDA in 1997 as a drug for Wilson's disease.[25] These facts suggest the safety of zinc supplementation in therapy.

In our previous study, the potential of EGCG to protect hepatocytes from cytotoxin (BB, RB)-induced cell death in cultivation was found (to be published elsewhere). In the present study, we attempted to promote the hepatoprotective activity of EGCG by using metals, with the aim of discovering effective and safe hepatoprotective agents for the treatment of hepatic failure. UV-VIS spectrometry revealed that the absorption of EGCG increased and its peak became large by adding zinc. These results suggested that EGCG complexed with zinc. In the cases of iron and copper, EGCG was also found to complex with them (Fig. 3). Among the metals examined, zinc was found to significantly enhance the hepatoprotectivity of EGCG (Fig. 4). Since the addition of only zinc did not protect hepatocytes from BB toxicity, it is supposed that EGCG formed a complex with zinc, thereby becoming more effective in protecting the cells from BB toxicity. The contribution of metallothionein induced by zinc can not be assumed. To our knowledge, this report is the first to show that zinc enhances the hepatoprotective activity of EGCG.

The hepatoprotective effects of Zn–EGCG prepared at different $[ZnCl_2]$/[EGCG] ratios were examined. The hepatoprotective effect was the highest when the ratio of $[ZnCl_2]$/[EGCG] in the complex was 0.57, as shown in Fig. 5. This suggests that the Zn–EGCG complex may have the structure $Zn(EGCG)_2$. Yoshioka *et al.* proposed the structure of the Cu(II)–epigallocatechin (EGC) complex based on a spin trapping study. According to their model, the ratio of Cu(II) : EGC in the complex was 1 : 2 and the two OH groups at the B-ring of EGC were used for coodination with Cu(II).[26] The corresponding complex structure was proposed for $Zn(EGCG)_2$. The transition metals in the complex with a bioflavonoid are thought to acquire additional superoxide dismuting centers and therefore may become more effective free radical scavengers than the parent bioflavonoid.[10] By a similar mechanism, zinc was considered to enhance the radical scavenging activity of EGCG, resulting in the enhancement of the hepatoprotective activity of EGCG against BB toxicity in the form of $Zn(EGCG)_2$. In our study, however, the increase in the radical scavenging activity of EGCG by zinc was very small (Fig. 9). The redox property of Zn–EGCG was similar to that of EGCG (Fig. 8). Therefore not the physicochemical properties of Zn–EGCG but biological ones are thought to contribute to enhancing the hepatoprotective activity of EGCG. One possible mechanism is that the complexation of EGCG with zinc prevents the inactivation of EGCG by metabolism or auto-oxidation *etc*., thereby keeping its bioactivity for longer periods.

In conclusion, we demonstrated that the hepatoprotective activity of EGCG was clearly enhanced by zinc and Zn–EGCG could be a potent therapeutic agent for the liver. Our next step is to conduct *in vivo* studies of Zn–EGCG for clinical use.

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