Synergic Anticandidal Effect of Epigallocatechin-*O*-gallate Combined with Amphotericin B in a Murine Model of Disseminated Candidiasis and Its Anticandidal Mechanism

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In the present study, we investigated synergic anticandidal effect of epigallocatechin-*O*-gallate (EGCG) in a murine model of disseminated candidiasis caused by *Candida albicans*. In addition, its mechanism was examined. In the animal system, EGCG-given BALB/c mice group intraperitoneally (i.p.) before intravenous (i.v.) inoculation with viable *C. albicans* yeast cells survived longer than diluent-received (control) mice group ($p<0.05$). EGCG treatment inhibited the hyphal formation from the yeast form of *C. albicans*, causing growth-inhibition of the candidal cells. In experiments determining synergic effect, mice given diluent (control), Amp B (amphotericin B; 0.5 mg/kg of body weight), or EGCG (2 mg/kg) had mean survival times (MST) of approximately 10.9, 11.7, and 13.9 d, respectively. However, mice administered combination of Amp B (0.5 mg/kg) plus EGCG (2 mg/kg) had a MST value of 42.1 d, surviving an average of app. 30 d longer than the Amp B alone-received mice groups. The MST value from the combination-treated mice groups was much greater than MST value from mice groups that received four times the Amp B dose. These results indicate that EGCG, which has anticandidal activity causing blockage of the hyphal formation, has the synergism combined with Amp B against disseminated candidiasis.

Key words *Candida albicans*; epigallocatechin-*O*-gallate (EGCG); amphotericin B; hyphal formation; synergism; disseminated candidiasis; mice

Candida albicans, a dimorphic fungus, is the most common cause of fungal disease in humans.[1] This fungus is known as the fourth leading cause of nosocomial infections.[2,3] Over the last few decades, incidence of candidal infections due to *C. albicans* has been increasing, paralleling the growing numbers of immune-compromised patients.[2—5] To the patients, disseminated candidiasis is sometimes a serious disease which often results in death.[6] In addition, the fungus also causes local infections such as vaginitis and thrush. For medical treatment of the fungal infections, amphotericin B (Amp B) that has been considered as the drug of choice[7,8] and the azoles are mainly used in common clinical situations. However, toxicity and resistance to these antifungal drugs are a major problem.[2,3] In case of Amp B, an increased amount of Amp B must be administered to patients due to its poor permeability across the membrane,[9] which can result in severe side effects, for example, renal damage.[10,11] To lessen severity of the side effects, Amp B is often combined with other antifungal drugs such as the azoles,[12—14] but data reporting resistance of *C. albicans* to the azoles have been recently increasing.[15—17] Therefore, reduction of Amp B dose by combining it with a new product having anticandidal activity seems to be very important.

It has been reported that polyphenolic compounds[18] have antifungal activity. Okubo, *et al.*[19] reported that black tea extract containing the polyphenols inhibited the growth of *Tricophyton mentagrophytes* and *T. rubrum*. In recent, other researchers showed that a polyphenol, epigallocatechin gallate (EGCG), had growth-inhibitory effect on clinical isolates of *Candida* species, but not on *C. albicans*.[20] However, Hirasawa and Takada reported that EGCG inhibited *C. albicans* growth, revealing that the anticandidal activity was pH-dependant, and they also displayed a synergic effect of EGCG combine with Amp B as well.[21] The above observations were all done under *in-vitro* conditions. In our Lab, we also had a similar observation of inhibitory effect with EGCG on *C. albicans* growth (our unpublished data).

In this current study, we investigated anticandidal effect of EGCG on its own and furthermore combination effect with Amp B in a murine model of disseminated candidiasis due to *C. albicans*. In addition, we examined if EGCG could block the hyphal formation from yeast form of *C. albicans* as a possible anticandidal mechanism.

MATERIALS AND METHODS

Organisms and Culture Conditions *C. albicans* CA-1 that was characterized previously[22—24] was each grown in glucose–yeast extract–peptone (GYEP) broth at 37 °C as mentioned before.[22,23] Yeast form of *C. albicans* was collected from the broth cultures, washed with sterile cold Dulbecco's phosphate-buffered saline (DPBS; Sigma, St. Louis, MO, U.S.A.) solution and enumerated with use of hemocytometer to obtain desired numbers of yeast cells.

Mice BALB/c female mice (Charles River Labs, U.S.A.) at 6 to 7 weeks of age were used. The mice were maintained in the animal facility under Dongduk Women's University's regulation.

EGCG The EGCG was obtained from Sigma. Stock solution of EGCG was prepared in DPBS and sterile-filtered with use of syringe filter (pore size=0.45 μm, Corning, U.S.A.). Desired concentrations of the EGCG were diluted in sterile DPBS at pH 7.4.

Anticandidal Effect of EGCG in Mice The anticandidal effect was determined in a murine model of disseminated candidiasis as previously characterized.[22,23,25] The EGCG at 1, 2, and 4 mg/kg of body weight, respectively, were intraperitoneally (i.p.) administered to mice. One hour after the

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administration, the mice were intravenously (i.v.) inoculated with viable *C. albicans* yeast cells (5×10^5 yeast cells per mouse). Control mice received 100 μl of the DPBS. Their survival rates were then measured.

Assay of Hyphal Formation It is known that in the pathogenesis of *C. albicans* the hyphal form is more pathogenic than the yeast form[26]; thus, as one of possible mechanisms of the EGCG anticandidal activity, the morphological transition was examined. In experiments, the yeast form of *C. albicans* prepared as described the above was suspended in RPMI 1640 tissue culture medium (Sigma). The final concentration of the suspension was 2×10^5 cells per ml. To a designated flask containing the suspension, EGCG at concentrations of 10 and 20 μg/ml, respectively, was added and incubated at 37 °C in 5% CO_2 for 24 h. A control culture suspension received diluent instead of EGCG. The concentrations were selected based on our previous *in-vitro* assay with EGCG. That is, EGCG at above 20 μg/ml caused almost 100% killing of the candidal cells (data not shown). After the 24 h incubation, hyphal forming cells were counted by using hemocytometer. The ratio of the hyphal cells to yeast cells were calculated as follows: the percentage of hyphal cells=[(total number of cells−number of yeast cells)/(total number of cells)]×100. In addition, the transition was examined under a bright microscope (Olympus CXPCD, Japan).

Assessment of Synergic Effect against Disseminated Candidiasis Forty mice were grouped into four subgroups. Each subgroup was given a mixture of EGCG and Amp B, EGCG, Amp B, or diluent, respectively, by i.p.-route. One hour after the treatment, all of the mice were challenged, i.v., with 0.2 ml of live *C. albicans* yeast cells (25×10^5 cells/ml), and their survival rates were measured. Amounts of EGCG and Amp B in the combination were 2 and 0.5 mg/kg of body weight, respectively. The kidney is a target organ in experimental disseminated candidiasis; therefore, the number of CFU (colony forming unit) in kidney tissue may be used as an indicator of disease severity.[22,25] The CFU determination was performed by homogenizing the kidneys with homogenizers as described previously[22] and plated onto Mycosel agar (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD, U.S.A.).

Prior to these experiments, a minimal anticandidal dose of Amp B against the disseminated disease was determined as previously described.[24]

Statistics Statistical significance of differences in survival times was calculated through the Kaplan–Meier method (New Statistic for Windows; SPSS, Chicago, U.S.A.). For all other analyses, Student's *t* test was used.

RESULTS

EGCG Has Anticandidal Effect, *in Vivo* The anticandidal effect was determined in a murine model of disseminated candidiasis due to *C. albicans*. The resulting survive curve showed that the mice treated with EGCG survived longer than control mice that received the diluent, instead of the EGCG ($p<0.05$) (Fig. 1). These experiments were repeated three times. Results from the experiments were very similar. In one experiment, mice that received none, 1, 2, and 4 mg/kg EGCG of body weight had mean survival times (MST) of 11.0±2.7, 12.6±4.6, 17.4±5.5, and 29.0±12.5 [MST±standard error (S.E.)], respectively, resulting in that EGCG treatment enhanced resistance of mice against the disseminated candidiasis. Two out of the five mice given the dose of 4 mg survived until the end of 40 d-observation.

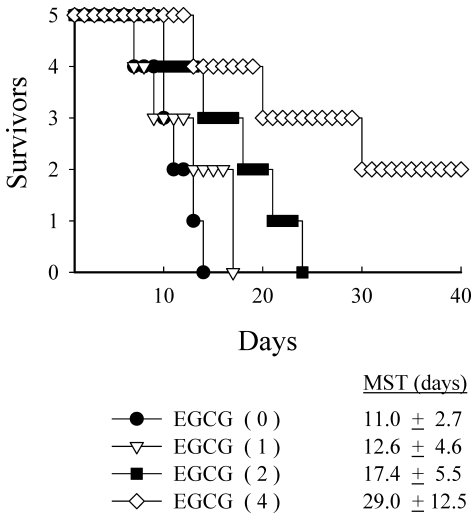

Fig. 1. EGCG Enhances Resistance of Mice against Disseminated Candidiasis

Mice given EGCG at various doses by i.p.-route were inoculated with *C. albicans* yeast cells, i.v., and their survival times were measured. EGCG at 2 resulted in marginal protection of mice, whereas EGCG at 4 enhanced resistance of the mice as compared to survival rates of untreated (control) mice groups ($p<0.05$). For determining synergy effect of the compound, dose of 2 was chosen. MST stands for mean survival time. MST stands for mean survival time. Unit: mg/kg of body weight.

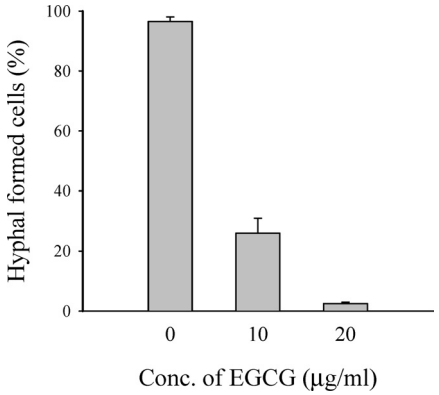

Fig. 2. EGCG Inhibits Hyphal Formation of *C. albicans* Yeast Cells

EGCG (none, 10, 20 μg/ml) was added to yeast for of *C. albicans* in RPMI 1640 culture medium and incubated at 37 °C in 5% CO_2 for 24 h. After the incubation, the hyphal forming cells were measured. EGCG treatment caused blocking the hyphal formation, decreasing the cell numbers. The data represent mean values of three repeated experiments. Error bar: S.E.

Based on these results, the dose of 2 mg/kg EGCG of body weight was chosen as a minimal dose resulting in marginal protection of mice against the disseminated disease for determining a synergic effect of EGCG in animals.

EGCG Blocks the Hyphal Formation Ratio of the hyphal formation of the EGCG-treated yeast cells was far less as compared with ratio of EGCG-untreated (control) yeast cells (Fig. 2). The EGCG treatment at 20 μg/ml inhibited the candidal growth up to more than 97%, and as compared with the control yeast cells (Fig. 3A), hyphal formation from the remained viable cells was almost blocked (Fig. 3C).

EGCG Synergy Combined with Amp B to Disseminated Canidiasis Before examining the synergic effect,

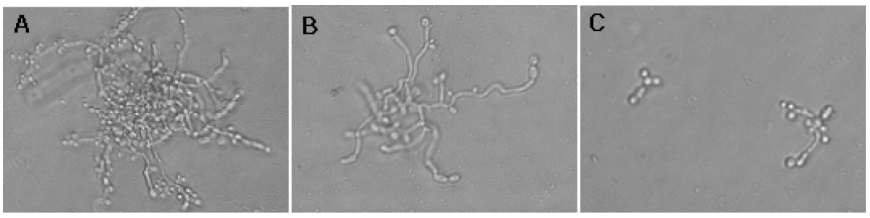

Fig. 3. Microscopic Observation Confirms the Blockage of Hyphal Formation

EGCG-treated *C. albicans* yeast cells as described in Fig. 2—legend were also examined microscopically (×400). EGCG-untreated (control) yeast cells (A) mostly produced hyphae, increasing cell numbers, whereas the EGCG-treated cells (B, C) had reduced ratios of hyphal formation as compared to the control. At higher dose of EGCG, most of the remained cells had no hyphae formed. Notice that cell numbers are decreased as dose of EGCG increases. Panels: EGCG-treated with; (A) none; (B) 10 μg/ml; (C) 20 μg/ml.

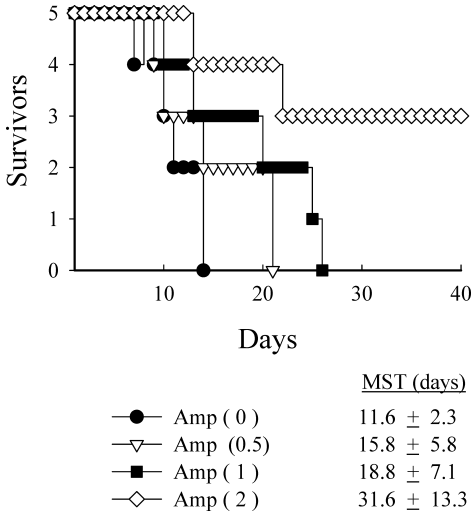

Fig. 4. Against the Disseminated Disease Amp B at 0.5 Results in a Minimal Dose

Amp B-treatment at the various doses displayed different degrees of protection of mice. Amp B at 0.5 was chosen for the synergic effect. MST stands for mean survival time. Unit: mg/kg of body weight.

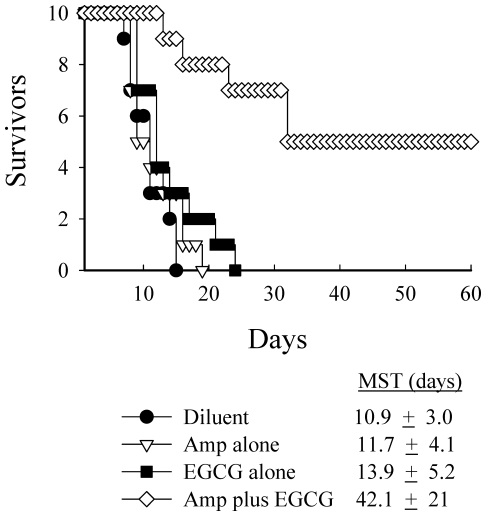

Fig. 5. EGCG Combined with Has Synergic Effect When Combined with Amp B against the Disseminated Candidiasis

Mice were given Amp B (0.5), EGCG (2), or a combination of EGCG (2) plus Amp B (0.5), respectively, before the i.v.-challenge with the yeast cells, and their survival times were measured. When the survival rates of mice given the combination were compared to survival rates from untreated (diluent)-received mice, the differences were statistically significant ($p<0.05$). Mice given either Amp B or EGCG had similar survival days as the diluent-given mice groups. The combination-treated mice survived around 32 d longer than the untreated mice groups during the period of 60 d-observation ($p<0.05$). These data indicate that EGCG has synergic effect with Amp B. MST stands for mean survival time. Unit: mg/kg of body weight.

anticandidal activity of Amp B alone at various concentrations against the disseminated disease was measured for determining a minimal antifungal dose and comparing the anticandidal effect of the combination effect. Results showed that mice given diluent (control), 0.5, or 1 mg/kg Amp B of body weight had MST (±S.E.) values of 11.6 (±2.3), 15.8 (±5.8) and 18.8 (±7.1) d, respectively (Fig. 4). The survivability at Amp B dose at 0.5 mg/kg of body weight was statistically almost similar to survivability of the control mice groups (Fig. 4). Thus, the dose of 0.5 mg/kg Amp B was chosen as a minimal dose for examining the synergic effect. For examining the synergy, we gave mice the combination of EGCG and Amp B before the i.v.-inoculation with yeast cells. Mice that were given DPBS (negative control), Amp B, EGCG alone, or Amp B plus EGCG resulted in a MST (±S.E.) of 10.9 (±3.0), 11.7 (±4.1), 13.9 (±5.2), and 42.1 (±21) d, respectively (Fig. 5). As before, mice that received Amp B (0.5 mg/kg of body weight) had similar survival days as the control mice groups. Mice treated with a double dose of Amp B (1 mg/kg of body weight) in the absence of EGCG survived longer, but their survivability was still less than the combination-treated mice group (Fig. 5). However, the animals treated with the combination survived significantly ($p<0.01$) longer than the control mice (Fig. 5). The 60-d survivors were sacrificed, and their kidneys were plated for candidal CFU. The kidneys from three of the five survivors showed CFU development (45.2×10^3/g, 27.5×10^3/g, and 72.1×10^3/g), whereas no CFU were detected in undiluted homogenates of kidneys from two of the mice.

DISCUSSION

Previously, we examined anticandidal effect of EGCG under an *in-vitro* condition. By agar diffusion and broth susceptibility tests, our results showed that EGCG inhibited growth of *C. albicans* yeast cells (data not shown). Furthermore, we determined that EGCG had synergism when it was combined with Amp B (data not shown). All of our *in-vitro* results confirmed the *in-vitro* results from Hirasawa and Takadas' report.[21)]

Continuing our investigation of anticandidal effect of EGCG, we examined if the anticandidal activity and synergic anticandidal effect of EGCG could occur under *in-vivo* condition. As an animal model, we used the murine model of disseminated candidiasis that was applied for determining candidal vaccines formulae,[22,23)] and synergic activities.[24)] In case of examination of the synergic activity, we chose Amp B, an antifungal drug, because of the reasons mentioned in

the introduction. In this study for checking antifungal effect of EGCG in the murine system, our results showed that EGCG enhanced resistance of mice against the disseminated disease. This test demonstrates that EGCG anticandidal activity can be observed even in the *in-vivo* system. The activity was dose-dependent, confirming the compound has a potential as an anticandidal agent although its activity is less efficient as compared with Amp B. We then attempted to find possible mechanism responsible for the activity. Other researcher has reported that EGCG disrupts ergosterol synthesis for the replication of *C. albicans*.[27] Until now, EGCG effect on the germ-tube formation, which is one of major virulence factors of the fungus[26,28,29] has been not investigated. From our current study, we observed that the compound itself inhibited the hyphal production when determined at EGCG concentration below 20 μg/ml by causing decrease of the cell numbers. Above the concentration, the EGCG-treated cells were entirely killed. This observation suggests that the inhibition might influence the ergosterol synthesis. How the two mechanisms are related to each other is beyond the aim of this study, though.

In examinations of synergic effect against disseminated candidiasis, mice given the combination therapy of EGCG plus Amp B at such minimal doses had strikingly high survival rates than survival rates resulted from mice groups that received EGCG or Amp B only, respectively. The survivability of the combination-treated mice groups was much greater than survival rates resulted from mice groups given four times the Amp B dose. The amount of Amp B in the combination therapy was a kind of marginal dose protecting mice against the disseminated disease when administered to mice in the absence of EGCG. All these results strongly suggest that EGCG has the synergic effect with Amp B against the experimental disseminated candidasis. From these data, a conclusion can be drawn that in clinical situations dose of Amp B may be reduced by combining it with EGCG that is far safe even up to a single dose of 1600 mg in human.[30]

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