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Report

Anti-tumor effect of gallic acid on LL-2 lung cancer cells transplanted in mice

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We previously reported that gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol, can induce apoptosis in four kinds of human lung cancer cell lines *in vitro*. The present study further investigated the *in vivo* anti-tumor effects of orally administered gallic acid. Gallic acid reduced cell viability of LL-2 mouse lung cancer cells *in vitro* dose dependently, with a 50% inhibitory concentration (IC_{50}) value of around 200 μM. C57Black mice were transplanted with LL-2 cells, and administered gallic acid (1 mg/ml in drinking water, *ad libitum*) and/or cisplatin (4 mg/kg i.p. injection, once a week). The average weight of the transplanted tumors, obtained at 29 days after transplantation, in the mice of control, gallic acid-treated cisplatin-treated and cisplatin plus gallic acid-treated groups was 4.02, 3.65, 3.19 and 1.72 g, respectively. The average tumor weight of the mice treated with cisplatin combined with gallic acid was significantly smaller than that of the control group ($p < 0.05$). The amount of apoptotic cells in the tumor tissues of mice treated with gallic acid and/or cisplatin was significantly higher than those of the control mice. Combination of gallic acid and cisplatin increased the tumor cell apoptosis compared with the treatment with cisplatin alone. The present findings suggest that the combination of gallic acid with an anti-cancer drug, including cisplatin, may be an effective protocol for lung cancer therapy. [© 2001 Lippincott Williams & Wilkins.]

Key words: Apoptosis, gallic acid, lung cancer.

Introduction

Treatment of patients with lung cancer still remains a vexing problem, because about 70% of all patients have advanced disease incurable with localized treatment (surgery and radiotherapy) alone. Cisplatin-based chemotherapy has been the standard systemic therapy for both non-small cell and small cell lung cancers for the past two decades, although the efficacy and benefit remain modest.[1–4] To improve the efficacy of chemotherapy for the treatment of advanced lung cancer, many regimens of combination chemotherapy have been introduced. Combinations with new chemotherapeutic agents and cisplatin produced a higher response rate than conventional cisplatin-based chemotherapy and improved survival was observed in some randomized trials.[5–8]

We previously demonstrated in *in vitro* experiments that gallic acid (3,4,5-trihydroxybenzoic acid), which is a naturally occurring plant phenol obtained by the hydrolysis of tannins, has a property to induce apoptosis in human lung cancer cells.[9] The apoptosis-inducing efficacy of gallic acid was not altered by the acquisition of cisplatin resistance in lung cancer cells. Gallic acid can be taken orally from food and medicinal herbs, and appears to be applied more safely to cancer patients compared with toxic chemotherapeutic agents. The *in vivo* anti-tumor effects of oral administration of gallic acid have not been reported. It is, therefore, important to clarify the *in vivo* anti-tumor effects of gallic acid against lung cancer cells and its additive or synergistic activity in combination with cisplatin.

In the present study, the effects of orally administered gallic acid were investigated on the growth of LL-2 mouse lung cancer cells transplanted into mice. We demonstrated that oral administration of gallic acid suppressed the growth of transplanted lung cancer by inducing tumor cell apoptosis *in vivo*. The efficacy of cisplatin on the apoptosis induction and tumor growth suppression was enhanced by gallic acid, suggesting the combination of cisplatin and gallic acid to be a promising protocol for the treatment of lung cancer.

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Materials and Methods

Cell cultures and drug treatment

A mouse lung cancer cell line LL-2 was obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). Cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (Filton, Tasmania, Australia) and antibiotics (50 μg/ml of streptomycin and 50 U/ml of penicillin). Gallic acid was obtained from Nakalai Tesque (Kyoto, Japan). Cisplatin was obtained from Bristol-Myers Squibb (Park Avenue,NY).

Cytotoxicity assay

Cells were seeded at 5×10^4 cells/well in 96-multiwell plates and precultured for 24 h before treatment. Drugs (cisplatin or gallic acid) were added in the medium at various concentrations. After 48 h of treatment, the relative viable cell number was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.[10] All the assays were carried out in triplicate. Each experiment was repeated at least 3 times.

Transplantation of LL-2 cells

C57Black male mice were obtained from Clea Japan (Tokyo, Japan). They were kept under sterile conditions in autoclaved cages with filter bonnets in a laminar flow unit, and were fed sterilized food pellets and distilled water. Mice were used for experiments when they were 6–8 weeks old. LL-2 cells [4×10^6 cells/ml in phosphate-buffered saline (PBS), pH 7.4, 50 μl/mouse] were transplanted s.c. into the backs of C57Black mice. The mice were then divided into the following four groups (n=8, for each group): (i) control group, (ii) gallic acid-treated group, in which gallic acid was administered by *ad libitum* drinking of water containing gallic acid (1 mg/ml) everyday after transplantation, (iii) cisplatin-treated group, in which mice were administered with cisplatin (4 mg/kg) i.p. on 1, 8, 15 and 22 days after transplantation, and (iv) gallic acid plus cisplatin group, in which mice drank water containing gallic acid (1 mg/ml) *ad libitum* and were administered with cisplatin (4 mg/kg) i.p. on 1, 8, 15 and 22 days after transplantation (Figure 1). Body weights were recorded once a week. Mice were sacrificed on day 29, and tumors were excised and weighed.

DNA nick end-labeling

The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method was perfomed according to the protocols described by Gavrieli *et al.*[11] with slight modifications. Tumor tissues were fixed with 4% paraformaldehyde for 12 h and 4-μm tissue sections were made. Tissue sections were treated with 20 μg/ml of proteinase K for 10 min. The slides were then washed with 0.5% Triton X-100 (Sigma, St Louis, MO) in PBS for 5 min at room temperature and incubated in a 1% glycine–PBS solution. After being rinsed

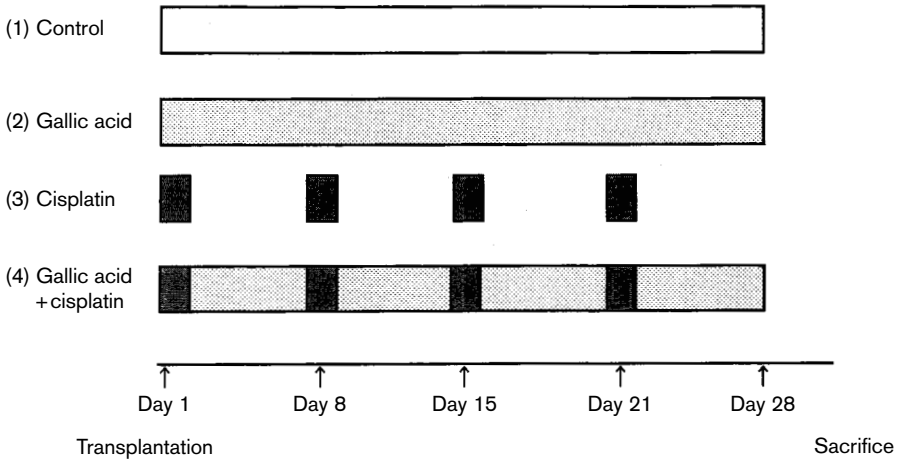

Figure 1. Experimental protocol and drug administration. LL-2 cells (4×10^6 cells/ml in PBS, 50 μl/mouse) were transplanted s.c. into the back of C57Black mice. The mice were divided into following four groups (n=8, for each group): (i) control group, (ii) gallic acid-treated group, in which mice drank water containing gallic acid (1 mg/ml) *ad libitum* everyday, (iii) cisplatin-treated group, in which mice were administered cisplatin (4 mg/kg) i.p. on 1, 8, 15 and 22 days after transplantation, and (iv) gallic acid plus cisplatin combination group.

with distilled H_2O, the slides were immersed in terminal deoxynucleotidyl transferase (TDT) buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate and 1 mM cobalt chloride). TDT (Gibco/BRL, Gaithersburg, MD) (0.3 U/ml) and biotin-16-dUTP (Boehringer Mannheim) in TDT buffer were then added to cover the sections and incubated in a humid atmosphere at 37°C for 60 min. The slides were washed with PBS and incubated with streptavidin–alkaline phosphatase conjugate (Gibco/BRL) at a concentration of 1 U/ml for 30 min at 37°C. After being rinsed with PBS, the slides were washed 3 times for 5 min each in 0.5% Triton in PBS, followed by three 5-min washes in PBS and three 5-min washes in Ap 9.6 [0.1 M Tris HCl (pH 9.6), 0.1 M NaCl and 0.1 M $MgCl_2$]. They were then immersed in AP 9.6 containing 5-bromo-4 chloro-3-indole phosphate (BCIP) (Sigma), 169 μg/ml, and nitro blue tetrazolium (NBT) (Sigma), 331 μg/ml, and left in the dark for 30 min. The color reaction was stopped by washing with Na_2-EDTA and the slides were examined under a light microscope. For the quantification of TUNEL expression, positive cells were counted in 20 random 0.159-mm^2 fields at $\times 100$.

Statistical methods

All values were expressed as mean $\pm$ SD. All data were analyzed by analysis of variance (ANOVA). Statistical significance was analyzed with the one- or two-tailed Student's *t*-test.

Results

In vitro effects of gallic acid and cisplatin on the viability of LL-2 cells

Cultured LL-2 cells were treated with various concentrations of gallic acid or cisplatin for 48 h and cell viability was evaluated by MTT assay. Both drugs reduced cell viablity of LL-2 cells dose dependently with a 50% inhibitory concentration (IC_{50}) value of around 25 μM for cisplatin and 200 μM for gallic acid (Figure 2). The cell death induced by cisplatin and gallic acid was demonstrated to be apoptosis, which was confirmed by characteristic morphology such as chromatin condensation and nuclear fragmentation (data not shown).

In vivo effects of gallic acid and cisplatin on the growth of transplanted tumors in mice

Gallic acid and/or cisplatin were administered until day 28 after transplantation. The average weight of the tumors at 29 days after transplantation in the control, gallic acid-treated, cisplatin-treated and gallic acid plus cisplatin-treated groups was 4.02 ± 0.45, 3.65 ± 0.68, 3.19 ± 0.44 and 1.72 ± 0.57 g, respectively. Transplanted tumors were significantly smaller in mice treated with the combination of gallic acid and cisplatin than those of the control mice (Figure 3).

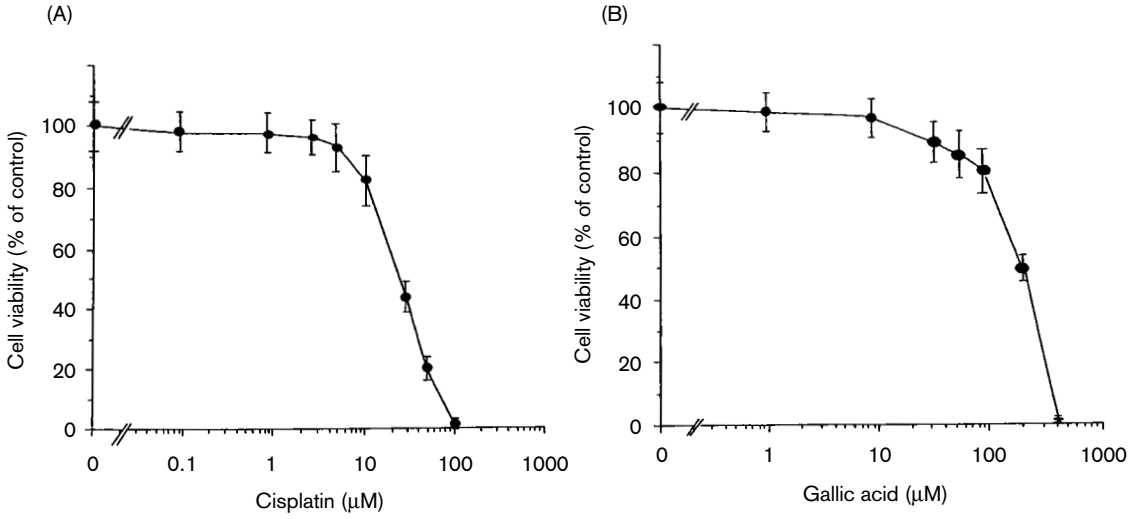

Figure 2. Effects of cisplatin (left panel) or gallic acid (right panel) on the cell viability of LL-2 cells. Cells were cultured in the presence of varying concentrations of gallic acid or cisplatin for 48 h. Cell viability was assessed with MTT assay and the values were plotted as a percentage of non-treated control culture. Values are presented as the mean $\pm$ SD.

The TUNEL method histologically detects DNA strand breaks of cells which indicates apoptotic cell death. The numbers of TUNEL-positive cells per microscopical high-power field view in the tumor tissues of control, gallic acid-treated, cisplatin-treated and gallic acid plus cisplatin-treated mice were 2.8 ± 1.7, 7.4 ± 4.1, 6.5 ± 3.3 and 9.7 ± 3.3, respectively (Figure 4). The numbers of apoptotic cells in the tranplanted tissues were significantly higher in mice treated with gallic acid ($p < 0.01$), cisplatin ($p < 0.01$) or gallic acid plus cisplatin ($p < 0.0001$) than the control group. The average number of apoptotic cells in the tumors of mice treated with cisplatin plus gallic acid was significantly higher than those of mice treated with cisplatin alone ($p < 0.01$).

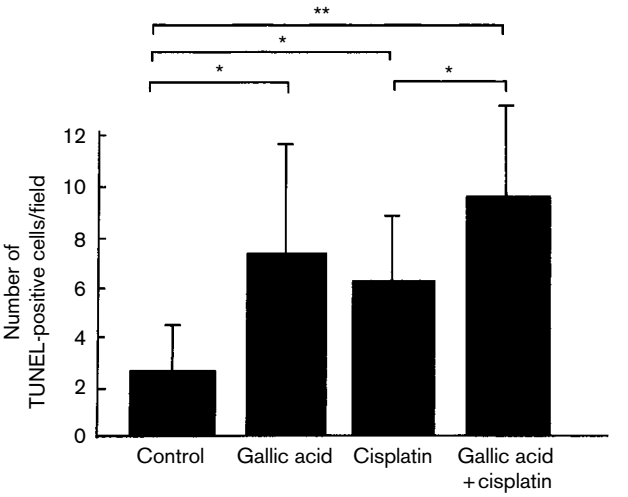

Figure 4. Quantification of apoptotic cells in LL-2 tumor tissue transplanted in mice. Numbers of TUNEL-positive cells were measured from 20 random 0.159-mm^2 fields at $\times 100$. Values are presented as the mean $\pm$ SD. *Significantly different at $p < 0.01$. **Significantly different at $p < 0.0001$.

Discussion

Plant polyphenoles are secondary metabolites widely distributed in various groups of the higher plant kingdom.[12] Many kinds of plant polyphenols, such as tannins and flavonoids, have been shown to be effective not only against tumor initiation, but also against the promotion phase of tumorigenesis. The mechanisms by which plant polyphenols inhibit carcinogenesis include anti-oxidative effects, and inhibition of activities of proliferation-associated enzymes such as ornithine decarboxylase, DNA polymerase, ribonucleotide reductase, etc.[13–15] These biological properties of plant polyphenols are now recognized as reasons why a polyphenol-rich diet, supplements or herbal medicines are effective in cancer prevention.

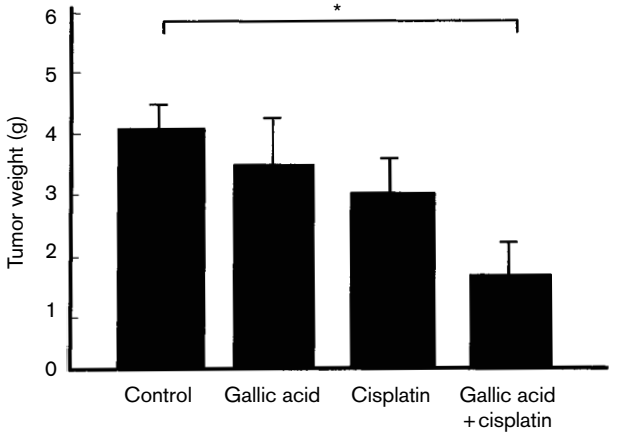

Figure 3. Average weight of the tumor at 29 days after transplantation in mice treated with gallic acid and/or cisplatin. The weight of the transplanted tumor in mice treated with the combination of gallic acid with cisplatin was signficantly smaller than that of control mice (*$p < 0.05$).

Some plant polyphenols have also been shown to induce apoptosis in cancer cells. For example, the tea polyphenol epigallocatechin gallate induces apoptosis by inhibiting S phase progression in many kinds of cancer cells;[16–19] genistein, the major isoflavone in soybean, inhibits the growth of prostate cancer cells *in vitro* by affecting the cell cycle and inducing apoptosis.[20,21] Inoue *et al.* reported that gallic acid, a plant phenol obtained by the hydrolysis of tannins, had a property to induce apoptosis with higher selectivity in cancer cells than in normal cells.[22,23] We previously reported that gallic acid induced apoptosis in four human lung cancer cell lines: SBC-3 (small cell carcinoma), EBC-1 (squamous cell carcinoma), A549 (adenocarcinoma) and SBC-3/CDDP (cisplatin-resistant subclone of SBC-3).[9] The susceptibility of cancer cells to gallic acid-induced apoptosis is not altered by the acquisition of cisplatin resistance, suggesting a possible synergistic anti-tumor activity by the combination of gallic acid and cisplatin.

These accumulating findings suggest gallic acid to be a promising candidate in cancer treatment. To verify this possibility, we have investigated the anti-tumor effect of gallic acid against LL-2 cells *in vivo*. We demonstrated in the present study that gallic acid induced apoptosis in transplanted tumor tissue and suppressed the growth of the tumor significantly

compared to controls. Tumor growth suppression and apoptosis induction by cisplatin were both enhanced by combination with gallic acid. Tumor growth depends on the balance between cell proliferation and apoptosis, and, therefore, the apoptosis-inducing activity of gallic acid may be associated with its anti-cancer activity. However, the mechanisms by which gallic acid induced apoptosis in tumor tissue remains to be elucidated. It has been reported that the formation of reactive oxygen species such as hydrogen peroxide and superoxide anions may be involved in gallic acid-induced cell death.[12,24–28] Effects on the signal transduction system should also be investigated.

An improved efficacy for growth suppression was demonstrated by the combination of cisplatin and gallic acid in a transplanted tumor model. Combination of cisplatin and gallic acid reduced tumor weight at low doses, which were not effective in reducing tumor weight by single use. Apoptotic cells were significantly higher in the tumor treated with gallic acid and/or cisplatin compared with that of controls. The appearance of apoptotic cells was increased by the combination of gallic acid plus cisplatin compared with the treatment with cisplatin alone. Increased tumor cell apoptosis generally represents increased treatment efficacy. Thus, the present observations suggest the more benefical effect of the combination of cisplatin and gallic acid in the treatment of lung cancer.

Cisplatin-based chemotherapy has been the standard systemic therapy for lung cancer and many regimens of combination with new chemotherapeutic agents have been introduced to improve the efficacy of chemotherapy for the treatment of advanced lung cancer.[29] However, the combination of many kinds of toxic chemotherapeutic agents often results in severe side effects when used at high doses. Since gallic acid is less toxic compared with other toxic chemotherapeutic agents, and has anti-mutagenic and anti-carcinogenic activities,[30,31] gallic acid may be useful for cancer treatment, prevention of recurrence and improvement of long-term survival. Further studies are needed to determine whether gallic acid and cisplatin combinations improve survival in comparison with other cisplatin-containing regimens.

Conclusion

The *in vivo* anti-tumor effects of orally administered gallic acid were investigated using LL-2 mouse lung cancer cells transplanted into mice. Oral administration of gallic acid was shown to suppress the growth of transplanted lung cancer by inducing tumor cell apoptosis *in vivo*, and significantly enhance the efficacy of cisplatin on the apoptosis induction and tumor growth suppression. It was suggested that the combination of cisplatin and gallic acid may be a promising protocol for the treatment of lung cancer.

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