Selective Induction of Cell Death in Cancer Cells by Gallic Acid

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Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring plant phenol obtained by the hydrolysis of tannins and is known to show some pharmacological activities. In screening anti-cancer agents in traditional Chinese medicines, gallic acid was found to show cytotoxicity against all cancer cells that we examined in this study (IC50= 4.8—13.2 μg/ml). Gallic acid, however, showed no cytotoxicity against primary cultured rat hepatocytes and macrophages, and lesser cytotoxicity against fibroblasts and endothelial cells. Cell death in DRl.h-84 cells occurred within 6 h after gallic acid treatment at a concentration of more than 20 μg/ml. A study of structurally related compounds suggested that the cytotoxicity shown by gallic acid was not a common feature in phenolic compounds, but was a fairly specific characteristic of gallic acid. That is, three adjacent phenolic hydroxyl groups of gallic acid were responsible for the cytotoxicity, and the carboxyl group was not responsible, but seemed to be implicated in distinguishing between normal cells and cancer cells.

Key words gallic acid; plant phenol; selective cytotoxicity; antioxidant

Plant polyphenols are well known to show biological and pharmacological activity, such as antimutagenicity, anticarcinogenicity, antiviral activity and antioxidative activity.1 Gallic acid, a naturally occurring plant phenol, was also found to exhibit similar activities.2–4 Furthermore, biochemical research has demonstrated that gallic acid inhibits the induction of ornithine decarboxylase activity,5 DNA polymerase activity,6 ribonucleotide reductase activity7 and so on. However, such activities of gallic acid are usually much smaller than those of tannins, tannic acid or gallic acid derivatives.8–10 As for antimutagenic activity, its efficacy was comparable to that of ascorbic acid, but less than 1% of that of tannic acid.11 Many agents with antioxidative activity have been shown to be effective in suppressing cancer expansion in a rodent hepatocarcinogenesis model. Antioxidants are considered to act as anticarcinogens or antimutagens by interacting with carcinogen itself or a carcinogen-produced reactive oxygen species. On the other hand, tannins, one of several plant phenols with antioxidative activity, are reported to show anticancer activity by modulating host immunity.11 Recently, a concept that agents for cancer therapy are required to act as a stimulator of cell death in cancer cells by apoptosis rather than as a growth inhibitor has been proposed and widely accepted.12 In addition, agents with selectivity for cancer cells rather than normal cells, for an originator of cancer or for a specific phase of the cell cycle must be further explored.13 We therefore set out to search for agents that show some kind of selectivity. First of all, we have focused on traditional Chinese medicine used for the treatment of chronic hepatitis or hepatic cancer. In this report, we represented that gallic acid, a plant phenol, was found to be a candidate as a cancer-selective agent.

MATERIALS AND METHODS

Chemicals Gallic acid (Nakashii Tesque Co., Kyoto, Japan) was recrystallized from water and used for the following experiment. Heparin, protocatechuic acid, 3,5-dihydroxybenzoic acid, p-hydroxybenzoic acid, pyrogallol, l-shikimic acid and tannic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). 4-O-Methylgalate was prepared according to the method of Schopf.14 Fetal bovine serum, bovine serum, RPMI 1640, Medium 199-Earle's salts and Eagle's minimum essential medium (MEM) were from Irvine Scientific Co. (Santa Ana, CA). William's E medium, MTI (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and antibiotics (penicillin and streptomycin) were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin was from Merck Co. (Frankfurt, Germany). Collagenase was purchased from Nitta Gelatin Co. (Osaka, Japan).

Cells PLC/PRF/5 cell (human hepatoma), HL-60RG (human promyelocytic leukemia), dRL.h-84 cell (rat hepatoma), KB cell (human epidermoid carcinoma), HeLa cell (human epithelial carcinoma), P388-D1 cell (mouse lymphoid neoplasma) and IMR-90 cell (human fibroblast) were provided by Japan Cancer Research Resources Bank. Primary cultured rat hepatocytes were isolated according to the method of Seglen.15 Rat macrophages were obtained from the rat peritoneal cavity 4 d after i.p. injection of 10 ml of 3% thioglycolate broth and were cultured in RPMI1640 medium containing 10% fetal bovine serum. Human umbilical vein endothelial cells were isolated by trypsin digestion and were maintained in Medium-199, 20% fetal bovine serum, endothelial cell growth supplement (Sigma, St. Louis, MO) at 50 μg/ml, and heparin at 25 μg/ml.

Cytotoxicity Assay 0.1 ml of cell suspension at the concentration of 3 × 104—1.5 × 105 cells/ml was incubated in a 96-multi-well plate and cultured for 24 h. After washing the cells with PBS (phosphate-buffered saline), 0.1 ml of medium containing gallic acid at appropriate concentrations was added and the mixture was then incubated for another 6 or 48 h. The culture medium was discarded and the remaining gallic acid was removed by

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thorough washing with fresh medium. The surviving cell number was determined by the MTT method. In the case of HL-60RG cells, cytotoxicity was determined by the trypan blue dye exclusion method.

RESULTS

In our phytochemical study, we isolated gallic acid as a component with anti-cancer activity demonstrated by cytotoxicity from chestnut bark (Juglans mandshurica Maxim. Cortex), which has been used as an ingredient in traditional Chinese medicine for the therapy of hepatocarcinoma or chronic hepatitis. Various anticancer agents are known to show cytotoxicity or cell growth inhibitory activity against cultured cells in vitro. We therefore examined the cytotoxicity of gallic acid to various kinds of cancer cells, as shown in Fig.1. Treatment with gallic acid for 48 h showed cytotoxicity against all cancer cells that we examined in the present study, suggesting that the cytotoxicity is independent of the origins of cancer cells, that is, animal species, organ, tissue. The IC_{50} for P388-D1, HL-60RG, HeLa, dRLh-84, PLC/PRF/5 and KB were 4.8, 5.4, 6.1, 6.2, 6.6 and 13.2 µg/ml, respectively. In the subsequent study, we used rat hepatoma dRLh-84 cells because the objective of this study was to find a potent agent against hepatic cancer and to compare sensitivity to normal cells, which are available as primary cultured rat hepatocytes. Figure 2 revealed that 6 h of treatment was sufficient to kill dRLh-84 cells at the concentration more than 20 µg/ml. To determine whether the cytotoxic activity showed selectivity for cancer cells, we studied the effect of gallic acid on hepatocytes, fibroblasts, macrophages and endothelial cells as normal cells. As shown in Fig. 3, hepatocytes and macrophages were not killed by 6 h, the time at which dRLh-84 cells were completely killed, whereas endothelial cells and fibroblasts were killed, although their IC_{50}s were about three times higher than that for dRLh-84 cells. When gallic acid at concentrations up to 100 µg/ml was incubated with 4% (v/v) rat erythrocyte suspension at 37°C for 15 min, no hemolytic activity was observed. These results indicate that normal cells are less sensitive to gallic acid than cancer cells. To shed light on the mechanism by which gallic acid shows cytotoxicity, we attempted to examine the relationship between the structure and the cytotoxic activity of gallic acid and to clarify whether the cytotoxic activity is a common feature of phenol compounds. Among the compounds tested in this study (Fig. 4), compounds structurally similar to gallic acid (l-shikimic acid, protocatechuic acid, p-hydroxybenzoic acid, 3,5-dihydroxybenzoic acid) and derivatives of gallic acid (3,5-methoxy-4-hydroxy-benzoic acid, 3,5-dihydroxy-4-methoxybenzoic acid) did not show any cytotoxicity to cancer cells. However, ethylgallate and tan-
nic acid showed cytotoxic activity similar to gallic acid as shown in Fig. 5A, but rather weak activity (Fig. 5B). Furthermore, pyrogallol killed dRLh-84 cells to the same degree as gallic acid. However, we found that 48 h treatment with pyrogallol killed hepatocytes (Fig. 6), although the IC_{50} value was much bigger than those for cancer cells.

**DISCUSSION**

In this study, we have represented that gallic acid induces selective cell death in cancer cells compared with normal cells. As gallic acid didn’t show hemolytic activity against erythrocytes at concentrations up to 100 μg/ml, the death of cancer cells triggered by gallic acid did not seem to be due to perturbation of the membrane. Further-
more, the selectivity shown by gallic acid was not merely ascribed to a difference in growth speed between normal and cancer cells, because gallic acid didn’t act at a specific phase of the cell cycle, as evidenced by flow cytometric analysis. Thus, gallic acid is found to induce cell death in cancer cells with higher sensitivity than normal cells, which are likely to have some protective mechanisms against gallic acid. According to the study of the relationship between the structure and the cytotoxicity of phenolic compounds, three adjacent phenolic hydroxyl groups should be essential to the cytotoxicity, since the methylation or deletion of phenolic hydroxyl groups completely removed the cytotoxicity. On the other hand, considering the facts that ethylgallate and tannic acid, an ethylerester of gallic acid, show cytotoxicity and that decarboxylation of gallic acid induces cytotoxicity for normal cells in addition to cancer cells, a carboxyl group is likely to be necessary in order for gallic acid to show selectivity to cancer cells. Several types of tannins have been reported to show cytotoxicity to cancer cells, whereas the addition of serum in the culture diminished the cytotoxicity to about 4% of the initial activity, suggesting that such tannins may not show cytotoxicity to cancer cells directly in vivo. Therefore, the cytotoxicity shown by some tannins is considered to be expressed through potentiation of the host-immune defense, for example, NK cell activation or the augmentation of interleukin secretion. On the other hand, since gallic acid didn’t show any effect on antibody production by determining the hemagglutinin titer in serum after intravenous injection of sheep red blood cells into mice, the cytotoxicity shown by gallic acid is not identical with that shown by such tannins. Gallic acid shows antioxidative activity and inhibitory activity of hydroperoxide production, which are usually considered to protect cells from cell death by oxidative stress. However, some kinds of antioxidants are known to act as pro-oxidants in the presence of metal ions. Considering the activities so far reported in phenolic compounds, we couldn’t find any relationship among the degrees of cytotoxicity shown in this study, and antioxidative and anticancer activities which have been reported so far. Consequently, these observations suggest that the cytotoxic activity shown by gallic acid is not a common feature observed in phenolic compounds, but a fairly specific characteristic of gallic acid. Ito et al. reported that gallic acid suppresses the induction of pre-neoplastic GST-P positive foci in rats using the modified method of Solt and Farber. In their study, gallic acid-containing diet was given rats for 6 weeks from 2 weeks after diethylnitrosamine administration, and the chemopreventive effect of gallic acid was confirmed. Although they considered that the anticancer action of gallic acid should be due to antioxidative activity, our data supported the idea that selective cytotoxicity against cancer cells might be also implicated in the reduction of cancer. Furthermore, gallic acid has been reported to be easily metabolized into 3,5-dihydroxy-4-methoxybenzoic acid and pyrogallol after oral administration and then excreted into urine. Schelme also reported that the methylation of gallic acid didn’t occur when the acid was incubated with intestinal flora, suggesting that gallic acid may be largely transformed in the liver. We are therefore attempting to establish the usefulness of agents such as gallic acid which have selective cytotoxicity against cancer cells for cancer therapy, especially liver cancer, by examining the routes of administration or by application to various animal cancer models. At present, the detailed mechanism by which gallic acid shows selective cell death against cancer cells is under investigation.

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