Cell Death-Inducing Activity by Gallic Acid Derivatives

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In this study, the cytotoxic activity of gallic acid derivatives (GDs) was studied using some cancer cell lines. Among them, 3,4-methylenedioxyphenyl 3,4,5-trihydroxybenzoate (GD-1) and S-(3,4-methylenedioxyphenyl)-3,4,5-trihydroxy-thiobenzoate (GD-3) were found to induce cell death in cancer cell lines with IC50 ranging from 2.9 to 114.4 μM, a concentration comparable with or lower than that of gallic acid. On the other hand, although gallic acid did not show any cytotoxicity against primary cultured rat hepatocytes and human keratinocytes, GD-1 and -3 showed slightly higher sensitivity against such normal cells, when compared with gallic acid. The cell death induced by gallic acid and GD-1 was accompanied by internucleosomal DNA fragmentation characteristic of apoptosis, whereas only smear DNA degradation was detected following GD-3 treatment. When the mechanism by which GD-1 and -3 caused cell death in HL-60RG cells was examined, GD-1 and -3-induced cell death was inhibited by the intracellular Ca2+ chelator, bis-(o-aminophenoxo)-N,N,N',N'-tetracetic acid acetoxymethyl ester (BAPTA-AM), calmodulin inhibitor, W-7, and the Ca2+/Mg2+-dependent endonuclease inhibitor zinc sulfate. In contrast, catalase, N-acetylcysteine (NAC), and ascorbic acid inhibited gallic acid-induced apoptosis in HL-60RG cells, whereas they had no effect on GD-1- and -3-induced cell death. This result suggests that GD-1 and -3 induced cell death in a different manner to gallic acid. In conclusion, esterification of gallic acid with a 3,4-methylenedioxyphenyl group yielded potent agents to treat cancer with a different signaling pathway from gallic acid, although selectivity was lost.

Key words gallic acid; gallic acid derivative; cytotoxicity; signaling pathway

Apoptosis and necrosis are regarded as morphologically and biochemically distinct modes of cell death. Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA degradation and apoptotic body formation, and is believed to play a critical role in controlling normal embryogenesis, development of the immune system, elimination of virus-infected cells and the maintenance of tissue homeostasis. On the other hand, necrosis is characterized by cell swelling and plasma membrane disruption, and is regarded as a degenerative phenomenon produced by injury. Recent extensive studies have revealed that the mode of cell death is markedly varied and that apoptosis and necrosis appear to be extremes of a continuum of multiple forms of death. Both types of cell death can occur simultaneously in tissue or cell cultures exposed to the same stimulus, and often the intensity of the same initial insult decides the prevalence of either apoptosis or necrosis. In fact, many signals as diverse as oxidative stress, nitric oxide, protein synthesis inhibitor, and glutamate can elicit both apoptosis and necrosis. Although the destiny of cells which undergo apoptosis or necrosis is dependent on the stimuli, the physiological condition of cells is also an important determinant.

Gallic acid is a naturally occurring plant phenol, which has been reported to show a number of biological activities. We have previously found that gallic acid induces apoptosis in promyelocytic leukemia HL-60RG cells. Its cytotoxic activity is mediated by reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion and intracellular Ca2+. Although gallic acid has three hydroxyl groups and one carboxyl group, structure-activity relationship studies reveal that methylation of the phenolic hydroxyl group and esterification of the carboxyl group markedly reduces cytotoxic activity. A recent study has suggested that apoptosis-inducing activity might be paralleled by the intensity of both the gallate radical and oxidation potential, although such radicals were determined at pH 9.0, but not under physiological conditions. Furthermore, the distinctive characteristic of gallic acid-induced cell death is that normal cells such as primary cultured rat hepatocytes and macrophages are resistant to gallic acid, while endothelial cells and fibroblasts are less sensitive to gallic acid than cancer cells. Therefore, accumulating evidence prompted us to develop gallic acid derivatives (GDs) that are more potent agents with selectivity against cancer cells. In this study, we examined the cytotoxic activity of GDs, the mechanism by which they induce cell death, the mode of cell death and the signaling pathway.

MATERIALS AND METHODS

Chemicals Gallic acid (Nacalai Tesque Co., Kyoto, Japan) was recrystallized from hot water and used in the following experiments. Donor bovine calf serum (BS), fetal calf serum (FCS), minimal essential medium (MEM)-Eagle's salts (with non-essential amino acids) and RPMI1640 were purchased from Irvine Scientific Co. (Santa Ana, CA). Antibiotics (penicillin and streptomycin) were from Life Technologies Inc. (Grand Island, NY, U.S.A.). Trypsin (pancreas protease) was obtained from Merck Co. (Frankfurt, Germany). Proteinase K and RNase A were obtained from Sigma Chemical Co. (St. Louis, MO). Bis-(o-aminophenoxy)-N,N,N',N'-tetracetic acid acetoxymethyl ester (BAPTA-AM), catalase, and N-acetylcysteine (NAC) were from Wako Pure Chemical Industries (Osaka, Japan). N-(6-Amino-hexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was obtained from Funakoshi Co. (Tokyo, Japan). Ascorbic acid was from Katayama Chemical Co. (Osaka, Japan) and zinc sulfate (ZnSO4) was purchased from Nacalai Tesque Co. (Kyoto, Japan).

Cell Culture HL-60RG (human promyelocytic leukemia), dRLh-84 (rat hepatoma), S-180 (mouse sarcoma), and
HeLa (human epithelial carcinoma) cells were provided by the Japan Cancer Research Resource Bank. Human keratinocytes were purchased from Oriental Yeast Industry (Tokyo, Japan). HL-60RG cells were cultured in RPMI1640 medium supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. dRLH-84 cells were in MEM-Eagle's salts supplemented with 20% BS, 50 U/ml penicillin and 50 μg/ml streptomycin. S-180 cells were in MEM-Eagle's salts supplemented with 10% BS, 50 U/ml penicillin and 50 μg/ml streptomycin. HeLa cells were in MEM-Eagle's salts supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. Keratinocytes were cultured in serum-free medium, K110 type-II (Kyokuto Pharm. Industry Inc., Tokyo, Japan).

Hepatic parenchymal cells were isolated from male SD rats (300 g) by the two-step collagenase perfusion method of Seglen.\(^{20}\) Cell viability was determined by the 0.25% trypan blue dye exclusion method and cells with viability more than 91.3 ± 0.10% (n = 9) were used in this study. Hepatocytes were seeded into collagen-coated 96-multi-well culture dishes at a density of 1.5 × 10⁶ cells/well and incubated in William's E medium (pH 7.4) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 10⁻⁷ M insulin and 10⁻⁷ M dexamethasone. After incubation for 4 h at 37°C in an atmosphere of 95% air and 5% CO₂, hormone-containing medium was removed from the wells. The cells were washed twice with phosphate-buffered saline (PBS) and then incubated in William's E medium supplemented with 10% FCS for the cytotoxic assay.

GDs The GDs described in Fig. 1 were kindly provided by Tsumura Co., Ltd. The derivatives that we chose were 3,4-methylenedioxyphenyl-3,4,5-trihydroxybenzoate (GD-1), N-(3,4-methylenedioxyphenyl)-3,4,5-trihydroxybenzamide (GD-2), S-(3,4-methylenedioxyphenyl)-3,4,5-trihydroxythiobenzoate (GD-3), N-acetyl-S-(3,4,5-trihydroxybenzoyl)-l-cysteine methyl ester (GD-4), N-(3,4,5-trihydroxybenzoyl)-S-methyl l-cysteine methyl ester (GD-5), N-(3,4,5-methylenedioxyphenyl)-N-(3,4,5-trihydroxybenzoyl) glycine methyl ester (GD-6), and 3’,4’-methylenedioxy-N-(3,4,5-trihydroxybenzoyl)-S-methyl l-cysteine anilide (GD-7).

Cytotoxicity Assay 0.1 ml cell suspension of rat primary cultured hepatocytes, keratinocytes, HL-60RG, dRLH-84, S-180, and HeLa cells were seeded into 96-multi-well plates at concentrations of 3 × 10⁴–1.2 × 10⁵ cells/ml and cultured for 24 h. After washing the cells with PBS, 0.1 ml medium containing gallic acid or its derivatives dissolved in dimethyl sulfoxide (DMSO, less than 0.1% in an assay mixture to prevent the concentration of which did not show cytotoxicity against any of the cells used in this study) were added to the cell culture at appropriate concentrations, followed by incubation in 5% CO₂ in air for 48 h. The cytotoxic activity was determined by measuring the number of surviving cells using the 3′,4′,5′-tris(methylenehiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)\(^{21}\) or trypan blue dye exclusion methods.

In a study to examine the effects of various kinds of inhibitors, inhibitors (catalase, 10 U/ml; NAC, 5 mM; ascorbic acid, 0.28 mM; BAPTA-AM, 25 μM; W-7, 50 μM; ZnSO₄, 0.2 mM) were added to HL-60RG cells 30 min before the addition of gallic acid or its derivatives. Following 48 h culture, cell viability was determined by the trypan blue dye exclusion method.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis HL-60RG cells (6 × 10⁶ cells/10 ml/dish) exposed to gallic acid or its derivatives for 48 h were collected in tubes and then washed with PBS. The cells were incubated for 10 min in 500 μl lysis buffer (20 mM Tris–HCl pH 7.4, 10 mM EDTA, 0.2% Triton X-100) at room temperature and centrifuged at 10000 g for 10 min at 4°C. The supernatant was incubated overnight at 50°C with 100 μg/ml proteinase K. DNA was extracted with 1 vol. chloroform/phenol (1:1), precipitated from the aqueous phase with 1 vol. isopropanol and 500 mM NaCl at −20°C overnight, and collected by centrifugation at 14000 g for 30 min at 0°C. The pellet was suspended in 70% ethanol and centrifuged at 14000 g for 10 min at 0°C. The pellet was then dried under reduced pressure and incubated in 25 μl 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA for 1 h at 37°C with 1 μg/ml RNase A. Samples were heated at 65°C for 10 min and subject to agarose gel

![Fig. 1. Structures of GDs (GD-1, 2, 3, 4, 5, 6, 7)](image-url)
electrophoresis after the addition of a loading buffer. Horizontal electrophoresis was performed for 1 h at 80 V in 1.5% agarose gel with Tris–borate–EDTA buffer (TBE) (×0.5) as a running buffer. After treatment of the gel with 0.5 μg/ml ethidium bromide for 10 min, DNA was visualized under UV light.

RESULTS

We previously found that gallic acid shows cytotoxic activity against cancer cells with a higher sensitivity, around 35 μM of IC_{50}, than normal cells such as primary cultured rat hepatocytes, macrophages, endothelial cells, and fibroblasts. Structure–activity relationship studies revealed that methylation of the phenolic hydroxy group abolished this activity and esterification of the carboxyl group with an ethyl group markedly attenuated the cytotoxic activity (IC_{50} against dRLh-84 cells: more than 150 μM).

In the present study we synthesized the GDs depicted in Fig. 1, which had more hydrophobic residues than ethyl ester, and examined their cytotoxicity against several cancer cell lines and normal cell lines. Among the derivatives, GD-1 and -3 showed more potent cytotoxic activity against dRLh-84 cells than gallic acid (Fig. 2), and other derivatives exhibited comparable or less activity than gallic acid. GDs were first dissolved in DMSO and then added to the culture medium at appropriate concentrations. The DMSO concentration in the assay mixture was less than 0.1%, at which level no cytotoxicity was detected.

In this study, the existence of an amido bond appeared to reduce the cytotoxic activity of GDs. Therefore, we investigated the cytotoxic activity of GD-1 and -3 in detail. GD-1 and -3 showed more potent cytotoxic activity than gallic acid against all cancer cell lines tested in this study, although HeLa cells were less sensitive to GD-1 and -3 (Fig. 3). On the other hand, when the cytotoxic activity against normal cells was examined, GD-3 caused damage to primary cultured rat hepatocytes, which are not cancer cells and do not have dividing ability in this culture condition, whereas GD-1 hardly showed cytotoxicity as gallic acid. However, against human keratinocytes, which are also not cancer cells but have dividing ability, GD-1 and -3 were cytotoxic at lower IC_{50} of 72.7 and 42.8 μM compared with gallic acid (Fig. 3). The IC_{50} obtained in this study are summarized in Table 1.

When the activities of GD-1 and GD-3 were compared, thioester GD-3 showed more potent cytotoxicity than GD-1 against cell lines tested.

To clarify the mode of cell death caused by GD-1 and -3, we first examined the effects of GD-1 and -3 on the internucleosomal DNA fragmentation characteristic of apoptosis. GD-1 and gallic acid elicited internucleosomal DNA cleavage, as shown by the agarose gel electrophoresis of DNA extracted from the treated HL-60RG cells (Fig. 4). In contrast, GD-3 treatment failed to show such DNA fragmentation, but the smear DNA degradation pattern characteristic of necrosis was seen.

We previously showed that gallic acid-induced apoptosis in HL-60RG cells was mediated by ROS and intracellular Ca^{2+}. This was supported by the fact that catalase, NAC, and ascorbic acid inhibited it, as did the intracellular Ca^{2+} chela-

<table>
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<tr>
<th>Hepatocyte</th>
<th>Keratinocyte</th>
<th>HL-60RG</th>
<th>dRLh-84</th>
<th>HeLa</th>
<th>S-180</th>
</tr>
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<td>GA</td>
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<td>524.3</td>
<td>31.7</td>
<td>36.4</td>
<td>72.3</td>
</tr>
<tr>
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<td>24.5</td>
<td>14.5</td>
<td>114.4</td>
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<tr>
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<td>42.8</td>
<td>8.5</td>
<td>3.9</td>
<td>90.8</td>
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</tbody>
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This table summarizes the IC_{50} of gallic acid, GD-1 and GD-3 on hepatocytes, keratinocytes, HL-60RG, dRLh-84, HeLa and S-180 cells.

Fig. 2. Cytotoxicity of Gallic Acid and Its Derivatives against dRLh-84 Cells

dRLh-84 cells (3×10^5 cells/ml) were incubated with gallic acid and its derivatives at various concentrations for 48 h. After washing with fresh medium, the number of viable cells was determined by the MTT method. ○, GD-1; ●, GD-2; □, GD-3; ■, GD-4; △, GD-5; ▲, GD-6; ○ (broken line), GD-7 and ● (broken line), gallic acid. Each value represents the mean±S.E. of 8 wells.

Fig. 3. Cytotoxicity of Gallic Acid, GD-1 and GD-3 against Hepatocytes, Keratinocytes, and Various Cancer Cells

Cells were incubated for 48 h at various concentrations of gallic acid, GD-1 and GD-3. After incubation, viability was determined by the MTT method, except for HL-60RG cells whose viability was determined by the trypan blue dye exclusion method. ○, Hepatocytes (1.5×10^5 cells/ml); △, keratinocytes (1.2×10^5 cells/ml); ●, HL-60RG cells (6×10^5 cells/ml); ■, dRLh-84 cells (3×10^5 cells/ml); ▲, HeLa cells (4×10^5 cells/ml); □, S-180 cells (6×10^5 cells/ml). Each value represents the mean±S.E. of 4–8 wells.
tor, BAPTA-AM, the calmodulin inhibitor, W-7, and the Ca^{2+}/Mg^{2+}-dependent endonuclease inhibitor zinc sulfate, ZnSO_4.

Next, we investigated the effects of these inhibitors or antioxidants on GD-1-and -3-induced cell death. As shown in Fig. 5, BAPTA-AM and W-7 inhibited GD-1- and -3-induced cell death completely in the same way that they inhibited gallic acid-induced cell death. However, ZnSO_4 inhibited GD-3-induced cell death incompletely and did not prevent GD-1-induced cell death whereas gallic acid-induced cell death was completely suppressed. In addition, catalase, ascorbic acid and NAC, all of which prevented gallic acid-induced cell death, failed to inhibit GD-1- and -3-induced cell death (Fig. 6). These results indicate that the mechanism by which gallic acid, GD-1 and -3 induced cell death differed as far as their signaling pathway was concerned. Thus, GD-1 and -3 did not require ROS as a mediator of cell death. In addition, the activity of GD-1 and -3 also differ, as shown by the difference in response to ZnSO_4 and in their induction of DNA fragmentation.

DISCUSSION

In this study we have found that the GDs, GD-1 and -3, whose hydrophobicity was increased by esterification of the carboxyl group with a 3,4-methylenedioxyphenyl group, induced cell death against cancer cells in a different manner from gallic acid, although we previously reported that esterification markedly reduced this cytotoxic activity. Modification of the carboxyl group by introducing the 3,4-methylenedioxyphenyl group appeared to be more effective in increasing the cytotoxic activity than using other residues, and the thioster derivative showed more potent activity than the ester and amido derivatives. In fact, IC_{50} of GD-3 and GD-2 was about 10- and 2-fold lower, respectively, than that of gallic acid, except for HeLa cells. Although the reason why

Fig. 4. Agarose Gel Electrophoresis of DNA Extracted from HL-60RG Cells Treated with Gallic Acid, GD-1 and GD-3

HL-60RG cells (6x10^5 cells/10ml/dish) were exposed to gallic acid (25 μg/ml), GD-1 (50 μg/ml) and GD-3 (50 μg/ml) for 48h. DNA was isolated and subjected to electrophoresis on 1.5% agarose gel as described in Materials and Methods. Lane 1, gallic acid; lane 2, GD-1; lane 3, GD-3; lane 4, MW standard.

Fig. 5. Effect of Intracellular Ca^{2+} Chelator, Calmodulin Inhibitor and Ca^{2+}/Mg^{2+}-Dependent Endonuclease Inhibitor on Gallic Acid- , GD-1- , or GD-3-Induced HL-60RG Cell Death

BAPTA-AM (●, 25 μM), W-7 (●, 50 μM), ZnSO_4 (●, 0.2 mM) or nothing (●) were added to HL-60RG cells 30 min before addition of gallic acid, GD-1 and GD-3 at various concentrations. After 48 h incubation, cell viability was determined by the trypan blue dye exclusion method. Each value represents the mean±S.E. of 4 wells.

Fig. 6. Effect of Antioxidants and Catalase on Gallic Acid- , GD-1- , or GD-3-Induced HL-60RG Cell Death

Catalase (●, 10 U/ml), NAC (●, 5 mM), ascorbic acid (●, 0.28 mM) or nothing (●) were tested for gallic acid- , GD-1- , or GD-3- induced HL-60RG cell death. The experimental procedure was the same as described in Fig. 5. Each value represents the mean±S.E. of 4 wells.
HeLa cells were more resistant to GDs and gallic acid than other cancer cells is not obvious at present, it may be that HeLa cells generally exhibit resistance to the Ca²⁺-mediated signaling pathway leading to cell death.

Primary cultured rat hepatocytes, which are not cancer cells and do not have essential dividing ability, were resistant to GD-1 and gallic acid, whereas they were killed by GD-3 at a high IC₅₀ of 117.5 μM. On the other hand, keratinocytes with normality and dividing ability were sensitive to GD-1 and GD-3, but not to gallic acid. These data suggest that GD-1 and -3, which resulted from esterification of the carboxyl group, have lost the selectivity inherent in gallic acid. Hence, GD-1 and GD-3 damaged both normal and cancer cells, indicating that the presence of a free carboxyl group is necessary for a lack of cytotoxicity against normal cells.

Cell death induced by GD-1 and -3 was inhibited by BAPTA-AM and W-7, suggesting that GD-1 and -3 elevated intracellular Ca²⁺, leading to activation of calmodulin as gallic acid did. Although Ca²⁺ is not regarded as a common signal in the signaling pathway leading to death, it may play a pivotal role in GD-1, -3, and gallic acid-induced cell death. In addition, antioxidant NAC, ascorbic acid, and catalase failed to inhibit GD-1 and -3-induced cell death, although all of them suppressed gallic acid-induced cell death. This indicates that ROS does not cause cell death induced by GD-1 and -3 and/or is not involved in the signaling pathway associated with GD-1 and -3. In contrast, in the case of gallic acid, the combined effect of ROS and intracellular Ca²⁺ may accelerate the induction of cell death. The mode of cell death differed between GD-1 and -3 in terms of the pattern of DNA degradation and sensitivity to ZnSO₄ treatment. An endonuclease responsible for DNA degradation might be activated in a different manner by gallic acid, GD-1, and GD-3, or different endonucleases might be involved in DNA degradation. Recent evidence indicates that apoptosis and necrosis share common features in the early death signaling pathway and some signals such as oxidative stress, nitric oxide, protein synthesis inhibitor, and glutamate can elicit both apoptosis and necrosis, depending on their intensity or the physiological conditions under which the cells are maintained. Furthermore, apoptosis and necrosis can be regarded as extremes of a continuum of multiple forms of death, and a variety of modes of cell death could coexist in some pathological situations. Taking into consideration that necrosis can elicit inflammation at the site where cell death occurs, apoptosis-inducing agents which possess a moderate ability to induce cell death in target cells under specific physiological conditions are suitable anticancer agents. Accordingly, the development of GDs that possess a different intensity and are highly selective against cancer cells may pave the way to identifying interesting anticancer drugs. In conclusion, we found GDs with more potent activity than gallic acid itself and a different signaling pathway from that of gallic acid, although selectivity was lost. We are now undertaking an experiment to clarify in detail the differences in mechanism by which gallic acid and its derivatives induce cell death. We hope this will allow us to develop a range of potent derivatives.

REFERENCES