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Reactive Oxygen Species and Intracellular Ca^{2+}, Common Signals for Apoptosis Induced by Gallic Acid

*Nahoko Sakaguchi, Makoto Inoue and Yukio Ogihara**

Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467, Japan

ABSTRACT. Gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol, induces cell death in apparently different manners, depending on cell lines. Flow cytometric analysis and agarose gel electrophoresis indicated that internucleosomal breakdown of chromatin DNA was observed in HL-60RG cells but not in dRLh-84, HeLa, and PLC/PRF/5 cells, and that the action of gallic acid was independent of cell cycle. A detailed study of signal transduction revealed that the gallic acid-induced cell death of all cells tested in this study was prevented by treatment with the intracellular thiol antioxidant *N*-acetyl-L-cysteine, catalase, and the intracellular calcium chelator bis-(*o*-aminophenoxy)-*N*,*N*,*N*,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM). However, the effects of ascorbic acid, superoxide dismutase, EGTA, the endonuclease inhibitor zinc sulfate, the calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), and the NADPH oxidase inhibitor diphenyleneiodonium chloride on cell death were different depending on the cell type, suggesting that the death signal induced by gallic acid was diverse among different cell types, although the production of reactive oxygen species, such as H_2O_2, and the elevation of intracellular calcium concentration were required as common signals. BIOCHEM PHARMACOL **55**;12:1973–1981, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. gallic acid; apoptosis; reactive oxygen species; ROS; signal transduction

Cell death is regarded not only as a degenerative phenomenon produced by injury, but also as active self-destruction to control normal embryogenesis, the development of the immune system, the elimination of virus-infected cells, and the maintenance of tissue homeostasis. An alternative mode of cell death to necrosis is apoptosis, well known to be a process by which organisms eliminate damaged, precancerous, or excessive cells. However, increasing evidence suggests that apoptosis and necrosis share common features in the early death signaling pathway, and that many signals such as protein synthesis inhibitors [1], glutamate [2], nitric oxide [3], and oxidative stress [4] can induce both apoptosis and necrosis. In addition, both types of cell death appear to coexist in many pathological situations such as ischemic brain damage [5] and liver damage [6], and are blocked completely by treatment with caspase inhibitors in an *in vivo* model of TNF-α†-mediated liver failure [7]. These observations support the idea that apoptosis and necrosis may be extremes of a continuum of multiple forms of death and that the destiny of cells that undergo apoptosis or necrosis would be determined by the intensity and type of insult, in addition to metabolic conditions in the cells [8].

Thus far, anticancer agents are known to induce apoptosis in cancer and normal cells by distinct mechanisms. The topoisomerase I inhibitor camptothecin [9], the topoisomerase II inhibitor etoposide (VP-16) [10], cisplatin [11], 1-β-D-arabinofuranosylcytosine [12], vincristine [13], methotrexate [11], taxol [14], and Adriamycin® [15] induce cell death accompanied by cell shrinkage, chromatin condensation, apoptotic body formation, and DNA degradation characteristic of apoptosis. One of the mechanisms by which such agents induce apoptosis is to produce ROS such as H_2O_2, which is already known to be an intracellular second messenger at a low concentration in both animal and plant cells [16, 17]. In fact, H_2O_2 can cause activation of transcription factors such as NF-κB [18], AP-1 [19, 20], and phosphorylation of mitogen-activated protein (MAP) kinase [21]. In addition, a slightly high concentration of H_2O_2 induces inositol-1,4,5-trisphosphate production within 10 sec after exposure to lymphocytes [22]. On the

* Corresponding author: Dr. Y. Ogihara, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan. Tel. 81-52-836-3415; FAX: 81-52-836-3415.

† *Abbreviations:* BAPTA-AM, bis-(*o*-aminophenoxy)-*N*,*N*,*N*,*N*′-tetraacetic acid acetoxymethyl ester; BS, bovine calf serum; DPhI, diphenyleneiodonium chloride; FBS, fetal bovine serum; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; LAH, lactalbumin hydrolysate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; PDTC, dithiocarbamate; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; and W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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other hand, antioxidants can protect various kinds of cells from apoptosis by scavenging ROS [23, 24]. TNF-α-induced apoptosis, which can stimulate ROS production, is inhibited by NAC, the iron chelator PDTC, or enforced expression of Mn-SOD. Fas-induced apoptosis of Jurkat T cells is also mediated by ROS, and its action is abolished completely by NAC and glutathione [25, 26].

Plant polyphenols are well known to show antioxidative activity and also to protect cultured cells from oxygen stress [23, 24]. Furthermore, phenolic antioxidants exhibit anti-inflammatory, anti-atherosclerotic, and anti-carcinogenic activities [27]. The anti-carcinogenic activity is due, in part, to the induction of the glutathione *S*-transferase Ya subunit and the quinone reductase gene through an antioxidant response element [28]. We found previously that gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol and an antioxidant, induces death in cancer cells, such as HL-60RG, HeLa, dRLh-84, PLC/PRF/5, and KB cells, with higher sensitivity than normal cells, such as rat primary cultured hepatocytes, macrophages, endothelial cells, and fibroblasts [29, 30]. Furthermore, the cytotoxic activity shown by gallic acid was found not to be a common feature observed in phenolic compounds, but a fairly specific characteristic of gallic acid. Therefore, in this study we attempted to clarify the mechanism by which gallic acid induced cell death by studying whether death signals converged on common pathways in various kinds of cells.

MATERIALS AND METHODS

Materials

Gallic acid (Nakalai Tesque Co.) was recrystallized from hot water and used for the following experiment. Donor BS, FBS, MEM-Eagle's salts (with nonessential amino acids), and RPMI-1640 were obtained from the Irvine Scientific Co. LAH and antibiotics (penicillin and streptomycin) were from Life Technologies Inc. Trypsin (pancreas protease) was from the Merck Co. BAPTA-AM, SOD, catalase, NAC, verapamil, and DPhl were purchased from Wako Pure Chemical Industries. Proteinase K and RNase A were obtained from the Sigma Chemical Co. H-7, H-89, W-7, and genistein were obtained from the Funakoshi Co. Ascorbic acid was from the Katayama Chemical Co. Zinc sulfate and EGTA were obtained from the Nakalai Tesque Co. The TUNEL assay kit was purchased from Trevigen Inc.

Cell Culture

HL-60RG (human promyelocytic leukemia), dRLh-84 (rat hepatoma), HeLa (human epithelial carcinoma), and PLC/PRF/5 cells (human hepatoma) were provided by the Japan Cancer Research Resources Bank. HL-60RG cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 50 U/mL of penicillin, and 50 μg/mL of streptomycin. PLC/PRF/5 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 6.5 mg LAH, 50 U/mL of penicillin, and 50 μg/mL of streptomycin. dRLh-84 cells were cultured in MEM-Eagle's salts (with nonessential amino acids) supplemented with 20% BS, 50 U/mL of penicillin, and 50 μg/mL of streptomycin. HeLa cells were cultured in MEM-Eagle's salt supplemented with 10% FBS, 50 U/mL of penicillin, and 50 μg/mL of streptomycin.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis

HL-60RG cells (6×10^6 cells/10 mL/dish) exposed to 50 μg/mL of gallic acid for 6 hr were collected into tubes and then washed with PBS. The cells were incubated for 10 min in 500 μL of lysis buffer (20 mM of Tris–HCl, pH 7.4, 10 mM of EDTA, 0.2% Triton X-100) at room temperature and centrifuged at 10,000 *g* for 10 min at 4°. The supernatant was incubated overnight at 50° with 100 μg/mL of proteinase K. DNA was extracted with 1 vol. of chloroform: phenol (1:1), precipitated from the aqueous phase with 1 vol. of isopropanol and 500 mM of NaCl at −20° overnight, and collected by centrifugation at 14,000 *g* for 30 min at 0°. The pellet was suspended in 70% ethanol and centrifuged at 14,000 *g* for 10 min at 0°. Then the pellet was dried under reduced pressure and incubated in 25 μL of 10 mM of Tris–HCl, pH 7.5, and 1 mM of EDTA for 1 hr at 37° with 1 μg/mL of RNase A. Samples were heated at 65° for 10 min and applied to agarose gel electrophoresis after the addition of loading buffer. Horizontal electrophoresis was performed for 1 hr at 80 V in 1.5% agarose gel with Tris-borate/EDTA (× 0.5) as running buffer. After treating the gel with 0.5 μg/mL of ethidium bromide for 10 min, DNA was visualized by UV illumination.

Flow Cytometric Analysis

Cells (1×10^6) exposed to gallic acid (50 μg/mL) for the indicated time were harvested by centrifugation and washed with PBS. The cells were fixed with ice-cold 70% methanol for 30 min, washed with PBS, and then treated with 1 mL of 1 mg/mL of RNase A solution (containing 0.112 mg/mL of trisodium citrate) at 37° for 30 min. Cells were harvested by centrifugation at 400 *g* for 5 min and stained with 250 μL of nuclear staining solution (10 mg of propidium iodide, 0.1 mg of trisodium citrate, and 0.03 mL of Triton X-100 were dissolved in 100 mL H_2O) at room temperature for 30 min in the dark. After adding 750 μL of PBS, the DNA content in each cell cycle phase was determined by FACScan (Becton Dickinson).

Cytotoxic Assay

dRLh-84 (3×10^4 cells/mL), HeLa (4×10^4 cells/mL), PLC/PRF/5 (6×10^4 cells/mL), and HL-60RG (6×10^4 cells/mL) cells were seeded into a 96-multiwell plate and cultured for 24 hr. Various kinds of inhibitors were added to the culture 30 min before the addition of gallic acid at the

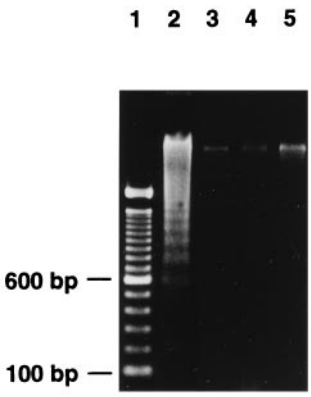

FIG. 1. Agarose gel electrophoresis of DNA extracted from various cells treated with gallic acid. HL-60RG (lane 2), dRLh-84 (lane 3), HeLa (lane 4), and PLC/PRF/5 (lane 5) cells were treated with 50 μg/mL of gallic acid for 6 hr. DNA was isolated and subjected to electrophoresis on a 1.5% agarose gel as described in Materials and Methods. Lane 1 represents molecular weight markers.

concentrations indicated. Following a 6-hr culture, cell viability was determined by the MTT method [31], and the effects of the inhibitors were examined.

In Situ *Nick-End Labeling*

The terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay was used to detect DNA fragmentation *in situ* according to the manufacturer's instructions with a slight modification.

RESULTS

We previously reported that gallic acid shows cytotoxic activity against HL-60RG, dRLh-84, HeLa, and PLC/PRF/5 cells with IC_{50} values of 5.4, 6.2, 6.1, and 6.6 μg/mL, respectively [29, 30], and that the death of HL-60RG cells was by apoptosis, evidenced by internucleosomal DNA fragmentation, cell shrinkage, disappearance of microvilli, chromatin condensation, and apoptotic body formation [30]. In this study, therefore, we examined the death of other types of cells and the signal transduction leading to cell death by gallic acid. First, DNA extracted from cells treated with gallic acid at a concentration of 50 μg/mL for 6 hr was subjected to agarose gel electrophoresis. Internucleosomal DNA cleavage of 180–200 bp multiples was observed in HL-60RG cells, but not in dRLh-84, HeLa, and PLC/PRF/5 cells (Fig. 1), regardless of the fact that the morphological examination by light microscopy revealed the appearance of cell shrinkage and partial chromatin condensation (data not shown). A fluorescence micrograph, after staining with Hoechst 33258, showed chromatin condensation and nuclear fragmentation in HL-60RG cells, but not in the other cells. Cells that are undergoing apoptosis sometimes produce large DNA fragments from 50 to 300 kbp prior to internucleosomal DNA cleavage of 180–200 bp multiples. Therefore, in order to detect large DNA fragments, dRLh-84, HeLa, and PLC/PRF/5 cells treated with gallic acid at 50 μg/mL for 6 hr were stained by the TdT-mediated dUTP nick end-labeling (TUNEL) method. These three cell lines were all labeled by the TUNEL method, indicating that dRLh-84, HeLa, and PLC/PRF/5 cells underwent apoptosis with DNA cleavage into large fragments (data not shown).

Flow cytometric analyses revealed that the smaller DNA, which resulted from DNA degradation, was detected at 2 hr after gallic acid treatment of HL-60RG cells and that the cell cycle distribution was not changed during the incubation (Fig. 2). On the other hand, although dRLh-84, HeLa, and PLC/PRF/5 cells were killed completely by gallic acid at a concentration of 50 μg/mL during the 6-hr incubation, the appearance of DNA with low fluorescent intensity, which was observed in the HL-60RG cells, did not occur. Their cell cycle distributions were not changed during the incubation, indicating that the cytotoxic activity of gallic acid is independent of the cell cycle.

We therefore examined the effects of various inhibitors on gallic acid-induced DNA fragmentation of HL-60RG cells, which reflected cell death in this cell line. As shown in Fig. 3, internucleosomal DNA cleavage was prevented by the calmodulin inhibitor W-7, the intracellular Ca^{2+} chelator BAPTA-AM, ascorbic acid, the thiol antioxidant NAC, catalase, SOD, and the Ca^{2+}/Mg^{2+}-dependent endonuclease inhibitor $ZnSO_4$. These results indicated that intracellular Ca^{2+} and ROS, in addition to endonuclease, may play a critical role in gallic acid-induced cell death in HL-60RG cells. We therefore studied the effects of various inhibitors on gallic acid-induced cell death in other cells that did not show the classical characteristics of apoptosis. First, the effects of Ca^{2+} chelator and Ca^{2+}-dependent enzyme inhibitors on gallic acid-induced cell death were investigated (Fig. 4). BAPTA-AM abrogated cell death in any cells tested in this study. W-7 and EGTA inhibited the death of dRLh-84 cells, whereas their effects were not observed against HeLa and PLC/PRF/5 cells. The Ca^{2+} channel blocker verapamil, the Ca^{2+}-dependent cysteine protease calpain inhibitor calpeptin, and the NADPH oxidase inhibitor DPhI failed to show inhibitory activity against gallic acid-induced cell death in the cells used in this study. Second, when the effects of antioxidants and radical scavengers were studied, NAC and catalase protected against gallic acid-induced cell death (Fig. 5). Furthermore, ascorbic acid abolished the death of dRLh-84 and PLC/PRF/5 cells, whereas it slightly stimulated the death of HeLa cells.

Since the involvement of protein kinases in death signals has been well investigated in a variety of apoptosis-inducing agents, the effects of various inhibitors were examined against gallic acid-induced cell death. The protein kinase C inhibitor H-7, the protein kinase A inhibitor H-89, and the tyrosine kinase inhibitor genistein failed to protect against gallic acid-induced cell death or showed only a weak stimulatory or inhibitory effect that was not significant (Fig. 6). These results, summarized in Table 1, indicated that

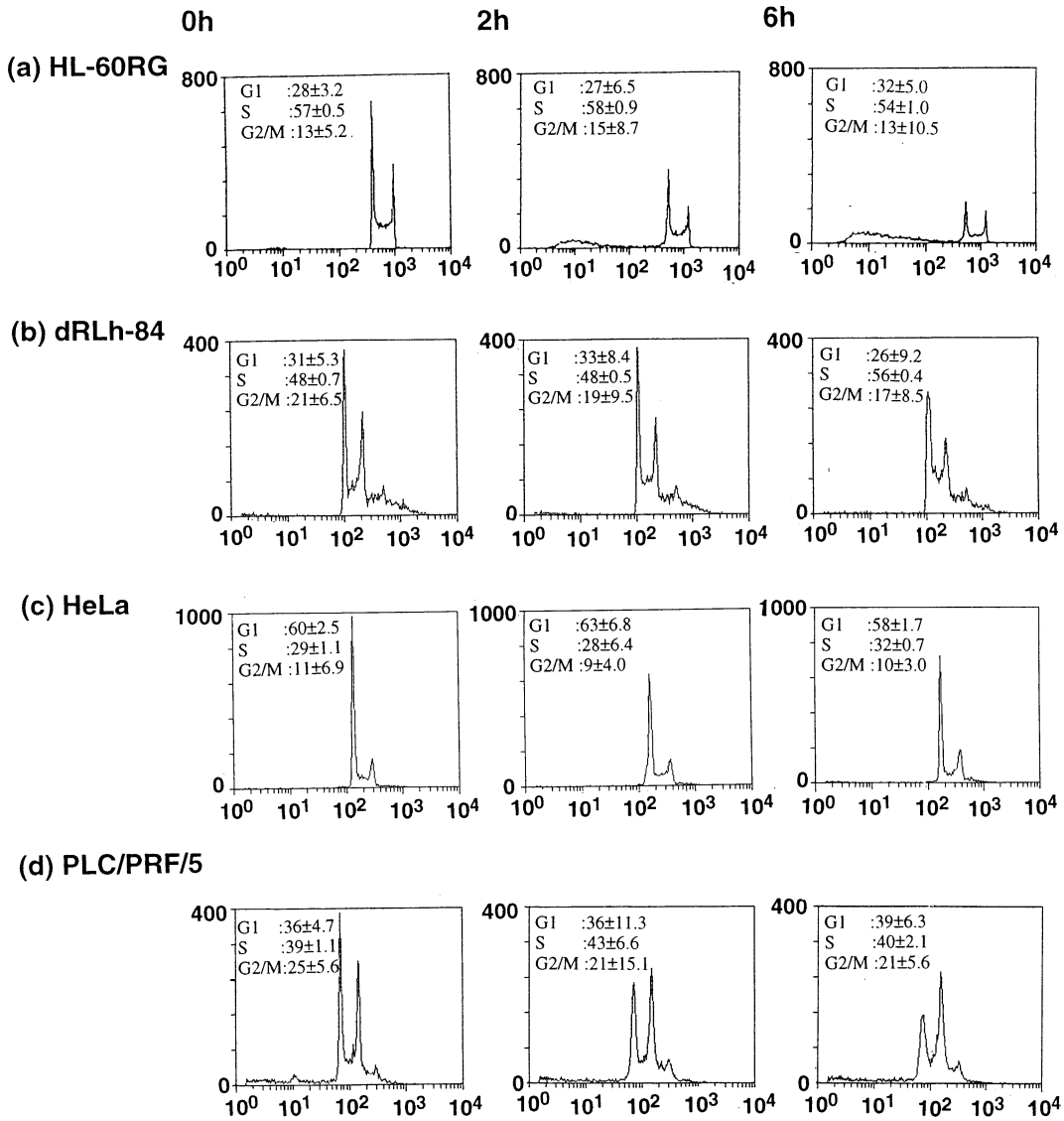

FIG. 2. Flow cytometric analysis of cell cycle phase distribution. Cells tested in this study were treated with 50 μg/mL of gallic acid for 2 or 6 hr. DNA content in each cell cycle phase was determined by FACScan as described in Materials and Methods.

intracellular Ca^{2+} and ROS were involved in the cell death induced by gallic acid as common signals and that the death of these cells was mediated by active signal transduction, regardless of the presence or absence of the classical characteristics of apoptosis.

DISCUSSION

Diverse factors are known to elicit apoptosis, including hormones, toxins, chemotherapeutic drugs, carcinogens, growth factor withdrawal, and physical trauma. The cellular signaling events leading to apoptosis are also quite varied. They include increased Ca^{2+} levels, activation of protein kinase C or protein kinase A, activation of tyrosine kinase, the production of ceramide, and caspase activation. In this study, we found that gallic acid induced apoptosis in four kinds of cell lines via intracellular Ca^{2+} elevation and the production of ROS as common denominators. When the effects of antioxidants, SOD, and catalase were investigated, NAC and catalase suppressed apoptosis in the four kinds of cells, whereas the others showed diverse effects, depending on the cell type. These results suggested that gallic acid-induced apoptosis would be mediated by H_2O_2 or intracellular ROS. NAC has been suggested to act as an antioxidant by raising the intracellular concentration of glutathione, which protects against cell damage induced by ROS and scavenges oxidant species. As NAC reacts with hydroxyl radical and slowly with H_2O_2, but not with superoxide [32], bioactive ROS as gallic acid-elicited signals would be H_2O_2 or hydroxyl radical.

Recent evidence suggests that moderate concentrations of intracellular ROS influence gene expression as well as posttranslational modification of proteins, although ROS cause damage to cells by oxidizing lipids in the cell membrane or by attacking DNA directly when produced in excess. Furthermore, several observations suggest that ROS

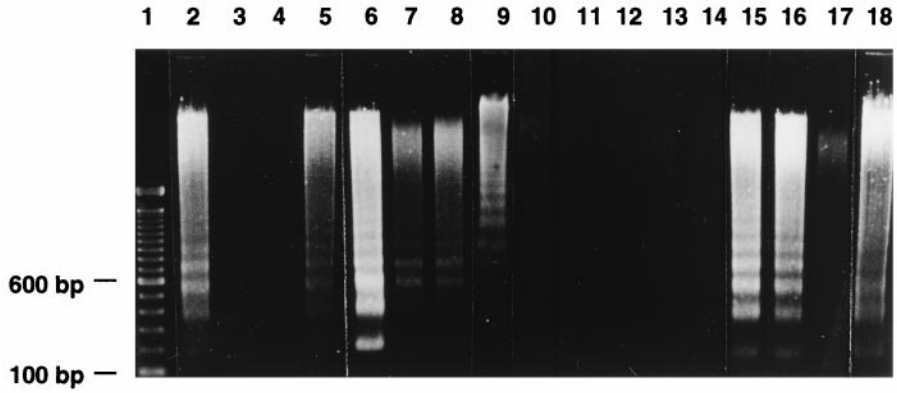

FIG. 3. Effects of various inhibitors on gallic acid-induced DNA fragmentation in HL-60RG cells. HL-60RG cells (6×10^6) were treated with various inhibitors at the concentrations described below for 30 min and then incubated with gallic acid (50 μg/mL) for 6 hr in the presence of the inhibitors. DNA was extracted according to the method described in Materials and Methods and was subjected to electrophoresis on a 1.5% agarose gel. Lane 1, molecular weight markers; lane 2, calpeptin (0.25 μM); lane 3, W-7 (50 μM); lane 4, BAPTA-AM (25 μM); lane 5, EGTA (0.5 mM); lane 6, verapamil (50 μM); lane 7, H-7 (10 μM); lane 8, H-89 (0.1 μM); lane 9, genistein (30 μM); lane 10, ascorbic acid (56.8 μM); lane 11, NAC (5 mM); lane 12, catalase (10 U/mL); lane 13, SOD (10 U/mL); lane 14, catalase (10 U/mL) + SOD (10 U/mL); lane 15, 2-mercaptoethanol (50 μM); lane 16, DPhI (1 μM); lane 17, $ZnSO_4$ (0.2 mM); and lane 18, no inhibitor.

may mediate apoptosis and are now recognized as potential modulators of apoptosis, evidenced by the finding that addition of ROS or depletion of endogenous antioxidants can induce apoptosis. Conversely, experiments under hypoxic conditions have suggested that apoptosis could occur in the absence of ROS, and ROS are unlikely to be a common intermediary and an essential part in the induction of apoptosis [33–35]. Several oxidases and reductases are known to produce superoxide anion and H_2O_2 in cells. Since NADPH oxidase, one such enzyme, is regulated by intracellular Ca^{2+}, the involvement of NADPH oxidase in ROS production by gallic acid was studied using the inhibitor DPhI. However, it did not inhibit apoptosis induced by gallic acid, thus indicating that ROS production by gallic acid was not ascribed to NADPH oxidase. In addition, phorbol myristate acetate, a protein kinase C activator, can modulate ROS production in many different cells [36, 37] by way of NADPH oxidase activation. However, in this study, an inhibitor of protein kinase C, H-7 (10 μM), did not influence gallic acid-induced apoptosis, indicating that protein kinase C and NADPH oxidase were irrelevant to its cytotoxicity. Consequently, the enzyme responsible for ROS production induced by gallic acid remains to be determined. However, there is a possibility that gallic acid acts as a prooxidant, thus damaging cells. If superoxide anion, among the ROS, plays a pivotal role in cell death, the presence of SOD, which catalyzes superoxide anion into H_2O_2, suppresses cell death. However, SOD did not show any protective effect in three of the four cell lines studied (dRLh-84, HeLa, and PLC/PRF/5 cells), the exception being HL-60RG cells, and the presence of both SOD and catalase prevented apoptosis of any of the cells. Furthermore, ascorbic acid, the most effective soluble antioxidant, did not protect HeLa cells from gallic acid. These results suggested that superoxide anion was not a causal radical as a common signal and that H_2O_2, hydroxyl radical, or other radicals were responsible for gallic acid-induced cell death. However, taking into account the permeability of ROS, extracellular hydroxy radical is not allowed to pass the plasma membrane and should not act as a second messenger leading to apoptosis. On the other hand, extracellular H_2O_2 may be a signal in gallic acid-induced cell death, whereas the addition of H_2O_2 to cultured cells did not take the place of gallic acid

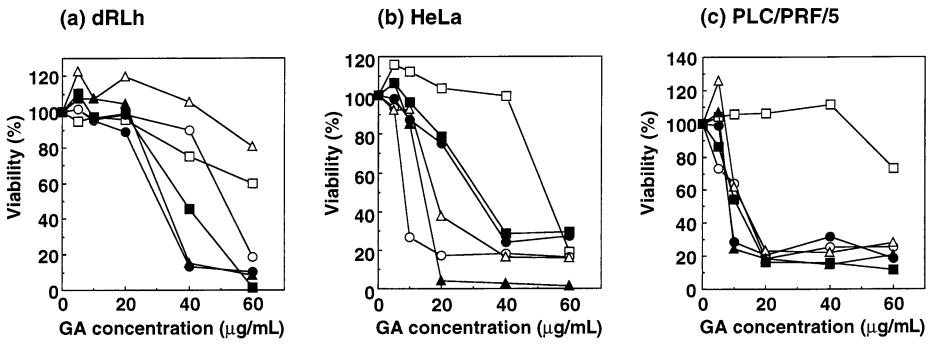

FIG. 4. Effect of Ca^{2+} chelator or Ca^{2+}-dependent enzyme inhibitors on gallic acid-induced cell death. 25 μM of BAPTA-AM (□), 25 μM of calpeptin (■), 50 μM of W-7 (△), 50 μM of verapamil (▲), 1 mM of EGTA (○), and no inhibitor or chelator (●) were added into (a) dRLh-84, (b) HeLa, and (c) PLC/PRF/5 cells 30 min before the addition of gallic acid (GA) at concentrations indicated in the figure. After a 6-hr incubation, cell viability was determined by the MTT method. Each value is the mean ± SEM of 8 wells.

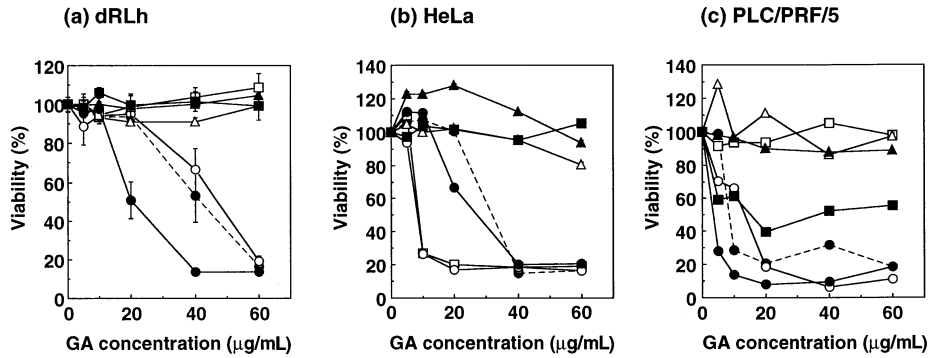

FIG. 5. Effects of antioxidants, SOD, and catalase on gallic acid-induced cell death. 0.28 mM of ascorbic acid (□), 5 mM of N-acetyl-L-cysteine (■), 10 U/mL of catalase (△), 10 U/mL each of catalase + SOD (▲), 10 U/mL of SOD (○), 50 μM of 2-mercaptoethanol (●, solid line), and untreated cells (●, broken line) were tested for gallic acid-induced cell death. The experimental procedure was the same as described in the legend of Fig. 4. Each value is the mean ± SEM of 8 wells.

with respect to the response to various inhibitors (data not shown).

Taken together, ROS generated outside cells appear not to be the means by which gallic acid induces apoptosis. On the other hand, considering the difference of redox regulation in each cell line, there is a possibility that the different expression of enzymes involved in redox regulation may give rise to different patterns of resistance against various ROS in each cell line. In plant cells, salicylic acid plays an important role in protection against virus infection by inhibiting catalase, resulting in the accumulation of H_2O_2 [38]. Compounds related to salicylic acid, such as protocatechuic acid and 3,5-dihydroxybenzoic acid, with structures similar to that of gallic acid also suppressed catalase activity. Thus, the possibility that gallic acid inhibits catalase or ROS-generating enzymes to increase ROS is conceivable, but needs to be explored. Various oxidants stimulate tyrosine as well as serine/threonine phosphorylation by activating protein kinases directly or by inhibiting protein phosphatases, although the detailed mechanism is still not understood [39]. Phosphorylation of serine, threonine, and tyrosine residues on proteins is a fundamental posttranslational regulatory process for apoptosis as well as cell proliferation. Activation of specific protein kinases, in some circumstances, can protect against cell death, while in others it protects the cell against apoptosis [40]. In this study, we used only three inhibitors of protein kinase C, protein kinase A, and tyrosine kinase [H-7 (10 μM), H-89 (0.1 μM), and genistein (30 μM), respectively] to examine the involvement of phosphorylation in the signaling pathway elicited by gallic acid. However, these inhibitors did not affect gallic acid-induced cell death significantly, suggesting that gallic acid could not directly activate or inactivate protein kinases leading to cell death and that stimulation of protein kinases was not implicated in the signaling pathway following elevated ROS production. The effects of the calcium chelator BAPTA-AM (25 μM), EGTA (0.5 mM), the calmodulin inhibitor W-7 (50 μM), the calcium-dependent protease inhibitor calpeptin (0.25 μM), and the calcium channel blocker verapamil (50 μM) were diverse, depending on the cell type. However, BAPTA-AM efficiently inhibited gallic acid-induced apoptosis, suggesting that the increase of intracellular Ca^{2+} was required for apoptosis. The sources of Ca^{2+} may be derived from intracellular stores, because EGTA and Ca^{2+} channel blockers did not affect gallic acid-induced apoptosis uniformly in the cancer cells used in this study. The role of Ca^{2+} in apoptosis induction is complicated, and the elevation of intracellular Ca^{2+} is not likely to be a common signal leading to apoptosis, evidenced by the observation that glucocorticoid activates apoptosis in thymocytes through an elevation of cytosolic Ca^{2+} concentration, but in human CEM lymphocytes it activates apoptosis in a Ca^{2+}-independent manner [41, 42]. In addition, there are some reports that the elevation of intracellular Ca^{2+} concentration both induced [43, 44] and did not induce [45] apoptosis. However, Ca^{2+} does act as a crucial signal in gallic acid-induced apoptosis. The Ca^{2+}/Mg^{2+}-dependent endonuclease inhibitor $ZnSO_4$ (0.2 mM) inhibited the death of HL-60RG cells, which showed internucleosomal DNA fragmentation, but not that of the other cells, thus indicating that DNase I or NUC18, confirmed to be

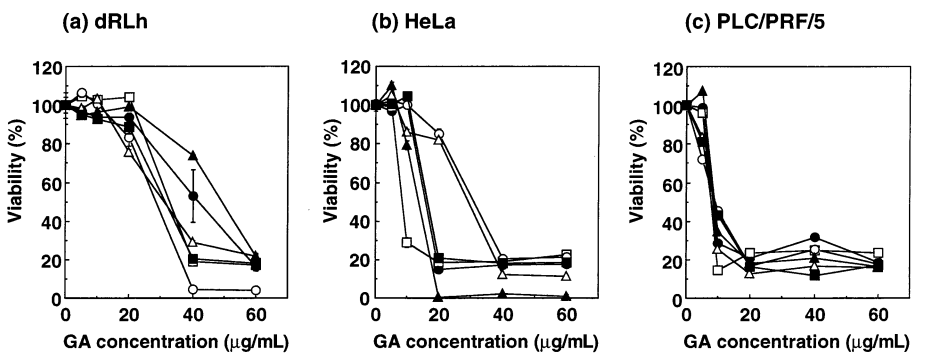

FIG. 6. Effects of various kinds of enzyme inhibitors on gallic acid-induced cell death. 10 μM of H-7 (□), 0.2 mM of $ZnSO_4$ (■), 30 μM of genistein (△), 1 μM of DPhI (▲), 0.1 μM of H-89 (○), and no inhibitor (●) were tested for gallic acid-induced cell death. The experimental procedure was the same as described in the legend of Fig. 4. Each value is the mean ± SEM of 8 wells.

TABLE 1. Effects of various agents on gallic acid-induced cell death of HL-60RG, dRLh-84, HeLa, and PLC/PRF/5 cells

Cell line	Calpeptin	W-7	BAPTA-AM	EGTA	Verapamil	H-7	H-89	Genistein
HL-60RG	X	○	○	X	X	X	X	X
dRLh-84	X	○	○	○	X	X	X	X
HeLa	X	X	○	X	X	X	X	X
PLC/PRF/5	X	X	○	X	X	X	X	X

Cell line	Ascorbic acid	NAC	Catalase	SOD	CT/SOD	2ME	DPhI	$ZnSO_4$
HL-60RG	○	○	○	○	○	X	X	○
dRLh-84	○	○	○	X	○	X	○	X
HeLa	X	○	○	X	○	X	X	X
PLC/PRF/5	○	○	○	X	○	X	X	X

Symbols indicate that the agent did not inhibit (X) or did inhibit (○) gallic acid-induced cell death. 2ME = 2-mercaptoethanol.

inhibited by $ZnSO_4$ [46, 47], may play a role in DNA cleavage in HL-60RG cells. On the other hand, cells used in this study other than HL-60RG cells may undergo apoptosis without full activation of endonucleases or internucleosomal DNA cleavage.

Recently, the activation of cysteinyl aspartate-specific proteases, designated caspase, has become generally recognized as playing a central role during the induction of apoptosis [48]. Proteases of the ICE subfamily (caspase-1, -4, and -5) predominantly play a role in inflammation, whereas proteases of the CED-3 subfamily (caspase-2, -3, and -10) are intimately involved in apoptosis. The caspase inhibitors Ac-YVAD-CHO (up to 200 μg/mL) and Ac-DEVD-CHO (up to 200 μg/mL) are able to inhibit apoptosis induced by various agents, whereas they did not influence gallic acid-induced apoptosis (data not shown), indicating that at least caspase-1 and -3 are not involved in gallic acid-induced apoptosis. Despite the diversity of signal transduction shown by gallic acid in this study, these distinct signals leading to apoptosis by gallic acid seem to feed into two common denominators, Ca^{2+} and ROS. However, the relation between those intermediaries and apoptosis has only been explained by the routes reported thus far, such as involvement of protease, protein phosphorylation, and activation of endonuclease.

Three types of cells (excepting HL-60RG cells) did not show internucleosomal DNA fragmentation characteristic of apoptosis [49], although their cell death was accompanied by cell shrinkage, partial chromatin condensation, and large DNA fragmentation. Morphological changes in apoptotic cells are considered to be dissociated from internucleosomal DNA cleavage, and types of DNA fragmentation different from internucleosomal DNA cleavage, such as fragmentation into large lengths (50–300 kbp) and single-strand cleavage, have been reported during apoptosis [50, 51]. Internucleosomal DNA cleavage seems to occur consistently in immune cells, whereas it has not always been observed in transformed or epithelial cells, implying that various types of DNA cleavage events may be cell-type specific. In brief, morphological changes and the type of DNA cleavage in gallic acid-induced apoptosis appear to depend on cell types. Finally, we concluded that gallic acid induced apoptosis in cancer cells by producing intracellular ROS and Ca^{2+} as common denominators, independent of the morphological and biochemical characteristics of apoptosis. Further studies of the mechanism by which gallic acid induces apoptosis are currently in progress and may offer a new insight into the role of ROS in the signal transduction leading to cell death.

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