Ca\(^{2+}\)-Dependent Caspase Activation by Gallic Acid Derivatives

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Gallic acid (GA) derivatives, 3,4-methylenedioxyphenyl 3,4,5-trihydroxybenzoate (GD-1) and S-(3,4-methylenedioxyphenyl)3,4,5-trihydroxythiobenzoate (GD-3), were previously reported to induce apoptosis in tumor cells with IC\(_{50}\) s of 14.5 \(\mu\)M and 3.9 \(\mu\)M, respectively.

To elucidate the mechanism by which these gallic acid derivatives (GDs) induce apoptosis, we studied whether GD-1 and GD-3 can activate caspasces. When promyelocytic leukemia HL-60RG cells were treated with GD-1 and GD-3, poly(ADP-ribose)polymerase (PARP), a substrate of caspase-3, was cleaved into 85 kDa of degradative product with increasing incubation time. GA also activated PARP cleavage, which was inhibited by catalase, \(N\)-acetyl-L-cysteine (NAC), and intracellular Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxyethane)-\(N\),\(N\),\(N\)',\(N\)’-tetraacetic acid tetrakis (acetoxyethyl ester) (BAPTA-AM), in addition to a caspase inhibitor, Z-VAD-FMK. Its inhibitory pattern was identical with that of hypoxanthine/xanthine oxidase. On the other hand, GD-1- and GD-3-induced PARP cleavage was not suppressed by catalase or NAC, but by BAPTA-AM. This suggested that the GD-elicited signaling pathway is different from GA’s. Taken together, GDs activated caspase-3 following intracellular Ca\(^{2+}\) elevation independent of reactive oxygen species. Thus, it became evident that the signaling pathway leading to apoptosis was regulated by GDs in a different manner from GA.

Key words: gallic acid derivative; apoptosis; caspase; intracellular Ca\(^{2+}\); reactive oxygen species

Apoptosis is regulated by a series of biochemical events that commit a cell to death. A common feature of cells undergoing apoptosis is the activation of caspases, a family of aspartate-specific cysteiny1 proteases. Caspase-mediated proteolysis of specific proteins results in an irreversible commitment of cells to undergo apoptosis characterized by cytoplasmic shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation. To date, many caspase substrates have been reported which contribute to the apoptotic responses.\(^{1–3}\)

We have already reported that gallic acid (GA; 3,4,5-trihydroxybenzoic acid) induces apoptosis in various kinds of tumor cells with higher sensitivity than normal cells.\(^{4,5}\) Its biochemical study further found that reactive oxygen species (ROS) generation and intracellular Ca\(^{2+}\) play important roles in the early signaling pathway in GA-induced apoptosis.\(^{6,7}\) Recent reports also indicated that GA induced apoptosis in stomach, colon, and lung cancer cells.\(^{8,9}\) Furthermore, we previously reported that GA derivatives, 3,4-methylenedioxyphenyl 3,4,5-trihydroxybenzoate (GD-1) and S-(3,4-methylenedioxyphenyl) 3,4,5-trihydroxythiobenzoate (GD-3), induced apoptosis in various kinds of tumor cells with lower IC\(_{50}\) s than that of GA,\(^{10}\) although a previous study of the structure activity relationship revealed that methylation of the phenolic hydroxyl group and esterification of the carboxyl group markedly reduced cytotoxicity.\(^{9,15}\) In addition, other GA derivatives such as the alkyl ester of GA,\(^{11}\) galloyl monosaccharides,\(^{12}\) hydrogenated farnesyl gallate, and cholesteryl gallate,\(^{13}\) were also reported to induce apoptosis in tumor cells, whereas their mechanisms were not fully explained. Reported biological activity of GA derivatives to date, in addition to cytotoxicity, includes antibacterial activity,\(^{14}\) antitumor-promoting activity,\(^{15}\) inhibitory activity of 5-lipoxygenase\(^{16}\) and squalene epoxidase.\(^{17}\) However, these activities do not appear to be related to the cytotoxicity shown by GA derivatives. Our previous study demonstrated that an intracellular Ca\(^{2+}\) change was implicated in apoptosis induced by GD-1 and GD-3. In the present study, we therefore studied not only the mechanism by which GD-1 and GD-3 induced apoptosis, but also the difference in the intracellular signaling pathway induced by GDs or GA, focusing on caspase activation.

MATERIALS AND METHODS

Chemicals Gallic acid (Nacalai Tesque Co., Kyoto, Japan) was recrystallized from hot water and used in the following experiments. RPMI1640 and fetal calf serum (FCS) were purchased from Irvine Scientific Co. (Santa Ana, CA, U.S.A.). Antibiotics (penicillin and streptomycin) were from Life Technologies, Inc. (Grand Island N.Y. 14072 U.S.A.). 1,2-Bis-(2-aminophenoxyethane)-\(N\),\(N\),\(N\)',\(N\)’-tetraacetic acid tetrakis (acetoxyethyl ester) (BAPTA-AM), catalase, and \(N\)-acetyl-L-cysteine (NAC) were from Wako Pure Chemical Industries (Osaka, Japan). Mouse anti-poly (ADP-ribose) polymerase monoclonal antibody was purchased from Biomol. Research Lab., Inc. (PA, U.S.A.). Carbobenzoxy-t-valyl-t-alanyl-\(\beta\)-methyl-t-amap-1-yl-fluoromethane (Z-VAD-FMK) was from Peptide Institute, Inc. (Osaka, Japan).

Cell Culture HL-60RG (human promyelocytic leukemia) cells were provided by the Japan Cancer Research Resource Bank and were cultured in RPMI1640 medium supplemented with 10% FCS, 50 \(\mu\)g/ml penicillin and 50 \(\mu\)g/ml streptomycin.

GA Derivative\(^{10}\) GA derivatives used in the present study, 3,4-methylenedioxyphenyl 3,4,5-trihydroxybenzoate (GD-1) and S-(3,4-methylenedioxyphenyl)-3,4,5-trihydroxythiobenzoate (GD-3), were kindly provided by Tsumura Co., Ltd.

Western Blotting Analysis HL-60RG cells (2\(\times\)10\(^{6}\) cells) were incubated with GDs at 50 \(\mu\)g/ml for 0.5, 1, 2, or 3 h in 5% CO\(_2\) in air. Then, cells were solubilized in 50 \(\mu\)l of lysis buffer containing 62.5 mm Tris/HCl, pH 6.8/6 M urea/10% glycerol/2% SDS/5% \(\beta\)-mercaptoethanol/0.00125% bro-

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mophenol blue by sonication for 15 s, followed by incubation at 65 °C for 15 min. Twenty microliters of cell extract were subjected to 10% SDS-PAGE. After separation, proteins were transferred to PVDF membranes using a transfer buffer (25 mM Tris–HCl/192 mM glycine/0.02% SDS/20% methanol). Membranes were incubated with a blocking buffer (1% powdered skimmed milk in phosphate-buffered saline (PBS)) for 2 h and incubated with a 1:500 dilution of anti-PARP mouse monoclonal antibody (Biomol Research, Lab. Inc., PA, U.S.A.) at 4 °C overnight. The membranes were washed in four changes of wash buffer (0.1% Tween-20 in PBS), then incubated with biotin-conjugated anti-mouse IgG antibody (Zymed Laboratories Inc., South San Francisco, CA, U.S.A.) in 1% powdered skimmed milk/0.1% tween-20 in PBS for 1 h at room temperature. Finally, the membrane was incubated with streptavidin–alkaline phosphatase conjugate (Zymed Laboratories Inc., South San Francisco, CA, U.S.A.) for 1 h at room temperature. Membranes were washed in five changes of wash buffer and proteins were detected using CDP-Star™ (Roche Diagnostics, Inc., Penzberg, Germany) as a substrate of alkaline phosphatase.

RESULTS

We previously reported that the GDs, GD-1 and GD-3, whose hydrophobicity was increased by esterification of the carboxyl group with a 3,4-methylenedioxyphenyl group, induced apoptosis against tumor cells in a different manner from GA.10) In the present study, we further questioned how the signaling pathway leading to apoptosis differed between GDs and GA. The structures of GA, GD-1 and GD-3 used in the present study are depicted in Fig. 1. First of all, to study the mechanism by which GDs induce apoptosis, we examined whether GDs stimulate caspases by measuring the cleavage of poly (ADP-ribose) polymerase (PARP), a substrate of caspase-3. HL-60RG cells (2 × 10⁶/ml) were treated with 50 µg/ml of GD-1 or GD-3 for the indicated time, then PARP cleavage was assessed by western blotting analysis as described in Materials and Methods. GD-1 and GD-3 activated PARP cleavage 2 and 0.5 h after the treatment, respectively, as evidenced by the disappearance of a 116 kDa intact form and the appearance of an 85 kDa degradative form (Fig. 2). This suggested that both GDs were able to stimulate caspase-3 in a way similar to GA, as reported elsewhere.18) ROS and intracellular Ca²⁺ play important roles in GA-induced apoptosis. Therefore, we first examined the effects of various kinds of inhibitors on GA, hydrogen peroxide or hypoxanthine–xanthine oxidase (HX/XO)-elicited PARP cleavage (Fig. 3). Hydrogen peroxide and HX/XO were also able to stimulate PARP cleavage as well as GA, and the cleavage was completely abolished by a caspase inhibitor, Z-VAD-FMK. Catalase and an anti-oxidant, NAC, also inhibited PARP cleavage by GA, hydrogen peroxide, and HX/XO, although NAC inhibited hydrogen peroxide-induced PARP cleavage only slightly, as compared with HX/XO-induced PARP cleavage. Interestingly, an intracellular Ca²⁺ chelator, BAPTA-AM, effectively inhibited PARP cleavage by GA, hydrogen peroxide and HX/XO. These results suggested that the change in intracellular Ca²⁺ levels was secondary to ROS generation in GA-induced apoptosis. To address the mechanism underlying GD-induced apoptosis, the effects of catalase, NAC, and BAPTA-AM were determined, as shown in Fig. 4. GD-1 or GD-3-induced PARP cleavage was abolished by BAPTA-AM, whereas catalase and NAC failed to do so. This result supports the idea that a GD-induced intracellular Ca²⁺ change, which is a cause of GD-induced apoptosis, was independent of ROS generation and caused by a mechanism distinct from that of GA. Thus, although GA and GDs triggered PARP cleavage, it became evident that the intracellular
signaling pathway, which regulates caspase activation, was definitely different between GA and GDs.

DISCUSSION

Our previous report indicated that modification of the carboxyl group in GA by introducing a 3,4-methylenedioxyphenyl group increased the cytotoxic activity to a greater extent than by using other residues, and the thioester derivative (GD-3) showed more potent activity than the ester and amido derivatives. In addition, the study of the signaling pathway indicated that intracellular Ca\(^{2+}\) may play an important role in GD-1 and GD-3-induced apoptosis as well as GA-induced apoptosis, but ROS was involved only in GA-induced apoptosis. To clarify the mechanism by which GDs induce apoptosis and the difference in the signaling pathway elicited by GDs or GA, we asked in the present study whether GDs activate caspase-3, a downstream caspase in the signaling pathway leading to apoptosis. Our results indicated that GDs stimulated PARP cleavage as well as GA. This demonstrated that GDs activated caspase-3 in the early signaling pathway. GA-induced PARP cleavage was prevented by catalase and NAC, which inhibited hydrogen peroxide and HX/XO-induced PARP degradation, whereas GD-induced PARP cleavage was not inhibited by catalase or NAC. The inhibitory pattern of PARP cleavage indicated that the action of GA appeared to be similar to that of HX/XO. In addition, BAPTA-AM prevented GA- and GD-induced PARP cleavage in addition to hydrogen peroxide- and HX/XO-induced PARP cleavage. These results demonstrated that Ca\(^{2+}\) played a critical role in GD-induced caspase activation and apoptosis in a manner independent of ROS, thus suggesting that the signaling pathway involved an intracellular Ca\(^{2+}\) change which appeared to be regulated differently in GD- and GA-induced apoptosis. Ca\(^{2+}\) is known to regulate a diverse range of cellular processes, and the spatial, temporal and amplitude pattern of Ca\(^{2+}\) signals are important in encoding the specificity of cellular responses. In the signaling pathway leading to apoptosis, the role of Ca\(^{2+}\) is complicated and the elevation of intracellular Ca\(^{2+}\) is not likely to be a common denominator. However, recent accumulating evidence indicates that Ca\(^{2+}\) triggers a mitochondrial permeability transition, resulting in the release of cytochrome c into cytoplasm through a mitochondrial permeability transition (MPT) pore. Therefore, cytochrome c activates procaspase-9, an upstream caspase of caspase-3, in combination with Apaf-1, followed by the activation of downstream caspases to orchestrate the biochemical execution of cells. The MPT pore is known to be regulated by Ca\(^{2+}\), pH, adenine nucleotides, free radicals, and mitochondrial membrane potential. In addition, the MPT pore was found to be regulated by the Bcl-2 protein family. Bad, which is a Bcl-2 protein family and bears only a BH3 domain, was dephosphorylated by Ca\(^{2+}\) and a calmodulin-dependent serine/threonine protein phosphatase, calcineurin, and it interacts with the anti-apoptotic proteins Bcl-2 and Bcl-x, resulting in the opening of the MPT pore.

The result that GD-induced apoptosis and caspase activation were mediated by Ca\(^{2+}\) suggests that GDs may activates caspase via mitochondria. In addition, caspase-12 was recently found to exist in the endoplasmic reticulum and to be activated by intracellular Ca\(^{2+}\), although the importance of caspase-12 in apoptosis induction was not fully elucidated. There is a possibility that GDs activate caspase-3, resulting from the activation of caspase-12 by Ca\(^{2+}\). On the other hand, GA-induced apoptosis was inhibited by BAPTA-AM and ROS scavenger; further, the change in intracellular Ca\(^{2+}\) levels was secondary to ROS generation. ROS have been identified as central mediators in certain signaling events. Among them, hydrogen peroxide increases the levels of both diacylglycerol and IP\(_3\), through enhanced hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C. The level of phosphatidic acid through endothelial cell phospholipase D activation, protein tyrosine phosphorylation, and agonist-sensitive Ca\(^{2+}\)-influx. Taken together, GA-induced intracellular Ca\(^{2+}\) changes seemed likely to be caused by ROS and to be differentially regulated from GD-induced intracellular Ca\(^{2+}\) changes.

In conclusion, our findings suggested that modification of the carboxyl group of GA abolished ROS-generating activity and increased hydrophobicity, resulting in the GDs being able to enter cells easily and induce caspase activation and apoptosis in a Ca\(^{2+}\) dependent manner. However, the signaling pathways in GD- and GA-induced apoptosis finally converged at the point of intracellular Ca\(^{2+}\) change, and thereafter appeared to proceed to caspase-3 activation and apoptosis induction via the identical pathway. That is, we found that GD-1 and GD-3 were cytotoxic agents with characteristics definitely different from GA.

REFERENCES